Environmental Toxicology and Chemistry

Environmental Toxicology

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Environ Toxicol Chem., **Accepted Article** • DOI: 10.1002/etc.4211

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Characterization of Cadmium and Calcium Fluxes Along the Gut, Malpighian Tubules and Anal Papillae of the Dipteran, *Chironomus riparius*

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Submitted 18 January 2018; Returned for Revision 10 April 2018; Accepted 14 June 2018

Abstract: Chironomids are often one of the dominant organisms in significantly polluted freshwater. Many invertebrate studies have characterized whole organism mechanisms of toxicity, for example, assessing cadmium (Cd) uptake via calcium (Ca) channels. However, with the use of the Scanning Ion-selective Electrode Technique (SIET) and the use of an innovative Cd-selective microelectrode, we analyze this relationship at the organ level using a realistic concentration of Cd and Ca in the hemolymph (blood). Generally, Cd fluxes follow the same directional pattern as Ca, although Ca fluxes are ~5x higher than those of Cd. These results correlate well with previous studies indicating that chironomids have a higher affinity for Ca over Cd which affords them tolerance to Cd toxicity. When saline Ca concentration was increased to 10x physiological levels, Cd fluxes from the gut lumen into the cells of the midgut regions were reduced by 50-80%. Transport of Cd from hemolymph to tissue for the posterior midgut, Malpighian tubule and proximal caeca was also reduced by ~50%. Our results indicate that Cd fluxes into or across the gut and Malpighian tubules are reduced by high Ca, suggesting that Cd may be transported in some cells by similar mechanisms. However, Cd was actively excreted at the anal papillae after a 48-h waterborne exposure to Cd, but this process was independent of Ca and instead may involve a P-glycoprotein-related pump to detoxify Cd.

Keywords: Cadmium, Calcium, Scanning ion-selective electrode technique (SIET), Chironomid and anal papillae

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INTRODUCTION

Larval chironomids are known for their tolerance to metals (Béchard et al., 2008; Gillis and Wood, 2008b; Leonard and Wood, 2013; Canfield et al., 1994). Indeed, 4th instar *Chironomus riparius* larvae are the least sensitive to Cd of all aquatic organisms in the U.S. EPA's Species Sensitivity Distribution (U.S. EPA, 2000). In general, aquatic organisms limit metal toxicity by (1) reducing uptake via branchial and/or dietary routes, (2) sequestering the metal within target tissues, thus reducing its ability to interfere with biological processes, and (3) secreting the metal via excretory organs such as the renal system. Much work has evaluated how water chemistry integrates with biotic ligands of various species to elicit Cd binding and incipient toxicity, for example in the development of the Biotic Ligand Model (DiToro et al., 2001). However, this study's focus is on the latter two aspects of metal tolerance: sequestration and elimination. We have also examined the interplay of Cd with its physiologically relevant transport analog, calcium (Ca).

Sequestration of metals within cellular organelles or concretions can contribute to metal tolerance, and several studies have highlighted the importance of the chironomid gut as a site of Cd sequestration (Craig et al., 1998; Krantzberg and Stokes, 1990; Leonard et al., 2009). The suggestion that the Malpighian tubules and rectum contribute to metal trafficking in chironomids (Krantzberg and Stokes, 1990; Postma et al., 1996) was examined directly in our previous study (Leonard et al., 2009). Using the Scanning Ion-selective Electrode Technique (SIET), Cd transport rates were calculated from Cd activity gradients measured in the unstirred layer next to the surface of the isolated gut segments and Malpighian tubules. There was evidence of Cd sequestration within the posterior midgut and analysis of the Malpighian tubule Cd content and the Cd concentration within samples of fluid secreted by isolated Malpighian tubules, set up in

the Ramsay assay, indicated that the tubules both secrete Cd (from hemolymph to lumen) and sequester Cd within the cells.

Cd was absorbed (gut lumen to hemolymph) across the anterior midgut, which would expose the muscles, nervous system and other tissues to the toxic ion Cd. We suggested in our earlier study that this absorption of Cd may be a consequence of physiological mechanisms for Ca absorption in that segment (Leonard et al., 2009). Cd is known to compete with Ca for transport in many organisms (Craig et al., 1999; Pedersen and Bjerregaard, 1995; Ahearn et al., 2004). High levels of Ca (10 mmol/L) inhibit Cd uptake in *C. staegeri* and the calcium channel blockers, lanthanum and verapamil, reduce Cd uptake by the whole larvae (Craig et al., 1999). Additionally, Cd transport rates are affected by pharmacological agents targeting calcium transport systems in aquatic insects (Buchwalter and Luoma, 2005) as well as mussels (Vercauteren and Blust, 1999; Wang and Fisher, 1999).

The anal papillae, in addition to the midgut, Malpighian tubules and rectum, are thought to take up ions from the external medium, thereby contributing to ionoregulation (Belowitz and O'Donnell, 2013). Ion uptake by the papillae has been observed in *Chironomus riparius* (Nguyen and Donini, 2010) as well as in another dipertan, *Aedes aegypti* (Donini and O'Donnell, 2005; Donini et al., 2007), and the caddis fly *Hydropsyche slossonae* larvae (Tessier et al., 2000). However, the role of the anal papillae in Cd and Ca regulation is currently unknown. Earlier studies on the whole organism established that the low affinity for Cd allows *C. riparius* larvae to maintain internal Ca levels even when exposed to Cd (Gillis et al., 2008b), however, it is not known what occurs at a cellular or organ level.

