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Outcomes of Chronic Arsenic Exposure on Aquatic Insects

A Dissertation submitted in partial satisfaction  
of the requirements for the degree of

Doctor of Philosophy

in

Entomology

by

Christina Loraine Mogren

June 2013

Dissertation Committee:

Dr. John T. Trumble, Chairperson  
Dr. Ring T. Cardé  
Dr. David R. Parker  
Dr. William E. Walton

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The Dissertation of Christina Loraine Mogren is approved:

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JT Trumble directed and supervised the research which serves as the basis for this dissertation. GR von Kiparski and SM Webb provided technical expertise. DR Parker and WE Walton provided the resources necessary for the published research to be conducted.



## ABSTRACT OF THE DISSERTATION

### Outcomes of Chronic Arsenic Exposure on Aquatic Insects

by

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Doctor of Philosophy, Graduate Program in Entomology  
University of California, Riverside, June 2013  
Dr. John T. Trumble, Chairperson

Arsenic is a widespread contaminant of aquatic systems worldwide, and though the toxic effects on vertebrates are well understood, the impacts of chronic arsenic exposure on lower trophic levels of insects have received little attention. The overall goal of this dissertation was to investigate the sublethal effects of arsenic on aquatic primary consumers, and how they could transfer arsenic from aquatic to terrestrial environments.

I investigated the effects of arsenic on survival, growth, and reproduction of *Chironomus riparius*. There was a significant increase in the time between male and female emergence when exposed to 1000 µg/l as larvae, and a significant decrease in the number of eggs produced per egg mass. Total arsenic body burdens decreased 72% between larval and adult stages.

Larvae of *Culex tarsalis* and *Culex quinquefasciatus* exposed to arsenate or arsenite were assayed against *Bacillus thuringiensis* var. *israelensis* or *Lysinibacillus sphaericus* microbial pathogens to determine shifts in survival curves resulting from arsenic accumulation. Arsenic did not affect growth and survival of these mosquitoes

compared to controls. *Culex tarsalis* was the more sensitive species, but both species were tolerant to arsenic exposure.

X-ray absorption spectroscopy was used to map the localization and biotransformation of arsenic in various life stages of *C. riparius* and *Cx. tarsalis*.

*Chironomus riparius* larvae accumulated arsenic in the midgut, while adults had arsenic distributed throughout the exoskeleton. There was evidence for reduction of arsenate to arsenite, and production of an As-thiol. In *Cx. tarsalis*, arsenic was found throughout the larval and adult bodies.

Behavioral assays on *Cx. tarsalis* revealed minimal effects of prolonged arsenic exposure on the measured behaviors, including time spent resting, gliding, diving, and swimming. There was a limited effect of exposure on predator avoidance behaviors, but interactions made patterns difficult to discern.

The trophic transfer potential of arsenic from aquatic to terrestrial environments was evaluated by feeding contaminated *Cx. tarsalis* to aquatic and terrestrial predators. Results suggest that overall the trophic transfer of arsenic is more likely to occur within an aquatic system, although transfer to terrestrial predators may play an important role in arsenic cycling during periods of mass emergence of insect prey.

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## **CHAPTER 1**

### **Introduction**

The history of the toxic properties of arsenic can be traced back millennia, with records as far back as 5000 BC indicating extensive arsenic poisoning in the ancient Chinchorro people of Chile (Arriaza et al., 2010). The toxic properties of arsenic were recognized by numerous ancient civilizations, including the Greeks, who are believed to have projected the deformities of blacksmiths exposed to arsenic in copper ores in their likenesses of the god Hephaestus (Nriagu, 1994). In more recent centuries, various medicines were developed using arsenic to treat a range of medical conditions, including ulcers, malaria, diabetes, arthritis, and cancer, among many others (Henke, 2009; Nriagu, 1994; Ravenscroft et al., 2009, and references therein). The insecticidal properties were first documented in the *Chinese Encyclopedia of Medicine*, in which it is noted that certain arsenic compounds were effective at killing pests of rice (Nriagu, 1994). The development of lead-arsenate and calcium-arsenate pesticides for use against insects eventually led to resistance by insect pests and has left a legacy of contamination in agricultural soils (Creger and Peryea, 1994; Schooley et al., 2008).

This "poison of kings" currently affects hundreds of millions of people worldwide through contamination of surface and ground water supplies (Ravenscroft et al., 2009), particularly in Southeast Asia where irrigation with arsenic laden water has led to contamination of rice and other crops (e.g. Meharg and Rahman, 2003; Rahaman et al., 2013). Environmental contamination is largely the result of natural processes, such as volcanism and weathering and oxidation of parent materials, which releases inorganic arsenic into the environment (Nriagu, 1994; Ravenscroft et al., 2009; Smedley and Kinniburgh, 2002). However, these processes can be exacerbated by anthropogenic

disturbances, such as mining (Eisler, 2004). Weather events and runoff then transport arsenic to aquatic environments. The toxic and widespread nature of this contaminant prompted the United States Environmental Protection Agency (US EPA) to categorize it as a priority toxic pollutant of natural waters, and the maximum safe concentration for chronic exposure for aquatic life is 150  $\mu\text{g/l}$  (US EPA, 2006). Concentrations exceeding 300,000  $\mu\text{g/l}$  have been found at the Iron Mountain superfund site in the United States (Nordstrom et al., 2000; Smedley and Kinniburgh, 2002), though concentrations near 1000  $\mu\text{g/l}$  and below are more commonly encountered (Ravenscroft et al., 2009).

The most commonly occurring forms of arsenic in the environment are inorganic arsenate [ $\text{As(V)}$ ] and arsenite [ $\text{As(III)}$ ], though arsenate is the more thermodynamically favorable form in freshwater systems (Rahman and Hasegawa, 2012). Arsenate, which is a chemical analogue for phosphate, is taken up into cells via phosphate transporters (Zangi and Filella, 2012) and disrupts glycolysis by altering the structures of molecular intermediates during ATP synthesis (Hughes, 2002). Arsenite is more toxic than arsenate (National Research Council, 1999) and interacts with the sulfhydryl bonds of proteins, disrupting tertiary structures and enzymatic function (Hughes, 2002). These inorganic forms may be further transformed into numerous organic forms (Reimer et al., 2010; Smith et al., 2005) through the actions of plants (Meharg and Hartley-Whitaker, 2002; Zaman and Pardini, 1996; Zhang et al., 2008) or microbial activity in the aquatic environment (Lloyd and Oremland, 2006). Thus, insects are exposed to a number of arsenic species in the environment.

Despite a broad general knowledge of how arsenic affects humans and the transformations that occur in the environment, relatively little is known about how insects are able to tolerate environmental exposures, particularly in aquatic systems where insects spend their lives in direct contact with contaminated substrates. The purpose of this dissertation research was to fill these gaps in the literature with experiments investigating a variety of sublethal effects of arsenic on aquatic insects.

In Chapter 2 of this dissertation, I investigated the effects of arsenic exposure on development of the midge *Chironomus riparius* Meigen (Diptera: Chironomidae), a ubiquitous aquatic detritivore. By exposing *C. riparius* to arsenic at various environmentally relevant concentrations, I was able to quantify the effects of prolonged exposure on time to adult emergence for both sexes, and the reproductive consequences of exposure. I also developed a protocol for the digestion and analysis of insect tissues in order to quantify actual concentrations of arsenic within individual insects. This technique was essential in follow up studies on arsenic accumulation in other insects.

