INVESTIGATIONS INTO CADMIUM TOLERANCE IN *CHIRONOMUS RIPARIUS*: SPATIAL PATTERNS OF TRANSPORT AND SEQUESTRATION

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ABSTRACT

Chironomus riparius are the least sensitive aquatic species in the U.S. EPA (2000) species sensitivity distribution (SSD) for cadmium (Cd). LC50 values over 25,000 times the Criterion Maximum Concentration suggest that chironomids have an extraordinary capacity to excrete or sequester cadmium as a means of increasing their overall tolerance to toxic metals. Using atomic absorption spectroscopy, we have shown saturable uptake of cadmium by whole chironomids and isolated guts. The transport of Cd from the gut lumen to the hemolymph exposes other tissues such as the nervous system and muscles to Cd. To quantify the movement of Cd^{2+} from the main point of entry, the digestive tract, we have identified regional differences of Cd transport along the gut using a Cd^{2+}-selective microelectrode in conjunction with the Scanning Ion-Selective Electrode Technique (SIET). Cd^{2+} fluxes were determined in high mucosal or serosal Ca^{2+} to analyze the contribution of Ca^{2+} transporters to Cd^{2+} uptake. The major tissues responsible for elimination of Cd^{2+} from the hemolymph are the posterior midgut and the ileum. In addition, experiments using an isolated Malpighian tubule preparation (the Ramsay assay) have shown that the Malpighian tubules both sequester and secrete Cd^{2+}. The tubules can secrete the entire hemolymph burden of Cd^{2+} in ~ 18 hours.

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State of Mind:

If you think you are beaten, you are.

If you think you dare not, you don't.

If you'd like to win but think you can't.

It's almost a cinch that you won't.

If you think you'll lose, you've lost.

For out in the world, you'll find

Success begins with a person's will —

It's all in the state of the mind.

- Author unknown

THESIS ORGANIZATION AND FORMAT

This thesis is presented as an "open-faced sandwich thesis" with a general introduction and objectives of the research presented in chapter 1 followed by two research papers which are in the format of manuscripts to be submitted for publication in peer-reviewed journals.

Chapter 1: Introduction and project objectives

Chapter 2: Investigation into Cd tolerance in *Chironomus riparius*: spatial patterns of Cd transport and sequestration.
Authors: Erin M. Leonard, Laura M. Pierce, Patricia L. Gillis, Chris M. Wood and Michael J. O'Donnell

Chapter 3: The effect of calcium on cadmium transport across the gut of *Chironomus riparius*
Authors: Erin M. Leonard, David Irwin, Chris Wood and Michael J. O'Donnell

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CHAPTER 1:

General Introduction

Cadmium in the aquatic environment- sources and chemistry

Cadmium (Cd) is a divalent metal found in the environment as impurities of

metallic ores, mainly those of zinc, copper and lead (Godt et al., 2006; Martelli et al.,

2006). Cd is released into the air during mining and smelting of these ores and can be

transported from its origin as suspended atmospheric particles. Cd is therefore present in

many bodies of water in the Northern Hemisphere and enters these aquatic systems by

being absorbed onto particles and settling on the surficial sediments leading to the

accumulation within benthic organisms (Naimo, 1995; Martelli et al., 2006). Cd can also

enter into the environment by leaching from industrial products. These include nickel-

cadmium (NiCd) batteries, colour pigments in paints and plastics, coating by

electroplating and polyvinyl chloride (PVC) products (Godt et al., 2006; Naimo, 1995;

Martelli et al., 2006).

Recently, there has been more attention paid to the toxicity of cadmium to plants,

animals and humans, because of increased dependence on industrial metal consumption

(Lee et al., 2006). Studies have highlighted cadmium's ability to displace or mimic

essential ions within organisms, mainly calcium, zinc and iron (Martelli et al., 2006). The

displacement of these ions from proteins leads to loss of function and the production of

reactive oxygen species from the free iron molecules (Lee et al., 2006). Under biological

conditions, cadmium maintains a Cd^{2+} oxidation state and acts as a Lewis acid by

accepting electrons allowing for easy binding to many sulfur containing proteins and

enzymes (Martelli et al., 2006). In doing so, cadmium perturbs redox homeostasis and ion transport systems (Martelli et al., 2006), thereby disrupting many essential cellular processes. Following exposure to Cd, invertebrates have displayed alterations in intracellular organelles including nuclei, mitochondria, endoplasmic reticulum and plasma membranes (Seidman et al., 1986). In aquatic invertebrates, Cd exposure can result in physiological and behavioural changes affecting mating, oviposition, egg development and pupation (Lee et al., 2006).

Chironomids as highly tolerant bioindicator species

The midge *Chironomus riparius* (Order Diptera, Family Chironomidae) is often found in bodies of water highly contaminated with toxic metals (Postma et al., 1996). This insect has therefore become of key interest as a bioindicator of metal contamination and a model species for toxicological studies pertaining to Cd. The aquatic larvae have four instars which spend most of their time in the sediment compartment below the sediment-water interface. This interface is the primary location for Cd accumulation in aquatic environments (Lee et al., 2006). Aquatic invertebrates such as chironomids are important in terms of their ecological health, especially with respect to metal toxicity within the environment (Buchwalter and Luoma, 2005). Chironomids are an ideal species for toxicological experiments because they have a short life-cycle (approximately 27-29 days at 21° C), they are easily cultured in the laboratory, they are a common aquatic species found around North America and the world (Gillis et al., 2002) and they are an important food for many fish and bird species (Lee et al., 2006).

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Toleration of toxic metals by C. riparius

Chironomids have been found in waters heavily contaminated with metals and are one of the first species to repopulate areas where industrial activities have subsided (Postma et al., 1994). The concentrations of Cd, Cu, Pb, Ni and Zn associated with 50 % survival (LC50) for first instar chironomids are 25 thousand to 500 thousand times higher than the levels permitted in the environment by the United States Environmental Protection Agency (U.S. EPA) and the Canadian Councils of the Ministers of the Environment (CCME), respectively (Béchard et al., 2008). This extraordinary tolerance suggests that chironomids have a significant capacity to excrete or sequester Cd as a means of coping with exposure to high levels of metals. It should be noted that the first instars have been shown to be the most sensitive to Pb toxicity (Williams et al., 1986; Nebeker et al., 1984; Postma et al., 1994). Therefore, even when using the most sensitive larval phase, chironomids display LC50 values orders of magnitude higher than environmentally relevant levels. The LC50 value for Cd for chironomids is 200 times higher than that of *Daphnia carinata* (Khan and Nugegoda, 2007) and approximately 420 times higher than that of *Oncorhynchus mykiss* (Sloman et al., 2003).

Mechanisms involved in toxic metal detoxification

At high levels of toxic metal exposure, there are three methods for protection: formation of a physical barrier, detoxification by binding to complexes, and elimination (Naimo, 1995; Krantzberg and Stokes, 1990). Detoxification methods include sequestration by proteins (Amiard et al., 2006) and incorporation into inorganic

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crystalline concretions (Naimo, 1995). Both of these detoxification mechanisms render metals incapable of exerting their toxic effects.

For marine invertebrates the concretions consist of lead carbonate, zinc phosphate or ferric phosphate whereas in freshwater species calcium salts are more common (Naimo, 1995). Organisms demonstrate several changes following exposure to Cd. One such change at the molecular level is the production of metallothionein-like proteins (MTLP) which are cysteine rich, low-molecular weight proteins which bind available metals, rendering them inactive (Seidman et al., 1986). MTLP's have been studied as a possible biomarker to assess toxicity in benthic invertebrates (Gillis et al., 2002). MTLP have been found in several *Chironomus* species such as *C. thummi* (Seidman et al., 1986) and *C. yoshimatsui* (Yamamura et al., 1983) following exposure to Cd. MTLP are not limited to *Chironomus* species, and have been identified in the freshwater oligochaete, *Tubifex tubifex* (Gillis et al., 2002) and the nereid polychaete, *Perinereis aibuhitensis* (Ng et al., 2008). As well, metallothionein production has been determined in the teleost, *Onchorhynchus mykiss* (Chowdhury et al., 2005).

Anatomy of the gut

Several studies have described changes in the gut when chironomids are exposed to Cd in the environment (e.g. Craig et al., 1998; Krantzberg and Stokes, 1990). The chironomid gut consists of seven segments. Posterior to the mouth is the esophagus (ESO) which connects to the caecae. The caecae consists of finger-like projections extending from the esophagus-midgut junction. The segment of the caeca which attaches

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to the rest of the gut is the proximal segment (PC), whereas the distal segment (DC) makes up the component of the caeca further away from the gut. Anterior (AMG) and posterior (PMG) midguts follow the caeca. These two segments are morphologically distinct in that the anterior midgut has small projections to increase surface area whereas the posterior midgut is thinner and the surface is smoother. Four individually attached Malpighian tubules (MT) are attached at the junction between the midgut and the hindgut (see below). The ileum is considerably thinner than the midgut segments and connects to the anterior rectum. The anterior rectum (AR) has thick projections followed by the posterior rectum (PR) and anal papillae.

Anatomy of the Malpighian tubules

The excretory system of *C. riparius* consists of the Malpighian tubules and the hindgut. The four Malpighian tubules are individually attached to the junction between the midgut and hindgut. They are one cell layer thick and can be divided into two main segments: the lower segment (LMT) which attaches to the midgut-hindgut junction and the distal segment (DMT). Urine in insects is produced from the active transepithelial movement of ions from the hemolymph to the Malpighian tubule lumen with osmotically obliged water (O'Donnell et al., 1998). Ions can be reabsorbed in the lower Malpighian tubules or in the hindgut.

Methods for analysis of Cd^{2+} transport in Chironomids

The small size of insects has necessitated the modification or development of micro methods for measuring the concentrations of ions and toxins, including some organic anions and organic cations, in cells, fluid samples or in the unstirred layer near the surface of isolated tissues such as the gut and the Malpighian tubules. I have applied these approaches to the development of techniques for analysis of Cd^{2+} transport by chironomids and these methods are described briefly in Chapter 2 and Chapter 3. However, those chapters have been written in the format of papers for submission to journals, and thus the space available for methodological descriptions is limited. I have therefore provided a more extensive description of the methods used in this thesis in this section.

Radiolabelled probes have long been used to measure the concentration of inorganic ions and organic compounds in fluid samples collected from Malpighian tubules *in vitro* (e.g. Maddrell and Gardiner, 1974). Although ^{109}Cd could, in theory, be used to measure Cd transport by the tubules, there is a potential drawback because of the very small size of samples secreted by Malpighian tubules of small insects such as chironomids (~ 10 nl).

An alternative approach is the use of ion-selective microelectrodes. Ion-selective microelectrodes have been used extensively for analysis of physiological ions (H$^+$, Na$^+$, K$^+$, Ca^{2+}, Cl$^-$) inside cells of the Malpighian tubules or in samples of secreted fluid (O'Donnell and Maddrell, 1995; Ianowski et al., 2002). In this thesis I have applied a Cd^{2+}-selective microelectrode originally developed for analysis of Cd^{2+} uptake by plant

roots (Piñeros et al., 1998) to the analysis of Cd^{2+} transport by isolated tissues of the larvae of *Chironomus riparius*. Ion-selective microelectrodes have also been used to study transport of the prototypical organic cation tetraethylammonium (TEA) and the organic anion salicylate (Rheault and O'Donnell, 2004; O'Donnell and Rheault, 2005). In both cases, the detection limit for micromolar concentrations in sub-nanoliter samples is well below that achievable using radioisotopic methods. Moreover, these microelectrode methods are cheaper, faster and safer when compared to radioisotopic methods. In addition, the non-invasive scanning ion-selective electrode technique (SIET) can be applied to measure the fluxes of Cd^{2+} across isolated tissues such as the gut and the Malpighian tubules of insects, using methods similar to those used for other ions (Rheault and O'Donnell, 2004; O'Donnell and Rheault, 2005).

SIET exploits ionic concentration gradients created in the unstirred layer by ion transport across cell membranes or epithelial layers (Fig. 2A). The microelectrode is positioned by an orthogonal array (X, Y, Z) of computer-controlled stepper motors and is moved between two points at each measurements site (Fig. 2B). The first point is close (within 5 to 10 μm) of the cell surface and the second point is 30 – 100 μm farther away, at right angles to the surface. The voltage difference between the two limits of the microelectrode excursion (ΔV) is used to calculate a corresponding concentration difference (ΔC) using the electrode calibration curve. The concentration difference is then converted to net flux (mols cm^{-2} s^{-1}) using Fick's Law: J = DΔC/ΔX, where D is the diffusion coefficient of the ion of interest and ΔX is the excursion distance. SIET is particularly useful for spatial and temporal analysis of ion transport. The role of different

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segments of the gut and the Malpighian tubules in Cd^{2+} transport can thus be determined even for small insects such chironomids that are not amenable to the Ussing chamber studies used for gut segments of larger species such as locusts and *Manduca sexta*.

I have also made use of ion-selective microelectrodes to measure the concentrations of ions in the fluid secreted by isolated Malpighian tubules set up in the Ramsay assay (Fig. 3). Multiple droplets can be collected from 10 or more tubules set up in the Ramsay assay. Transepithelial flux (pmol min^{-1}) for each tubule is calculated as the product of fluid secretion rate (nl min^{-1}) and ion concentration (mmol l^{-1}) determined by the ion-selective microelectrode. Ion concentration is determined from the voltage difference between the sample and a calibration droplet and from the electrode slope, using the following equation:

$$[Cd^{2+}]_{sf} = [Cd^{2+}]_{cal} \times 10^{(\Delta V/S)}$$

Where $[Cd^{2+}]_{sf}$ is the concentration of Cd^{2+} in the secreted fluid, $[Cd^{2+}]_{cal}$ is the concentration of Cd^{2+} in a calibration droplet, ΔV is the voltage difference between the calibration droplet and the secreted fluid and S is the slope of the electrode, measured as the voltage change for a 10-fold difference in Cd^{2+} concentration. Although ion-selective microelectrodes measure ion *activity* and not *concentration*, data can be expressed in terms of concentration if it is assumed that the ion activity coefficient is the same in the secreted fluid samples and the calibration solutions. Expression of data in terms of concentrations simplifies comparisons with other studies using techniques such as atomic absorption spectroscopy which measure ion concentration.

Cd^{2+}-selective microelectrodes have not been used previously for analysis of droplets secreted by insect Malpighian tubules, and part of this thesis thus concerns measurement of parameters such as the detection limit for Cd^{2+}, electrode slope and selectivity relative to other ions (e.g. Ca^{2+}) for small fluid samples containing levels of ions comparable to those in insect hemolymph, considerably higher than the media used in the media bathing plant root tips in the original study by Piñeros et al. (1998).

An additional problem arises when ion-selective microelectrodes are used for analysis of samples maintained under paraffin oil. The ionophore cocktail is made up in an organic solvent, typically nitrophenyl octyl ether. Since the cocktail is hydrophobic, the glass micropipettes must be made hydrophobic in order to retain the cocktail in the tip and prevent capillary rise of aqueous fluids into the microelectrode. The glass is made hydrophobic through exposure to the vapours of silane compounds such as dimethyl trimethyl silyl amine. Baking the glass at ~ 200 C after exposures to silane vapour produces a hydrophobic glass surface. However, ion-selective microelectrodes made in this way cannot be used for aqueous samples under paraffin oil, since the oil is also hydrophobic and it will thus tend to rise into the microelectrode tip by capillarity and displace the ionophore cocktail. This problem can be avoided by dipping the tip of the ion-selective microelectrode in a solution of polyvinyl chloride in tetrahydrofuran. Evaporation of the tetrahydrofuran leaves a very thin coating of polyvinyl chloride around the tip of the microelectrode. Polyvinyl chloride prevents entry of oil into the microelectrode but allows the permeation of ions from aqueous samples to the ionophore

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cocktail. It is worth noting that the membranes used for conventional macroscopic ion-selective electrodes are fabricated from polyvinyl chloride.