In this study, we have used SIET to measure Cd and Ca flux across isolated guts, Malpighian tubules and anal papillae of *C. riparius*. As well, we have measured the effects of Ca

on Cd flux in these same tissues. We report Cd fluxes for tissues bathed in saline containing the physiological levels of Ca (1 mmol/L; Leonard et al., 2009) as well as Ca-rich (10 mmol/L) saline.

METHODS

Chironomid *larvae*

A *C. riparius* culture at McMaster University was initiated from cultures from J. Webber (Environment Canada, Burlington, Ontario, Canada). *Chironomus riparius* larvae were maintained in 20 L tanks with one part fine-grained silica sand and three parts Hamilton dechlorinated moderately hard tap water with an ionic composition of (in mmol/L) Na (0.6), Cl (0.8), Ca (1.0), K (0.4), Mg (0.4) and Cd ($<0.5 \times 10^{-7}$). Water hardness and alkalinity were 140 mg/L and 95 mg/L as $CaCO_3$ equivalents, respectively and pH was 7.8-8.0. The deionized water used in specific experiments described below was obtained by reverse osmosis (High Purity Water Services Inc., Mississauga, ON, Canada). Tanks were continuously aerated with an air stone and the culture room was maintained at 21 ± 2ºC under a 16:8 h light:dark photoperiod. *C. riparius* larvae were fed *ad libitum* on ground Nutrafin™ fish flakes (45% protein, 5% crude fat, 2% crude fibre, 8% moisture; Big Al's Aquarium Supercentres, Woodbridge, ON, Canada). 3rd and 4th instar larvae were used for analysis.

Ion-selective microelectrodes

Cd fluxes across isolated tissues were determined using Cd-selective microelectrodes and the Scanning Ion Electrode Technique (SIET). Microelectrodes were constructed from borosilicate glass capillaries (TW150-4; WPI, Sarasota, FL, USA) pulled to a tip diameter of approximately 5-8 µm on a P-97 Flaming-Brown pipette puller (Sutter Instruments Co., Novato, CA, USA). Micropipettes were placed on a hot plate heated to 200 ºC and N,N-

dimethyltrimethylsilylamine was added to silanize the micropipettes. Following silanization, micropipettes were kept over desiccant until use. Micropipettes were backfilled with a solution of 1 mmol/L $Cd(NO_3)_2 \cdot 4H_2O$ (Fisher Scientific) and 75 mmol/L of KCl and tips were loaded via capillary action with a 50-100 µm length of Cd ionophore cocktail containing 10% cadmium ionophore I (Fluka), 10% potassium tetrakis (3,5 bis-[trifluoromethyl] phenyl) borate (Fluka) and 80% 2-nitrophenyl octyl ether (Fluka; Leonard et al., 2009; Piñeros et al., 1998). Cd-selective microelectrodes were calibrated in 0.1, 0.01 and 0.001 mmol/L of Cd in *C. riparius* saline or dechlorinated water. The saline contained (in mmol/L): KCl (5), NaCl (74), $CaCl_2 \cdot 2H_2O$ (1), $MgCl_2 \cdot 6H_2O$ (8.5), $NaHCO_3$ (10.2), PIPES (1) and glucose (20). Saline containing higher Ca (10 mmol/L) was prepared by adding one part *C. riparius* saline containing 1 mmol/L Ca to one part *C. riparius* saline containing 19 mmol/L Ca, the latter prepared by substitution of NaCl with $CaCl_2$. All salines were titrated to pH 7 immediately before use (Leonard et al., 2009). The slope of the Cd-selective microelectrodes was 28-29 mV/decade between 0.1 and 0.01 mmol/L Cd and 24 mV/decade between 0.01 mmol/L and 0.001 mmol/L Cd. Microelectrode slope was not affected by changes in bathing saline Ca concentration from 1 to 10 mmol/L.

Ca-selective microelectrodes were based on the Ca ionophore ETH1001 (Calcium Ionophore 1 – cocktail A; Sigma–Aldrich, St. Louis, MO, USA) and were prepared as described by Browne and O'Donnell (2016). Ca selective microelectrodes were calibrated in saline or dechlorinated water containing Ca at concentrations bracketing the range of interest. Slopes per 10-fold change in Ca concentration were 27 to 29 mV.

Reference electrodes were made from 10 cm borosilicate glass capillaries that were bent at a 45º angle, 1-2 cm from the end, to facilitate placement in the sample dish. Capillaries were

filled with boiling 3 mol/L KCl solution containing 3% - 5 % agar and were stored at 4 ºC in 3 mol/L KCl solution.

Speciation of cadmium in chironomid saline was calculated using Visual MINTEQ ver 3.1. (https://vminteq.lwr.kth.se/) developed by Jon Petter Gustafsson at KTH Royal Institute of Technology, Sweden. Approximately 20% of the cadmium was present in its ionic form (Cd^{2+}), whereas the remaining was complexed with chloride. The speciation did not change in the high 10 mM Ca saline as the chloride concentration was kept constant in both conditions (see above).

Tissue dissections

The gut, rectum and attached Malpighian tubules were isolated by grasping the larva at the head and anal papillae with forceps and applying slight tension. Tissues were transferred to a Petri dish coated with poly L-lysine (Sigma) to facilitate adherence. The apical surface of the gut lumen was accessed by cutting the gut open along its long axis and pinning out the gut in a Sylgard lined Petri dish. SIET measurements of Cd flux across isolated tissues were determined in saline containing the physiological levels of calcium (1 mmol/L; Leonard et al., 2009) and in calcium-rich saline (10 mmol/L) , as described below.