The goal of the research described in Chapter 3 was to determine if the addition of a stressor, in this case the microbial biocontrol agents *Bacillus thuringiensis* var. *israelensis* and *Lysinibacillus sphaericus* would lead to an increase in mortality of *Culex tarsalis* Coquillett and *Culex quinquefasciatus* Say (Diptera: Culicidae) that were exposed to either arsenate or arsenite. Although arsenic exposures did not lead to mortality, downward shifts in survival curves would indicate that there is still a physiological cost to arsenic exposure and accumulation in these insects.

The next step in evaluating the sublethal effects of arsenic in *C. riparius* and *Cx. tarsalis* was to determine where arsenic was accumulated in the insects and to determine if the arsenic was being transformed by the insects after ingestion. In Chapter 4, I utilized X-ray absorption spectroscopy to map the distribution of arsenic, in addition to other elements, in whole insect samples. X-ray absorption near-edge structure (XANES) analysis was applied to determine the oxidation states of arsenic in the samples. The fluorescence peaks of the detected oxidation states then led to further elucidation about the potential modes of arsenic reduction, excretion, and ultimately, detoxification, in these insects.

Chapter 5 offers an in depth investigation on the effects of metals and metalloids on insect behaviors, specifically ingestion behaviors, taxis behaviors, and oviposition behaviors, and is organized by terrestrial and aquatic insects. Conducting this literature review provided a valuable basis for the behavioral assays conducted in Chapter 6, which were used as a sublethal indicator of arsenic induced stress in *Cx. tarsalis*. Measuring changes in insect behavior has been useful in detecting sublethal effects of chronic exposures to contaminants. In addition to measuring changes in the duration and frequency of various taxis behaviors (resting, gliding, swimming, shallow dives, deep dives, and ascending), I also evaluated the oviposition preferences of adult females in choice tests between control, arsenic water, and phosphate water (used as a sodium ion control treatment).

In the final chapter of this dissertation, I quantify the transfer of arsenic that was accumulated in the larval stages of *Cx. tarsalis*, but retained as adults, to two terrestrial

predators: the Chinese mantid *Tenodera aridifolia sinensis* (Mantodea: Mantidae) and the spider *Tidarren haemorrhoidale* (Araneae: Theridiidae). I then compared the rates of transfer via accumulation in the terrestrial predators to accumulation in an aquatic predator, the backswimmer *Buenoa scimitra* (Hemiptera: Notonectidae). These generalist predators are widespread in the United States, and represent two feeding strategies: piercing sucking and engulfing. I hypothesized that the piercing sucking predators would accumulate less arsenic than the engulfing predator due to the relative bioavailability of arsenic within the prey item. The results from this chapter have implications for the movement of arsenic, as well as other environmental contaminants, from the aquatic to adjacent terrestrial environments by insect biovectors, such as *Cx. tarsalis*.

Together, these chapters provide valuable information on the biological effects of arsenic in aquatic systems, and contribute to the growing body of literature with regards to how aquatic pollutants in general exert sublethal effects and alter the ecosystem services provided by important links in aquatic food webs.

## **CHAPTER 2**

**Survival, reproduction, and arsenic body burdens in *Chironomus riparius* exposed  
to arsenate and phosphate**



## Abstract

Despite the increasing awareness of arsenic (As) contamination in surface waters worldwide, little is known about how As alone and in the presence of other chemicals affects aquatic insects. Larvae of *Chironomus riparius* were exposed in a laboratory investigation to factorial combinations of 0, 10, 150, 400, and 1000  $\mu\text{g As/l}$  and 0, 14, and 1400  $\mu\text{g PO}_4\text{/l}$  throughout development from first instar to pupal emergence. The time between male and female emergence increased from  $1.8 \pm 0.17$  days to  $2.9 \pm 0.34$  days with exposure at higher As levels. The highest As exposure also decreased the number of eggs per egg mass, which may affect population maintenance. For these parameters, there was no effect from  $\text{PO}_4$ , and no interaction between As and  $\text{PO}_4$ . Total As determination of larval and adult tissues was conducted using Hydride Generated Atomic Absorption Spectroscopy (HGAAS) and revealed concentrations ranging from  $2.48 \pm 0.363 - 30.5 \pm 0.473 \mu\text{g/g}$  and  $1.03 \pm 0.286 - 8.97 \pm 0.662 \mu\text{g/g}$ , respectively, indicating elimination of approximately 72% of total As body burdens between the fourth instar and adult stages. There was no effect of  $\text{PO}_4$ , indicating  $\text{PO}_4$  does not alter uptake of As in *C. riparius*. The potential for movement of As to terrestrial systems exists, though trophic transfer may be more likely during the aquatic larval stage.

## Introduction

Background concentrations of arsenic (As) in the environment can be elevated as a result of both natural (geothermal and weathering processes) and anthropogenic contamination. Smedley and Kinniburgh (2002) review worldwide concentrations of arsenic in natural waters, which range from near zero up to 10,000 µg/l in naturally enriched areas, and up to 850,000 µg/l in anthropogenically disturbed areas. However, concentrations up to 1000 µg/l are more typical. In the US, As is considered a priority toxic pollutant of natural waters, and the US Environmental Protection Agency (US EPA) has set the maximum safe concentration for chronic exposure at 150 µg/l for freshwater life (US EPA, 2006). Despite this, how As affects freshwater life, specifically aquatic insects, is still not well understood.

Arsenic is unique among the common metal and metalloid contaminants given its solubility at neutral pH (Tamaki and Frankenberger, 1992). Arsenic exists in several oxidative states, but inorganic arsenite [As(III)] and arsenate [As(V)] are most common in natural waters. Distinguishing species in environmental analyses is crucial to fully understanding toxicity, as arsenite and arsenate, in addition to organic forms, have different modes of action and varying bioavailabilities. Arsenate, the less toxic of the two (Hughes, 2002; Irving et al., 2008; Jeyasingham and Ling, 2000) but more environmentally prevalent (Tamaki and Frankenberger, 1992), replaces phosphate in biochemical reactions, thus disrupting glycolysis by altering the structures of molecular intermediates and inhibiting ATP synthesis (Hughes, 2002).

Given the widespread nature of As, there is a dearth of information regarding effects on aquatic life and potential interactions with other pollutants. Arsenate and phosphate are chemical analogues, and have been shown to compete for the same uptake carriers in the plasmalemma of plant roots (Meharg and Hartley-Whitaker, 2002). The resulting uptake of As can be variable, however, due to phosphate affecting As solubility by competitive adsorption reactions. Creger and Peryea (1994) documented increased uptake of As(V) from soils when apricot rootstocks were exposed to PO<sub>4</sub> in fertilizers. This synergistic interaction could be particularly devastating in parts of Southeast Asia where groundwater exceeding safe levels is used for crop irrigation (250-500 µg/l; Meharg and Rahman, 2003). Rahman et al. (2008) documented the aquatic macrophyte, *Spirodela polyrhiza*, as having a negative correlation between arsenate and PO<sub>4</sub> uptake. Although this interaction may be variable in plants as a result of competitive adsorption, this relationship has not been evaluated in animals.