Putative cadmium transporters:

The divalent metal transporter 1 (DMT1; also known as SLC11A2-HUGO and Nramp2) is a proton-coupled divalent cation transporter (Southon et al., 2008) which can transport Fe^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Ni^{2+} and Pb^{2+} (Gunshin et al., 1997). In mammalian systems, DMT1 is found on the brush border membrane of enterocytes and is responsible for iron homeostasis (Martini et al., 2002). In teleost fish, DMT1 is thought to be responsible for Cu (Nadella et al., 2007) and Cd (Cooper et al., 2006) transport in the gut of *Oncorhynchus mykiss* and *Danio rerio*, respectively. However, more recent studies have shown the prevalence of DMT1 in several other tissues. In the invertebrate, *Drosophila melanogaster*, Malvolio (the DMT1 homolog found in *Drosophila*), is located in the Malpighian tubules, midgut, testes and brain (Southon et al., 2008; Folwell et al., 2006). DMT1 homologs are also found in the Malpighian tubules, midgut and testes of the mosquito, *Anopheles albimanus* (Martinez-Barnetche et al., 2007). Cd has been shown to be transported by DMT1, in the yeast species, *Saccharomyces cerevisiae* (Rosakis and Koster, 2005) and in the duodenum of rats (Garrick et al., 2006).

Cd may also be transported by P-glycoproteins, which are a type of ATP-dependent efflux pump found within the membranes of cells making up the tissues of the gut, kidney, liver and blood-brain barrier (Leader and O'Donnell, 2005). P-glycoproteins are associated with the development of cancer drug therapy resistance. With the

expansion of chemotherapies and other cancer related drugs that carry out their function inside the cells, there has been more attention to this set of genes coding for multidrug resistance transporters. They actively pump out a wide array of neutral or cationic toxins from the interior of cells (Leader and O'Donnell, 2005). P-glycoprotein-like transporters have been found in the cuticle of the tobacco budworm, *Heliothis virescens* and in the Malpighian tubules of the tobacco hornworm, *Manduca sexta,* the cricket, *Teleogryllus commodus,* and the fly, *Drosophila melanogaster* (Podsiadlowski et al., 1998; Leader and O'Donnell, 2005). As well, colchicine, a known P-glycoprotein substrate, increases expression of the genes for two P-glycoproteins (mdr49 and mdr65), in the gut and brain of *Drosophila melanogaster* larvae (Tapadia and Lakhotia, 2005). P-glycoproteins have also been implicated in Cd resistance in invertebrates; Callaghan and Denny (2002) exposed *Drosophila melanogaster* to Cd and a known P-glycoprotein blocker, verapamil, and demonstrated a significant reduction in adult emergence. Podsiadlowski et al. (1998) demonstrated the presence of P-glycoprotein ATP-ase activity in the anal papillae of *C. riparius* larvae. The anal papillae are one of the major organs responsible for ion homeostasis in the larvae of *C. riparius* (Podsiadlowski et al., 1998). Taken together, the studies of Callaghan and Denny (2002) and Podsiadlowski et al. (1998) raise the possibility that a P-glycoprotein-like efflux pump may play a role in *C. riparius* tolerance to high levels of cadmium exposure.

Cd has a similar ionic radius to calcium and may compete with calcium at transporter sites (Craig et al., 1999; Martelli et al., 2006). Craig et al. (1999) investigated the role of Ca^{2+} channels on Cd uptake in *Chironomus staegeri*, where Cd uptake was

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inhibited when Ca^{2+} concentration is elevated. As well, Cd uptake is perturbed in the presence of calcium channel blockers such as verapamil and lanthanum (Craig et al., 1999).

Calcium is absorbed primarily across the anterior midgut epithelium of dipteran insects where the low pH favours Ca^{2+} solubility (Taylor, 1985). However, in contrast to mammals, regulation of hemolymph Ca^{2+} levels in the blowfly, *Calliphora vicina*, involves excretion rather than absorption (Taylor, 1985). Hemolymph Ca^{2+} concentration are stabilized by regulation of Malpighian tubule excretion and not by regulation of Ca^{2+} absorption through the midgut epithelium. The Malpighian tubules of *Drosophila melanogaster* are able to transport large amounts of Ca^{2+} either by secretion or sequestration (Dube et al., 2000). Given the high rates of Ca^{2+} transport by the anterior gut epithelium (Taylor, 1985), the capacity of the Malpighian tubules to cope with a large Ca^{2+} burden (Dube et al., 2000), and the potential for Cd to displace Ca^{2+} in membrane transport (Craig et al., 1999), further studies of the tissues involved in Cd transport in the Cd tolerant species, *C. riparius*, and the competition between Cd and Ca^{2+} are warranted.

In particular, this thesis therefore examines sequestration and secretion of Cd by the gut, Malpighian tubules and rectum. Cd levels have also been measured in the hemolymph to determine if the gut and cuticle prevent access of Cd into the hemolymph. Entry into the hemolymph means that other tissues such as the nervous system and muscles are exposed to Cd, and also that the Malpighian tubules and the basolateral (i.e. hemolymph-facing) surface of the gut might therefore play roles in minimizing Cd

toxicity through secretion and/or sequestration of Cd. The following specific questions are addressed:

1. What are the levels of Cd^{2+} in the hemolymph of chironomid larvae exposed to Cd^{2+} in the water?

2. Is Cd^{2+} absorbed across the anterior midgut (i.e. from gut lumen to hemolymph)?

3. Do the Malpighian tubules sequester and/or secrete Cd^{2+}?

4. Do other segments of the gut such as the ileum and rectum transport Cd^{2+}?

5. Do the anal papillae excrete Cd^{2+}?

References

Amiard, J.C., Amiard-Triquet, C., Barka, S., Pellerin, J., Rainbow, P.S., 2006.
Metallothioneins in aquatic invertebrates: their role in metal detoxification and
their use as biomarkers. Aquat. Toxicol. 76, 160-202.

Béchard, K.M., Gillis, P.L., Wood, C.M., 2007. Acute toxicity of waterborne Cd, Cu, Pb,
Ni and Zn to first-instar *Chironomus riparius* larvae. Arch. Environ. Contam.
Toxicol. 54, 454-459.

Buchwalter, D.B., Luoma, S.N., 2005. Differences in dissolved cadmium and zinc uptake
among stream insects: mechanistic explanations. Environ. Sci. Technol. 39, 498-
504.

Callaghan, A., Denny, N., 2002. Evidence for an interaction between p-glycoprotein and
cadmium toxicity in cadmium-resistant and -susceptible strains of *Drosophila
melanogaster*. Ecotoxicol. Environ. Saf. 52, 211-213.

Chowdhury, M.J., Grosell, M., McDonald, D.G., Wood, C.M., 2005. Tissue-specific
cadmium and metallothionein levels in rainbow trout chronically acclimated to
waterborne or dietary cadmium. Arch. Environ. Contam. Toxicol. 48, 381-390.

Cooper, C.A., Handy, R.D., Bury, N.R., 2006. The effects of dietary iron concentration on gastrointestinal and branchial assimilation of both iron and cadmium in zebrafish (*Danio rerio*). Aquat. Toxicol. 79, 167-175.

Craig, A., Hare, L. Charest, P.M., Tessier, A., 1998. Effect of exposure regime on the internal distribution of cadmium in *Chironomus staegeri* larvae (Insecta, Diptera). Aquat. Toxicol. 41, 265-275.

Craig, A., Hare, L. and Tessier, A., 1999. Experimental evidence for cadmium uptake via calcium channels in the aquatic insect *Chironomus staegeri*. Aquat. Toxicol. 44, 255-262.

Dube, K., McDonald, D.G., O'Donnell, M.J., 2000. Calcium transport by isolated anterior and posterior Malpighian tubules of *Drosophila melanogaster*: roles of sequestration and secretion. J. Insect Physiol. 46, 1449-1460.

Folwell, J.L., Barton, C.H., Shepherd, D., 2006. Immunolocalisation of the *D. melanogaster* Nramp homologue Malvolio to gut and Malpighian tubules provides evidence that Malvolio and Nramp2 are orthologous. J. Exp. Biol. 209, 1988-1995.

Garrick, M.D., Kuo, H.-C., Vargas, F., Singleton, S., Zhao, L., Smith, J.J., Paradkar, P., Roth, J.A., Garrick, L.M., 2006. DMT1: Which metals does it transport? Biol. Res. 39, 79-85.

Gillis, P.L., Diener, L.C., Reynoldson, T.B., Dixon, D.G., 2002. Cadmium-induced production of a metallothioneinlike protein in *Tubifex tubifex* (Oligochaeta) and *Chironomus riparius* (Diptera): correlation with reproduction and growth. Environ. Toxicol. Chem. 21, 1836-1844.

Godt, J., Grosse-Siestrup, C., Scheidig, F., Esche, V., Brandenburg, P., Reich, A., Groneberg, D.A., 2006. The toxicity of cadmium and resulting hazards for human health. J. Occup. Med. Toxicol. 1, 22.

Gunshin, H., Mackenzie, B., Berger. U.V., Gunshin, Y. Romero, M.F., Boron, W.F., Nussberger, S., Gollan, J.L., Hediger, M.A., 1997. Cloning and characterization of a mammalian proton-coupled metal-ion transporter. Nature 388, 482-488.

Ianowski, J.P. Christensen, R.J., O'Donnell, M.J., 2002. Intracellular ion activities in Malpighian tubule cells of *Rhodnius prolixus*: evaluation of Na^+-K^+-$2Cl^-$ cotransport across the basolateral membrane. J. Exp. Biol. 205, 1645-1655.

Leader, J.P., O'Donnell, M.J., 2005. Transepithelial transport of fluorescent p-glycoprotein and MRP2 substrates by insect Malpighian tubules: confocal microscopic analysis of secreted fluid droplets. J.Exp. Biol. 208, 4363-4376.

Khan, S., Nugegoda, D., 2007. Sensitivity of juvenile freshwater crayfish *Cherax destructor* (Decapoda: Parastacidae) to trace metals. Ecotoxicol. Environ. Saf. 68, 463-469.

Krantzberg, G., Stokes, P.M., 1990. Metal concentrations and tissue distributions in larvae of *Chironomus* with reference to X-ray microprobe analysis. Arch. Environ. Contam. Toxicol. 19, 84-93.

Lee, S.E., Yoo, D.H., Son, J., Cho, K., 2006. Proteomic evaluation of cadmium toxicity on the midge *Chironomus riparius* Meigen larvae. Proteomics 6, 945-957.

Maddrell, S.H.P., Gardiner, B.O., 1974. The passive permeability of insect Malpighian tubules to organic solutes. J. Exp. Biol. 60, 641-652.

Martelli, A., Rousselet, E. Dycke, C., Bouron, A., Moulis, J.M., 2006. Cadmium toxicity in animal cells by interference with essential metals. Biochimie 88, 1807-1814.

Martinez-Barnetche, J., Solache M.G., Lecona, A.N., Lopez, A.T.T., Rodriguez M.C., Gamba, G., Vazquez N., Rodriguez, M.H., Lanz-Mendoza, H., 2007. Cloning and functional characterization of the *Anopheles albimanus* DMT1/NRAMP homolog: implications in iron metabolism in mosquitoes. Insect Biochem. Mol. Biol. 37, 532-539.

Martini, L.A., Tchack, L., Wood, R.J., 2002. Iron treatment downregulates DMT1 and IREG1 mRNA expression in Caco-2 cells. J. Nutr. 132, 693-696.

Nadella, S.R., Grosell, M., Wood, C.M., 2007. Mechanisms of dietary Cu uptake in freshwater rainbow trout: evidence for Na-assisted Cu transport and a specific metal carrier in the intestine. J. Comp. Physiol. B 177, 433-446.

Naimo, T.J., 1995. A review of the effects of heavy metals on freshwater mussels. Ecotoxicol. 4, 341-362.

Nebeker, A.V., Cairns, M.A., Wise, C.M., 1984. Relative sensitivity of *Chironomus tentans* life stages to copper. Environ. Toxicol. Chem. 3, 151.

Ng, T.Y.-T., Wood, C.M., 2008. Trophic transfer and dietary toxicity of Cd from the oligochaete worm to the rainbow trout. Aquat. Tox. 87, 47-59.

O'Donnell, M.J., Maddrell, S.H.P., 1995. Fluid reabsorption and ion transport by the

    lower Malpighian tubules of adult female Drosophila. J. Exp. Biol. 198, 1647-

    1653.

O'Donnell, M.J., Rheault, M.R., Davies, S.A., Rosay, P., Harvey, B.J., Maddrell, S.H.P.,

    Kaiser, K., Dow, J.A.T., 1998. Hormonally controlled chloride movement across

    Drosophila tubules is via ion channels in stellate cells. Am. J. Physiol. Regulatory

    Integrative Comp. Physiol. 274, 1039-1049.

Piñeros, M.A., Shaff, J.E., Kochian, L.V., 1998. Development, characterization, and

    application of cadmium-selective microelectrode for the measurement of

    cadmium fluxes in roots of *Thlaspi* species and wheat. Plant Physiology 116,

    1393-1401.

Podsiadlowski, L., Vladimir, M., Vilcinskas, A., 1998. Detection of a P-glycoprotein

    related pump in *Chironomus* larvae and its inhibition by verapamil and

    cyclosporine A. Comp. Biochem. Physiol. B 121, 443-450.

Postma, J.F., van Nugteren, P., Buckert-de Jong, B., 1996. Increased cadmium excretion

    in metal-adapted populations of the midge *Chironomus riparius* (Diptera).

    Environ. Toxicol. Chem. 15, 332-339.

Rheault, M.R., O'Donnell, M.J., 2004. Organic cation transport by *Drosophila melanogaster*: application of two novel electrophysiological methods. J. Exp. Biol. 207, 2173-2184.

Rosakis A., Koster W., 2005. Divalent metal transport in the green microalga *Chlamydomonas reinhardtii* is mediated by a protein similar to prokaryotic Nramp homologues. BioMetals. 18, 107-120.

Seidman, L.A., Bergtrom, G., Gingrich, D.J., Remsen, C.C., 1986a. Accumulation of cadmium by the fourth instar larva of the fly *Chironomus thummi*. Tissue and Cell 18, 395-405.

Seidman, L.A., Bergstrom, G., Remsen, C.C., 1986b. Structure of the larval midgut of the fly *Chironomus thummi* and its relationship to sites of cadmium sequestration. Tissue Cell. 18, 407-418.

Sloman, K.A., Baker, D.W., Ho, C.G., McDonald, D.G., Wood, C.M., 2003. The effects of trace metal exposure on agonistic encounters in juvenile rainbow trout, *Oncorhynchus mykiss*. Aquat. Tox. 63, 187-196.

Southon, A., Farlow, A., Norgate, M., Burke, R., Camakaris, J., 2008. Malvolio is a copper transporter in *Drosophila melanogaster*. J. Exp. Biol. 211, 709-716.

Tapadia, M.G., Lakhotia, S.C., 2005. Expression of mdr49 and mdr65 multidrug resistance genes in larval tissues of *Drosophila melanogaster* under normal and stress conditions. Cell Stress Chaperones. 10, 7-11.

Taylor, C.W., 1985. Calcium regulation in blowflies: absence of a role for midgut. Am. J. Physiol. Regulatory Integrative Comp. Physiol. 249, 209-213.

Williams, K.A., Green, D.W.J., Pascoe, C., Gower, D.E., 1986. The acute toxicity of cadmium to different larval stages of *Chironomus riparius* (Diptera: Chironomidae) and its ecological significance for pollution regulation. Oecologia (*Berlin*) 70, 362.

Yamamura, M. Suzuki, K.T. Hatakeyama, S., Kubota, K., 1983. Tolerance to cadmium and cadmium-binding proteins induced in the midge larva, *Chironomus yoshimatsui* (Diptera, Chironomidae). Comp. Biochem. Physiol. C 75, 21-24.

Figure 1. Schematic representation of the *C. riparius* gut. 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.