Anal papillae of *C. riparius* were dissected under *C. riparius* saline and isolated from 4th instar larvae using a technique similar to that used by Donini and O'Donnell (2005). Briefly, the larvae was pinched at the 9th abdominal segment (just anterior to the papillae) with fine forceps, making an incision with microscissors and sealing the open end with Vaseline to prevent loss of hemolymph. The tissue was then positioned in a 35-mm Petri dish coated with poly-L-lysine to facilitate adherence of the preparation. Cd fluxes were measured in dechlorinated tap water with the addition of a stock Cd solution made from $Cd(NO_3)_2 \cdot 4H_2O$ and dissolved in 1% HNO_3.

To test for both the health of the tissue and the accuracy of anal papillae dissection, Na transport rates were measured. Na influxes in *C. riparius* were ~-70 pmol cm^{-2} s^{-1}, comparable in magnitude and direction to those measured previously (Nguyen and Donini, 2010).

Experimental set-up

Basolateral fluxes of Cd and Ca in the gut, rectum, and Malpighian tubules for Fig. 1 and 2 were conducted in saline mimicking the physiological levels of ions in the hemolymph (as determined by Leonard et al., 2009). Positive values from the basolateral surfaces in the gut, rectum and Malpighian tubules indicate movement of Cd or Ca in the direction of lumen to hemolymph, whereas negative values indicate movement in the direction of the hemolymph into the cells (Figs. 1 and 2). As the levels of ions within the gut are not known, we used physiological saline for measurements conducted on the apical surface of the gut (Fig. 2A). Positive fluxes at the apical surface indicate movement of Cd from the gut lumen in the direction of the hemolymph. Cd fluxes at the anal papillae were performed in 10 µmol/L Cd following a 48-h waterborne exposure to either dechlorinated water containing no Cd, 10 µmol/L Cd (Fig. 3A). In the latter condition, we would expect the hemolymph Cd concentration following a 48-h exposure to be ~10 µmol/L, based on earlier experiments (see Leonard et al., 2009). We also exposed *C. riparius* larvae to 72-h of 100 µmol/L Cd and measured Cd fluxes in 10 µmol/L. Ca fluxes were measured following 48-h exposure to either dechlorinated tap water or deionized water. For the anal papillae, positive values represent movement of Cd or Ca from the lumen of the papilla into the water, whereas negative values indicate the movement of ions from bath into the papilla.

Scanning ion-selective electrode technique (SIET)

SIET measures ion concentration gradients within the unstirred layer adjacent to the surface of transporting epithelia. Gradients measured across a known excursion distance of the ion-selective microelectrode (typically 50 µm) within the unstirred layer can then be used to estimate corresponding ion fluxes using Fick's law. SIET measurements were made using hardware from Applicable Electronics (Forestdale, MA, USA) and Automated Scanning Electrode Technique (ASET) software (version 2.0, Science Wares Inc., East Falmouth, MA, USA). An orthogonal arrangement of computer-controlled stepper motors positioned the Cd-selective microelectrode 5-10 µm from the surface of the isolated tissue and then moved 50 µm away, perpendicular to the tissue surface. At each limit of the 50 µm excursion distance, the voltage was sampled for 0.5 s after a 5.5 s wait during which voltages were not recorded. Voltage differences were measured three times at each of the five sites along a tissue segment. This was repeated two times for each tissue. To determine the effects of increasing the level of Ca in the saline, the preparations were first scanned in saline containing 1 mmol/L Ca. Half of the saline was then removed from the Petri dish and replaced with the same amount of *C. riparius* saline containing 19 mmol/L calcium to produce a saline containing 10 mmol/L Ca. Care was taken not to move the preparation during this exchange and Cd fluxes were measured at the same sites for each segment in physiological and high calcium conditions.

Voltage differences (ΔV) were converted to the corresponding change in Cd or Ca concentration by the following equation (Leonard et al., 2009):

$$\Delta C_{Ion} = C_B * 10^{(\Delta V/S)} - C_B \qquad \text{Equation 1}$$

where ΔC_{Ion} is the Cd or Ca concentration difference between the two limits of the excursion distance (µmol/cm^3); C_B is the background Cd or Ca concentration in the Petri dish (µmol/L); ΔV is the voltage gradient (µV); and S is the slope of the electrode. The flux of Cd or Ca across the tissue was calculated using Fick's law:

$$J_{Ion} = D_{Ion}(\Delta C)/\Delta X \qquad \text{Equation 2}$$

where J_{Cd} is the net flux in pmol cm^{-2} s^{-1}; D_{Ion} is the diffusion coefficient (Cd, 7.20 x 10^{-6} cm^2 s^{-1}; Ca, 7.9x10^{-6} cm^2 s^{-1}); ΔC is the Cd or Ca concentration gradient (µmol cm^{-3}) and ΔX is the excursion distance between the two points (cm). Total Cd or Ca flux across each gut segment or tubule was calculated by multiplying the flux in pmol cm^{-2} s^{-1} by tissue surface area (A; cm^2) estimated as ($A = 2\pi rl$, where r is the radius of the gut or tubule segment and l is the corresponding length). More extensive descriptions of SIET for isolated insect tissues are provided in a review (O'Donnell, 2009).

Statistical analysis

Comparisons between two treatment groups in the 1 mM and 10 mM Ca experiments (Fig. 2) employed two–tailed paired t-tests (GraphPad InStat, GraphPad Software, Inc. San Diego, CA, USA). Fluxes from the anal papillae (Fig. 3) were considered to be negligible when the SEM of the mean overlapped with zero. Values significantly different than zero were indicative of net transport (unpaired t-test (GraphPad InStat, GraphPad Software, Inc. San Diego, CA, USA). Statistical significance was allotted to differences with $p < 0.05$. Data have been reported as means ± SEM (*N*).