Many toxicity studies do not explore the possible sublethal effects of metals and metalloids (Mogren and Trumble, 2010), and instead rely on death as a toxicological endpoint (Stark and Banks, 2003). The few studies that focus on As effects in aquatic insects report on development of LC<sub>50</sub>s (Canivet et al., 2001; Jeyasingham and Ling, 2000; Liber et al., 2011) or accumulation of As by insects collected from contaminated streams (Burgehelea et al., 2011; Lavilla et al., 2010). While presenting valuable information with regards to As accumulation at a single point in time, these studies do not provide information on how insects respond throughout their life cycles, which has the potential to inform upon population level effects. We examine how arsenate and PO<sub>4</sub>

alone and combined affect the chronic survival of *Chironomus riparius* Meigen (Diptera: Chironomidae), a ubiquitous aquatic insect. We also evaluate how exposure as larvae affects elemental As concentrations in the terrestrial adults. Finally, we discuss how adults exposed to arsenate as larvae could transfer As to higher trophic levels.

## **Methods**

### *Chironomid survival assay*

Egg masses of *Chironomus riparius* maintained in a colony were purchased from Environmental Consulting and Testing, Inc. (Superior, WI). After two days at 23° C, the eggs began hatching. First instar larvae (14-20 individuals per beaker) were transferred to 600 ml glass beakers containing 300 ml of reconstituted water (described below) and factorial combinations of As(V) (at 0, 10, 150, 400, and 1000 µg/l) as sodium hydrogenarsenate heptahydrate, 99.998% (Sigma-Aldrich, St. Louis, MO, USA) and PO<sub>4</sub> (at 0, 14, and 1400 µg/l, as potassium dihydrogen phosphate, 99.99% (Sigma-Aldrich, St. Louis, MO, USA)). The As concentrations were chosen because they represent the World Health Organization's recommendation for drinking water (10 µg/l) (WHO, 2008); the US Environmental Protection Agency's recommended maximum concentration for indefinite exposure of aquatic life (150 µg/l) (US EPA, 2006); the median As concentration in Hot Creek (Mono Co., CA, USA) (400 µg/l), a geologically active stream (Mariner and Willey, 1976), and; the LC<sub>50</sub> of *Baetis tricaudatus* nymphs after chronic exposure (1000 µg/l) (Irving et al., 2008). All of these concentrations are environmentally relevant and fall below the maximum values reported elsewhere (Smedley and Kinniburgh, 2002). The PO<sub>4</sub> levels chosen represent low concentrations

typical in aquatic systems (14 µg/l) (Rahman et al., 2008), or high concentrations such as a pulse of phosphate as a result of agricultural runoff (1400 µg/l).

A thin layer of pre-rinsed quartz sand (Repti Sand, Zoo Med Laboratories, Inc., San Luis Obispo, CA, USA) was added to the beakers to cover the bottom, which provided a substrate for the immatures while facilitating counting of the larvae. Reconstituted water was prepared using calcium chloride hexahydrate, 98%, sodium bicarbonate ACS reagent, 99.7-100.3%, calcium sulfate, ≥ 99.9% trace metals basis, magnesium sulfate heptahydrate, 98+%, ACS reagent (Sigma-Aldrich, St. Louis, MO, USA), and potassium chloride (Fisher Scientific, Pittsburgh, PA, USA), mixed in Milli-Q HPLC grade water. The chemistry of this water follows the recommendations established by the US EPA (1994) (Table 2.1). A single batch of this reconstituted water was mixed initially and 300 ml were transferred to each of the 15 beakers. Amounts of PO<sub>4</sub> and/or arsenate were added from premixed stock solutions to create and maintain the required treatment concentrations.

Beakers were covered with cheesecloth and placed in an environmental rearing chamber at 23°C and 16L:8D with constant aeration. Compressed air was filtered through a one-way glass microfiber Whatman air filter before reaching the test beakers. Water temperature throughout the experiment averaged 22.4 ± 0.5 °C, and pH was maintained between 7.1-7.8. Larvae were fed a slurry of TetraMin® Tropical Fish Flakes (United Pet Group, Inc., Cincinnati, OH, USA) made by adding 1 gram of flakes to 10 ml of deionized water at a rate of 3 drops every other day, through pupation. Thus, *C. riparius*

was exposed chronically in all treatments from first instar larva through pupal emergence, approximately two weeks.

**Table 2.1** Water chemistry used during chironomid larval survival assays.

	Cations				Anions	
Alkalinity	Ca	K	Mg	Na	SO <sub>4</sub>	Cl
	-----mEq-----					
1.14	1.64	0.05	0.50	1.14	1.23	1.64

Evaporative water loss was replenished daily by adding Milli-Q HPLC-grade water to maintain a 300 ml total volume in the beakers. Beginning on day 5, one third of the water in the beakers was replaced daily and arsenate and/or PO<sub>4</sub> added to maintain the test concentrations. Water replacement was delayed until day 5 to minimize injury to early instars. Arsenic concentrations in the 0 and 10 µg/l arsenate treatments were validated using Hydride Generated Atomic Absorption Spectroscopy (HGAAS). Actual concentrations were within 15% agreement of the target concentrations. Arsenic concentrations in the 150, 400, and 1000 µg/l arsenate treatments were validated using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES), and all were within 5% agreement of the target concentrations. Phosphorus concentrations were also validated using ICP-OES, and actual concentrations fell within 1% agreement for 1400

µg/l. Actual concentrations for the 14 µg/l treatments were 4x higher than expected, possibly resulting from residual dissolved fish flakes present in the analyzed solution.

Larval survival and pupation were monitored daily starting on day five. Adults were counted and sexed as they emerged. Egg masses were removed daily and the number of eggs per egg mass counted. At 72 hours post oviposition, egg masses were monitored for hatch percentage. Using these observations, the net reproductive rate ( $R_0$ ), generation time ( $G$ ), and intrinsic rate of increase ( $r$ , estimated using the equation  $r \approx \frac{\ln R_0}{G}$ ) (Gotelli, 2008) were calculated for each of the 15 treatments. This experiment was replicated five times through time.

#### *Arsenic analysis*

As adults expired in the survival assay, they were removed from the treatments and stored in 1.5 ml centrifuge tubes. Prior to digestion, the chironomid adults were washed with 1 ml of 0.25 M  $\text{KH}_2\text{PO}_4$  solution and rinsed twice with 1 ml ultra pure water to remove any adsorbed As. This rinse procedure effectively removed surface bound As from biological tissues in a concurrent experiment. The chironomid adults were then oven-dried at 50° C to constant mass.

The digestion procedure was modified from Ringmann et al. (2002). Preliminary digestions of oyster tissue standard reference material (NIST 1566b, Gaithersburg, MD, USA) resulted in only 10% recovery of As using the published protocol of US EPA Method 200.8 (US EPA, 1999). The arsenobetaines (AB) that comprise the majority of As in oyster tissue are incapable of being broken down without extended hold periods at high temperatures and pressures (Fecher and Ruhnke, 1998), which exceeded the

operating limits of our equipment. Though Andrahennadi and Pickering (2008) reported that insects are not likely to create AB to sequester As, detoxification mechanisms are still unknown and could involve the production of hard-to-breakdown organoarsenicals. To validate the As values for unknowns, we therefore adapted a protocol (Ringmann et al., 2002) that would breakdown all putative As species in the standard reference material.

