Aquatic Toxicology 92 (2009) 179–186

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Cadmium transport by the gut and Malpighian tubules of *Chironomus riparius*

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ARTICLE INFO

Article history:
Received 4 November 2008
Received in revised form 21 January 2009
Accepted 24 January 2009

Keywords:
Cadmium
Chironomus riparius
Cd^{2+}-selective microelectrodes
Malpighian tubules
Cd secretion
Cd sequestration

ABSTRACT

Many aquatic insects are very insensitive to cadmium in short-term laboratory studies. LC50 values for larvae of the midge *Chironomus riparius* are over 25,000 times the Criterion Maximum Concentration in the United States Environmental Protection Agency (U.S. EPA (2000)) species sensitivity distribution (SSD). Excretion or sequestration of cadmium may contribute to insensitivity and we have therefore examined cadmium transport by isolated guts and renal tissues of *C. riparius* larvae. Regional differences of Cd transport along the gut were identified using a Cd^{2+}-selective microelectrode in conjunction with the Scanning Ion-Selective Electrode Technique (SIET). Cd is transported into the anterior midgut (AMG) cells from the lumen and out of the cells into the hemolymph. The transport of Cd from the gut lumen to the hemolymph exposes other tissues such as the nervous system and muscles to Cd. The gut segments which remove Cd from the hemolymph at the highest rate are the posterior midgut (PMG) and the ileum. In addition, assays using an isolated Malpighian (renal) tubule preparation have shown that the Malpighian tubules (MT) both sequester and secrete Cd. For larvae bathed in 10 μmol l^{-1} Cd, the tubules can secrete the entire hemolymph burden of Cd in ~15 h.

1. Introduction

Relative to many other groups of organisms, insects have been shown to be relatively insensitive to cadmium, copper, lead, nickel and zinc in acute laboratory toxicity tests. The LC50 values are typically about 4 orders of magnitude higher than concentrations found in nature (Brix et al., 2005; Buchwalter et al., 2007). Larval chironomids in particular are well known for their ability to survive harsh environmental conditions including exposure to industrial byproducts such as metals (Bervoets et al., 1996; Mousavi et al., 2003; Brinkhurst, 1974; Gillis and Wood, 2008b). Béchard et al. (2007) found that the 24-h LC50 values for first instar *Chironomus riparius* in soft water are 25,000–500,000 times above the environmental guidelines outlined by the Canadian Council of the Ministers of the Environment (CCME) and the United States Environmental Protection Agency (U.S. EPA) for the five metals tested (Cd, Cu, Pb, Ni and Zn). In moderately hard water (100–110 mg l^{-1} $CaCO_3$) the 48 h LC50 values for Cd exceed 6 mmol l^{-1} for 4th instar larvae (Williams et al., 1986). Such extreme LC50 values suggest that chironomids may have an extraordinary capacity to excrete (Timmermans and Walker, 1989) or sequester (Gillis et al., 2002; Gillis and Wood, 2008b) Cd as a means of increasing their overall short-term insensitivity to toxic metals.

At high levels of metal exposure, three strategies aid survival: formation of a physical barrier (i.e. reducing metal uptake), elimination of metals in the urine or feces and metal detoxification (Hall, 2002; Naimo, 1995; Krantzberg and Stokes, 1990).

Detoxification involves the sequestration by proteins and/or incorporation into inorganic crystalline concretions, rendering the metal incapable of exerting its toxic effects (Naimo, 1995). In general, metal sequestration occurs in the organs responsible for digestion, storage and ion regulation (Krantzberg and Stokes, 1990).

Sequestration within the gut was suggested as the dominant mechanism for Cd insensitivity in *Chironomus staegeri*, based on the results of a microautoradiographic study (Craig et al., 1998). Although noting the gut as an important site for metal accumulation in chironomids, Krantzberg and Stokes (1990) proposed that detoxification was not limited to this organ. They highlighted the importance of the rectum and Malpighian tubules (MT) in ion transport and homeostasis, as well as in metal metabolism. Other studies have also implicated the Malpighian tubules in detoxification of metals in the housefly, *Musca* (Sohal et al., 1976) and silkworm larvae, *Bombyx mori* (Suzuki et al., 1984).

Although Cd is a non-essential metal, it has been shown to compete for transport with calcium in aquatic insect larvae, including chironomids (Craig et al., 1999; Buchwalter and Luoma, 2005; Martelli et al., 2006; Gillis and Wood, 2008a,b). Cd uptake by *C. staegeri*, is inhibited by 88% by high levels (10 mmol l^{-1}) of Ca, and

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doi:10.1016/j.aquatox.2009.01.011

Cd uptake is perturbed in the presence of calcium channel blockers such as verapamil and lanthanum (Craig et al., 1999). In *C. riparius*, Gillis and Wood (2008a,b) concluded that the mechanism of inhibition was by simple competition for the same transport sites. In the blowfly, *Calliphora vicina*, calcium is absorbed primarily across the anterior midgut (AMG) epithelium where the lower pH favours Ca solubility (Taylor, 1985). However, in contrast to mammalian systems, regulation of hemolymph Ca levels in *C. vicina* involves the renal system rather than the intestine (Taylor, 1985). Dube et al. (2000) determined that the Malpighian tubules of *Drosophila melanogaster* can secrete the entire Ca content of the body within approximately 9 h and that the distal segments of the Malpighian tubules can sequester the same amount in approximately 2 h.

In this study we report the first measurements of the directions and rates of cadmium transport by different anatomical segments of the isolated gut and Malpighian tubules of 4th instar larvae of *C. riparius*. These *in vitro* data helps us to assess whether secretion and/or sequestration of cadmium by these tissues contribute to the insensitivity to cadmium that has been observed in short-term toxicity studies of chironomids and other insects.