RESULTS

Regionalization of Cd and Ca fluxes across the gut and Malpighian tubules

Cd is absorbed, from lumen to hemolymph, across the distal caecum and anterior midgut, but is secreted, from hemolymph towards the gut lumen, across all other gut regions and the Malpighian tubules (Figure 1A). The net Cd flux across each region, in pmol/s, was calculated by multiplying the area specific fluxes (pmol cm^{-2} s^{-1}) from Figure 1A by the corresponding surface area (cm^2) of each tissue region and is shown in Figure 1B. The largest fluxes are across the anterior and posterior midgut (Figure 1B). Summing of fluxes across all regions indicates that there is net absorption of Cd of 0.015 pmol/s into the hemolymph (Fig. 1B).

The animals were not exposed to Cd prior to dissection, raising the question of the source of Cd which is absorbed (from gut lumen to bath) across the anterior midgut and distal caecum. Cd may be recycled (i.e. secreted across esophagus, proximal caecum and posterior midgut) before absorption across the anterior midgut, or there may be entry of Cd from the bath through the cut end of the esophagus, as has been proposed in comparable studies of thallium transport by the chironomid gut (Belowitz and O'Donnell, 2013).

Fluxes of Ca were ~5-fold larger than those of Cd in saline containing 1 mM Ca and 10 µM Cd (Figure 1C). As for Cd, Ca was absorbed across the anterior midgut and secreted by the posterior midgut (Fig. 1C). However, the directions of Ca transport across the proximal and distal caecum were opposite to those of Cd (Fig. 1C). Multiplication of area specific fluxes (pmol/cm/s) from Figure 1C by the corresponding surface area of each tissue region indicated that the largest fluxes of Ca are across the midgut (Fig. 1D). Summing of fluxes across all regions indicates that there is net absorption of Ca of 0.1 pmol/s into the hemolymph (Fig. 1D).

Effects of high Ca on Cd flux along the gut

The influence of physiological (1 mmol/L) and high Ca levels (10 mmol/L) on Cd fluxes along the gut and Malpighian tubules in saline containing 10 µmol/L Cd are shown in Figure 2. Scans of the apical surface of the gut showed that absorption of Cd from the lumen side of the anterior and posterior midgut was reduced by ~50% across the anterior midgut and by ~80% across the posterior midgut when saline Ca concentration was increased from 1 mmol/L to 10 mmol/L (Fig. 2A).

Scans of the basolateral surface of the gut and tubules showed that Cd fluxes into the posterior midgut from the hemolymph were reduced by ~50% when bathing saline Ca concentration was increased from 1 mmol/L to 10 mmol/L (Fig. 2 B). A similar reduction was observed in the Cd fluxes for the proximal Malpighian tubule. However, a more marked decline in Cd flux of 90% was measured across the proximal caeca when saline Ca concentration was increased from 1 mmol/L to 10 mmol/L (Fig. 2B). Cd fluxes were unchanged in the esophagus, anterior midgut, ileum and anterior rectum when saline Ca concentration was changed from 1 mmol/L to 10 mmol/L.

Ca and Cd fluxes across the anal papillae

Fluxes were considered to be negligible when the SEM of the mean overlapped with zero (0-h time point), whereas values significantly different than zero were indicative of net transport. Exposure of chironomid larvae to 10 µmol/L Cd in dechlorinated tap water for 48-h was associated with a significant increase of Cd flux from the papillae lumen into the water (0.16 ± 0.03 pmol cm^{-2} s^{-1}; Figure 3A). Additionally, in a separate series of experiments, when larvae were exposed for 72-h to 100 µmol/L, Cd effluxes significantly increased in comparison to the 10 µmol/L exposure (0.31 ± 0.03 pmol cm^{-2} s^{-1}; N = 5). Ca fluxes across the papillae in dechlorinated tap water were negligible (0-h; Fig. 3B). Ca flux across the papillae was also

negligible when larvae had been maintained in deionized water for 48 hours to create conditions favourable for Ca uptake (Figure 3B).

DISCUSSION

Regionalization of Cd and Ca fluxes across the gut and Malpighian tubules

Cd is transported into the anterior midgut cells from the lumen and out of the cells into the hemolymph, confirming our previous results showing absorption of Cd from lumen to hemolymph across the anterior midgut (Leonard et al., 2009). The magnitude of Cd flux across the anterior midgut was of similar magnitude in the two studies with a flux of 0.01 pmol/s (Leonard et al., 2009) vs. 0.02 pmol/s in the current study (Fig. 1B). Studies by Krantzberg and Stokes (1989) and Timmermans and Walker (1989) indicate that chironomids do not regulate metal uptake. Indeed, the anterior midgut is known to be the site of Ca absorption from the diet in another species of dipteran, the blowfly, *Calliphora vicina* (Taylor, 1985). In contrast to mammals, blowflies do not regulate hemolymph Ca levels through changes in the rate of Ca absorption across the midgut. Rather, Ca is absorbed at a high and relatively constant rate, even when the diet contains levels of Ca in excess of the insect's needs. Regulation of hemolymph Ca levels in blowflies is accomplished by secretion and/or sequestration of excess Ca by the Malpighian (renal) tubules.