Hemolymph

Cd^{2+}

ESO   PC/DC   AMG   DMT   LMT   PMG   Ileum   AR   PR

Hemolymph
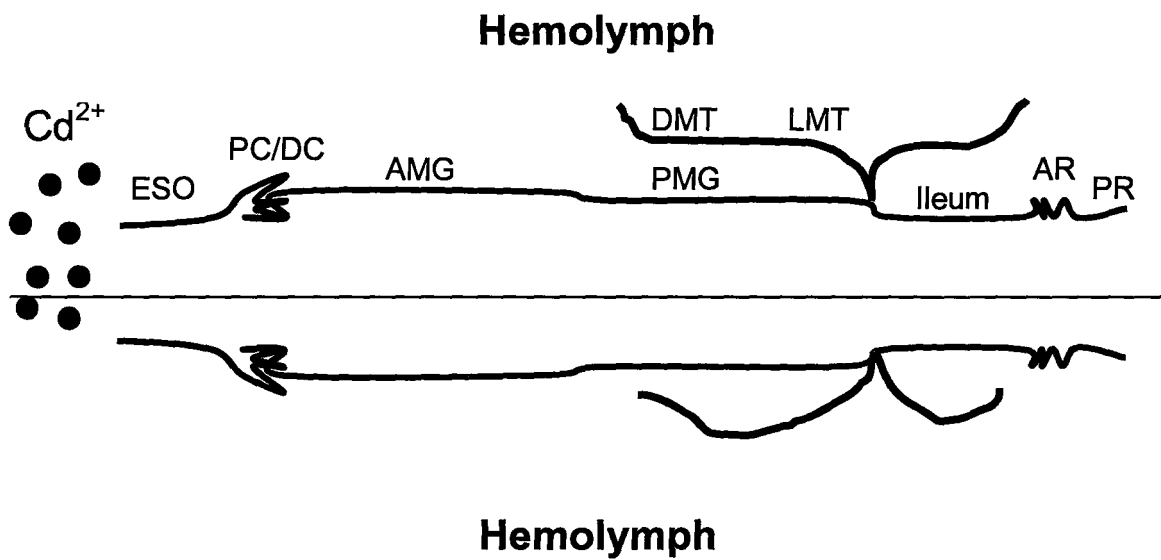
Figure 2. Non-invasive measurement of Cd^{2+} fluxes by the scanning ion-selective

electrode technique. (A) The technique exploits gradients in ion activity within the

unstirred layer that is created by ion transport across cells or epithelia. In this example,

secretion of Cd^{2+} across the epithelium in the direction of the arrows reduces the

concentration of Cd^{2+} close to the epithelial surface relative to that farther away. The size

of the labels and the density of the shading correspond to the concentration of Cd^{2+}. (B)

Schematic diagram of equipment used for SIET measurements. The isolated tissue and

the ion-selective microelectrode are observed through a microscope equipped with a

CCD camera. A computer-controlled motion control system drives an orthogonal (X, Y,

Z) array of stepper-motors which move the amplifier headstage and attached ion-selective

microelectrode to sites along the tissue and then at two points orthogonal to the tissue

surface, as indicated in (A). Voltage differences between the two limits of excursion are

recorded by the data acquisition system. Voltage gradients at different sites can be

overlaid as vectors on an image of the tissue captured by the frame grabber connected to

the CCD camera (as seen in Figure 3A in Chapter 2).

A

60 µm away

10 µm from
surface

Cd^{2+} $Cd^{2+,}$ Cd^{2+}

Cd^{2+} Cd^{2+} Cd^{2+}

Cd^{2+} Cd^{2+} Cd^{2+}

Unstirred
layer

Epithelium

B

CCD Camera

Microscope

Frame
Grabber

Motion
Control
System

X 10

Main Amp
X 100

Data
Acquisition
System

Computer

Reference
electrode

Ion-selective
microelectrode

Bathing
saline

Isolated
tissue

25
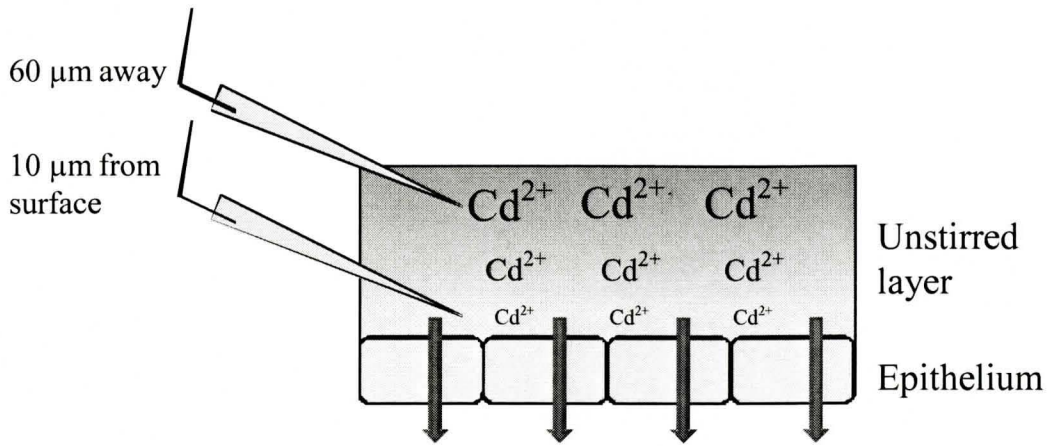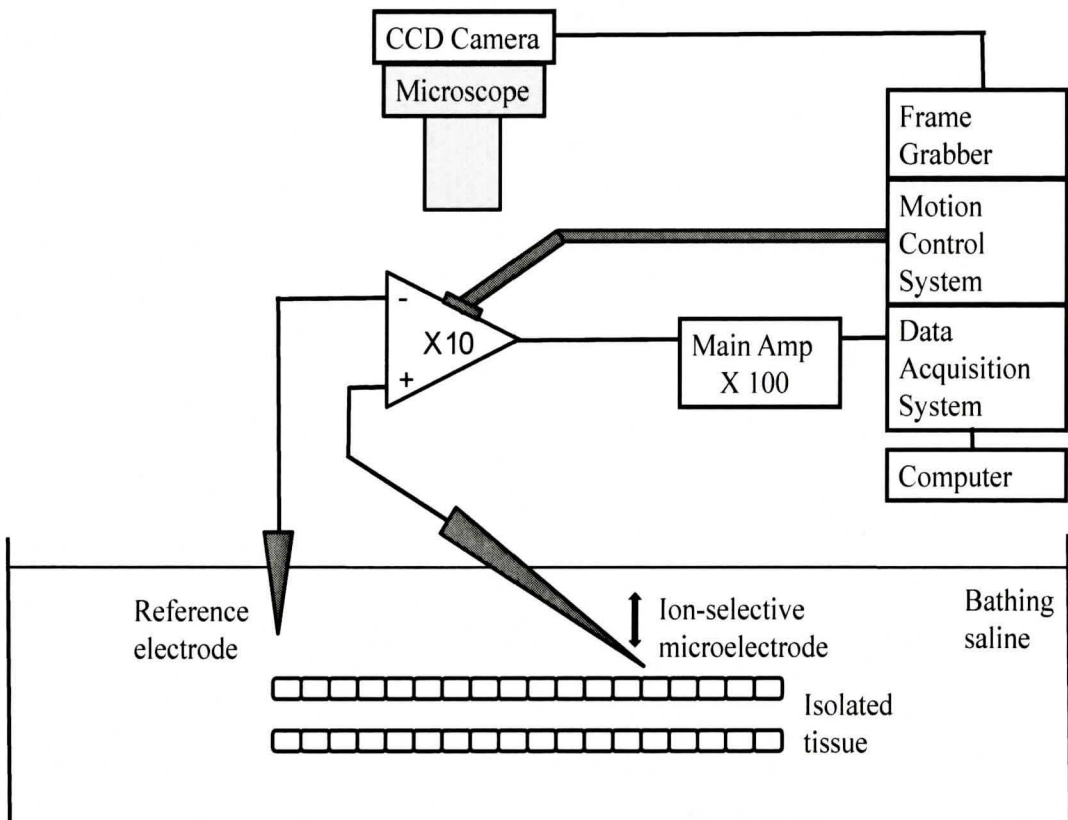
Figure 3. Measurement of the concentration of Cd^{2+} in droplets of fluid secreted by isolated Malpighian tubules in the Ramsay assay. (A) An isolated Malpighian tubule is placed in a droplet of bathing saline under paraffin oil. The open end (formed where the tubule was separated from its junction with the gut) is pulled out and wrapped around a stainless steel pin embedded in the Sylgard-line base of a Petri dish. Secreted fluid droplets which form at the ureter, positioned just outside the bathing saline droplet, are collected on glass rods and placed on the bottom of the dish adjacent to calibration droplets containing known concentrations of Cd^{2+} in *C. riparius* saline. For each droplet, the potential difference between the Cd^{2+}-selective microelectrode (Cd^{2+} ME) and the reference microelectrode (Ref ME) is measured by a high impedance ($>10^{15} \Omega$) operational amplifier and recorded on a PC-based data acquisition system. Cd^{2+} concentration in the secreted droplets is calculated from the voltage difference between the secreted droplet and the calibration droplets, as described in the Rheault and O'Donnell (2005). The tip of the Cd^{2+}-ME is coated with a solution of ~10% polyvinyl chloride (PVC) in tetrahydrofuran to avoid capillary rise of the paraffin oil into the silanized microelectrode. (B) Photograph showing 20 tubules set up in a Ramsay assay in a 55 mm diameter Petri dish. One of the 20 µl bathing droplets is indicated by the white arrow. Each tubule is secured to a black minutien pin anchored in the Sylgard to the right of each bathing saline droplet. Three secreted droplets collected at 30 min intervals are shown within the ellipse in the center of the frame. Reference and Cd^{2+}-selective microelectrodes (dark arrows) are positioned in a bathing saline droplet.
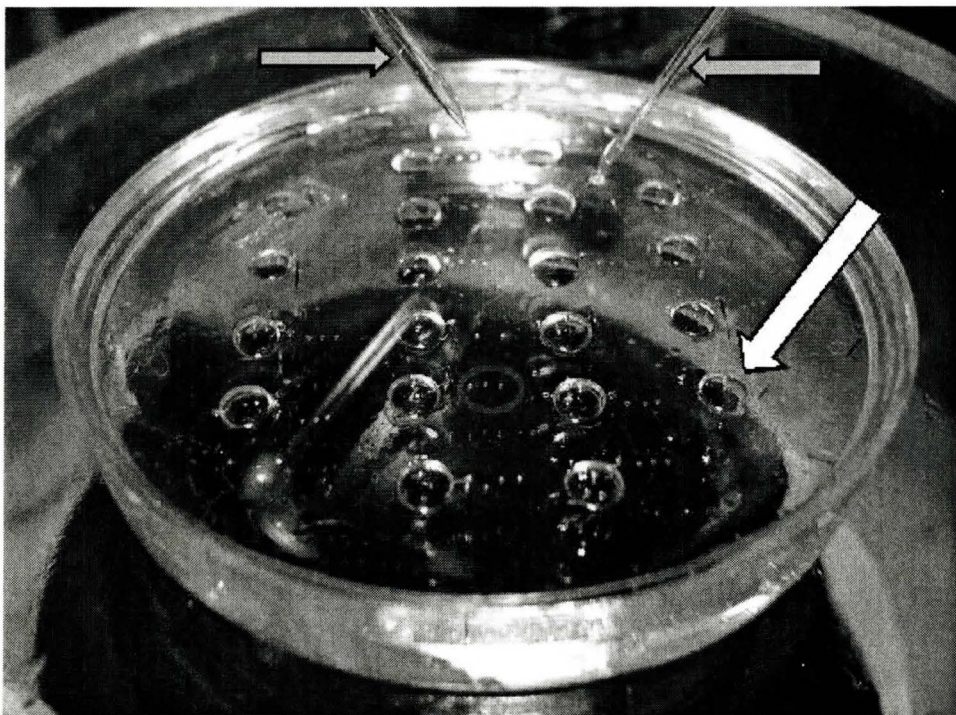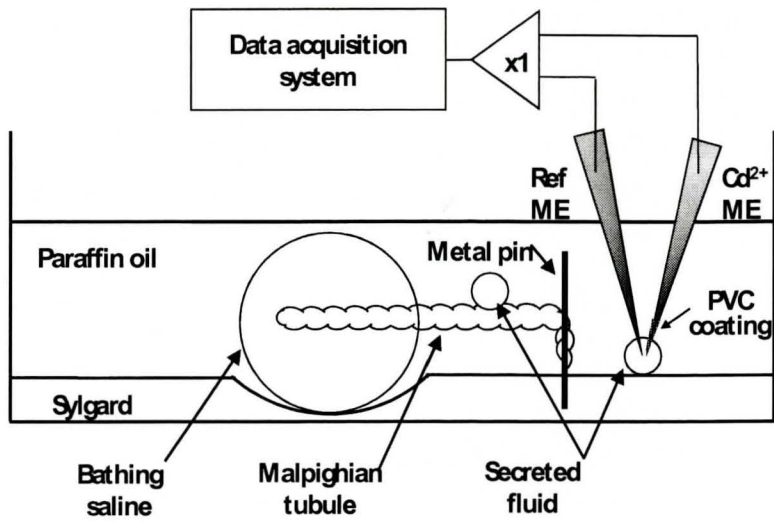
CHAPTER 2:

Abstract

C. riparius larvae are the least sensitive aquatic species in the U.S. EPA (2000) species sensitivity distribution (SSD) for cadmium (Cd). LC50 values over 25,000 times the Criterion Maximum Concentration suggest that chironomids have an extraordinary capacity to excrete or sequester cadmium as a means of increasing their overall tolerance to toxic metals. Using atomic absorption spectroscopy, we have shown saturable uptake of cadmium by whole chironomids and guts isolated from exposed intact chironomids. The transport of Cd from the gut lumen to the hemolymph exposes other tissues such as the nervous system and muscles to Cd. To quantify the movement of Cd^{2+} from the main point of entry, the digestive tract, we have identified regional differences of Cd transport along the gut using a Cd^{2+}-selective microelectrode in conjunction with the Scanning Ion-Selective Electrode Technique (SIET). Cd^{2+} is transported into the anterior midgut cells from the lumen and out of the cells into the hemolymph. The major tissues responsible for elimination of Cd^{2+} from the hemolymph are the posterior midgut and the ileum. In addition, experiments using an isolated Malpighian tubule preparation (the Ramsay assay) have shown that the Malpighian tubules both sequester and secrete Cd^{2+}. For larvae bathed in 10 μmol l^{-1} Cd^{2+}, the tubules can secrete the entire hemolymph burden of Cd^{2+} in ~ 6 hours.

Introduction

Chironomus riparius are the least sensitive aquatic species in the U.S. EPA

(2000) species sensitivity distribution for Cd. They are 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). In fact Béchard et al. (2007) found that the 24-h LC50 values for first instar *C.*

riparius are 25,000 to 500,000 times above the environmental guidelines outlined by the

United States Environmental Protection Agency (U.S. EPA) and the Canadian Council of

the Ministers of the Environment (CCME) for the five metals tested (Cd, Cu, Pb, Ni and

Zn). High 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 tolerance 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). Sequestration entails binding of Cd^{2+} to proteins or precipitation

as insoluble salts, thereby reducing the amount of freely diffusible Cd^{2+}. In general,

metal sequestration occurs in the organs responsible for digestion, storage and ion

regulation (Krantzberg and Stokes, 1990). Cd has been shown to accumulate in

association with low-molecular weight proteins inside cells in both terrestrial and aquatic

invertebrates, such as in the midge-larvae, *Chironomus yoshimatsui* (Yamamura et al., 1983), the stonefly, *Eusthenia spectabilis* (Everard and Swain, 1983), the earthworm, *Eisenia foetida* (Suzuki et al., 1980), the freshwater oligochaete, *Tubifex tubifex* (Gillis et al., 2004) and some plant species (Hall, 2002). These metallothionein-like proteins (MTLP), bind toxic metals, thereby protecting cells against toxicity (Roesijadi, 1992).

The potential role of metal sequestration within the gut tissues of *Chironomus staegeri* was shown by Craig et al. (1998) using microautoradiography. They suggested that sequestration within the gut was the dominant mechanism of Cd tolerance in this species. 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 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 (Suzuki et al., 1984).