All glassware used for digestions and analysis was acid washed prior to use. Digestions were carried out using Microwave Accelerated Reaction System (MARS) 5.0 (CEM Corporation, Matthews, NC, USA) HP-500 Teflon PFA digestion vessels. The maximum operating temperature and pressure for these vessels are 210° C and 2413 kPa (350 psi), respectively. The digestion chemistry and microwave programs are shown in Table 2.2. Saturated solutions of sodium persulfate  $\geq 98\%$  and sodium fluoride  $\geq 99\%$  (Sigma-Aldrich, St. Louis, MO, USA) were mixed by adding 55 g of  $\text{Na}_2\text{O}_8\text{S}_2$  and 5 g NaF to 100 ml of Milli-Q HPLC grade water, respectively.

After the second digestion, sample digestates were diluted to a 25 ml final volume using Milli-Q HPLC-grade water. An aliquot of 6 ml of this diluted digestate was then transferred to 15 ml transport tubes and prereduced overnight using 2 ml concentrated HCl and 2 ml 5%/5% w/w KI/L-ascorbic acid solution. Preliminary digestion trials of chironomid adults revealed very small concentrations in composite samples, and thus a larger digestate volume was analyzed for the unknowns to maximize the likelihood of As detection. The masses of oven dried adults that were digested for analysis were determined using a microbalance accurate to 0.00001 g (Sartorius model 1712 MP 8,



**Table 2.2** Digestion chemistry and microwave programs for first and second digestions, following Ringmann et al. (2002).

	Digestion 1	Digestion 2 <sup>a</sup>
Digestion Chemistry	6 ml Na <sub>2</sub> O <sub>8</sub> S <sub>2</sub> 2 ml NaF 0.2 ml HNO <sub>3</sub>	2 ml Na <sub>2</sub> O <sub>8</sub> S <sub>2</sub> 1 ml NaF
Microwave Program	10 min ramp to 200° C Hold at 200° C for 15 min 5 min cool down	10 min ramp to 200° C Hold at 200° C for 15 min 5 min cool down

<sup>a</sup>Ringmann et al. (2002) uses a second digestion step in which an additional 3 ml of digestion reagents are added after the digestate has cooled completely.

Goettingen, Germany), and ranged between 1.30 and 5.12 mg. There were between 7 and 19 adults analyzed per sample, with an average of 13.

In order to determine whole body As concentrations in larvae, a separate cohort was reared to the 4<sup>th</sup> larval instar as described above with three replicates of each of the five arsenate treatments. Preliminary data from adult As accumulation revealed no significance of PO<sub>4</sub> additions, and thus larvae were reared in the arsenate treatments alone. When larvae reached the 4<sup>th</sup> instar, they were frozen and stored in 1.5 ml centrifuge tubes until they were digested and prereduced as described for the adults. Eleven to 17 individuals were analyzed per sample.

The digested samples of adults and larvae were analyzed using a Perkin-Elmer (Waltham, MA, USA) Analyst 800 Atomic Absorption Spectrophotometer, with a Perkin-Elmer FIMS 400 flow injection mercury system coupled with an As-90 autosampler. The minimum detection limit of the HGAAS was determined by analyzing five samples of 1 µg As/l and multiplying the standard deviation of the results by the one-sided t-distribution. This was calculated to be 0.050 µg/l for As. Digestion and prerelution blanks (containing only prerelution solutions and 6 ml Milli-Q HPLC-grade water) were also included for analysis, as well as digestions of 10 mg NIST oyster tissue standard reference material for validation. Arsenic bioaccumulation factors were calculated by subtracting any As recovered in controls from the concentration recovered in tissues and dividing by the original exposure concentrations.

#### *Statistical analysis*

All statistical analyses were conducted using SAS v. 9.2 (SAS Institute, 2008). Data were assessed for normality (Kolmogorov-Smirnov test) and homoscedasticity (Bartlett's or Levene's test) prior to analysis. Data upholding these assumptions were analyzed using two-way ANOVA (PROC GLM procedure) with arsenate and PO<sub>4</sub> as the independent variables. When data violated these assumptions and could not be corrected using a transformation, Friedman's non-parametric test was used. When necessary, Wilcoxon two sample tests and two sample Kolmogorov-Smirnov tests were applied for comparisons between treatments, Wilcoxon two sample tests being applied when data were not normal but had equal variances and two sample Kolmogorov-Smirnov being applied when data were not normally distributed and had unequal variances. For post hoc

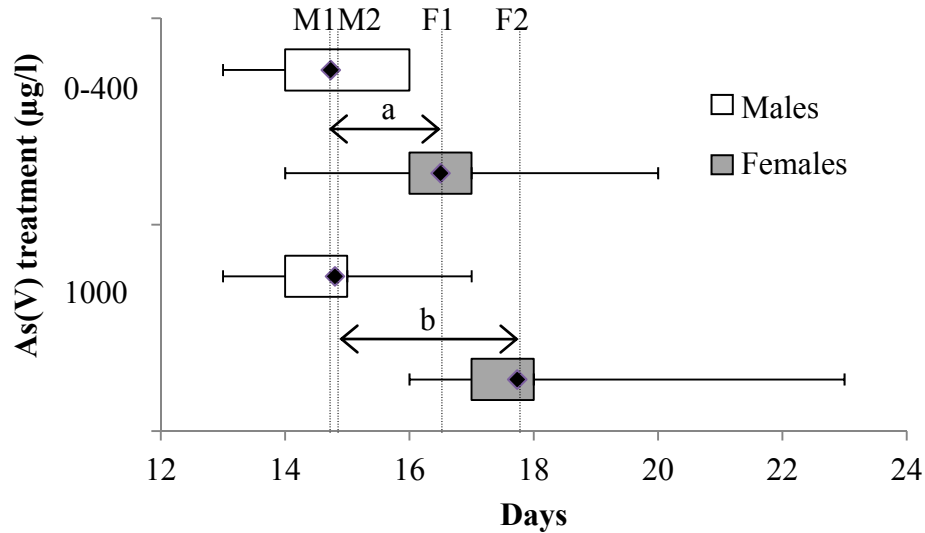
comparisons of ANOVA results, Tukey's test was applied. Sidak's correction was applied to adjust the  $\alpha$  value for post hoc comparisons (Abdi, 2007).