Results of the current study are consistent with this proposal, where it appears that Ca is absorbed across the anterior midgut of *Chironomus* larvae, however, transport by the more posterior regions of the gut and renal system is much less pronounced. It appears that the direction of Cd movement across various epithelia as well as the overall net movement of Cd follows a similar trend in most segments as the physiological ion, Ca. As the anterior and

posterior midgut sections have the largest surface area, these regions are primarily responsible for absorption and secretion, respectively, of both Cd (Fig. 1B, 2A) and Ca (Fig. 1D).

Effects of high Ca on Cd flux along the gut

Previous studies have shown that Cd competes for uptake with Ca across various epithelial surfaces in invertebrates. In crustaceans, Cd is taken up by the gill via voltage-independent non-selective Ca channels (Bjerregaard and Depledge, 1994; Ahearn et al., 2004), driven by electrochemical gradients. Basolateral transport of Cd into the hemolymph has been suggested to occur via Ca^{2+}-ATPase and/or Na^{+}/ Ca^{2+} exchanger (Flik et al., 1994). In perfused gills of *Carcinus maenas*, lanthanum, a calcium channel blocker, reduced both Ca and Cd uptake (Pedersen and Bjerregaard, 1995; Lucu and Obersnel, 1996). In whole animal studies of the caddisfly, *Hydropsyche californica*, increasing Ca concentration from 178 µmol/L to 1.42 mmol/L decreases Cd uptake by 34% (Buchwalter and Luoma (2005). In *C. staegeri*, Craig et al. (1999) found that both high levels of external Ca and Ca channel blockers reduced the uptake of Cd suggesting that Cd enters through Ca channels. In *C. riparius*, exposure to high external Ca levels caused a significant decrease in Cd uptake from the water (Bervoets et al., 1995).

In the current study, Cd fluxes from the gut lumen into the cells of both the anterior and posterior midgut are reduced when saline Ca concentration is increased, consistent with competition between Cd and Ca from transport (Fig. 2A). However, movement of Cd from the anterior midgut cells and distal caecum to hemolymph is unaffected by changes in bathing saline Ca concentration. This may reflect a transport mechanism (e.g. P-glycoproteins) that does not involve competition between Ca and Cd for transport (Fig. 2B). This is not the first study to demonstrate the independent mechanisms of Ca and Cd transport. In the Trichoptera, *Hydropsyche sparna* (another metal tolerant aquatic insect) (Poteat and Buchwalter, 2014a), total

accumulation and uptake of Cd remained consistent in higher Ca treatments and pharmacological agents, such as verapamil, nifedipine and carboxyeosin, did not alter Cd uptake (Poteat et al., 2012). Additionally, there was no significant correlation between Ca uptake rates and the rate constants for Cd uptake in the mayfly (*Ephemeralla invaria*) or the caddisfly (*H. sparna*), also suggesting independent mechanisms of uptake (Poteat and Buchwalter, 2014a). Indeed, recent evidence suggests that the traditional model where Cd out-competes Ca for entry resulting in osmoregulatory disturbance does not appear to apply to invertebrates (Rainbow and Black, 2005; Vercauteren and Blust, 1999; Gillis and Wood, 2008b; Poteat et al., 2012; Poteat and Buchwalter, 2014a,b).

Transport of Cd from hemolymph to tissue by the posterior midgut, proximal segment of the Malpighian tubule and proximal caecae is reduced by high Ca concentration (Fig. 2B). It thus appears that there may be competition between Cd and Ca for transport into these tissues as well. The small size of the tissues other than the midgut precludes dissecting them open so that SIET scans of the apical surface can be performed. We cannot therefore determine whether hemolymph to tissue Cd fluxes indicate transepithelial Cd flux (from hemolymph to lumen) or sequestration within the tissue, as occurs in the posterior midgut where there is absorption of Cd from both apical and basolateral surfaces (Fig. 2). There was no effect of high Ca saline on Cd flux from hemolymph to cell for the esophagus, the ileum or the anterior rectum. In these cases as well, Ca-independent mechanisms may be involved (Fig. 2B).

Ca and Cd fluxes across the anal papillae

This study provides the first look at a possible role of the anal papillae in Cd tolerance through the use of ion-selective microelectrodes and SIET (Fig. 3A). It also adds another important piece to the puzzle surrounding Cd tolerance in *C. riparius,* and completes the spatial

distribution pattern of Cd secretion along the gut tract published by Leonard et al. (2009). In the current study, Cd was excreted at the anal papillae (from anal papillae lumen to bathing solution) in *C. riparius* after a 48-h (Fig. 3A) or 72-h exposure to Cd.

Ca is neither absorbed nor secreted by the anal papillae of *C. riparius* (fluxes were not significantly different than 0; Fig. 3B), confirming a previous finding in larvae of another dipteran, *A. aegypti* (Donini and O'Donnell, 2005). It thus seems likely that the secretion of Cd that we have observed across the anal papilla does not involve Ca transporters. A role for the anal papillae in Cd detoxification was suggested earlier in a study by Podsiadlowski et al. (1998), who found evidence for a P-glycoprotein related ATP efflux pump at the anal papillae of *C. riparius*. This pump was shown to remove xenobiotic compounds from the hemolymph, and blocking it with P-glycoprotein inhibitors such as verapamil or cyclosporine A caused a synergistic increase in mortality of *C. riparius* towards the toxin ivermectin. Our results are consistent with Cd detoxification by active transport of Cd out of the anal papillae via a P-glycoprotein-related pump.