Although Cd is a non-essential metal, it has been shown to compete for transport with calcium (Craig et al., 1999; Martelli et al., 2006). Cd uptake by *Chironomus staegeri*, is inhibited by 88% by high levels (10 mmol l^{-1}) of Ca^{2+}, and Cd^{2+} uptake is perturbed in the presence of calcium channel blockers such as verapamil and lanthanum (Craig et al., 1999). Calcium is absorbed primarily across the anterior midgut epithelium where the lower pH favours Ca^{2+} solubility (Taylor, 1985). However, in contrast to mammalian systems, regulation of hemolymph Ca^{2+} levels in the blowfly, *Calliphora vicina*, involves the renal system rather than the intestine (Taylor, 1985). Hemolymph Ca^{2+} concentrations

are regulated by Malpighian tubule excretion and not by regulation of Ca^{2+} absorption across the midgut epithelium. Dube et al. (2000) determined that the Malpighian tubules of *Drosophila melanogaster* can secrete the entire Ca^{2+} content of the body within approximately 9 hours and that the distal segments of the Malpighian tubules can sequester the same amount in approximately 2 hours.

The aim of this study was to identify sites and rates of Cd transport and sequestration with a particular focus on the gut and Malpighian tubules. In particular, we wished to determine if the gut is a site for Cd transfer into the hemolymph, and also whether secretion and/or sequestration of Cd by the gut and Malpighian tubules contributes to the high tolerance of environmental Cd that is characteristic of *Chironomus*. We have also measured H^+ gradients near the gut and Malpighian tubules because changes in pH can alter the availability of divalent metals such as Cd^{2+}.

Methods

2.1. Chironomid larvae

The *Chironomus riparius* culture presently maintained 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 Hamilton dechlorinated moderately hard tap water. The ionic composition of Hamilton tap 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 ($<0.5 \times 10^{-7}$). Water hardness was 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. *C. riparius* larvae were fed *ad libitum* on ground Nutrafin^{TM} fish flakes (45% protein, 5% crude fat, 2% crude fibre, 8% moisture).

2.2. Chironomus riparius saline composition

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. Na^+, K^+, Ca^{2+} and Cl^- levels were determined using ion-selective electrodes (see 2.3.). The composition of *C. riparius* larval saline was as follows

(in mmol l^{-1}): KCl (5), NaCl (74), CaCl$_2$·2H$_2$O (1), MgCl$_2$·6H$_2$O (8.5), NaHCO$_3$ (10.2),

PIPES (1) and glucose (20). The saline was titrated to pH 7. Mg^{2+} and HCO$_3^-$ levels were

based on those in *Drosophila melanogaster* saline (O'Donnell et al., 1998).

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 complexation of the

buffer and the metal (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 to 7.5. Although PIPES was reported not to buffer Cd,

since the measurement by Scanning Ion Selective Electrode Technique (SIET) resolves

very small changes in Cd concentration, it was necessary to verify that there were no

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-

selective microelectrodes were determined between 0.1 and 0.01 mmol l^{-1} Cd in 1 mmol

l^{-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

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mmol l^{-1} PIPES, the voltage in 0.01 mmol 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 the *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 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 and silanized micropipettes were kept over desiccant until use. Micropipettes were backfilled and tips were loaded via capillary action with a column of the appropriate ionophore (~50-100 μm; Fluka, Buchs, Switzerland). Na^+ selective micropipettes were backfilled with 500 mmol l^{-1} NaCl and frontfilled with Na^+ ionophore II cocktail A. K^+ selective micropipettes were backfilled with 500 mmol l^{-1} KCl and frontfilled with K^+ ionophore I cocktail B. Ca^{2+} selective micropipettes were backfilled with 500 mmol l^{-1} $CaCl_2$ and frontfilled with Ca^{2+} ionophore I cocktail A. Detection limits 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 (Fluka ionophores http://sigmaaldrich.com/Brands/Fluka_Riedel_Home/Analytical/Sensoric_Applications.h tml). Electrodes 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. H$^+$ selective micropipettes were backfilled with a 100 mmol l^{-1} NaCl/100 mmol l^{-1} Na citrate (pH 6), frontfilled with H$^+$ Ionophore Cocktail B and calibrated in *C. riparius* saline at pH 6.5 and 7.5. Cl$^-$ ionophores are sensitive to several organic anions (e.g. SCN$^-$). To avoid interference from these organic anions, solid state Cl$^-$ selective micropipettes were constructed from silver wire. A 15 cm length of 0.005 inch 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$_3$.

Silanized Cd^{2+}-selective micropipettes, modified from Piñeros et al. (1998), were backfilled with a solution of 1 mmol l^{-1} Cd(NO$_3$)$_2$·4H$_2$O (Fisher Scientific) and 75 mmol l^{-1} of KCl. 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) was loaded into the tip of the electrode via capillary action. Selectivity of the Cd^{2+} microelectrodes for Cd^{2+} over other ions was determined by Piñeros et al. (1998). Selectivity for Cd^{2+} exceeds that for Ca^{2+} and Mg^{2+} by factors of $10^{10.8}$ and $10^{12.2}$, respectively. Electrodes were equilibrated in a 10 mmol l^{-1} Cd(NO$_3$)$_2$ solution for 30 min prior to calibration. Preliminary experiments showed that equilibration time increased if the ionophore column length was greater than 100 μm. Cd

microelectrodes were calibrated in 0.1, 0.01 and 0.001 mmol l^{-1} of Cd in *C. riparius* saline. Cd electrodes calibrated in 0.1, 0.01 and 0.001 mmol l^{-1} of Cd in *C. riparius* saline displayed a slope (mean ± SEM) 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 electrode. The slope decreased 24.22 ± 0.68 mV/decade between 0.01 mmol l^{-1} and 0.001 mmol l^{-1} Cd (*N* = 5).

For measuring 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).

Reference electrodes were constructed from 1.5 mm x 0.86 mm x 10 cm glass capillaries pulled to a tip diameter of 1-3 μm and filled with 500 mmol l^{-1} KCl. Voltages 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).

2.4. Atomic Absorption Spectroscopy

All experiments were performed on unfed larvae, starved for 24 hours prior to analysis to allow for gut clearance and avoid overestimation of fluxes (Gillis *et al.*, 2004, 2005). Cd concentrations in the exposure water were all within ± 22 % of the nominal values. Organisms were then 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, chironomids were

transferred to fresh water for five minutes to remove non-specifically bound Cd and blotted dry using filter paper (Gillis and Wood, 2008a). Whole chironomids were weighed in pre-weighed microcentrifuge tubes. Dissections of the gut and carcass were performed under *Chironomus* saline. All tissues were transferred to microcentrifuge tubes and 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. All experiments were performed in glass beakers because preliminary studies performed in polyethylene (PE) beakers at 0.3 mmol l^{-1} dissolved Cd revealed an 85% reduction in whole chironomid body burden of Cd. Similar results were documented by Norwood et al. (2007). There was no significant difference in Cd^{2+} concentrations determined by Cd^{2+}-selective microelectrodes in water contained in plastic versus glass beakers at the beginning or end of the 48-h period. Electrodes were placed both in the bulk water and near the surface of the beaker. We speculate that a compound leaching from the plastic alters the binding and/or uptake of Cd by the larvae.

2.5. Hemolymph Cd levels

Hemolymph was collected using the same technique as described in section 2.2. following a 48-h Cd waterborne exposure as outlined above in section 2.4. Hemolymph

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volume was calculated from optical measurement of droplet diameter. Hemolymph

droplets were then transferred by pipette to microcentrifuge tubes. Hemolymph samples

were digested using the protocol outline above for other tissues

2.6. Scanning Ion Electrode Technique (SIET) measurements of ion (Cd^{2+} and H^+) gradients adjacent to the gut and Malpighian tubules

Flux measurements across isolated tissues were made using the Scanning Ion

Electrode Technique. 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. More extensive descriptions

of SIET for isolated insect tissues are reported in Rheault and O'Donnell (2001, 2004)

and Donini and O'Donnell (2005).

The gut, rectum, Malpighian tubules and anal papillae were dissected out of the

carcass 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, Science Wares Inc., East Falmouth, MA, USA). SIET measurements within

the unstirred layer were done as described by Rheault and O'Donnell (2001, 2004).

Measurement and calculation of Cd^{2+} fluxes:

Cd^{2+} 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 seconds, 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 one hour period. Data were discarded if there was a greater than 25% difference in Cd^{2+} flux between the first and the fourth measurements over one hour.

Voltage differences (ΔV) obtained from the ASET software was converted to the corresponding change in Cd^{2+} concentration by the following equation (Donini and O'Donnell, 2005):

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

where ΔC is the Cd^{2+} concentration difference between the two limits of the excursion distance (μmol l^{-1} cm^{-3}); C_B is the background Cd concentration within the media (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 gradients are used to determine the Cd^{2+} flux using Fick's law of diffusion:

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

where J_{Cd} is the net flux in pmol cm^{-2} s^{-1}; D_{Cd} is the diffusion coefficient of Cd (7.20 x 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 πdl where d is the diameter of the gut or tubule segment and l is the corresponding length.

Measurement and calculation of H$^+$ fluxes:

Measurements of H$^+$ fluxes were conducted according to protocol outlined above with the following alterations. The excursion distance for the H$^+$-selective microelectrode was 20 μm. The "wait" and "sample" periods at each limit were 2 and 1 seconds, respectively.

The calculation of H$^+$ fluxes is different than other ions because the ion fluxes must be adjusted for the buffering capacity of PIPES. The following equations were used to determine J_{H+} for H$^+$ (Donini and O'Donnell, 2005; Kunkel et al., 2001):

$$J_{H+} = [(D_P + D_{H+})B_P] * (\Delta C/\Delta X), \qquad \text{Equation 3}$$

where D_P is the diffusion coefficient of PIPES and B_P is the buffering capacity of PIPES.

$$B_P = (C_P/C_B) * [F/(1+F)^2], \qquad \text{Equation 4}$$

where C_P is the concentration of PIPES (mmol l^{-1}), C_B is the background concentration of H$^+$ and F is given by:

$$F = \log_{10}(pK_{aP})/C_B \qquad \text{Equation 5}$$

where $\log_{10} pK_{aP}$ is the dissociation constant of PIPES ($pK_{aP} = 6.76$).

2.7. Measurement of Cd^{2+} secretion by Malpighian tubules in the Ramsay assay

Malpighian tubules are often studied using the Ramsay assay (Dow et al., 1994; Rheault and O'Donnell, 2004). Dissections were conducted under *C. riparius* saline as described above. Chironomid larvae contain four Malpighian tubules individually attached to the gut at the junction of the posterior midgut and hindgut. In a separate dish, 20 µl droplets of *C. riparius* saline containing a selected concentration of $Cd(NO_3)_2$ (nominally Cd free, 30, 100, 300 µmol l^{-1}) were suspended within the paraffin oil of a Sylgard lined Petri dish. To facilitate collection of sufficient volume of secreted fluid for analysis, tubules were stimulated with 1 mmol l^{-1} cAMP (Davies et al., 1995; Bijelic and O'Donnell, 2005). Stimulation of fluid secretion may give a closer representation of the *in vivo* conditions of freshwater organisms, such as chironomids. Osmotic water gain from the environment is expected to increase Malpighian tubule fluid secretion rate *in vivo*. Dissected tubules were transferred to the droplets within the paraffin oil and the lower end of the tubule was removed from the droplet and wrapped around a steel minuten pin inserted into the Sylgard (Dow Corning). The tubules were examined to ensure there was no damage. Secreted droplets were collected from the lower end of the tubule after 2-4 hours, the diameters were recorded using an ocular micrometer and the droplet volume was calculated as $\Pi d^3/6$. Fluid secretion rates were calculated by dividing the 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}).

2.8. Cd sequestration within the Malpighian tubules

Malpighian tubules were transferred from the dissection dish to saline droplets containing nominally Cd free, 30, 100, 300 μmol l^{-1} Cd suspended within paraffin oil in a Sylgard lined Petri dish. Tubules were left for a two hour period and then transferred to three consecutive saline droplets (2 min each) 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. 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 Cd sequestered within the tissue.

2.9. Percent secretion vs. sequestration of Cd in the Malpighian tubules

Malpighian tubule secretions in μmol l^{-1} were multiplied by the fluid secretion rate of *C. riparius* Malpighian tubules to determine Cd secretion per hour (μmol hr^{-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 one hour period (μmol hr^{-1}). Percent secretion was calculated as (Cd secretion rate)/(sum of Cd secretion rate and Cd sequestration rate) * 100. Percent sequestration was calculated similarly.

2.10. Statistical analysis

Michaelis-Menten kinetic parameters for Cd uptake by whole chironomids or selected tissues were calculated using the equation:

$$C_{max} = C_{max}Cd / K_t + Cd \qquad \text{Equation 6}$$

where C_{max} is the maximum concentration of Cd in the whole chironomid or selected tissue (mmol kg^{-1} wet weight), Cd is the concentration of Cd in the chironomid or selected tissue (mmol kg^{-1} wet weight) and K_t is the concentration of Cd in the water corresponding to half of the maximum concentration in the chironomid or selected tissue (μmol l^{-1}).

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:

$$[Cd]_{sf,max} = [Cd_{sf,max}][Cd]/K_t + [Cd] \qquad \text{Equation 7}$$

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 the secreted fluid (μmol l^{-1}) and K_t is the concentration of Cd in the hemolymph corresponding to half of the maximum concentration in the secreted fluid (μmol l^{-1}). Curves were fit 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 ± SEM (*N*).

Results

3.1. Cd uptake kinetics

Uptake of waterborne Cd into intact *C. riparius* and gut followed Michaelis-Menten kinetics (Fig. 1). Although the capacity of gut transport (C_{max}) was only half that of the whole chironomid, the affinity ($1/K_t$) was nine times higher. This may be because the K_t reported here for the whole chironomid is an apparent K_t and it has not been corrected for competing ions such as calcium. At the concentrations tested, uptake of Cd into the carcass was linear (non-saturable). The gut accumulated the largest amount of Cd per unit weight basis in comparison to the carcass and whole chironomid.

3.2. Hemolymph Cd levels

At the lower exposure levels (~0, 10 and 30 µmol l^{-1}), hemolymph Cd levels are similar to those in the water (Fig. 2), implying that the gut and/or body surface are not complete barriers to Cd. At exposure levels of 100 to 300 µmol l^{-1} Cd, hemolymph Cd concentrations were approximately one third those in the water. There was no significant difference between the hemolymph Cd levels at water concentrations between 30 and 1000 µmol l^{-1} suggesting regulation of Cd hemolymph levels at higher exposure concentrations (Fig. 2).

3.3. Spatial pattern of Cd^{2+} flux along the gut

We used a background Cd concentration that was representative of that in the haemolymph of chironomids exposed to waterborne Cd. 10 µmol l^{-1} Cd was the Cd

concentration of the hemolymph following a 48-h waterborne exposure and this concentration yielded Cd^{2+} fluxes which 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 anterior midgut (AMG) and posterior midgut (PMG) both absorb Cd^{2+} in a lumen to hemolymph direction (Fig. 3B). Scans of the basolateral surface showed a large efflux of Cd^{2+} from the AMG in a gut-to-hemolymph direction, with a small efflux from the distal caecae. Taken together, the apical and basolateral scans indicate that there is net absorption of Cd^{2+} from the lumen to hemolymph across the AMG. By contrast, the PMG absorbs Cd^{2+} from the gut lumen, as well as from the hemolymph (Fig. 3A), suggesting sequestration of Cd^{2+} in the PMG cells (Fig. 3C). There were influxes of Cd^{2+} across the esophagus (ESO), proximal caecae (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 flux across the PMG, for the clearance of Cd^{2+} from the hemolymph (Fig. 3B). The sum of the basolateral influxes across the tissues (9.9 fmol s^{-1}) is approximately equal to the sum of the basolateral effluxes across the DC and AMG (10 fmol s^{-1}; Fig. 3B). The data in Fig. 3B have been replotted in Fig. 3C, which shows the morphology in schematic form and summarizes the mean flux of Cd^{2+} across the tissue.

3.4. Contribution of the Malpighian tubules in Cd^{2+} clearance from the hemolymph

Fluid secretion rate was constant at approximately 1.9 nl min^{-1} for 2-4 hours (data not shown) in Cd^{2+} concentrations ranging from 0-300 μmol l^{-1} in the bathing saline.