2. Methods

2.1. Chironomid larvae

A culture of *C. riparius* at McMaster University was initiated with egg ropes obtained from Environment Canada, Burlington, Ontario. First instar larvae were placed in 10 L aquaria containing one part fine-grained silica sand and three parts aerated and dechlorinated Hamilton tapwater. The ionic composition of this water (in mmol l^{-1}) was Na^+ (0.6), Cl^- (0.8), Ca^{2+} (1.8), K^+ (0.4), Mg^{2+} (0.5) and Cd ($<0.5 \times 10^{-7}$). The water was moderately hard (140 mg l^{-1} $CaCO_3$) and pH was 7.8–8.0. The cultures were maintained at 21 ± 2 °C under a 16:8 h light:dark photoperiod. Larvae were fed *ad libitum* on ground Nutrafin™ fish flakes (45% protein, 5% crude fat, 2% crude fibre, 8% moisture).

2.2. Saline composition

2.2.1. Hemolymph ion levels

A recipe for *C. riparius* saline was not available in the literature and we therefore designed a saline based on measured hemolymph ion levels. Samples of hemolymph were collected under paraffin oil. The head and anal papillae of the chironomid were held with forceps and a slight tension was applied to pull the anal papillae and gut out the posterior end of the carcass causing the hemolymph to pool in a droplet. Each gut was checked for lesions to ensure that the hemolymph sample was not contaminated by leakage of the gut contents. Levels of Na^+, K^+, Ca^{2+} and Cl^- in hemolymph were determined using ion-selective electrodes (see Sections 2.3 and 3.1). The composition of *C. riparius* larval saline was as follows (in mmol l^{-1}): 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). The saline was titrated to pH 7.

2.2.2. Minimizing Cd buffering by pH buffers

Although "Good" buffers are widely used in biological salines, there is extensive research demonstrating that these buffers form complexes with metal ions, such as copper (Kandegedara and Rorabacher, 1999; Leverrier et al., 2007; Lleu and Rebel, 1991). One such "Good" buffer is HEPES, commonly used in insect saline (e.g. O'Donnell and Maddrell, 1995). "Better" buffers were designed so as to avoid this problem (Kandegedara and Rorabacher, 1999). "Better" buffers are tertiary amines that lack hydroxyl groups on their α, β and γ carbons, thereby increasing steric hindrance and preventing metal ions from complexing with the buffer (Kandegedara and Rorabacher, 1999). The "Better" pH buffer used in this study was PIPES, which works optimally in the range of pH 6.1–7.5. Although PIPES was reported not to buffer Cd, the measurement by Scanning Ion-Selective Electrode Technique (SIET) resolves very small changes in Cd concentration and it was necessary therefore to verify that there was no possibility of changes in Cd concentration produced by a pH-dependent shift in buffer-Cd complexation within the unstirred layer next to the tissue of interest. The slopes of Cd^{2+}-selective microelectrodes were determined between 10 and 100 μmol l^{-1} Cd in 1 and 10 mmol l^{-1} PIPES-buffered saline at pH 6.5 and 7.5. Preliminary results showed no difference of slopes in 1 mmol l^{-1} PIPES buffer. However, in saline containing 10 mmol l^{-1} PIPES, the voltage in 10 μmol l^{-1} Cd at pH 6.5 increased by ~15%, thereby, decreasing the electrode slope. A concentration of 1 mmol l^{-1} PIPES was therefore used in *C. riparius* saline.

2.3. Ion-selective microelectrodes

Ion concentrations in samples of hemolymph or fluid secreted by isolated Malpighian tubules set up in the Ramsay assay (modified from Ramsay, 1954 and previously described by Dow et al., 1994) were determined using ion-selective microelectrodes. Borosilicate glass capillaries (TW150-4; WPI, Sarasota, FL, USA) were 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 silanized with N,N-dimethyltrimethylsilylamine at 200 °C for 60 min. Silanization renders the glass surface hydrophobic and facilitates filling with, and retention of, the hydrophobic ionophore cocktail. Micropipettes were backfilled and tips were loaded via capillary action with a column of the appropriate ionophore cocktail (~50–100 μm; Fluka, Buchs, Switzerland). The following ionophore cocktails (Fluka, Buchs, Switzerland) and back-fill solutions (in parentheses) were used: K^+ ionophore I cocktail B (500 mmol l^{-1} KCl); Na^+ ionophore II cocktail A (500 mmol l^{-1} NaCl); Ca^{2+} ionophore I cocktail A (500 mmol l^{-1} $CaCl_2$). Detection limits listed by the manufacturer for microelectrodes based on these ionophores are $10^{-4.8}$, $10^{-2.5}$, $10^{-7.4}$ mol l^{-1} for K^+, Na^+ and Ca^{2+}, respectively. For use in hemolymph samples and Malpighian tubule secretions under paraffin oil, ion-selective microelectrodes based on ionophore cocktails were dipped in a solution of 10% polyvinylchloride (PVC, Fluka) in tetrahydrofuran (Fluka) to prevent displacement of the ionophore cocktail by the paraffin oil (O'Donnell and Rheault, 2005).

Microelectrodes were calibrated in solutions bracketing the concentration range of interest for each ion. Na^+, K^+ and Ca^{2+} electrodes were calibrated in solutions of NaCl/LiCl, KCl/LiCl and $CaCl_2$/NaCl, respectively.

Cl^- ionophores are sensitive to several organic anions (e.g. SCN^-). To avoid interference from organic anions, solid-state Cl^- selective micropipettes were constructed from silver wire. A 15 cm length of 0.005 in. silver wire was advanced down the barrel and through the tip of a micropipette broken to a diameter of ~50 μm. Hot melt glue was used to secure the silver wire in the micropipette tip so that a length of ~50 μm protruded. A razor blade was used to taper the end of the silver wire to a fine (~10 μm) point. The exposed tip of the silver wire was immersed in $FeCl_3$ solution (25 g $FeCl_3$ in 75 ml of 1 N HCl) to coat the tip with AgCl. Cl^- microelectrodes were calibrated in solutions of KCl/$KHCO_3$.