Implications for cadmium resistance

Industrial levels of Cd exposure to *C. riparius* have shown that Cd accumulates in the whole body to levels 5-fold greater than normal whole-body Ca concentrations (Béchard et al., 2008). Indeed, the level of Cd required to elicit a decline in whole body Ca was extremely high (Gillis and Wood, 2008b), allowing for a high tolerance to Cd without consequent hypocalcaemia. Michaelis-Menten kinetic analysis substantiated this phenomenon demonstrating that the affinity of Ca was much higher than that of Cd, as well as being much higher in comparison to other aquatic organisms (Gillis et al., 2008a). Therefore, the Cd-tolerant *C. riparius* larvae are able to maintain their internal Ca concentrations despite high levels of Cd

exposure (Gillis et al., 2008a). This has also become evident in other aquatic invertebrates such as mayflies (*E. invaria*), caddisflies (*H. sparna*; Poteat and Buchwalter, 2014a), and crabs (*Carcinus maenas* and *Eriocheir sinensis*; Rainbow and Black, 2005). This is further substantiated by evidence that in a Cd contaminated lake, *Chironomus* larvae were more efficient at sequestering Ca in their tissues than larvae from a "clean" lake (Krantzberg and Stokes, 1989). These authors suggested that the ability to sequester Ca during exposure to Cd would facilitate Cd tolerance. In the current study, we observe at a tissue level, in physiologically relevant conditions, that Ca fluxes are ~5-fold larger than Cd fluxes, further substantiating a mechanism of tolerance within this species to preferentially absorb Ca over Cd, avoiding hypocalcaemia at "lower levels" of Cd exposure (orders of magnitude below the lethal concentration causing 50% mortality for this species). In addition, to our knowledge, this is the first study to demonstrate the role of the anal papillae as a mechanism of metal excretion contributing to the tolerance of *Chironomus* larvae.

CONCLUSIONS

In summary, our results provide evidence that Cd fluxes into or across some of the compartments of the gut and Malpighian tubules are reduced by high Ca, suggesting that Cd may be transported in some cells by mechanisms which normally transport the physiological ion, Ca. Our measurements demonstrate that Cd is excreted from the isolated anal papillae of larvae pre-exposed to Cd for 48 or 72 h. In contrast to our analyses of the gut and Malpighian tubules, our measurements with isolated anal papillae indicate that the elimination of Cd across the anal papillae does not involve Ca transporters.

Future examination of the effects of agents known to alter Ca transport such as Ca channel blockers (e.g. diltiazem and lanthanum) may further identify regions of the gut and

Malpighian tubules where Cd transport is occurring through Ca channels. Verapamil, which is both a P-glycoprotein inhibitor and a Ca channel blocker, may reduce Cd flux through either pathway and may help decipher mechanisms of detoxification at the anal papillae.

Acknowledgment—We wish to thank D. Irwin for his input. Research was supported by an NSERC Discovery Grant awarded to M.J. O'Donnell.

Data Availability—Data and calculation tools are available from the corresponding author (leonarem@mcmaster.ca).

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Figure 1. Cadmium and calcium fluxes along the gut and Malpighian tubules of C. riparius. (A) Cadmium fluxes in saline containing 10 µM Cd and 1 mM Ca (physiological concentration) per unit area (pmol/cm2/s) at each tissue region (B) Total cadmium flux by each gut region (pmol/s), calculated as the product of flux per unit area (pmol/cm2/s) and the surface area (cm2) of the corresponding tissue. (C) Calcium fluxes in saline containing 1 mM Ca (with no Cd) per unit area (pmol/cm2/s) at each tissue region (D) Total calcium flux by each gut region (pmol/s), calculated as the product of flux per unit area (pmol/cm2/s) and the surface area (cm2) of the corresponding tissue. Positive values (absorption) represent movement from gut lumen to hemolymph. Negative values (secretion) indicate the movement from hemolymph to gut lumen. The sum of the fluxes for all gut regions is represented by the grey bar in parts B and D. Abbreviations: ESO , esophagus; PC, proximal caecum; DC, distal caecum; AMG, anterior midgut; PMG, posterior midgut; LMT, lower Malpighian tubule; DMT, distal Malpighian tubule; AR, anterior rectum; PR, posterior rectum. Values are means of S.E.M.; n = 5-10 per tissue.

Figure 2. Cadmium fluxes along the gut and Malpighian tubules of C. riparius in saline containing the physiological calcium level (1 mM) and in calcium-rich (10 mM) saline. Both salines contained 10 µM cadmium. (A) Apical cadmium fluxes from gut lumen occurred in the direction of the hemolymph. The apical surface of the gut was accessed by cutting the gut open, as described in Methods. (B) Basolateral cadmium fluxes measured from the tissue exposed in vivo to the hemolymph. Positive values (absorption) represent movement of cadmium from gut lumen to hemolymph. Negative values (secretion) indicate the movement of cadmium from hemolymph to gut lumen. *Asterisks denote significant differences (paired t-test; $p < 0.05$) in cadmium flux in saline containing 1 mM versus 10 mM calcium. Abbreviations: ESO , esophagus; PC, proximal caecum; DC, distal caecum; AMG, anterior midgut; PMG, posterior

midgut; LMT, lower Malpighian tubule; DMT, distal Malpighian tubule; AR, anterior rectum; PR, posterior rectum. Values are means of S.E.M.; n = 5-10 per tissue.