Above 300 µmol l^{-1} Cd^{2+}, the fluid secretion rate decreased significantly (data not shown). This presumably reflected toxicity and Cd^{2+} levels in the secreted fluid were therefore not measured. Transepithelial Cd^{2+} uptake was saturable for Cd^{2+} in the range of 0-300 µmol l^{-1} (Fig. 4A). MTs appear not to concentrate Cd^{2+} in the secreted fluid above the level in the hemolymph (Fig. 4A).

By contrast to Cd^{2+} secretion, the sequestration of Cd within the tubules does not appear to saturate between 10 and 300 µmol l^{-1} Cd^{2+} (Fig. 4B). For tubules exposed to 10 µmol l^{-1} Cd^{2+}, the concentration of Cd in the Malpighian tubules was equal to that in the bathing saline. At higher concentrations of Cd^{2+} (100 and 300 µmol l^{-1}) there is up to seven times more Cd in the tissue than in bathing saline (Fig.4B).

Cd^{2+} secretion predominated (65% of the total) for tubules bathed in 10 µmol l^{-1} Cd^{2+}. By contrast, at 100 and 300 µmol l^{-1} Cd^{2+}, more Cd is sequestered within the tubules (65% and 85% of the total, respectively) than is secreted during an equivalent period (Fig. 4C).

3.5. Measurement of H$^+$ ion fluxes using SIET

We wished to determine if the variation in ion transport along the chironomid gut resembled those of other better studied dipteran species such as the mosquito, *Aedes aegypti*. Transport of H$^+$ has been well characterized along the larval mosquito gut using SIET (Boudko, 2001). Midgut H$^+$ fluxes from the present study are comparable in size and direction to H$^+$ fluxes of *A. aegypti* (Boudko, 2001). Positive flux values of ~1-6 pmol cm^{-2} s^{-1} were shown in *A. aegypti*, suggesting a net efflux of H$^+$ ions (Boudko,

2001). Efflux of H^+ is also seen in the PMG of *C. riparius*. H^+ fluxes across the gut and Malpighian tubules are presented in Fig. 5 after correcting for the surface area of each tissue.

As for Cd transport, the largest H^+ fluxes after correction for surface area (fmol s^{-1}; Fig. 3A and 5) are the AMG and PMG. The implications of the differences in H^+ and Cd^{2+} flux across the posterior midgut are discussed below.

Discussion

This study reports the first measurements of Cd^{2+} fluxes across isolated animal tissues obtained through the use of Cd^{2+}-selective microelectrodes and the Scanning Ion Electrode Technique. In addition, we provide the first application of ion-selective microelectrodes for measurement of physiological ion levels and Cd in the hemolymph of chironomid larvae. As well, this is the first report of secretion of Cd^{2+} by insect Malpighian tubules set up in the Ramsay assay. In conjunction with measurements of Cd in whole larval chironomids and isolated tissues by GFAAS, these measurements allow us to develop a model describing the roles of specific tissues in Cd uptake and excretion.

4.1. Cd accumulation in C. riparius

Previous studies have proposed that most of the Cd accumulated within *Chironomus* species is sequestered within the gut cells; 85% of the total Cd was accumulated in the gut of *Chironomus thummi* (Seidman et al. 1986a,b) and 94% of the total in *Chironomus staegeri* (Craig et al. 1998). In the present study using *Chironomus riparius*, 45%-76 % of the whole chironomid Cd is accumulated in the gut. Carcass levels of Cd are relatively low in a per weight basis in comparison to the gut and whole body. These findings are not unexpected considering that the gut, along with the Malpighian tubules and rectum, comprise one third of the total weight of the organism.

In the present study, the whole body burden of Cd in chironomids exposed to 1000 μmol l^{-1} Cd in the water was 6.4 mmol kg^{-1}, which is comparable to the value of approximately 5.3 mmol kg^{-1} (after conversion from dry to wet weight) in the study of

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Gillis and Wood (2008b). The significantly lower K_t (284 ± 160 µmol l^{-1}; i.e higher

affinity) of Cd for the gut in comparison to whole chironomid (2280 ± 4430 µmol l^{-1})

again emphasizes the gut as the primary site of Cd accumulation.

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 water, 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 exposed to the free metal. At higher concentrations of

Cd (above 30 µmol l^{-1}) there was no further increase in hemolymph Cd levels, suggesting

some form of regulation. Regulation would involve a reduction in the rate of Cd entry or

increases in the rates of sequestration and/or excretion. It is worth pointing out that the

concentration of Cd in the hemolymph does not exceed ~100 µmol l^{-1} (Fig. 2), well

below the level of 300 µmol l^{-1} Cd associated with toxic effects on Malpighian tubule

fluid secretion rate.

4.3. Spatial pattern of Cd flux along the gut

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 either waterborne or dietary exposures.

The stabilization of Cd levels in the hemolymph (Fig. 2) indicates possible Cd regulation. The posterior midgut, along with the esophagus, Malpighian tubules, ileum and rectum show influxes of Cd^{2+} from the hemolymph to tissue. These segments may account for some of the Cd^{2+} clearance from the hemolymph and may therefore be involved in Cd^{2+} regulation (Fig. 3A).

The distal caeca transports Cd^{2+} in the direction of the hemolymph, whereas, the proximal caeca absorbs Cd^{2+} from the hemolymph. The significance of this pattern is unclear, but it is worth noting that another study has differences in ion motive ATPase distribution in the gastric caeca of *A. aegypti*; P-type Na^+/K^+ ATPases are located in the proximal two-thirds and V-type H^+ ATPases are located in the distal one-third (Patrick et al. 2006).

4.4. Contribution of the Malpighian tubules in Cd^{2+} 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 chironomids 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^{2+} can secrete 25 fmol min^{-1} of Cd^{2+} as calculated by multiplying the fluid secretion rate (nl min^{-1}) by the concentration of Cd^{2+} in the secreted fluid (μmol l^{-1}) and the number of tubules per chironomid (4). If Cd^{2+} is present in the hemolymph at 10 μmol l^{-1}, the quantity of Cd^{2+} present in the

hemolymph is 9 pmol as calculated by multiplying hemolymph Cd^{2+} concentration by volume ($\sim$ 0.9 μl). An approximation of the time required to eliminate this quantity of Cd^{2+} from the hemolymph can be calculated by dividing the secretion rate of Cd^{2+} by the quantity of Cd^{2+} in the hemolymph yielding a value of 6 hours. Cd^{2+} secretion by the Malpighian tubules can thus contribute to the elimination of Cd^{2+} from the hemolymph at a significant rate.

Cadmium is also sequestered within the Malpighian tubules, consistent with earlier proposals (Seidman et al., 1986, 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 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).

3.5. Measurement of H^+ ion fluxes using SIET

There are two reasons for measuring H^+ fluxes along the gut. We showed that there was no difference in Cd concentration produced by a pH-dependent shift in Cd-buffer complexation by 1 mmol l^{-1} PIPES between pH 6.5 and 7.5 (see methods). It was therefore important to show that pH gradients in the unstirred layer did not exceed one pH unit. The largest H^+ gradient was found for the PMG, where the difference between

the two limits of excursion of the pH microelectrode within the unstirred layer was 0.03 pH units. We are therefore confident that there is a negligible effect of pH changes in the unstirred layer on Cd-buffering by PIPES and that Cd fluxes therefore, are a consequence of transport into or across the tissues studied.

Secondly, we wished to determine if ion transport along the gut of chironomid larvae resembles the pattern seen in other dipteran species and whether the pattern of Cd^{2+} transport reflects changes in the availability of free Cd^{2+} in the gut lumen due to changes in pH. H^+ fluxes across the gut of *C. riparius* were in the direction same as those across the anterior midgut of the larvae of the mosquito *A. aegypti* (Boudko et al., 2001). However, whereas we observed an H^+ efflux across the posterior midgut of *C. riparius*, Boudko et al. (2001) detected H^+ influx across the posterior midgut of *A. aegypti*. The observed H^+ efflux may be due either as a consequence of midgut lumen alkalization or metabolic CO_2 production. In *A. aegypti*, for example, lumen pH is ~10 in the anterior midgut and ~8 in the posterior midgut (Boudko et al., 2001). The effect of an alkaline lumen is to decrease the availability of Cd^{2+} for uptake. Thus, some degree of protection from Cd^{2+} in the diet of dipteran insects may be provided by the lumenal alkalinization of the anterior midgut. It will be of interest in future studies to directly measure lumenal pH in the gut of *C. riparius* with a view to determining the extent of this effect.

In this study, we have further emphasized the gut as the primary site for cadmium accumulation; however, it is not the sole detoxification mechanism in *C. riparius*. The gut does not act as a complete barrier, allowing access of Cd^{2+} to the hemolymph. Using SIET, we have determined that the main site of Cd^{2+} entry into the hemolymph appears to

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be the anterior midgut, whereas the posterior midgut absorbs Cd^{2+} from the gut lumen as well as from the hemolymph, possibly to sequester cadmium within the tissue. The esophagus, Malpighian tubules, ileum and rectum may aid in Cd^{2+} clearance from the hemolymph. Our SIET measurements allow us to formulate a balance sheet, identifying sites and rates of Cd^{2+} influx and efflux. The movement of Cd^{2+} into the hemolymph across the anterior midgut is balanced by movement of Cd^{2+} towards the lumen by the remaining gut segments and by secretion and sequestration by the Malpighian tubules. As a result, there is no net flux into the hemolymph. Cd is a non-essential metal, raising the question as to why the anterior midgut absorbs the metal into the hemolymph. Cd^{2+} has been shown to compete for transport with Ca^{2+} (Craig et al., 1999) and the anterior midgut of dipterans is the primary site of Ca^{2+} absorption (Taylor, 1985). Cd^{2+} absorption may thus be an unavoidable consequence of the need to absorb the essential dietary ion, Ca^{2+}. Our companion paper examines patterns of Ca^{2+} transport across the gut of chironomids and the extent of competition between Ca^{2+} and Cd^{2+} in this organism.

References:

Béchard, K.M., Gillis, P.L., Wood, C.M., 2007. Acute toxicity of waterborne Cd, Cu, Pb, Ni and Zn to first-instar *Chironomus riparius* larvae. Arch. Environ. Contam. Toxicol. 54, 454-459.

Bervoets, L., Wils, C., Verheyen, R., 1996. Tolerance of *Chironomus riparius* larvae (Diptera: Chironomidae) to salinity. Bull. Environ. Contam. Toxicol. 57, 829-835.

Boudko, D.Y., Moroz, L.L., Linser, P.J., Trimarchi, J.R., Smith, P.J.S., Harvey, W.R., 2001. *In situ* alanlysis of pH gradients in mosquito larvae using non-invasive, self-referencing, pH-sensitive microelectrodes. J. Exp. Biol. 204. 691-699.

Bijelic, G., O'Donnell, M.J., 2005. Diuretic factors and second messengers stimulate secretion of the organic cation TEA by the Malpighian tubules of *Drosophila melanogaster*. J. Exp. Biol. 51, 267-275.

Brinkhurst, R.O., 1974. The benthos of lakes. St. Martin's Press, New York.

Craig, A., Hare, L. Charest, P.M., Tessier, A., 1998. Effect of exposure regime on the internal distribution of cadmium in *Chironomus staegeri* larvae (Insecta, Diptera). Aquat. Toxicol. 41, 265-275.

Craig, A., Hare, L., Tessier, A., 1999. Experimental evidence for cadmium uptake via calcium channels in the aquatic insect *Chironomus staegeri*. Aquat. Toxicol. 44, 255-262.

Davies, S.A., Huesmann, G.R., Maddrell, S.H., O'Donnell, M.J., Skaer, N.J., Dow, J.A., Tublitz, N.J., 1995. CAP$_{2b}$, a cardioacceleratory peptide, is present in *Drosophila* and stimulates tubule fluid secretion via cGMP. Am. J. Physiol. Regulatory Integrative Comp. Physiol. 269, 1321-1326.

Donini, A., O'Donnell, M.J., 2005. Analysis of Na$^+$, Cl$^-$, K$^+$, H$^+$ and NH$_4^+$ concentration gradients adjacent to the surface of the anal papillae of the mosquito *Aedes aegypti*: application of self-referencing ion-selective microelectrodes. J. Exp. Biol. 208, 603-610.

Dube, K., McDonald, D.G., O'Donnell, M.J., 2000. Calcium transport by isolated anterior and posterior Malpighian tubules of *Drosophila melanogaster*: roles of sequestration and secretion. J. Insect Physiol. 46, 1449-1460.

Everard, L.B., Swain, R., 1983. Isolation, characterization and induction of metallothionein in the stonefly *Eusthenia spectabilis* following exposure to cadmium. Comp. Biochem. Physiol. C. Pharmacol. Toxicol. Endocrinol. 75, 275-280.

Gillis, P.L., Chow-Fraser, P., Ranville, J.F., Ross, P.E., Wood, C.M., 2005. Daphnia need to be gut-cleared too: the effect of exposure to and ingestion of metal-contaminated sediment on the gut-clearance patterns of *D. magma*. Aquat. Toxicol. 71, 143-154.

Gillis, P.L., Diener, L.C., Reynoldson, T.B., Dixon, D.G., 2002. Cadmium-induced production of a metallothioneinlike protein in *Tubifex tubifex* (Oligochaeta) and *Chironomus riparius* (Diptera): correlation with reproduction and growth. Environ. Toxicol. Chem. 21, 1836-1844.

Gillis, P.L., Dixon, D.G., Borgmann, U., Reynoldson, T.B., 2004. Uptake and depuration of cadmium, nickel, and lead in laboratory-exposed *Tubifex tubifex* and corresponding changes in the concentration of a metallothionein-like protein. Environ. Toxicol. Chem. 23, 76-85.

Gillis, P.L., Wood, C.M., 2008a. Investigating a potential mechanism of cadmium resistance in *Chironomus riparius* larvae using kinetic analysis of calcium and cadmium uptake. Aquat. Toxicol. 89, 180-187.

Gillis. P.L., Wood, C.M., 2008b. The effect of extreme waterborne cadmium exposure on the internal concentration of cadmium, calcium, and sodium in *Chironomus riparius* larvae. Ecotoxicol. Environ. Saf. 71, 56-64.

Hall, J.L., 2002. Cellular mechanisms for heavy metal detoxification and tolerance. J. Exp. Botany. 53, 1-11.

Kandegedara, A., Rorabacher, D.B., 1999. Noncomplexing tertiary amines as "Better" buffers covering the range of pH 3-11. Temperature dependence of their acid dissociation constants. Anal. Chem. 71, 3140-3144.

Krantzberg, G., Stokes, P.M., 1990. Metal concentrations and tissue distributions in larvae of *Chironomus* with reference to X-ray microprobe analysis. Arch. Environ. Contam. Toxicol. 19, 84-93.

Kunkel, J.G., Lin, L., Xu, Y., Prado, A.M.M., Feijó, J.A., Hwang, P.P., Hepler, P.K., 2001. The strategic use of Good buffers to measure proton gradients around growing pollen tubes. Cell Biology of Plant and Fungal Tip Growth (ed. A. Geitmann, M. Cresti and I.B. Heath), pp. 81-94. Amerterdam, The Netherlands: IOS Press.

Leverrier, P. Montigny, C., Garrigos, M., Champeil, P., 2007. Metal binding to ligands: Cadmium complexes with glutathione revisited. Anal. Biochem. 371, 215-228.

Lleu, P.L., Rebel, G., 1991. Interference of Good's buffers and other biological buffers with protein determination. Anal. Biochem. 192: 215-218.

Maddrell, S.H.P., Whittembury, G., Mooney, R.L., Harrison, J.B., Overton, J.A., Rodriguez, B., 1991. The fate of calcium in the diet of *Rhodnius prolixus*: storage in concretion bodies in the Malpighian tubules. J. Exp. Biol. 157, 483-502.

Martelli, A., Rousselet, E. Dycke, C., Bouron, A., Moulis, J.M., 2006. Cadmium toxicity in animal cells by interference with essential metals. Biochimie 88, 1807-1814.

Mousavi, S.K., Primicerio, R., Amundsen, P.-A., 2003. Diversity and structure of Chironomidae (Diptera) communities along a gradient of heavy metal contamination in a subarctic watercourse. Sci. Total Environ. 307, 93-110.