Cd^{2+}-selective microelectrodes were backfilled with a solution of 1 mmol l^{-1} $Cd(NO_3)_2 \cdot 4H_2O$ and 75 mmol l^{-1} of KCl. A 50–100 μm length of Cd ionophore cocktail (Piñeros et al., 1998) containing 10% cadmium ionophore I (Fluka), 10% potassium tetrakis (3,5 bis-[trifluoromethyl] phenyl) borate and 80% 2-nitrophenyl octyl ether was loaded into the tip of the microelectrode via capillary action. Selectivity of these microelectrodes for Cd^{2+} exceeds that for the interfering ions Ca^{2+} and Mg^{2+} by factors of $10^{10.8}$

and $10^{12.2}$, respectively (Piñeros et al., 1998). Microelectrodes were conditioned in a 10 mmol l^{-1} $Cd(NO_3)_2$ solution for 30 min prior to calibration. Preliminary experiments showed that the time required for conditioning increased if the ionophore column length was greater than 100 μm. Cd microelectrodes were calibrated in 1, 10 and 100 μmol l^{-1} of Cd in *C. riparius* saline and displayed a slope (mean ± S.E.M.) of 28.61 ± 0.4 mV/decade between 0.1 and 0.01 mmol l^{-1} Cd (N = 5), close to the value of 29 mV/decade predicted by the Nernst equation for a perfectly Cd^{2+}-selective microelectrode. The slope decreased to 24.22 ± 0.68 mV/decade between 1 and 10 μmol l^{-1} Cd (N = 5).

Reference electrodes were constructed from 1.5 mm × 0.86 mm × 10 cm glass capillaries pulled to a tip diameter of 1–3 μm and filled with 500 mmol l^{-1} KCl. All voltages were measured on high-impedance (>10^{13} Ω) electrometers and were recorded and analyzed using a PC-based data acquisition system (PowerLab 4/25) with CHART version 5 software (ADInstruments Inc., Colorado Springs, CO, USA).

Speciation of cadmium in chironomid saline was calculated with the use of CHEAQS, a program written by W. Verweij for calculating chemical equilibria in aquatic systems and downloadable as freeware from http://home.tiscali.nl/cheaqs/. Over 64% of cadmium was complexed with chloride, and less than 20% remained uncomplexed. The activities of Cd^{2+} in saline containing 10 and 100 μmol l^{-1} total Cd were 0.71 and 7.12 μmol l^{-1}, respectively. The values predicted by CHEAQS agreed well with the values of 0.70 and 7.1 μmol l^{-1}, respectively, measured in saline with a Cd^{2+}-selective microelectrode which had been calibrated in solutions of 10 and 1 μmol l^{-1} Cd^{2+} in distilled water. Differences in Cl^- concentration between saline and the calibration solutions in the latter measurements necessitated the use of a free flowing 500 mmol l^{-1} KCl bridge (Neher, 1992) with a 20 μm tip as the reference electrode. The tip of the bridge was positioned 2 cm from the Cd^{2+} microelectrode in a Petri dish containing 5 ml of solution and leakage of KCl from the bridge (<5 μl min^{-1}) was sufficiently slow to avoid alteration of the Cl^- concentration near the tip of the Cd^{2+} microelectrode.

It was not possible to determine speciation of Cd^{2+} in hemolymph or in fluid samples collected from isolated Malpighian tubules because the concentrations of organic anions and bicarbonate in these fluids are not known. For convenience, therefore, we refer to the total dissolved Cd in the exposure medium or bathing saline in the results. Although Cd^{2+}-selective microelectrodes measure the activity of the free ion Cd^{2+}, the form in which cadmium crosses membranes is not well understood and we therefore refer to Cd transport in subsequent sections.

2.4. Hemolymph Cd levels

Larvae were transferred to 250 ml glass beakers containing 200 ml of aerated dechlorinated Hamilton tap water (25 chironomids per beaker) containing the desired $Cd(NO_3)_2$ concentration. Following a 48 h exposure period, larvae were transferred to fresh water for 5 min to remove non-specifically bound Cd and blotted dry using filter paper (Gillis and Wood, 2008a). Hemolymph droplets were collected as described in Section 2.2 and droplet volume was calculated from optical measurement of droplet diameter. Hemolymph droplets were then transferred by pipette to microcentrifuge tubes and were digested using full strength metal grade HNO_3 (10 μl/mg wet weight) for 168 h followed by H_2O_2 (4 μl/mg wet weight) for 24 h (Gillis and Wood, 2008a). All samples were analyzed by Atomic Absorption Spectroscopy using a Graphite Furnace (GFAAS; Varian SpectrAA-220 with graphite tube atomizer [GTA–110], Mulgrave, Australia). Cd recovery was 96 ± 0.72% as determined by an Environment Canada certified reference material, TM15 (a trace element fortified sample). Cd concentrations were not corrected for recovery.

2.5. Scanning ion electrode technique measurements of Cd gradients adjacent to the gut and Malpighian tubules

Transport of cadmium into or out of tissues produces gradients in Cd concentration in the unstirred layer adjacent to the surface of the tissue. These gradients can be calculated from the voltages recorded by a Cd^{2+}-selective microelectrode moved between two points within the unstirred layer. Cadmium flux can then be calculated from the concentration gradients using Fick's law, as described in detail below. Measurement of fluxes in this way is the basis of the Scanning Ion Electrode Technique, which allows fluxes to be repeatedly measured at sites along the length of an isolated tissue. The entire gut and the attached Malpighian tubules were dissected out of a 4th instar larva under physiological saline and transferred to a Petri dish coated with Poly L-lysine (Sigma) to facilitate adherence of the preparation. The apical surface of the gut lumen was accessed by splitting open and pinning out the gut in a Sylgard lined Petri dish.

SIET measurements were made using hardware from Applicable Electronics (Forestdale, MA, USA) and automated scanning electrode technique (ASET) software (version 2.0) from Science Wares Inc. (East Falmouth, MA, USA). Reference electrodes were made from 10 cm borosilicate glass capillaries that were bent at a 45° angle, 1–2 cm from the end, to facilitate placement in the sample dish. Capillaries were filled with boiling 3 mol l^{-1} KCl solution containing 3–5% agar and were stored at 4 °C in 3 mol l^{-1} KCl solution. Extensive descriptions of the use of SIET for isolated insect tissues are reported in Rheault and O'Donnell (2001, 2004) and Donini and O'Donnell (2005).