Figure 3. Cd and Ca flux at the anal papillae of C. riparius. (A) Cd flux at the anal papillae of C. riparius from papillae exposed to either Cd-free or 10 μM Cd for 48 hours. Positive values represent movement of cadmium from the lumen of the papilla into the water. (B) Ca flux at the anal papillae of C. riparius from papillae measured directly from dechlorinated tapwater or placed in deionized water for 48 hours. Fluxes were considered to be negligible when the SEM of the mean overlapped with zero. Values significantly different than zero (unpaired t-test; $p < 0.05$) were indicative of net transport. Values are means of S.E.M.; n = 5-7 for all groups.

Figure 4. Schematic diagram representing the gut segments, Malpighian tubules and anal papillae of C. riparius larvae and the tissues where high Ca decrease Cd fluxes. Grey shaded areas represent the areas where competition between Cd and Ca is occurring in C. riparius larvae. ESO = esophagus; PC and DC = proximal and distal caecae; AMG = anterior midgut; PMG = posterior midgut; ileum; PMT and DMT = proximal and distal Malpighian tubules; AR, anterior rectum; AP, anal papillae.

Figure 1

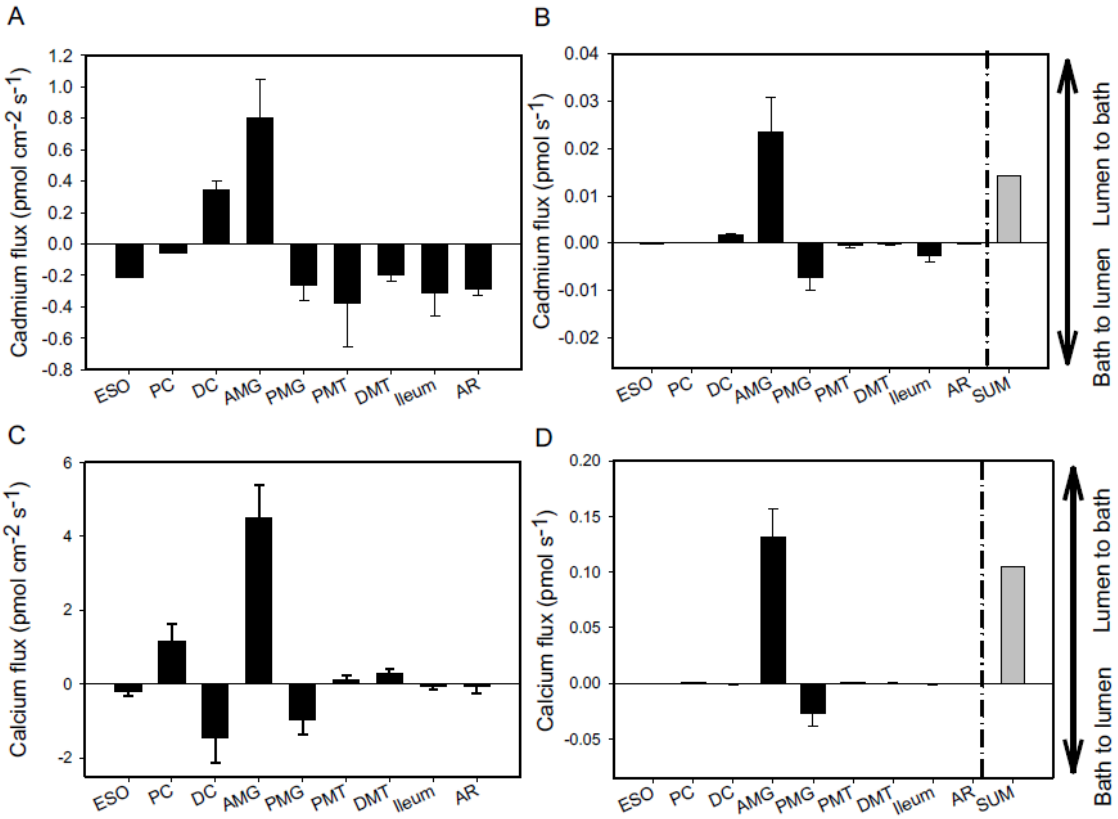

Figure 2

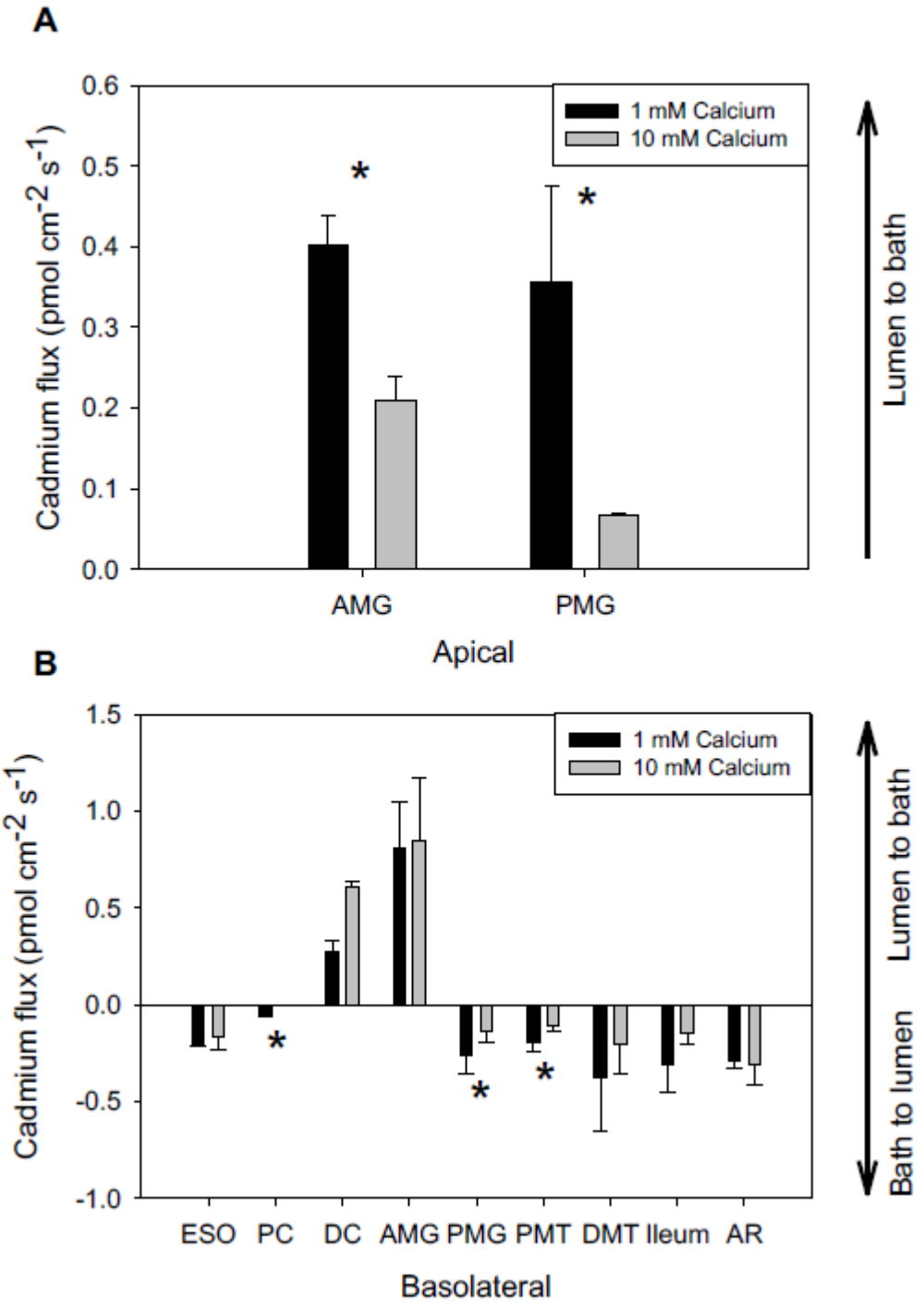

Figure 3

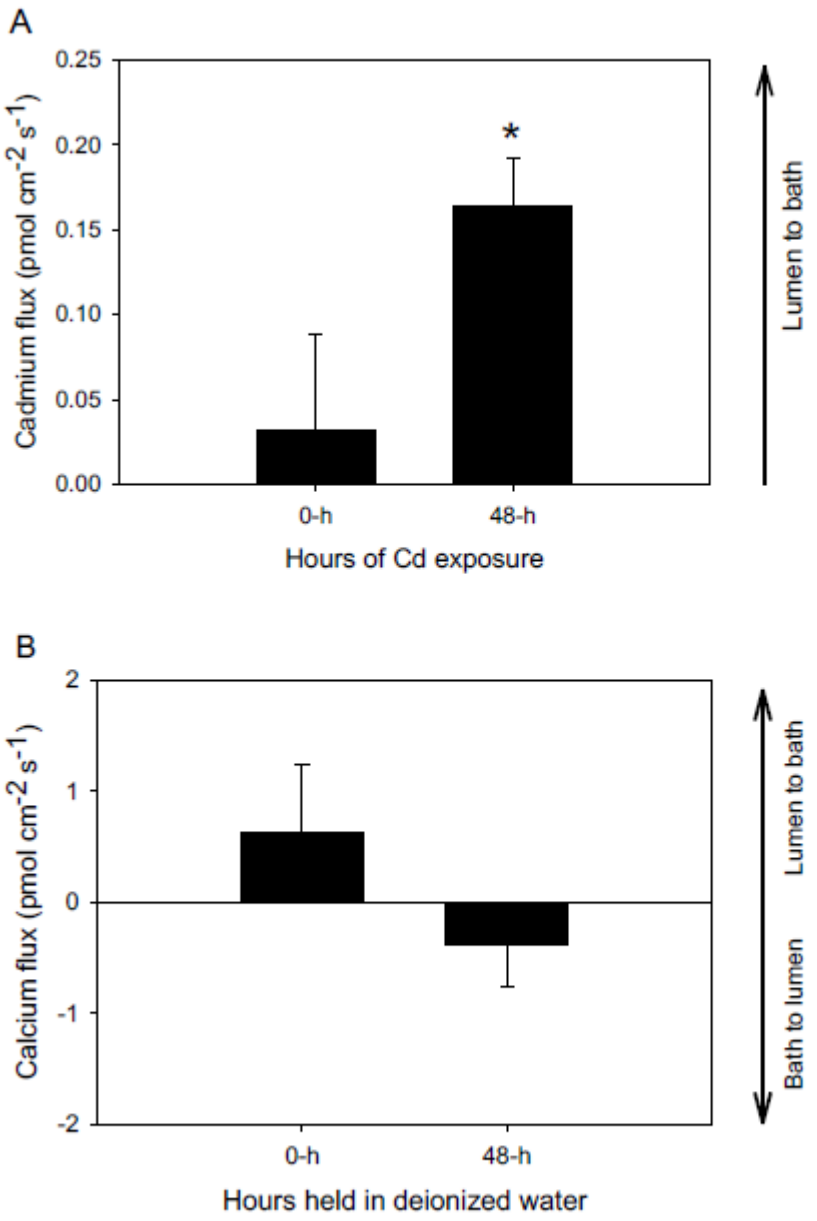

Figure 4