Naimo, T.J., 1995. A review of the effects of heavy metals on freshwater mussels. Ecotoxicol. 4, 341-362.

O'Donnell, M.J., Rheault, M.R., 2005. Ion-selective microelectrode analysis of salicylate transport by the Malpighian tubules and gut of *Drosophila melanogaster*. J. Exp. Biol. 208, 93-104.

O'Donnell, M.J., Rheault, M.R., Davies, S.A., Rosay, P., Harvey, B.J., Maddrell, S.H.P., Kaiser, K., Dow, J.A.T., 1998. Hormonally controlled chloride movement across *Drosophila* tubules is via ion channels in stellate cells. Am. J. Physiol. Regulatory Integrative Comp. Physiol. 274, 1039-1049.

Patrick, M.L., Aimanova, K., Sanders, H.R., Gill, S.S., 2006. P-type Na^+/K^+-ATPase and V-type H^+-ATPase expression patterns in the osmoregulatory organs of larval and adult mosquito *Aedes aegypti*. J. Exp. Biol. 209, 4638-4651.

Piñeros, M.A., Shaff, J.E., Kochian, L.V., 1998. Development, characterization, and application of cadmium-selective microelectrode for the measurement of cadmium fluxes in roots of *Thlaspi* species and wheat. Plant Physiol. 116, 1393-1401.

Postma, J.F., van Nugteren, P., Buckert-de Jong, B., 1996. Increased cadmium excretion in metal-adapted populations of the midge *Chironomus riparius* (Diptera). Environ. Toxicol. Chem. 15, 332-339.

Rheault, M.R., O'Donnell, M.J., 2001. Analysis of epithelial K^+ transport in Malpighian tubules of *Drosophila melanogaster*: evidence for spatial and temporal heterogeneity. J. Exp. Biol. 204, 2289-2299.

Rheault, M.R., O'Donnell, M.J., 2004. Organic cation transport by *Drosophila melanogaster*: application of two novel electrophysiological methods. J. Exp. Biol. 207, 2173-2184.

Roesijadi, G., 1992. Metallothionein in metal regulation and toxicology in aquatic animals. Aquat. Toxicol. 22, 81-114.

Seidman, L.A., Bergtrom, G., Gingrich, D.J., Remsen, C.C., 1986. Accumulation of cadmium by the fourth instar larva of the fly *Chironomus thummi*. Tissue and Cell 18, 395-405.

Seidman, L.A., Bergtrom, G., Remsen, C.C., 1986. Structure of the larval midgut of the fly *Chironomus thummi* and its relationship to sites of cadmium sequestration. Tissue Cell. 18, 407-418.

Sohal, R.S., Peters, P.D., Hall, T.A., 1976. Fine structure and x-ray microanalysis of mineralized concretions in the Malpighian tubules of the housefly, *Musca domestica*. Tissue Cell. 8, 447-458.

Suzuki, T.K., Aoki, Y., Nishikawa, M., Masui, H., Matsubara, F., 1984. Effect of cadmium-feeding on tissue concentrations of elements in germ-free silkworm (*Bombyx mori*) larvae and distribution of cadmium in the alimentary canal. Comp. Biochem. Physiol. C 79, 249-253.

Suzuki, T.K., Yamamura, M., Mori, T., 1980. Cadmium binding proteins induced in the earthworm. Arch. Environ. Contam. Toxicol. 9, 415-424.

Taylor, C.W., 1985. Calcium regulation in blowflies: absence of a role for midgut. Am. J. Physiol. Regulatory Integrative Comp. Physiol. 249, 209-213.

Timmermans, K.R., Walker, P.A., 1989. The fate of trace metals during metamorphosis of chironomids (diptera, chironomidae). Environ. Pollut. 62, 73-85.

Yamamura, M., Suzuki, K.T., Hatakeyama, S., Kubota, K., 1983. Tolerance to cadmium and cadmium-binding proteins induced in the midge larva, *Chironomus yoshimatsui* (Diptera, Chironomidae). Comp. Biochem. Physiol. C 75, 21-24.

Fig. 1. Cd content of isolated tissues and whole chironomid larvae after 48 hour waterborne exposure to the indicated Cd concentrations. Values are means ± S.E.M.; $N =$ 8-10 per treatment.

▲ Gut
K_t = 284 ± 160 µmol l⁻¹
C_{max} = 11.39 ± 2.39 mmol kg⁻¹
r^2 = 0.928

■ Whole Chironomid
K_t = 2280 ± 4330 µmol l⁻¹
C_{max} = 20.9 ± 28.8 mmol kg⁻¹
r^2 = 0.884

▼ Carcass
Slope = 4.9 ± 0.74 mmol kg⁻¹ per µmol l⁻¹

[Cd] (mmol kg⁻¹ wet wt.)

[Cd]$_{exposure}$ (µmol l⁻¹)
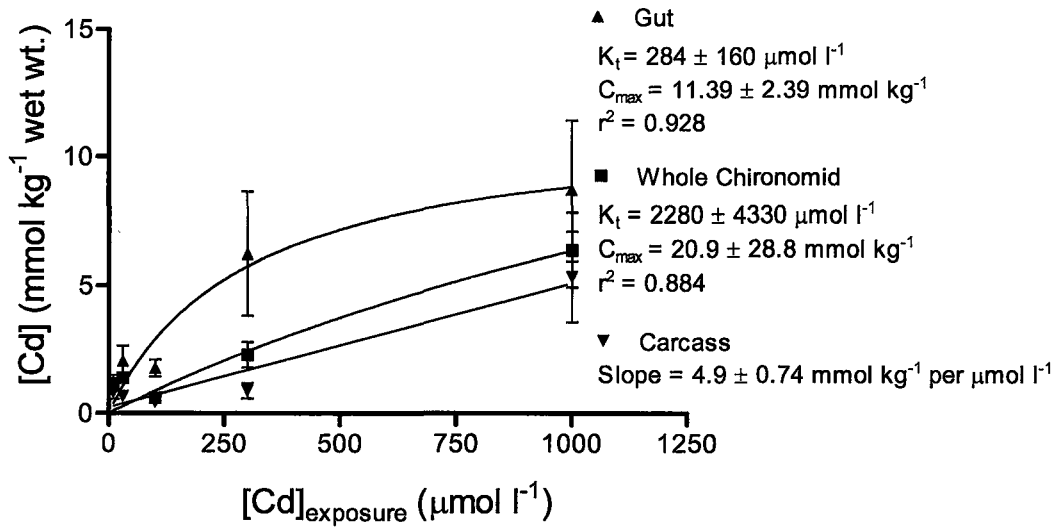
Fig. 2. Cd^{2+} concentration in the hemolymph ($[Cd^{2+}]_{hemolymph}$) following a 48 h waterborne exposure to concentrations indicated on the abscissa. Values are means of S.E.M.; $N = 10$. Columns labeled with the same letters are not significantly different (p>0.05).
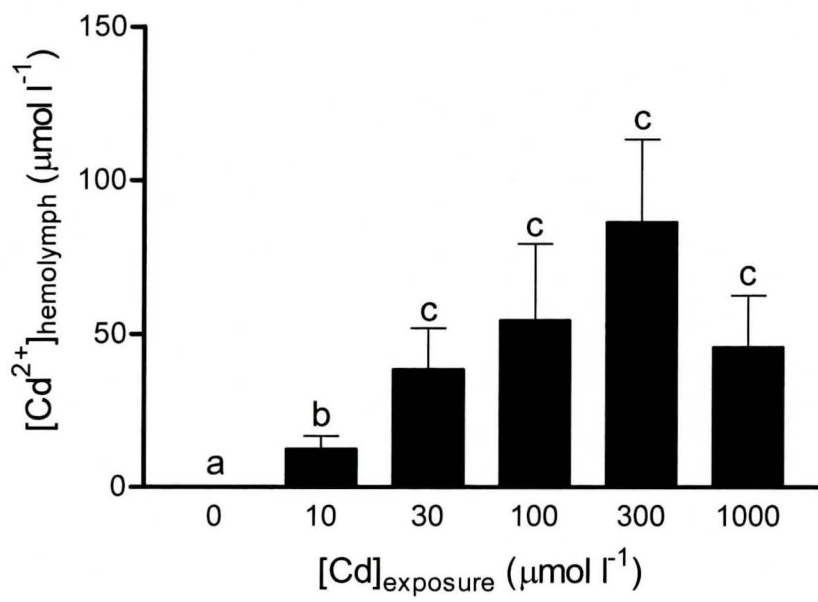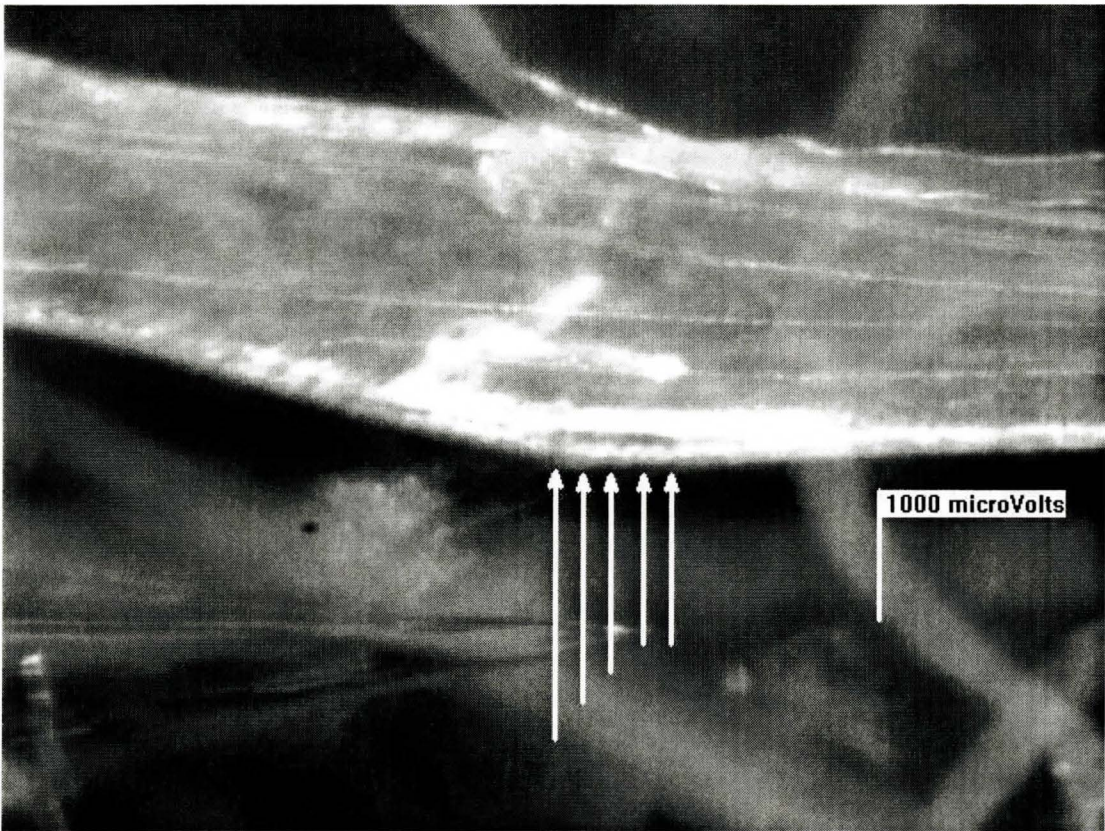
Fig. 3. (A) Representative example of SIET measurements along the posterior midgut. Arrows denote the relative size of the Cd^{2+} flux and the arrowheads show the direction of the Cd^{2+} flux. (B) SIET measurement of Cd^{2+} fluxes. Positive values represent movement of Cd^{2+} from gut lumen to hemolymph. Negative values indicate the movement of Cd^{2+} from hemolymph to gut lumen. Flux values have been corrected for the surface area of each tissue segment, as described in methods. Values are means of S.E.M.; $N = 5$-10 per tissue. (C) Schematic diagram of the *C. riparius* gut showing Cd^{2+} fluxes in the various gut segments. 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.

A

1000 microVolts

68

B

Apical   Basolateral

Cd^{2+} flux (fmol s^{-1})

12.5

10.0

7.5

5.0

2.5

0.0

-2.5

-5.0

-7.5

-10.0

AMG   PMG   ESO   PC   DC   AMG   PMG   LMT   DMT   Ileum   AR   PR

C

Hemolymph

ESO   PC/DC   AMG   DMT   LMT   AR

Cd^{2+} PMG   Ileum

Cd^{2+}

Cd^{2+}

Cd^{2+}
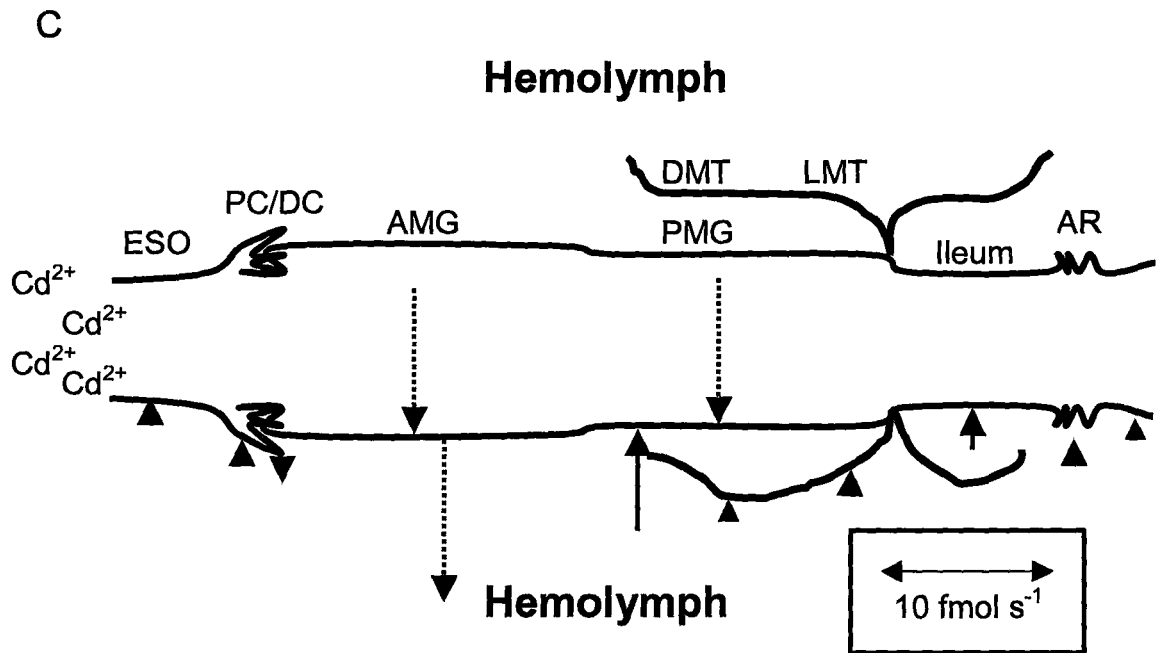
Hemolymph

10 fmol s^{-1}

69

Fig. 4. Contribution of the Malpighian tubules to Cd^{2+} clearance from the hemolymph. (A) Cd^{2+} concentration in the fluid secreted by isolated Malpighian tubules ($[Cd^{2+}]_{sf}$ as a function of bathing saline Cd ($[Cd^{2+}]_{bath}$). (B) Dependence of Cd sequestration by isolated Malpighian tubules on the bathing saline Cd^{2+} concentration. (C) Percent cadmium secretion and sequestration in the Malpighian tubules following one hour exposure to nominally Cd free (0), 10, 100 or 300 μmol l^{-1} $Cd(NO_3)_2$ in the bathing saline. Values are means of S.E.M.; N = 6-12 Malpighian tubules. Asterisks denote significant differences ($p<0.05$).