2.5.1. Measurement and calculation of Cd fluxes

Cd^{2+}-selective microelectrodes for use with SIET were calibrated in 1, 10 and 100 μmol l^{-1} Cd in *C. riparius* saline. All scans were performed in 10 μmol l^{-1} Cd. The Cd^{2+}-selective microelectrode was placed 5–10 μm from the surface of the tissue. The microelectrode was then moved a further 50 μm away, perpendicular to the tissue surface. The "wait" and "sample" periods at each limit of the 50 μm excursion distance were 5.5 and 0.5 s, respectively. Voltage differences were measured three times at each of the five sites for each tissue and a scan of the same sites was repeated four times over a 1-h period. Data were discarded if there was a greater than 25% difference in voltage differences between the first and the fourth measurements over 1 h.

Voltage differences (ΔV) obtained from the ASET software were converted to the corresponding Cd concentration difference by the following equation (Donini and O'Donnell, 2005):

$$\Delta C = C_B \times 10^{(\Delta V/S)} - C_B \quad (1)$$

where ΔC is the Cd concentration difference between the two limits of the excursion distance (μmol l^{-1} cm^{-3}); C_B is the background Cd concentration in the saline (μmol l^{-1}); ΔV is the voltage gradient (μV); and S is the slope of the electrode between 100 and 10 μmol l^{-1} Cd. Concentration differences are used to determine the Cd flux using Fick's law of diffusion:

$$J_{Cd} = \frac{D_{Cd}(\Delta C)}{\Delta X} \quad (2)$$

where J_{Cd} is the net flux in fmol cm^{-2} s^{-1}; D_{Cd} is the diffusion coefficient of Cd (7.20 × 10^{-6} cm^2 s^{-1}); ΔC is the Cd concentration gradient (μmol l^{-1} cm^{-3}) and ΔX is the excursion distance between the two points (cm).

Data were expressed in units of fmol s^{-1} by multiplying the flux in fmol cm^{-2} s^{-1} by the surface area of each tissue calculated as

$2\pi rl$ where r is the radius of the gut or tubule segment and l is the corresponding length.

2.6. Measurement of Cd secretion by Malpighian tubules in the Ramsay assay

Chironomid larvae contain four Malpighian tubules individually attached to the gut at the junction of the posterior midgut (PMG) and hindgut. Tubules were dissected under saline and fluid secretion rates were measured using the Ramsay assay (Dow et al., 1994; Rheault and O'Donnell, 2004). Droplets (20 μl) of *C. riparius* saline containing a selected concentration of $Cd(NO_3)_2$ (0, 30, 100, 300 μmol l^{-1}) were suspended in paraffin oil in a Sylgard lined Petri dish. Dissected tubules were transferred to the saline droplets and the lower end of the tubule was removed from the droplet and wrapped around a steel minuten pin inserted into the Sylgard. Tubules were stimulated with 1 mmol l^{-1} cAMP (Davies et al., 1995; Bijelic and O'Donnell, 2005) to facilitate collection of sufficient volume of secreted fluid for analysis. Stimulation of fluid secretion may give a closer representation of the *in vivo* conditions of freshwater organisms such as chironomids because osmotic water gain from the environment is expected to increase Malpighian tubule fluid secretion rate *in vivo*. Secreted droplets were collected from the lower end of the tubule after 2–4 h. Droplet diameters were measured using an ocular micrometer and droplet volume was calculated as $\pi d^3/6$. Fluid secretion rates were calculated by dividing droplet volume by the time (min) over which the droplet formed. Cd concentration (μmol l^{-1}) was determined by Cd-selective microelectrodes. Flux (fmol min^{-1} tubule) was calculated as the product of fluid secretion rate (nl min^{-1}) and ion concentration (μmol l^{-1}).

2.7. Cd sequestration within the Malpighian tubules

Malpighian tubules were transferred from the dissection dish to saline droplets containing 0, 30, 100, 300 μmol l^{-1} Cd suspended within paraffin oil in a Sylgard lined Petri dish. Tubules were left for a 2-h period and were then transferred for 2 min to each of three consecutive saline droplets to remove surface-bound Cd from the tissue (Krantzberg and Stokes, 1990). Malpighian tubule tissue samples as well as exposure and wash droplets were analyzed by Atomic Absorption Spectroscopy using a Graphite Furnace as described above for hemolymph samples. The concentration of Cd in the wash droplets declined with successive droplets. The Cd lost in the wash droplets for Malpighian tubules exposed to 30, 100 and 300 μmol l^{-1} Cd was 236%, 32% and 5%, respectively of the remaining Cd sequestered within the tissue.

2.8. Comparison of secretion and sequestration of Cd in the Malpighian tubules

The rate of Cd secretion by Malpighian tubules was expressed in μmol h^{-1}. Sequestration of Cd in the Malpighian tubules (μmol kg^{-1}) was multiplied by the average weight of the tubules (approximately 90 μg) and divided by the exposure time (2 h) to determine the total Cd sequestered over a 1-h period (μmol h^{-1}). Percent secretion was calculated as ((Cd secretion rate)/(sum of Cd secretion rate and Cd sequestration rate)) × 100. Percent sequestration was calculated similarly.