## **Results**

### *Chironomid survival assay*

Survival to adult emergence averaged  $84 \pm 4\%$  in controls, which exceeds Environment Canada's (1997) requirement for 70% survival of controls for a replicate to be valid. Thus, all replicates were valid and included in the analysis. There was a significant difference in the average time between male and female emergence (calculated as the first day to female emergence minus the first day to male emergence) for the arsenate treatments (Friedman's test controlling for  $\text{PO}_4$ ,  $F=13.6$ ,  $p=0.0086$ ). Because post hoc analyses showed no significant difference between the 0 through 400  $\mu\text{g/l}$  As treatments, they were pooled and compared to the 1000  $\mu\text{g/l}$  treatment. There was a significant increase in the average time between male and female emergence in the highest arsenate treatment (Wilcoxon,  $T=783$ ,  $p=0.0035$ ) as a result of female emergence being delayed (Figure 2.1). However, there was no significant differences detected between  $\text{PO}_4$  treatments (Friedman's test controlling for As,  $F=5.33$ ,  $p=0.0697$ ) (Table 2.3). There was also no difference in the proportion of adults emerging from each of the arsenate and  $\text{PO}_4$  treatments, and no interaction of arsenate and  $\text{PO}_4$ .

With regard to the reproductive potential of females, there was again no significant difference between the 0-400  $\mu\text{g/l}$  As treatments so they were pooled for analysis. The numbers of eggs per egg mass from these treatments were consistent with values reported for *C. riparius* elsewhere (Péry et al., 2002), although there were



**Figure 2.1** Box and whisker plot of the first day to emergence for males and females. The second quartile box values for the females and the third quartile box values for the males are zero. The black diamonds represent the respective means. The lower whisker value represents the earliest day that an individual emerged, and the upper whisker represents the longest it took for an individual to emerge. There was a significant difference between the first day to male and female emergence (females - males) between treatments, represented here by a (F1 minus M1) and b (F2 minus M2). N=60 for 0-400 µg/l and n=15 for 1000 µg/l.

significantly fewer eggs per egg mass in the 1000 µg/l treatment (Kolmogorov-Smirnov two-sample test,  $D=0.19$ ,  $p=0.0023$ ) (mean  $\pm$  SE: 0-400 µg/l treatments:  $299.3 \pm 6.0$  eggs; 1000 µg/l treatment:  $270.6 \pm 11.1$  eggs). There was no significant difference between  $\text{PO}_4$  treatments (Friedman's test controlling for As,  $F=0.25$ ,  $p=0.8832$ ) (Table 2.3).

**Table 2.3** Means and standard errors for parameters measured during the chironomid survival assay for the PO<sub>4</sub> treatments.

PO <sub>4</sub> treatment (µg/l)	Days between male and female emergence		Number of eggs per egg mass	
	n	Mean ± SE (d)	n	Mean ± SE
0	25	5.64 ± 0.36	215	290.4 ± 9.56
14	25	4.84 ± 0.23	207	295.7 ± 8.75
1400	25	4.60 ± 0.14	169	295.8 ± 9.26

**Table 2.4** Results from life history parameter analysis.

Arsenate Treatment, µg/l						
		0 <sup>a</sup>	10	150	400	1000
<b>R<sub>0</sub></b>	Mean	148.54	138.38	164.28	139.59	119.87
	SE	5.27	5.66	8.99	6.61	5.49
<b>G</b>	Mean	22.2	21.6	21.5	21.5	22.5
	SE	0.14	0.08	0.13	0.12	0.17
<b>r</b>	Mean	0.223	0.226	0.236	0.223	0.202
	SE	0.00079	0.0014	0.0040	0.0013	0.0021

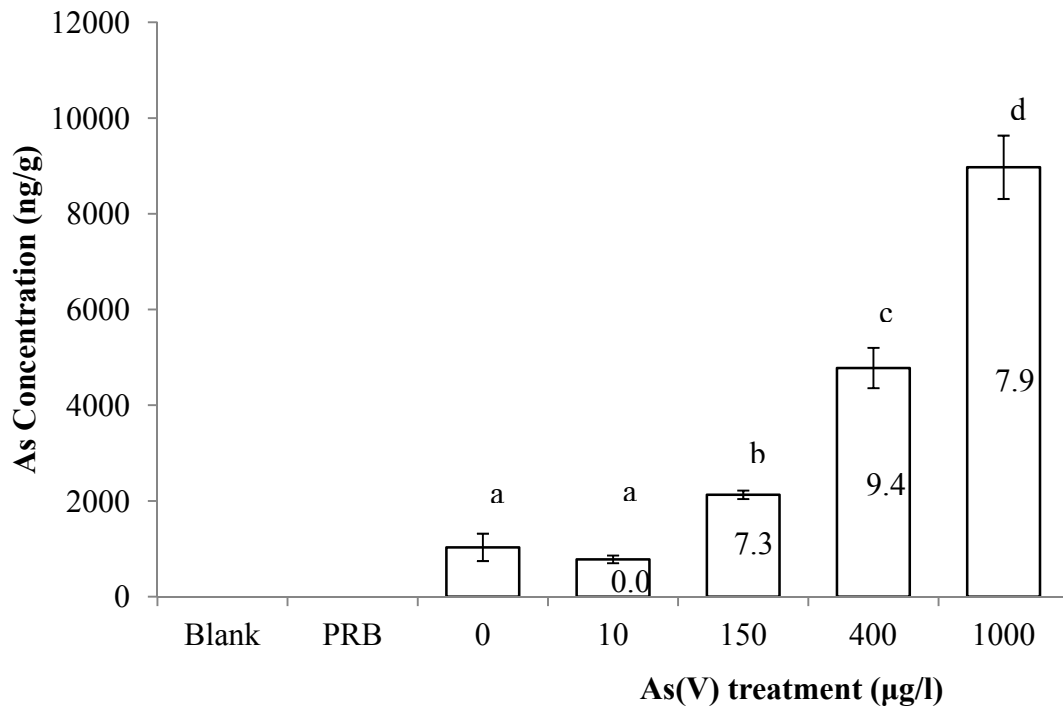
<sup>a</sup> N=15 for each variable in each treatment.

Analysis of the various life history parameters revealed no significant differences between treatments for  $R_0$  (2-way ANOVA,  $F=2.20$ ,  $df=4,2$ ,  $p=0.0952$ ),  $G$  (2-way ANOVA,  $F=0.55$ ,  $df=4,2$ ,  $p=0.8892$ ), and  $r$  (2-way ANOVA,  $F=0.69$ ,  $df=4,2$ ,  $p=0.7794$ ) (Table 2.4), although  $R_0$  approaches significance. This may be due to the negative effect of As on female fecundity. There was no difference between  $PO_4$  treatments and no significant interaction.