A

$K_t = 105.3 \ \mu mol \ l^{-1}$
$J_{max} = 46.71 \ \mu mol \ l^{-1}$
$r^2 = 0.9993$

y-axis: $[Cd^{2+}]_{sf} \ (\mu mol \ l^{-1})$
x-axis: $[Cd]_{bath} \ (\mu mol \ l^{-1})$

B

y-axis: $[Cd^{2+}]_{sequestration}$ $(\mu mol \ kg^{-1} \ wet \ wt.)$
x-axis: $[Cd]_{bath} \ (\mu mol \ l^{-1})$

C

Secretion
Sequestration

y-axis: % secretion or sequestration
x-axis: $[Cd]_{bath} \ (\mu mol \ l^{-1})$

71

Fig. 5. H$^+$ fluxes across the gut of *C. riparius*. Positive values represent movement of H$^+$ from gut lumen to hemolymph. Fluxes have been corrected for the surface area of the gut segment. Values are means of S.E.M.; $N = 6$ per gut segment. AMG = anterior midgut, PMG = posterior midgut, LMT = lower Malpighian tubule, DMT = distal Malpighian tubule, AR = anterior rectum, PR = posterior rectum.
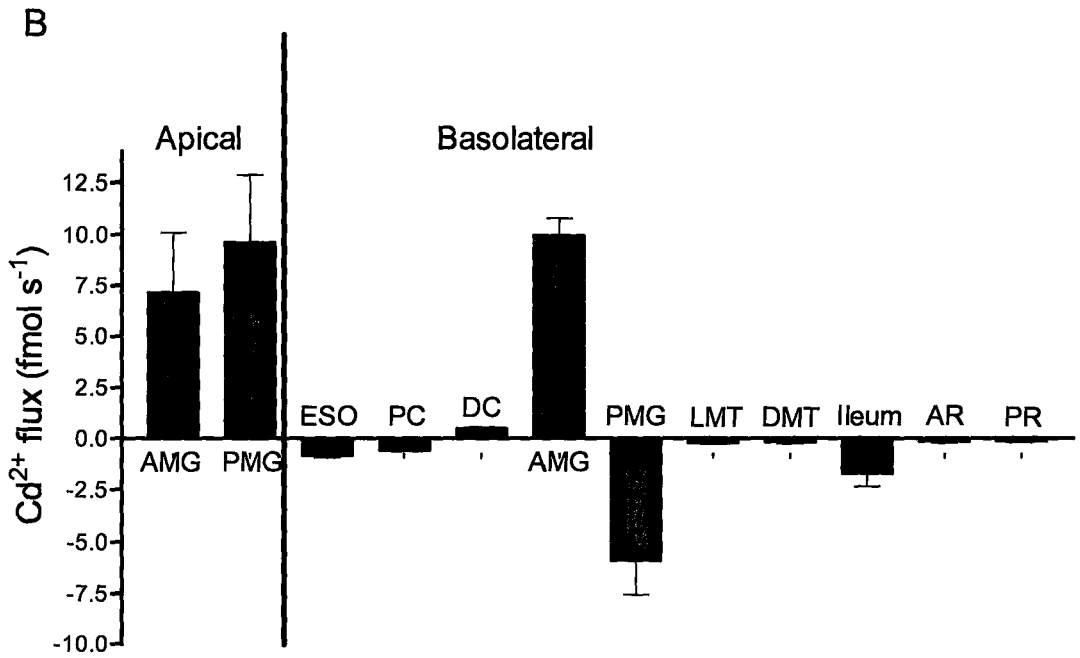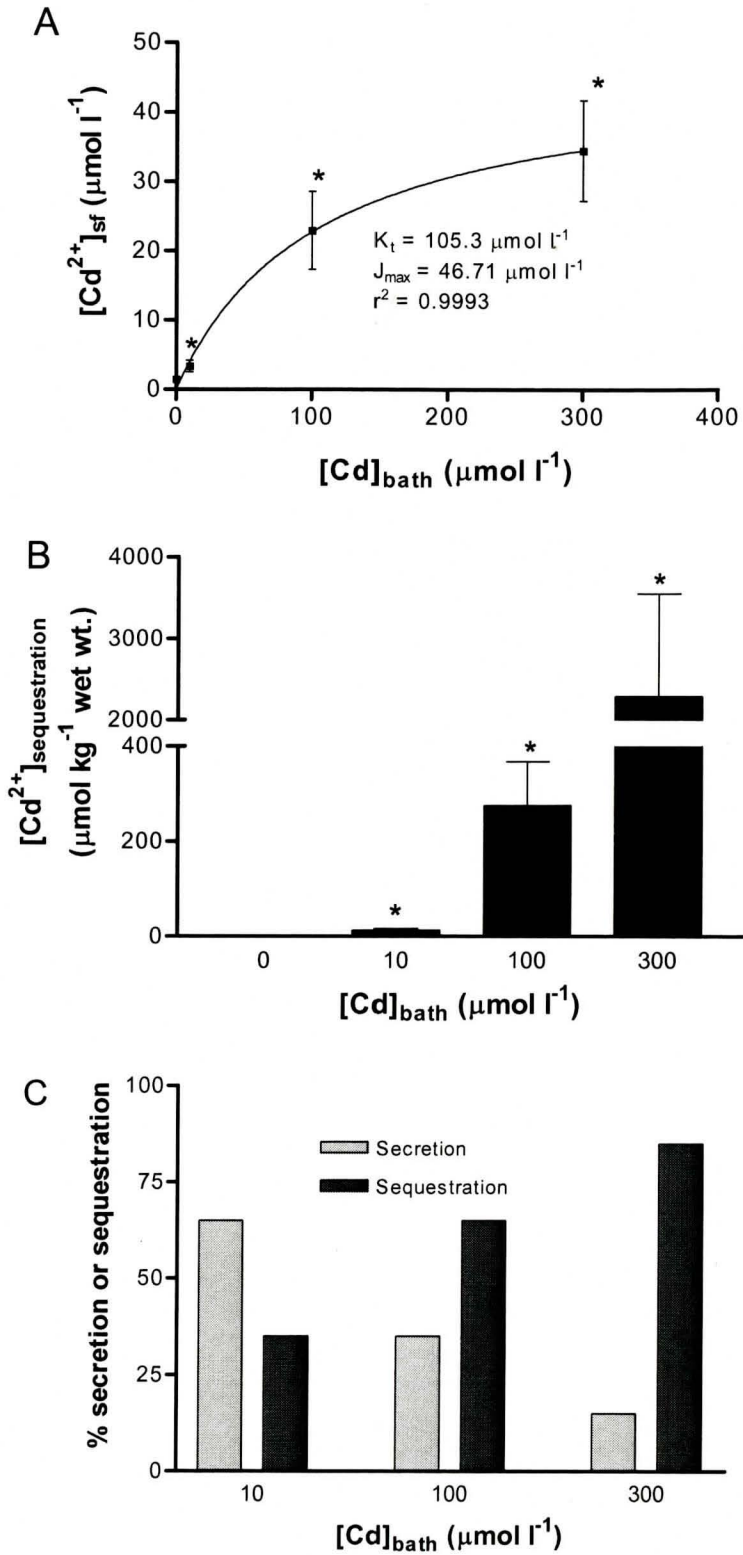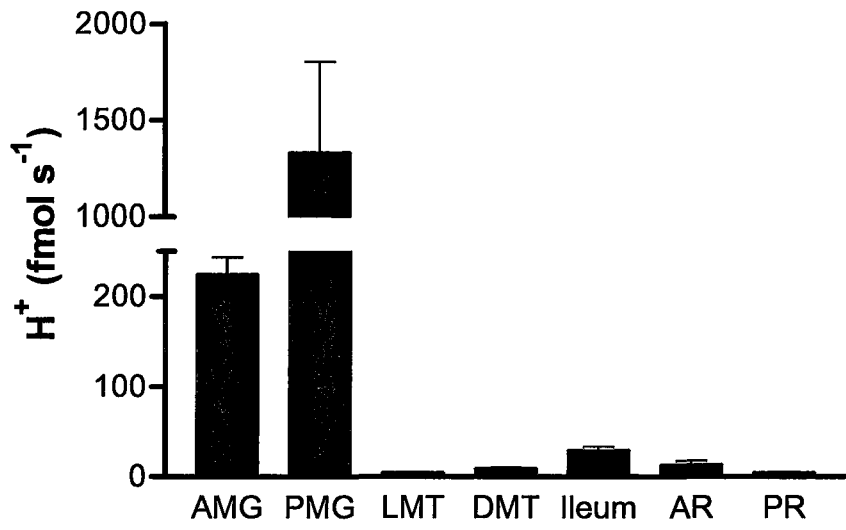
CHAPTER 3:

Abstract

Chironomids are an important bioindicator species and are known in particular for their tolerance of very high levels of toxic metals such as Ni, Cu, Pb, Zn and Cd (Béchard et al., 2008). In this study, we have used SIET to measure the effects of Ca^{2+} on Cd^{2+} flux across isolated guts and Malpighian tubules of *C. riparius*. Cd^{2+} fluxes from the gut lumen into the cells of both the anterior and posterior midgut are reduced by 50 % and 80 %, respectively, when saline Ca^{2+} concentration is increased, consistent with competition between Cd^{2+} and Ca^{2+} from transport. Transport of Cd^{2+} from hemolymph to tissue is reduced by approximately 50 % by high Ca^{2+} concentration for the posterior midgut, both lower and distal segment of the Malpighian tubule and both proximal and distal caecae. There was no effect of high Ca^{2+} saline on Cd^{2+} flux from hemolymph to cell for the esophagus, the ileum or the rectum. In this case as well, Ca^{2+}-independent mechanisms may be involved. We provide evidence that Cd^{2+} fluxes into or across the gut and Malpighian tubules are reduced by high Ca^{2+}, suggesting that Cd^{2+} may be transported in some cells by mechanisms which normally transport the physiological ion, Ca^{2+}.

Introduction

Chironomids are an important bioindicator species and are known in particular for their tolerance of very high levels of toxic metals such as Ni, Cu, Pb, Zn and Cd (Béchard et al., 2008). A number of studies have highlighted the importance of the gut as a site of Cd sequestration (Craig et al., 1998; Krantzberg and Stokes, 1990). The earlier suggestion that the Malpighian tubules and rectum contribute to metal metabolism in chironomids (Krantzberg and Stokes, 1990), was examined directly in our previous study (Leonard et al., 2008). Using the Scanning Ion-selective Electrode Technique (SIET), Cd^{2+} activity gradients were measured in the unstirred layer next to the surface of the isolated gut segments and Malpighian tubules. These measurements indicated net absorption of Cd^{2+} from the lumen of the anterior midgut into the hemolymph. There was also evidence of sequestration of Cd^{2+} within the posterior midgut and influxes of Cd^{2+} in a hemolymph to tissue direction for the esophagus, Malpighian tubules, ileum and rectum. Analysis of the Malpighian tubule Cd content and the Cd^{2+} concentration within samples of fluid secreted by isolated Malpighian tubules set up in the Ramsay assay indicated that the tubules both secrete Cd^{2+} (from hemolymph to lumen) and also sequester Cd within the cells. At levels of Cd at or below 10 μmol l^{-1} secretion is predominant, whereas sequestration predominates at higher levels of Cd. After accounting for the surface area of each tissue, the total movement of Cd^{2+} from the gut lumen to hemolymph, mainly across the anterior midgut, is balanced by hemolymph to tissue movements of Cd^{2+} across the remaining gut segments and the tubules. It therefore appears that the animal is in steady state with respect to Cd^{2+} at the exposure level of 10 μmol l^{-1}.

The absorption of Cd^{2+} across the anterior midgut is puzzling observation given the toxicity of Cd^{2+}. The muscles, nervous system and other tissues are thus exposed to Cd in the hemolymph. However, the anterior midgut is known to be the site of Ca^{2+} 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^{2+} levels through changes in the rate of Ca^{2+} absorption across the midgut. Rather, Ca^{2+} is absorbed at a high and relatively constant rate, even when the diet contains levels of Ca^{2+} in excess of the insect's needs. Regulation of hemolymph Ca^{2+} levels in blowflies is accomplished through the actions of the Malpighian (renal) tubules.

We suggested in our earlier study that the absorption of Cd across the anterior midgut of *Chironomus* may be a consequence of physiological mechanisms for Ca^{2+} absorption in that segment (Leonard et al., 2008). Cd^{2+} is known to compete with Ca^{2+} for transport in many organisms. High levels of Ca^{2+} (10 mmol l^{-1}) inhibit Cd^{2+} uptake in *C. staegeri* (Craig et al., 1999) and the calcium channel blockers, lanthanum and verapamil, reduce Cd^{2+} uptake by the whole larvae.

In this study, we have used SIET to measure the effects of Ca^{2+} on Cd^{2+} flux across isolated guts and Malpighian tubules of *C. riparius*. We report Cd^{2+} fluxes for tissues bathed in saline containing the physiological levels of Ca^{2+} (1 mmol l^{-1}; Leonard et al., 2008) as well as Ca^{2+}-rich (10 mmol l^{-1}) saline.

Methods

2.1. Chironomid larvae

Chironomus riparius larvae were maintained at McMaster University in 10 L

tanks containing fine-grained silica sand and Hamilton dechlorinated moderately hard tap

water with an ionic composition of (in mmol l^{-1}) Na^+ (0.6), Cl^- (0.8), Ca^{2+} (1.8), K^+ (0.4),

Mg^{2+} (0.5) and Cd^{2+} (<0.5 x 10^{-7}). Water hardness was 140 mg l^{-1} $CaCO_3$ and pH was

7.8-8.0. Tanks were continuously aerated with an air stone and the culture room was

maintained at 21 ± 2 °C 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).

2.2. Microelectrode system

Cd^{2+} fluxes across isolated tissues were determined using Cd^{2+}-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 °C 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^{-1} $Cd(NO_3)_2 \cdot 4H_2O$ (Fisher

Scientific) and 75 mmol l^{-1} 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%

77

potassium tetrakis (3,5 bis-[trifluoromethyl] phenyl) borate (Fluka) and 80% 2-nitrophenyl octyl ether (Fluka; Leonard et al., 2008; Piñeros et al., 1998). Cd^{2+}-selective microelectrodes were calibrated in 0.1, 0.01 and 0.001 mmol l^{-1} of Cd in *C. riparius* saline. 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). For high calcium conditions, one part *C. riparius* saline was added to one part 19 mmol l^{-1} calcium in *C. riparius* saline. All salines were titrated immediately before scans to reduce alkalization from CO_2 loss (Leonard et al., 2008). The slope of the Cd^{2+}-selective microelectrodes between 0.1 and 0.01 mmol l^{-1} Cd averaged 28.61 ± 0.4 mV decade^{-1} and 24.22 ± 0.68 mV decade^{-1} between 0.01 mmol l^{-1} and 0.001 mmol l^{-1} Cd. Microelectrode slopes were not affected by changes in bathing saline Ca^{2+} concentration from 1 to 10 mmol l^{-1} (data not shown).

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. More extensive descriptions of SIET for isolated insect tissues are reported in our previous study (Leonard et al., 2008) and in Rheault and O'Donnell (2001, 2004).

2.3. Effect of high Ca^{2+} on Cd^{2+} fluxes using Scanning Ion Electrode Technique (SIET)

Measurements of Cd^{2+} flux across isolated tissues were determined in saline containing the physiological levels of calcium (1 mmol l^{-1}) and in calcium-rich saline (10

mmol l^{-1}) using SIET. Chironomids were held at their head and anal papillae with forceps and a slight tension was applied to extract the gut, rectum and Malpighian tubules. Dissections were conducted under *C. riparius* saline and tissues were transferred to Petri dishes coated with poly-L-lysine 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, Science Wares Inc., East Falmouth, MA, USA).

Cd^{2+}-selective microelectrodes were placed 5-10 μm from the surface of the isolated tissue and then directed 50 μm away, perpendicular to the tissue surface. At the excursion distance limits, 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. The preparations were first scanned in saline containing 1 mmol l^{-1} Ca^{2+}. Half of the saline was then removed from the Petri dish and replaced with the same amount of 19 mmol l^{-1} calcium in *C. riparius* saline to produce a saline containing 10 mmol l^{-1} Ca^{2+}. Care was taken not to move the preparation during this exchange and identical sites were measured for each segment in physiological and high calcium conditions.