2.9. Statistical analysis

Curves relating the concentration of Cd in the fluid secreted by isolated Malpighian tubules as a function of Cd in the bathing saline were fit to the equation:

$$[Cd]_{sf} = \left[Cd_{sf,max}\left[\frac{Cd_{bath}}{K_t + [Cd]_{bath}}\right]\right] \quad (3)$$

where $[Cd]_{sf,max}$ is the maximum concentration of Cd in the secreted fluid of isolated tubules (μmol l^{-1}), [Cd] is the concentration of Cd in bathing saline (μmol l^{-1}) and K_t is the concentration of Cd in the bathing saline corresponding to half of the maximum concentration in the secreted fluid (μmol l^{-1}). Curves relating the rate secretion of Cd by isolated Malpighian tubules as a function of Cd in the bathing saline were fit to the equation:

$$J = \frac{J_{max}[Cd]_{bath}}{K_t + [Cd]_{bath}} \quad (4)$$

where J is the rate of Cd secretion in fmol min^{-1} $tubule^{-1}$, J_{max} is the maximum rate of Cd secretion, $[Cd]_{bath}$ is the concentration of Cd in the bathing saline (μmol l^{-1}) and K_t is the concentration of Cd in the bathing saline corresponding to half of the maximum rate of Cd secretion (μmol l^{-1}). Curves were fit to Eqs. (3) and (4) using a commercial graphics and analysis package (GraphPad InStat, GraphPad software Inc., San Diego, CA, USA).

Comparisons between two treatment groups employed Student's two-tailed unpaired t-test, whereas comparisons amongst multiple treatment groups were assessed using a one-way analysis of variance (ANOVA) followed by Tukey's post hoc 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 ± S.E.M. (N).

3. Results

3.1. C. riparius larval hemolymph ion levels

Ion concentrations in 40 samples of larval hemolymph under oil ion were measured using ion-selective microelectrodes. The values (in mmol l^{-1}, mean ± S.E.M.) were as follows, with the number of samples indicated in brackets: Na^+, 74 ± 3.0 (10); K^+, 5.0 ± 0.24 (10); Cl^-, 39 ± 2.2 (10); Ca^{2+}, 1.0 ± 0.14 (10).

3.2. Hemolymph Cd levels

At exposure levels of 0, 10 and 30 μmol l^{-1}, hemolymph Cd levels were similar to those in the exposure medium (Fig. 1), implying that the gut and/or body surface are not complete barriers to Cd.

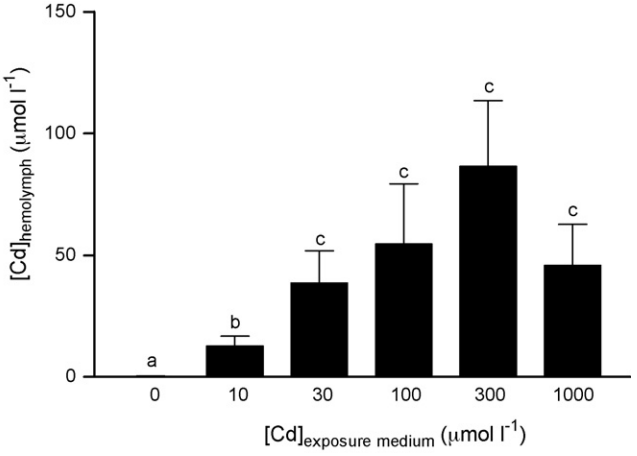

Fig. 1. Cd concentration in the hemolymph ($[Cd]_{hemolymph}$) following a 48 h waterborne exposure to concentrations indicated on the abscissa. Values are means ± S.E.M.; $N = 10$. Columns labeled with the same letters are not significantly different ($p > 0.05$).

At higher exposure levels of 100–300 μmol l^{-1} Cd, hemolymph Cd concentrations were approximately one third those in the exposure medium. Fig. 1 also shows that there was no significant difference between the hemolymph Cd levels at exposure medium concentrations between 30 and 1000 μmol l^{-1}, suggesting either regulation of Cd hemolymph levels at higher exposure concentrations or some degree of toxicity, as discussed below (Section 4.2).

3.3. SIET measurements of Cd transport by the gut and Malpighian tubules

We used an experimental Cd concentration (10 μmol l^{-1}) that was representative of the level in the hemolymph of chironomids exposed to 10 μmol l^{-1} waterborne Cd for 48 h (Fig. 1). At this concentration, voltage differences recorded by Cd^{2+}-selective microelectrodes positioned adjacent to isolated tissues were ~5–150 times above the background electrode signal recorded at sites distant (1600 μm) from the preparation.

SIET scans of the apical surface showed that the AMG and PMG both absorb Cd in a lumen to hemolymph direction (Fig. 2B). Scans of the basolateral surface showed a large efflux of Cd from the AMG in a gut-to-hemolymph direction, with a small efflux from the distal caecum. Taken together, the apical and basolateral scans indicate that there is net absorption of Cd from the lumen to hemolymph across the AMG. By contrast, the PMG absorbs Cd from the gut lumen, as well as from the hemolymph (Fig. 2A), suggesting sequestration of Cd in the PMG cells (Fig. 2B). There were influxes of Cd across the esophagus (ESO), proximal caecum (PC), Malpighian tubules (MT; distal and lower), ileum and anterior rectum (AR) in the hemolymph-to-lumen direction. These small influxes may be responsible, along with the influx across the PMG, for the clearance of Cd from the hemolymph (Fig. 2B). Consistent with this view is the finding that the sum of the basolateral influxes across the tissues (11.2 fmol s^{-1}) is approximately equal to the sum of the basolateral effluxes across the DC and AMG (10.5 fmol s^{-1}; Fig. 2B). The data in Fig. 2B have been replotted in Fig. 2C, which shows the morphology in schematic form and summarizes the mean flux of Cd measured for each anatomical segment of the gut and the Malpighian tubules.

3.4. Contribution of the Malpighian tubules to Cd clearance from the hemolymph

Fluid secretion rate for cAMP-stimulated tubules was constant at approximately 0.9 nl min^{-1} for 2–4 h (data not shown) in Cd concentrations ranging from 0 to 300 μmol l^{-1} in the bathing saline. Above 300 μmol l^{-1} Cd, the fluid secretion rate decreased significantly (data not shown). This presumably reflected toxicity and Cd levels in the secreted fluid were therefore not measured. Transepithelial Cd uptake was saturable for Cd in the range of 0–300 μmol l^{-1} (Fig. 3A and B). MTs appear not to concentrate Cd in the secreted fluid above the level in the bathing saline (Fig. 3B). Measurable levels of Cd were present in fluid secreted by Malpighian tubules bathed in Cd-free saline. This presumably reflects release of Cd that had been sequestered within the tissue prior to dissection.