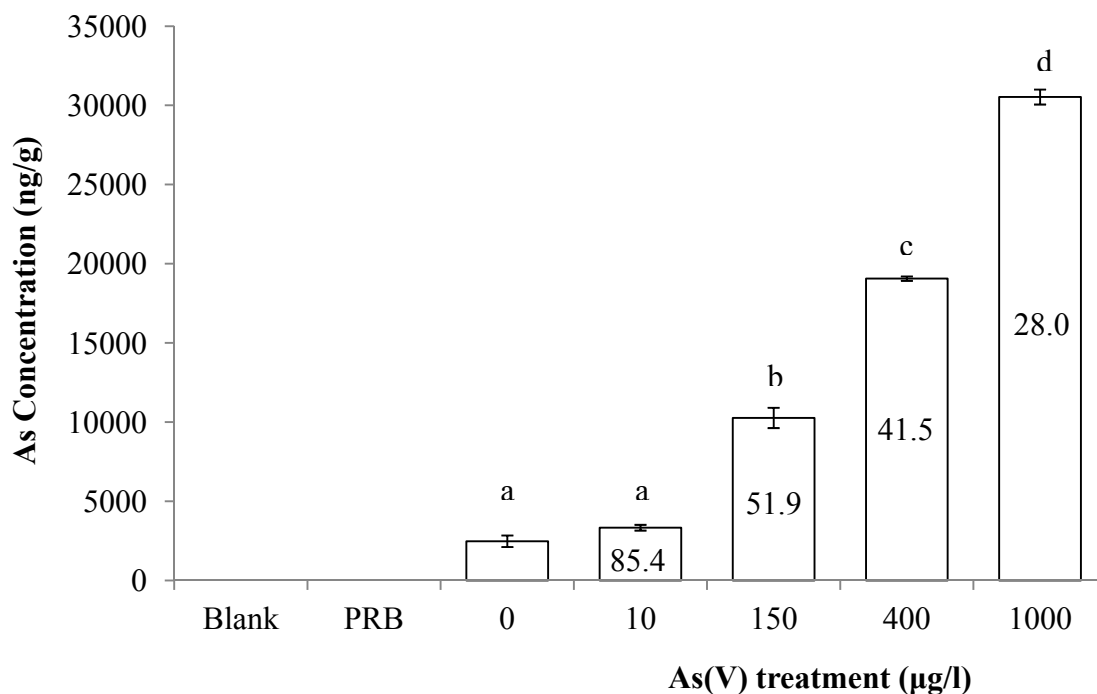
#### *Arsenic analysis*

Digestion and analysis of the NIST oyster tissue validated the protocol used to extract As from the digested chironomid tissues, with  $111.8 \pm 3.5\%$  recovery. Analysis of As accumulation in adults revealed significant differences between arsenate treatments (Friedman's test,  $F=56.2$ ,  $df=4$ ,  $p<0.001$ ) when controlling for  $PO_4$ . The presence of  $PO_4$  did not affect As accumulation and there was no interaction between the treatment variables. Comparisons between As(V) treatments showed a significant increase in As accumulation by adults with an increase in As(V) exposure concentration (Figure 2.2). Adults bioaccumulated approximately 8.2 times the As in which they were exposed as larvae. Significant As accumulation was also observed in larvae (ANOVA,  $F=918.3$ ,  $df=4$ ,  $p<0.0001$ ) (Figure 2.3). Larvae bioaccumulated approximately 51.7 times the As in which they were reared. Based on the average differences between As concentrations recovered in adults and larvae for each arsenate treatment, it is apparent that *C. riparius* eliminates approximately 72% of As body burdens between the 4<sup>th</sup> instar and adult stages.

There was no detectable As in the digestion or prereduction blanks; therefore, the presence of As in the controls for both the adults and larvae is the result of As contamination encountered prior to digestion. This may be due to small amounts of As being present in the TetraMin slurry fed to larvae. Our analysis of the fish flakes detected the presence of  $3.690 \pm 0.70 \mu\text{g As g}^{-1}$  (n=3).



**Figure 2.2** The mean ( $\pm$  SE) concentration of As recovered from digestion of chironomid adults. Letters indicate significant differences, with  $\alpha = 0.0102$  (Sidak's correction for 10 contrasts). There was no As detected in either digestion or prereduction blanks (PRB) (n=8 for each). Numbers inside the bars indicate the As bioaccumulation factor for that treatment.



**Figure 2.3** The mean ( $\pm$  SE) concentration of As recovered from digestion of chironomid larvae. Letters indicate significant differences using Tukey's test, with  $\alpha = 0.05$ . There was no As detected in either digestion or prereduction blanks (PRB) ( $n=2$  for each). Numbers inside the bars indicate the As bioaccumulation factor for that treatment.

## Discussion

### *Arsenate and Phosphate Interaction*

This is the first study to examine the interaction between arsenate and phosphate in an insect. Unlike in plants, arsenate and  $\text{PO}_4$  did not interact to alter reproduction, survival, or As accumulation in *C. riparius*. This suggests that the interaction may be important only to autotrophs. However, this interaction may still be of importance for herbivorous insects consuming plants containing both of these elements (e.g. *Azolla* sp.



(Zhang et al., 2008), *Hydrilla verticillata* (Srivastava et al., 2007), *Spirodela polyrhiza* (Rahman et al., 2008), and *Wolffia globosa* (Zhang et al., 2009)).

### *Survival and Reproduction*

This is the first time that the effects of chronic As exposure have been evaluated throughout the entire life cycle of an aquatic insect. Though studies have focused on acute effects of arsenic in aquatic systems, they report on alderflies (Croisetière et al., 2006), caddisflies (Canivet et al., 2001), dragonflies (Lavilla et al., 2010), and mayflies (Canivet et al., 2001; Irving et al., 2008), in addition to midges (Croisetière et al., 2006; Jeyasingham and Ling, 2000; Liber et al., 2011; Martinez et al., 2006). Certain studies have investigated how effluents containing metals induce mentum deformities in midges (e.g. Martinez et al., 2002, 2006), though the effects of As alone are impossible to deduce when working with metals mixtures. However, these do not address how chronic exposure affects survival and reproduction in aquatic insects.

We have shown that even though relatively high, ecologically relevant concentrations of arsenate exposure will not affect larval survival or the proportion of adults emerging between treatments, the highest arsenate level did increase the time between male and female emergence in *C. riparius* by delaying female emergence. In a study investigating survival of chironomids, Liber et al. (2011) found the LC<sub>50</sub> for *Chironomus dilutus* exposed for 96 h to As-spiked water to be 7.1 mg/l, approximately 7x the highest concentration tested here, though an acute toxicity threshold was reached at 3.31 mg/l. LC<sub>50</sub> values for *C. zealandicus*, *C. sp. a*, and *Polypedilum pavidus* were found to increase with age in 96 hr bioassays, and ranged from 33.1 to 4176 µg/l (Jeyasingham

and Ling, 2000). These elevated LC<sub>50</sub> values highlight the potential unsuitability of using short term LC<sub>50</sub>s (96 h or less) to gauge the long term effects of As on chironomids, particularly when assays do not evaluate potential sublethal effects.

In chironomids, males emerge before females to form mating swarms that ensure access to females (Ferrington et al., 2008). Because these are relatively short lived insects as adults, delayed female emergence as found in this study could result in males dying before females become receptive, particularly as adults do not feed. This may then lead to local extinctions of populations, as suggested for *Megaselia scalaris* (Diptera: Phoridae) exposed to selenium (Jensen et al., 2005). Reproduction as measured by the number of eggs per egg mass was also significantly reduced in the 1000 µg/l treatment, indicating that this concentration may be at or above a threshold for *C. riparius* at which sublethal effects become significant.