Voltage differences (ΔV) were converted to the corresponding change in Cd^{2+} concentration by the following equation (Leonard et al., 2008):

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

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where ΔC_{Cd} is the Cd concentration difference between the two limits of the excursion distance ($\mu mol\ l^{-1}\ cm^{-3}$); C_B is the background Cd concentration in the Petri dish ($\mu mol\ l^{-1}$); ΔV is the voltage gradient (μV); and S is the slope of the electrode between 0.1 and 0.01 mmol l^{-1} Cd. The flux of Cd across the tissue was calculated using Fick's law:

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

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

2.4. Statistical analysis

Comparisons between two treatment groups employed Student's two–tailed paired 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 $\pm$ SEM (N).

Results

Spatial pattern of Cd^{2+} flux along the gut

As a companion to our first paper determining the spatial pattern of Cd^{2+}, this study aims to determine the influence of high Ca^{2+} levels (10 mmol l^{-1}) on Cd^{2+} fluxes along the gut and Malpighian tubules in salines containing 10 µmol l^{-1} Cd^{2+}. Scans of the apical surface of the gut showed that absorption of Cd^{2+} by the anterior and posterior midgut was significantly reduced by 50 % across the anterior midgut and by 80 % across the posterior midgut when saline Ca^{2+} concentration was increased from 1 mmol l^{-1} to 10 mmol l^{-1} (Fig. 1A).

Scans of the basolateral surface of the gut and tubules showed that Cd^{2+} fluxes into the posterior midgut from the hemolymph were significantly reduced by 50 % in the when bathing saline Ca^{2+} concentration was increased from 1 mmol l^{-1} to 10 mmol l^{-1} (Fig. 1 B). A similar reduction was observed in the Cd^{2+} fluxes for the lower and distal Malpighian tubules. However, a more marked declined in Cd^{2+} flux of 90 % across the proximal caeca and 80% across the distal caeca was measured when saline Ca^{2+} concentration was increased from 1 mmol l^{-1} to 10 mmol l^{-1} (Fig. 1B). Cd^{2+} fluxes were unchanged in the esophagus, anterior midgut, ileum and anterior rectum when saline Ca^{2+} concentration was changed from 1 mmol l^{-1} to 10 mmol l^{-1}.

Discussion

Cd^{2+} 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^{2+} from lumen to hemolymph across the anterior midgut (Leonard et al., 2008)[1]. Similarly, Cd^{2+} moves from gut lumen into the cells of the posterior midgut and from the hemolymph into the cells, consistent with Cd^{2+} sequestration, as observed previously (Leonard et al., 2008).

Cd^{2+} fluxes from the gut lumen into the cells of both the anterior and posterior midgut are reduced when saline Ca^{2+} concentration is increased, consistent with competition between Cd^{2+} and Ca^{2+} from transport. This trend is consistent with the data of Gillis and Wood (2008a) that showed a 70 % reduction in whole body Ca^{2+} following a Cd^{2+} exposure in the LC50 range. Ca^{2+} and Cd^{2+} may out-compete each other for transport sites at in the midgut, thereby decreasing the limiting ion's uptake across the gut lumen. However, movement of Cd^{2+} from the anterior midgut cells to hemolymph is unaffected by changes in bathing saline Ca^{2+} concentration. This may reflect either the low (<0.1 μmol l^{-1}) levels of Ca^{2+} in cells or a transport mechanism (e.g. P-glycoproteins) that does not involve competition between Ca^{2+} and Cd^{2+} for transport.

Transport of Cd^{2+} from hemolymph to tissue is reduced by high Ca^{2+} concentration for the posterior midgut, both lower and distal segment of the Malpighian

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[1] In the earlier study, fluxes were measured in pmol cm^{-2} s^{-1} and were then multiplied by the surface area of each tissue (cm^2) so that the flux across each tissue (fmol s^{-1}) was represented. In this study we have reported the data as pmol cm^{-2} s^{-1}, for each tissue type or segment.

tubule and both proximal and distal caecae. It thus appears that there may be competition between Cd^{2+} and Ca^{2+} for transport into these tissues as well. The small size of the tissues other than the midgut precludes splitting them open so that SIET scans of the apical surface can be performed. We cannot therefore determine whether hemolymph to tissue Cd^{2+} fluxes indicate transepithelial Cd^{2+} flux (from hemolymph to lumen) or sequestration within the tissue, which occurs in the posterior midgut.

There was no effect of high Ca^{2+} saline on Cd^{2+} flux from hemolymph to cell for the esophagus, the ileum or the rectum. In this case as well, Ca^{2+}-independent mechanisms may be involved.

In summary, our results provide evidence that Cd^{2+} fluxes into or across the gut and Malpighian tubules are reduced by high Ca^{2+}, suggesting that Cd^{2+} may be transported in some cells by mechanisms which normally transport the physiological ion, Ca^{2+}.

Future Studies:

It will be of interest to use SIET and Ca^{2+}-selective microelectrodes to measure the pattern of Ca^{2+} transport across the gut segments and Malpighian tubules. If Cd^{2+} is transported by mechanisms normally used for Ca^{2+}, then we would expect the Ca^{2+} fluxes to mirror, at least in direction and relative magnitude, the Cd^{2+} fluxes reported in this and in our previous paper (Leonard et al., 2008).

It will also be useful to examine the effects of agents known to alter Ca^{2+} transport. Ca^{2+} channel blockers such as diltiagen and lanthanum may reduce Cd^{2+} fluxes

if Cd^{2+} is moving through Ca^{2+} channels. Verapamil is both a P-glycoprotein inhibitor and a Ca^{2+} channel blocker, and it might therefore be expected to reduce Cd^{2+} flux through either pathway. Changes in membrane potential produced by increases or decreases in bathing saline K^+ concentration would also be expected to alter Cd^{2+} flux through channels.

Lastly, it will be of interest to determine whether Cd^{2+} is transported across the anal papillae. The papillae are normally involved in the uptake of Na^+ and Cl^- in freshwater dipteran larvae, but they have also been implicated in the excretion of ammonia (Donini and O'Donnell, 2005). P-glycoproteins have been implicated in Cd^{2+} transport and there is evidence for P-glycoprotein activity in the anal papillae of chironomid larvae (Podsiadlowski et al., 1998). Our preliminary data (Appendix 1) of Cd^{2+} fluxes measured for the anal papillae of chironomid larvae reared in Cd^{2+}-rich (30 $\mu mol\ l^{-1}$) water suggest that Cd^{2+} is excreted across the papillae. Further studies are required to determine if active transport is involved and the nature of the transporters.
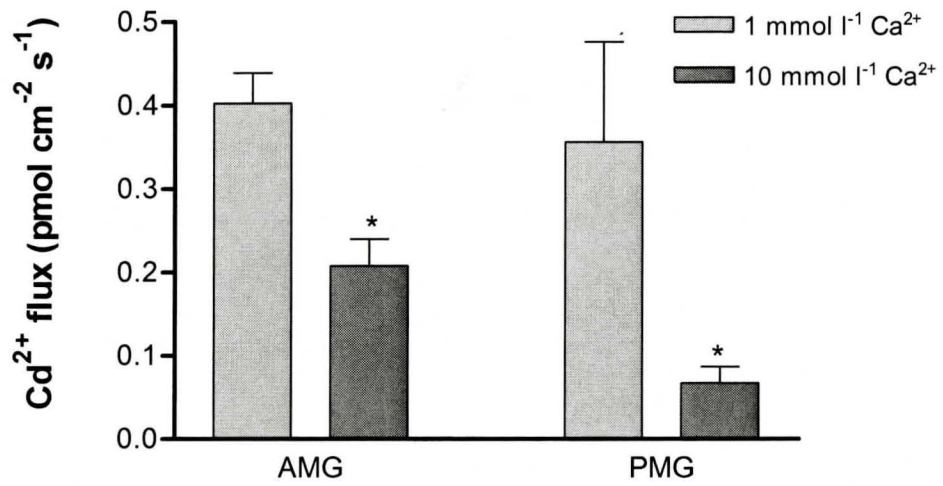
References

Béchard, K.M., Gillis, P.L., Wood, C.M., 2008. Acute toxicity of waterborne Cd, Cu, Pb, Ni and Zn to first-instar *Chironomus riparius* larvae. Arch. Environ. Contam. Toxicol. 54, 454-459.

Craig, A., Hare, L. Charest, P.M., Tessier, A., 1998. Effect of exposure regime on the internal distribution of cadmium in *Chironomus staegeri* larvae (Insecta, Diptera). Aquat. Toxicol. 41, 265-275.

Craig, A., Hare, L., Tessier, A., 1999. Experimental evidence for cadmium uptake via calcium channels in the aquatic insect *Chironomus staegeri*. Aquat. Toxicol. 44, 255-262.

Donini, A., O'Donnell, M.J., 2005. Analysis of Na^+, Cl^-, K^+, H^+ and NH_4^+ concentration gradients adjacent to the surface of the anal papillae of the mosquito *Aedes aegypti*: application of self-referencing ion-selective microelectrodes. J. Exp. Biol. 208, 603-610.

Krantzberg, G., Stokes, P.M., 1990. Metal concentrations and tissue distributions in larvae of *Chironomus* with reference to X-ray microprobe analysis. Arch. Environ. Contam. Toxicol. 19, 84-93.

Leonard, E.M., Pierce, L.M., Wood, C.M., O'Donnell, M.J., 2008. Investigations into Cd tolerance in *Chironomus riparius*: spatial patterns of Cd transport and sequestration.

Piñeros, M.A., Shaff, J.E., Kochian, L.V., 1998. Development, characterization, and application of cadmium-selective microelectrode for the measurement of cadmium fluxes in roots of *Thlaspi* species and wheat. Plant Physiol. 116, 1393-1401.

Podsiadlowski, L., Vladimir, M., Vilcinskas, A., 1998. Detection of a P-glycoprotein related pump in *Chironomus* larvae and its inhibition by verapamil and cyclosporine A. Comp. Biochem. Physiol. Part B 121, 443-450.

Rheault, M.R., O'Donnell, M.J., 2001. Analysis of epithelial K^+ transport in Malpighian tubules of *Drosophila melanogaster*: evidence for spatial and temporal heterogeneity. J. Exp. Biol. 204, 2289-2299.

Rheault, M.R., O'Donnell, M.J., 2004. Organic cation transport by *Drosophila melanogaster*: application of two novel electrophysiological methods. J. Exp. Biol. 207, 2173-2184.
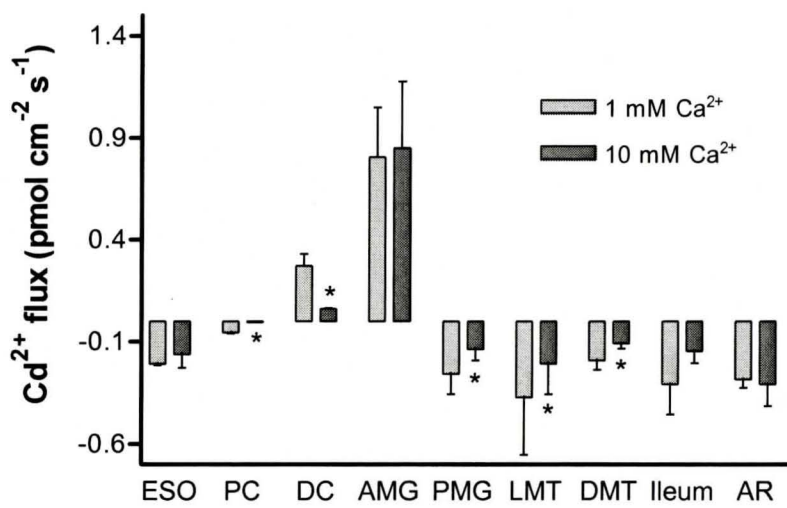
Taylor, C.W., 1985. Calcium regulation in blowflies: absence of a role for midgut. Am. J.

Physiol. Regulatory Integrative Comp. Physiol. 249, 209-213.

Fig. 1 SIET measurement of Cd^{2+} fluxes in saline containing the physiological calcium level (1 mmol l^{-1}) and in calcium-rich (10 mmol l^{-1}) saline. Both salines contained 10 µmol l^{-1} Cd^{2+}. (A) Apical Cd^{2+} fluxes. (B) Basolateral Cd^{2+} fluxes. Positive values represent movement of Cd^{2+} from gut lumen to hemolymph. Negative values indicate the movement of Cd^{2+} from hemolymph to gut lumen. Values are means of S.E.M.; $N = 5$-10 per tissue. *Asterisks denote significant differences (paired t-test; p<0.05) in Cd^{2+} flux in saline containing 1 mmol l^{-1} versus 10 mol l^{-1} Ca^{2+}. 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.
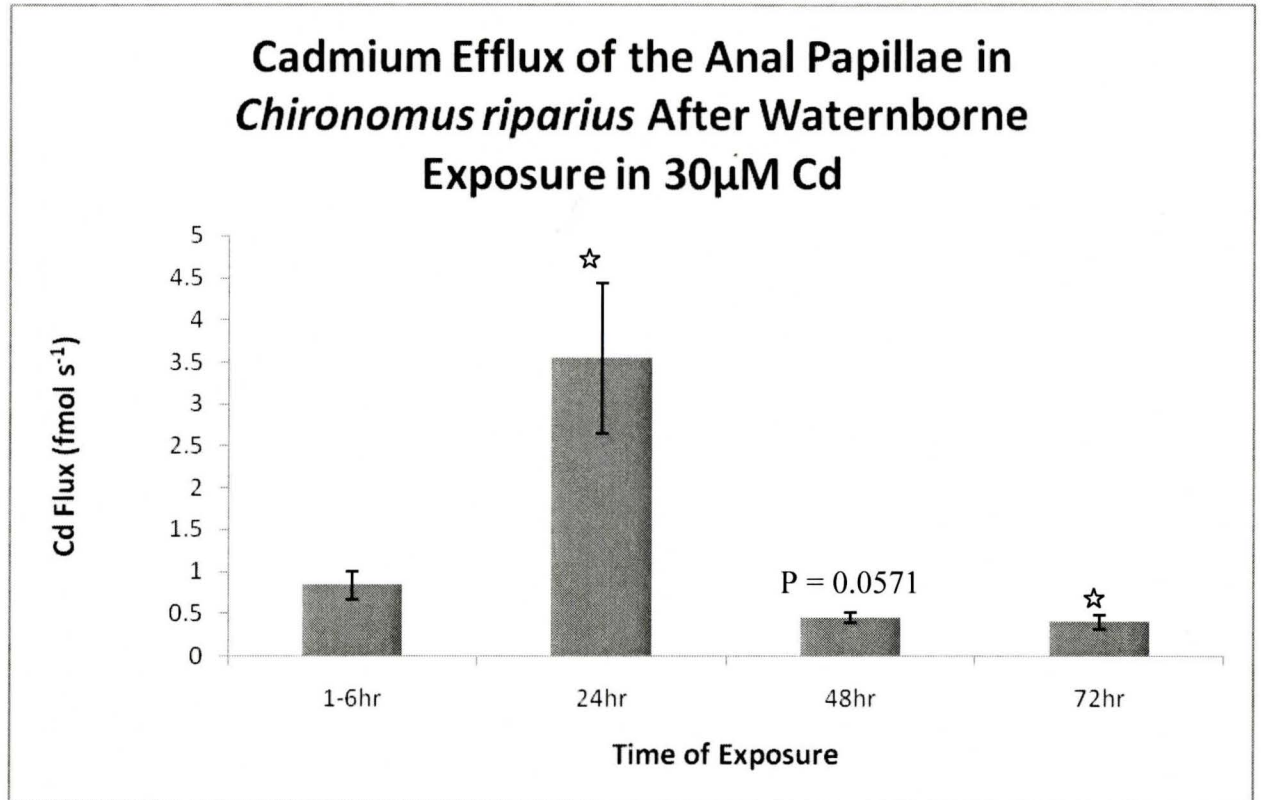
A

B

Appendix 1

Cadmium Efflux of the Anal Papillae in *Chironomus riparius* After Waternborne Exposure in 30μM Cd

Fig. 1. Cd^{2+} efflux from the anal papillae following waterborne exposure in 30 μmol l^{-1} Cd (in Hamilton tapwater) for 1-6, 24, 48 and 72 hours with a minimum of $N = 8$-17 preparations. ☆ denote statistical significance ($p < 0.05$) compared to the 1-6 h group.