By contrast to Cd secretion, the sequestration of Cd within the tubules does not appear to saturate between 10 and 300 μmol l^{-1} Cd (Fig. 3C). For tubules exposed to 10 μmol l^{-1} Cd, the concentration of Cd in the Malpighian tubules was equal to that in the bathing saline. At higher concentrations of Cd (100 and 300 μmol l^{-1}), the concentration of Cd in the tissue was up to seven times that in the bathing saline (Fig. 3C).

More Cd was secreted (65–70% of the total) than was sequestered for tubules bathed in 0 and 10 μmol l^{-1} Cd (Fig. 3D). By contrast, at 100 and 300 μmol l^{-1} Cd, more Cd was sequestered within the tubules (65% and 85% of the total, respectively) than was secreted during an equivalent period (Fig. 3D).

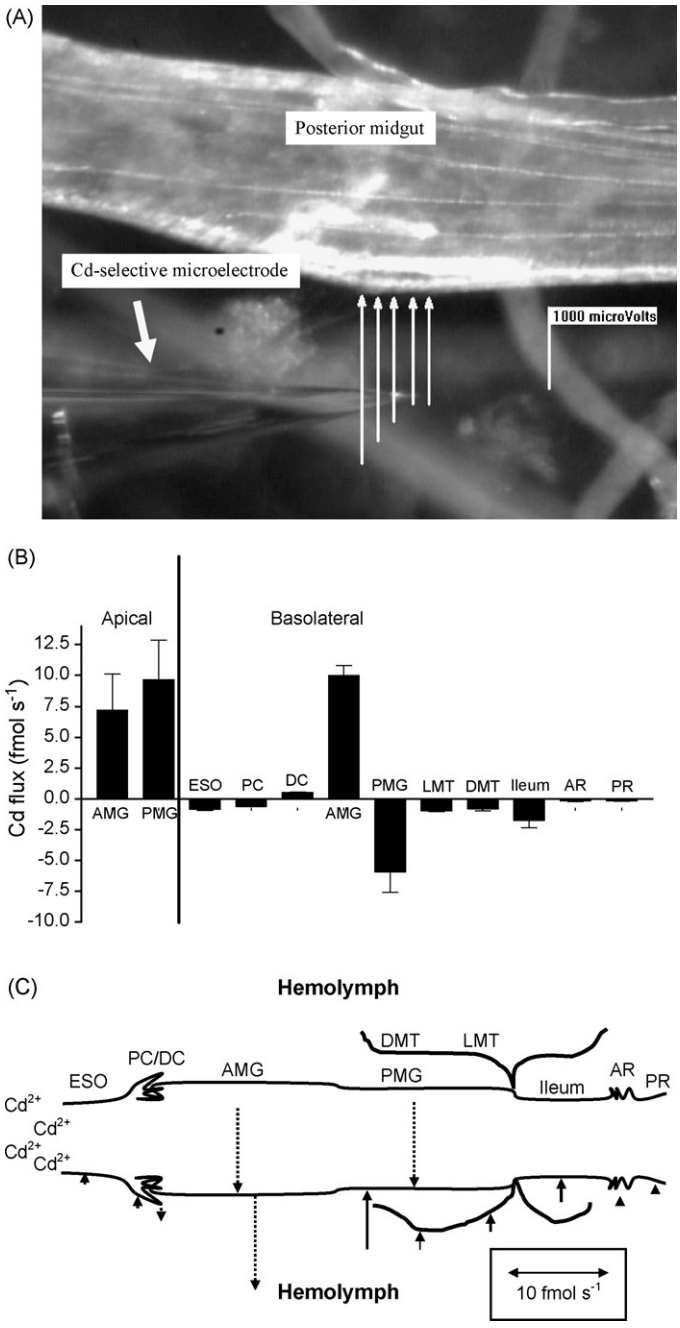

Fig. 2. (A) Representative SIET measurements along the posterior midgut. Arrow lengths denote the magnitude of the voltage gradient detected by the Cd^{2+}-selective microelectrode and the arrowheads indicate the direction of the Cd flux. (B) SIET measurement of Cd fluxes. Positive values represent movement of Cd from gut lumen to hemolymph. Negative values indicate the movement of Cd from hemolymph to gut lumen. Flux values have been corrected for the surface area of each tissue segment, as described in methods. DMT and LMT flux values were multiplied by four to show total flux by all 4 tubules. Values are means ± S.E.M.; $N = 5–10$ per tissue. All mean values are significantly different from 0 fmol s^{-1} ($p < 0.05$) determined at a reference position 1600 μm from the preparation. (C) Schematic diagram of the *C. riparius* gut showing Cd fluxes in the various gut segments. The scale is shown in the lower right box. ESO = esophagus, PC = proximal caecae, DC = distal caecae, AMG = anterior midgut, PMG = posterior midgut, LMT = lower Malpighian tubule, DMT = distal Malpighian tubule, AR = anterior rectum, PR = posterior rectum.

4. Discussion

This study reports the first measurements of Cd fluxes across isolated animal tissues obtained through the use of Cd^{2+}-selective microelectrodes and the Scanning Ion Electrode Technique. In