#### *Arsenic accumulation*

Arsenic accumulation within adults and larvae showed a significant dose response to increases in arsenate. The majority of what has been published examines accumulation in predators, with only a single study monitoring accumulation of As in *C. riparius* as a prey item (Croisetière et al., 2006). The authors transported laboratory reared 2<sup>nd</sup> instars to a contaminated lake containing 1.45 nmol As/l, where they were held for 1 wk before being fed to *Sialis velata* (Megaloptera: Sialidae). Larvae accumulated 17.2 µg As/g dry weight during this time, with an accumulation factor of  $1.1 \times 10^3$ . Studies examining As accumulation in other aquatic insects found concentration factors of 327 for *Sialis velata* when fed prey exposed to 1.56 µg As/l (Croisetière et al., 2006), 131 for *Pteronarcys*

*dorsata* (Plecoptera: Pteronarcyidae) exposed to 100 µg As/l and 33 when exposed to 1000 µg As/l (Spehar et al., 1980), 1.22 and 1093 for *Heptagenia sulphurea* and *Hydropsyche pellucidula*, respectively, when exposed to 100 µg As/l (Canivet et al., 2001), and 1, 1.28, and 1 for *Hydroglyphus pusillus*, *Laccophilus minutus*, and *Rhantus suturalis* (Coleoptera: Dytiscidae) when exposed to 0.32 µg As/l (Burghelea et al., 2011). However, the Croisetière et al. (2006) and Burghelea et al. (2011) studies were also field based, and analyzed As in addition to numerous other elements at the same time. Because of this, As accumulation may have been higher or lower as a result of undocumented synergistic or antagonistic interactions. The high variability between and within species highlights the need for caution when using bioaccumulation factors to assess the ability of an organism or group of organisms to accumulate As. When comparing between organisms or feeding guilds, only bioaccumulation factors from the same exposure concentration should be compared.

Interestingly, within *C. riparius* bioaccumulation factors change depending on the life stage being analyzed. In our study, bioaccumulation factors for adults and larvae were 8.2 and 51.7, respectively. This species clearly has the capacity to excrete large amounts of As body burdens between the last larval instar and the adult stage. Whether this is the result of As being shed as a meconium, in the pupal exuvia (e.g. as seen with selenium in *Cotesia marginiventris* (Hymenoptera: Braconidae) (Vickerman et al., 2004)), or through some other mechanism is still unknown. In this study, HGAAS analysis would not detect As in pupal exuvia because the sample mass was too small. Future studies are planned, however, to investigate As in pupal exuvia using micro X-ray

Absorption Spectroscopy. Chironomids have been documented elsewhere as excreting metals during the transition to the pupal and adult stages (e.g. cadmium (Groenendijk et al., 1999; Timmermans and Walker, 1989), uranium (Muscatello and Liber, 2009), and zinc (Groenendijk et al., 1999; Timmermans and Walker, 1989).

Chironomids are known in certain areas of the world to emerge en masse and when they do so, transport significant quantities of nitrogen and carbon to the surrounding terrestrial environment (Gratton et al., 2008). In their Icelandic study system at Lake Mývatn, 189 kg/ha/d of midge infall occurred over a 1 wk period. Based on the concentrations of As found in *C. riparius* adults in our study, it is possible for 1.70 g As/ha to be deposited onto terrestrial systems or consumed by terrestrial predators in a single day. This deposition could have substantial negative consequences over time (Lamberti and Chaloner, 2010; Morrissey et al., 2007), such as significant accumulation and trophic transfer of As within the food chain. Though there are small quantities of As per individual, As could also be bioconcentrated at the population level during periods of high emergence (Green, 2008). Higher concentrations recovered in larvae also indicate that trophic transfer of As to aquatic predators is highly likely, particularly in areas where chironomid larvae reach high densities.

Often the aquatic insects chosen for toxicity assays are those able to survive and reproduce readily in a laboratory setting, which may be because they are tolerant to variable environmental conditions. *Chironomus riparius* is a widely used toxicity assay organism and ranks as one of the more tolerant species, with regional tolerance values between 8.1 and 10, on a scale of 1 (low tolerance) to 10 (high tolerance) (Barbour et al.,

1999). We found significantly reduced reproduction in females and a significant increase in the difference between male and female emergence times at the highest As concentration tested. Both of these may have significant effects on population maintenance in wild populations of *C. riparius*. Arsenic accumulation increased significantly with increasing exposure concentrations in larvae and adults, and *C. riparius* is able to eliminate 72% of As body burdens before reaching the adult stage. However, given the generally high tolerance of *C. riparius* to pollution, this may be unrepresentative of other aquatic insects, and more research is needed to determine the sublethal effects of As for these less tolerant species.

### **CHAPTER 3**

**Tolerance to individual and joint effects of arsenic and *Bacillus thuringiensis* subsp.**

***israelensis* or *Lysinibacillus sphaericus* in *Culex* mosquitoes**

## Abstract

Arsenic contamination of global water supplies has come to the forefront in policy decisions in recent decades. However, the effects of arsenic on lower trophic levels of insects inhabiting contaminated ecosystems are not well understood. One approach to document both acute and sublethal effects of toxicants like arsenic is to assay them in combination with microbial pathogens to evaluate shifts in survival curves of the test organisms. Larvae of *Culex quinquefasciatus* and *Culex tarsalis* were reared in water containing 0 or 1000 µg/l of arsenate or arsenite. Fourth instars were then exposed to a range of doses of *Bacillus thuringiensis* subsp. *israelensis* (Bti) or *Lysinibacillus sphaericus* (Ls), with shifts in lethal concentrations determined. Arsenic accumulation in fourth instars was also quantified, and a Relative Growth Index (RGI) calculated for the treatments and compared to controls. Larvae of both species accumulated between  $4447 \pm 169$  and  $6983 \pm 367$  ng As/g, though RGI values indicated accumulation did not affect growth and development. In all cases, the  $LC_{50}$ s and  $LC_{90}$ s of *Cx. quinquefasciatus* exposed jointly with arsenic and Bti/Ls were higher than *Cx. tarsalis*. *Culex tarsalis* reared in arsenite showed a significant reduction in their Bti  $LC_{90}$  values compared to the control, indicating a sublethal effect of Bti. When exposed jointly with Ls, arsenite was more toxic than arsenate in *Cx. tarsalis*. Overall, these results indicate tolerance of these *Culex* species to arsenic exposures, and why this may occur is discussed.

## Introduction

In toxicological studies acute mortality is frequently used as the experimental endpoint (Mogren & Trumble 2010); however, this negates the more subtle effects of a toxicant that may occur at sublethal concentrations and the ecological processes that may be disrupted (Boyd 2010, Stark & Banks 2003). While acute mortality provides valuable information, some insects may appear to be resistant to pollutants at ecologically relevant concentrations (e.g. perchlorate, Sorensen et al. 2007; selenate, Jensen et al. 2007) if only mortality is considered. Thus, the population level effects depend on the parameters being measured, and it may be possible for a toxicant's sublethal effects to be missed entirely. One way to efficiently evaluate the effects of toxicants in insects is to assay them in combination with microbial pathogens to evaluate shifts in survival curves. This approach has been applied as a means of evaluating whether joint exposure of a pollutant and microbial control agent can lower the rates of microbial control applications needed in contaminated wetland areas (Sorensen et al. 2007). However, this joint exposure could also be applied as a means to test whether a particular toxicant is inducing a sublethal physiological stress.