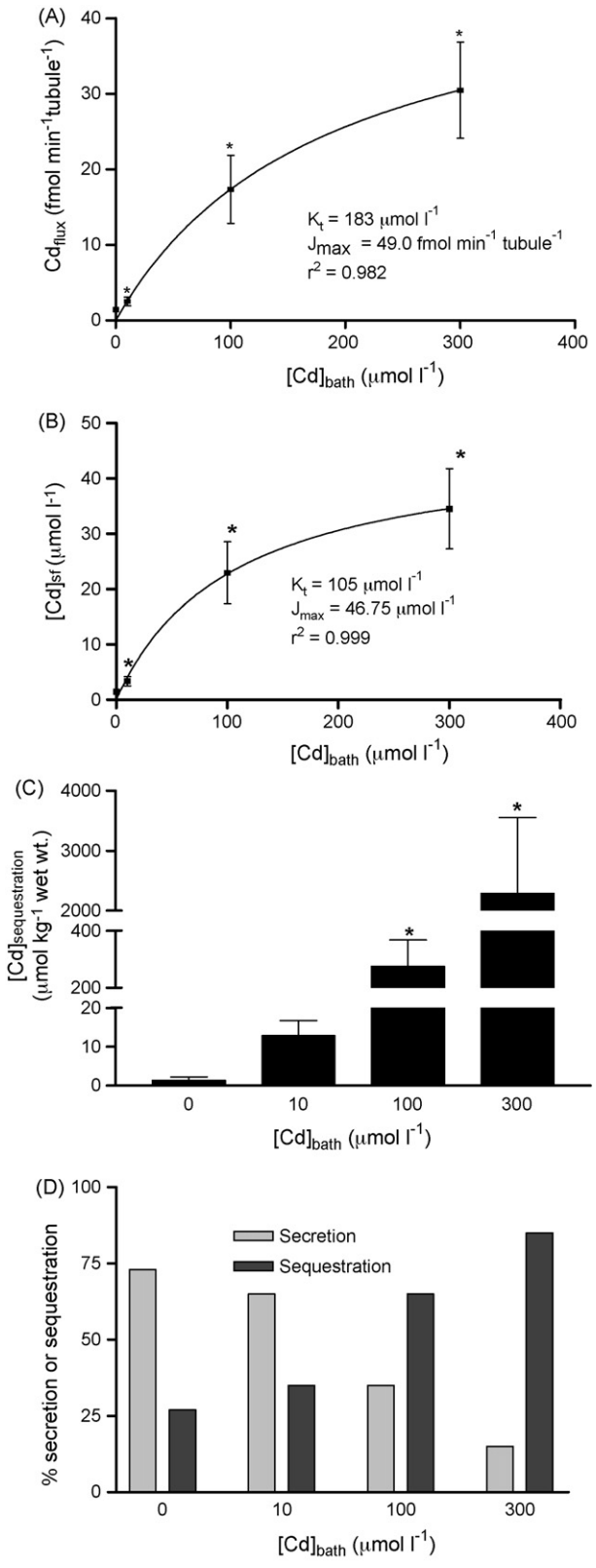

Fig. 3. Contribution of the Malpighian tubules to Cd clearance from the hemolymph. (A) Transepithelial Cd flux across isolated Malpighian tubules as a function of bathing saline Cd ($[Cd]_{bath}$). (B) Cd concentration in the fluid secreted by isolated Malpighian tubules ($[Cd]_{sf}$ as a function of bathing saline Cd. (C) Dependence of Cd sequestration by isolated Malpighian tubules on the bathing saline Cd concentration. (D) Percent cadmium secretion and sequestration in the Malpighian tubules following 1 h exposure to nominally Cd-free, 10, 100 or 300 μmol l^{-1} $Cd(NO_3)_2$ in the bathing saline. Values are means ± S.E.M.; N = 6–12 Malpighian tubules. Asterisks denote significant differences ($p < 0.05$) from the control.

addition, we provide the first application of ion-selective microelectrodes for measurement of physiological ion levels in hemolymph samples collected from chironomid larvae, as well as the first measurements of Cd levels in hemolymph samples collected from larvae that had been exposed to Cd in the water. We also provide the first direct measurement of secretion of Cd by insect Malpighian tubules set up in the Ramsay assay. Taken together, these measurements allow us to develop a model describing the roles of specific tissues in Cd uptake and excretion.

It is important to note that the concentrations of Cd used in this study are high relative to those found in most aquatic environments. There are two reasons for our choice of concentrations. Firstly, one goal of this study was to examine the extraordinary insensitivity of chironomids to environmental Cd in short-term toxicity studies. The concentration ranges used are thus well above the submicromolar levels of field situations (e.g. Martin et al., 2008) but well below those associated with toxicity over 48 h (>6000 μmol l^{-1}, Williams et al., 1986). Although the environmental relevance of our findings is thus limited, the data do indicate which tissues are likely to be involved in excretion of cadmium at very at the high concentrations that have been used in short-term toxicity tests in the laboratory. Secondly, the Cd^{2+}-selective microelectrodes used to measure cadmium transport by different segments of the gut and Malpighian tubules require the use of concentrations above 1 μmol l^{-1}. However, this limitation is countered by the ability of SIET and Cd^{2+}-selective microelectrodes to quantify Cd transport in near-real time with spatial resolution sufficient to define transport by specific anatomical segments of the gut and Malpighian tubules. Studies which infer Cd transport from accumulation of Cd within particular tissues such as the gut (e.g. Craig et al., 1998) do not provide information on the rates of transport, nor do they indicate from which side (lumen or hemolymph) the Cd was accumulated. Measurement of transport rates in isolated tissues is important because a gut segment which accumulates large amounts of Cd may be of less use in reducing Cd levels in the animal than a tissue which does not accumulate the metal but transports it at high rates into the lumen.

4.1. Hemolymph levels of Na^+, K^+, Ca^{2+} and Cl^-

The levels of Na^+ (74 mmol l^{-1}) and K^+ (5 mmol l^{-1}) in the hemolymph of larval *C. riparius* are slightly below those in the hemolymph of larval mosquitoes reared in freshwater. In *Aedes aegypti*, Na^+ and K^+ levels are 94 and 12 mmol l^{-1}, respectively (Donini et al., 2006). Na^+ and K^+ levels in hemolymph of larval *Ochlerotatus taeniorhyncus* are 111 and 11 mmol l^{-1}, respectively (Donini et al., 2006). The Na^+ and K^+ levels in *C. riparius* hemolymph are also well below those in salines used for terrestrial species such as adult *D. melanogaster* (133 mmol l^{-1} Na^+, 20 mmol l^{-1} K^+; O'Donnell et al., 1996). The reduction in levels of the predominant monovalent cations may serve to reduce hemolymph osmotic pressure and thereby reduce osmotic influx of water in freshwater.

The level of Cl^- in the hemolymph (39 mmol l^{-1}) is approximately one-half that of the sum of the cation levels, indicating a significant anion gap. This gap may be filled *in vivo* by bicarbonate and also by amino acids, whose concentrations in the hemolymph of dipterans and other insects are typically elevated (Wigglesworth, 1972).

We did not measure the concentrations of HCO_3^- and Mg^{2+} in hemolymph samples because of concerns over the limited selectivity of commercially available ionophores for use in microelectrodes. The levels of HCO_3^- and Mg^{2+} used in the saline in this study are those used in studies of the Malpighian tubules of mosquito larvae (Donini et al., 2006) and adult dipterans (Wigglesworth, 1972; O'Donnell et al., 1998).

4.2. Hemolymph Cd levels

At lower exposure concentrations (below 30 μmol l^{-1}), the concentration of Cd in the hemolymph is the same as that in the exposure medium, suggesting that the gut is not a complete barrier to Cd. Because Cd enters the hemolymph, this implies that the nervous system as well as the muscles and viscera are also exposed to Cd. At higher concentrations of Cd (above 30 μmol l^{-1}) there was no further increase in hemolymph Cd levels. This may indicate regulation of Cd levels via excretion or sequestration, suggesting some form of regulation. We think it less likely that the absence of an increase in hemolymph Cd levels reflects toxicity, given that the highest levels used (300 μmol l^{-1} Cd) were >20-fold less that the 48 h LC50 of 6450 μmol l^{-1} in 4th instar larvae (Williams et al., 1986).

4.3. Cd transport by the gut and Malpighian tubules

Our SIET measurements showed that the anterior midgut appears to be the primary gut segment involved in Cd absorption from the gut lumen into the hemolymph. By contrast the posterior midgut absorbs Cd into the tissue from both the gut lumen and from the hemolymph, consistent with sequestration of Cd in these gut cells. These results are in agreement with those of Craig et al. (1998), who showed that the anterior portion of the posterior midgut is the principal site of Cd accumulation in *C. staegeri* following waterborne exposures in the laboratory. High concentrations of Cd in the gut also account for the majority of Cd present in field-collected chironomids (Martin et al., 2008).

The low rate of Cd transport in the direction of the hemolymph by the distal caecum is counterbalanced by a similar rate of transport from the haemolymph by the proximal caecum.

The posterior midgut, along with the esophagus, Malpighian tubules, ileum and rectum show influxes of Cd from the hemolymph to tissue. These segments may account for some of the Cd clearance from the hemolymph and may therefore be involved in Cd regulation (Fig. 3A). Although the hindgut is important in salt absorption or excretion in insects and has been proposed as a site for the metabolism of toxic metals in larval *C. riparius* (Krantzberg and Stokes, 1990), it appears to play a negligible role in the elimination of cadmium by larval *C. riparius*.

4.4. Contribution of the Malpighian tubules to Cd clearance from the hemolymph

Our results also highlight the importance of the Malpighian tubules in secretion and sequestration of Cd. Secretion of metals by the Malpighian tubules of chironomid larvae was first proposed by Postma et al. (1996) and we have provided the first direct evidence for such secretion. Tubules bathed in 10 μmol l^{-1} Cd can secrete 10 fmol min^{-1} of Cd as calculated by multiplying the fluid secretion rate (nl min^{-1}) by the concentration of Cd in the secreted fluid (μmol l^{-1}) and the number of tubules per chironomid (4). If Cd is present in the hemolymph at 10 μmol l^{-1}, the quantity of Cd present in the hemolymph is 9 pmol (i.e. 9000 fmol) as calculated by multiplying hemolymph Cd concentration by volume (~0.9 μl). An approximation of the time required to eliminate this quantity of Cd from the hemolymph can be calculated by dividing the secretion rate of Cd by the quantity of Cd in the hemolymph, yielding a value of 15 h. Cd secretion by the Malpighian tubules can thus contribute to the elimination of Cd from the hemolymph at a significant rate.

Cd is also sequestered within the Malpighian tubules, consistent with earlier proposals (Maroni and Watson, 1985; Seidman et al., 1986a,b; Krantzberg and Stokes, 1990). One benefit of sequestration (both in the tubules and in the posterior midgut) is that the downstream tissues (the ileum and the rectum) are not exposed to high levels of free metals, as proposed by Maddrell et al. (1991). Our results suggest that it is the concentration of Cd which determines the dominant form of detoxification. Although sequestration is the major mechanism at high levels, secretion is the dominant form of detoxification by the Malpighian tubules at levels closer to those which are environmentally relevant (10 μmol l^{-1} or less).

In summary, our results emphasize important roles for both the gut and the Malpighian tubules in transport and sequestration of the toxic metal Cd in larval *C. riparius*. The gut does not act as a complete barrier, allowing access of Cd to the hemolymph. Using SIET, we have determined that the main site of Cd entry into the hemolymph appears to be the anterior midgut, whereas the posterior midgut absorbs Cd from the gut lumen as well as from the hemolymph, consistent with Cd sequestration within the tissue. Our SIET measurements allow us to formulate a balance sheet, identifying sites and rates of Cd influx and efflux. The movement of Cd into the hemolymph across the anterior midgut is balanced by movement of Cd towards the lumen by the remaining gut segments and by secretion and sequestration by the Malpighian tubules. As a result, levels of Cd do not tend to increase further.

Cd is a non-essential metal, raising the question as to why the anterior midgut absorbs the metal into the hemolymph. Cd has been shown to compete for transport with Ca (Craig et al., 1999; Gillis and Wood, 2008a,b) and the anterior midgut of dipterans is the primary site of Ca absorption (Taylor, 1985). Cd absorption may thus be an unavoidable consequence of the need to absorb the essential dietary metal, Ca. We are currently examining, therefore, patterns of Ca transport across the gut of chironomids and the extent of competition between Ca and Cd in this organism.

Acknowledgements

Funds were provided by Natural Sciences and Engineering Research Council of Canada in the form of a Discovery Grant to M.J.O. C.M.W. is supported by the Canada Research Chair Program.

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