This joint exposure technique is particularly suitable for aquatic systems that contain mosquitoes, such as *Culex quinquefasciatus* Say and *Culex tarsalis* Coquillett (Diptera: Culicidae), because biological control agents for these species, including the bacteria *Bacillus thuringiensis* subsp. *israelensis* (Bti) and *Lysinibacillus sphaericus* (Ls, formerly *Bacillus sphaericus*, Ahmed et al. 2007) are commercially available. Both mosquito species are of medical concern and are known to vector the causative agents of



important diseases such as St. Louis encephalitis, avian malaria, and West Nile fever (Farajollahi et al. 2011, Reisen 1993). However, these insects also play an important ecological function, serving as filter feeders of fine particulate organic matter and as food sources for higher trophic levels (Wallace & Walker 2008). Given their propensity for survival in moderately to severely polluted environments, they are good organisms to use for assessing both lethal and sublethal physiological effects of the aquatic pollutant, arsenic.

Arsenic is a common pollutant of surface waters worldwide as a result of geothermal and weathering processes and anthropogenic inputs (Nriagu 1994, Rahman & Hasegawa 2012, Ravenscroft et al. 2009), and is considered a priority toxic pollutant by the U.S. Environmental Protection Agency. Naturally occurring environmental concentrations may be as high as 10,000 µg/l (Smedley & Kinniburgh 2002), though the U.S. EPA has set the maximum safe concentration for chronic exposure as 150 µg/l for freshwater life (U.S. EPA 2006). In the environment, arsenic exists in numerous inorganic and organic forms as a result of complex redox chemistry, seasonal fluctuations, and prokaryotic transformations (Lloyd & Oremland 2006, Rahman & Hasegawa 2012). The arsenic forms most often encountered in aquatic environments are inorganic arsenate [As(V)] and arsenite [As(III)], with arsenate being the more thermodynamically favorable form in oxic waters and arsenite predominating in reducing environments (Rahman & Hasegawa 2012, Tamaki & Frankenberger 1992). Biologically, arsenite is considered to be the more toxic form (National Research Council 1999).

In this study, we test whether chronic arsenic exposure and accumulation is detrimental to *Cx. quinquefasciatus* and *Cx. tarsalis*. We hypothesized that arsenic accumulation induces sublethal physiological stress in larvae that will lower the LC<sub>50</sub>s (concentrations that will kill 50% of a population) and LC<sub>90</sub>s of Bti and Ls compared to controls, with the associated null hypothesis being no effect of arsenic. Further, we wanted to evaluate whether arsenite is more toxic than arsenate, and hypothesized that we would observe lower LC<sub>50</sub>s and LC<sub>90</sub>s of Bti and Ls in larvae exposed to arsenite than arsenate, with the null hypothesis being no difference in toxicity.

## **Materials and Methods**

### *Mosquito Rearing*

Egg rafts of *Cx. tarsalis* and *Cx. quinquefasciatus* were obtained from colonies maintained at the University of California, Riverside. Eggs were hatched in shallow white enamel pans (39 x 23 x 10 cm or 39 x 23 x 6 cm) containing 3 L of tap water. For both mosquito species, arsenic treatments contained 1000 µg/l of either sodium hydrogenarsenate heptahydrate, 99.998% (Sigma-Aldrich, St. Louis, MO, USA) or sodium arsenite (Fisher Scientific, Pittsburgh, PA, USA). This concentration was chosen because it represents a high, yet still ecologically relevant concentration of arsenic (As) encountered in aquatic systems (Smedley & Kinniburgh 2002). Concentrations of As in the pans (including controls) were validated using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES, Perkin-Elmer Optima 7300 DV, Waltham, MA, USA) and were found to be within 0.675% of the target concentration for the 1000 µg/l treatments for arsenate and arsenite, and below the limit of detection for controls.

Pans were maintained in an environmental rearing chamber under a 16:8 L:D cycle at  $28.0 \pm 0.25^\circ\text{C}$ . Larvae were fed daily a mixture of a 3:1 (wt:wt) ground mouse chow (mouse/rat diet, Harlan/Teklad, Madison, WI, USA) and brewer's yeast (MP Biochemicals, Aurora, OH, USA) as a 10% suspension in deionized water (after Van Dam & Walton 2008).

#### *Mosquito Growth Assays*

Larvae were transferred using an eyedropper to 100 ml glass jars containing 100 ml of deionized water and either 0 or 1000  $\mu\text{g/L}$  arsenate or arsenite, and covered with plastic lids. In order to avoid mortality to the young mosquito larvae, individuals were not transferred until they reached the second instar. For *Cx. tarsalis*, 15 individuals were assayed per arsenic treatment (control, arsenate, and arsenite) with three replicates per treatment in a static test system. Similarly, 20 individuals were assayed per arsenic treatment for *Cx. quinquefasciatus* with three replicates per treatment. Survival and instar were monitored daily until individuals became adults or expired. Larval instars were determined based on observations of head capsule size. Larvae were fed the mouse chow suspension described above with rates adjusted daily to maintain a light cloudiness in the water.

In order to determine if physiological stress induced by chronic arsenate and arsenite exposure affected growth and survival of *Cx. tarsalis* and *Cx. quinquefasciatus*, ten second instar larvae were transferred to a 100 ml jar containing either control or 1000  $\mu\text{g/L}$  of arsenate or arsenite water, and fed the mouse chow suspension daily. Mortality

and molting were assessed daily in order to calculate a Growth Index (GI) and Relative Growth Index (RGI), where:

$$GI = \frac{\sum_{i=1}^{i_{max}} [n_{(i)} \times i] + \sum_{i=1}^{i_{max}} [n_{(i)} \times (i - 1)]}{N \times i_{max}}$$

where  $i_{max}$  is the highest attainable instar by the insect and  $n$  is the total number of insects tested (after Zhang et al., 1993). The RGI was then calculated as:

$$RGI = \frac{\text{GI of test group}}{\text{GI of control group}}$$

The RGI values were calculated daily across treatments using the maximum control GI as the denominator in order to maintain continuity on the RGI plot (following Jensen et al. 2007). Differences between treatments were analyzed using a log likelihood ratio test (R Statistical Software, v.2.15.0) for each species, with day and treatment as the fixed variables, replicate as the random effect, and the RGI value as the response variable.

#### *Arsenic Accumulation*

Fourth instars of both species were reared as described above and analyzed for arsenic accumulation. Five replicates of ten individuals from each species for each treatment (control, arsenate, arsenite) were frozen and oven dried at 50°C to constant mass prior to microwave digestion and analysis using Hydride Generation Atomic Absorption Spectroscopy (HGAAS), as previously described (Mogren et al. 2012, Ringmann et al. 2002). Briefly, samples underwent a two step microwave digestion process with sodium persulfate, sodium fluoride (Sigma-Aldrich, St. Louis, MO, USA), and nitric acid in HP-500 Teflon PFA digestion vessels (CEM Corporation, Matthews, NC, USA). Once cooled, the digestate was diluted and an aliquot was pre-reduced using

concentrated HCl and a 5%/5% w/w KI (potassium iodide, Sigma-Aldrich, St. Louis, MO, USA)/L-ascorbic acid (Fisher Scientific, Pittsburgh, PA, USA) solution. Analysis was conducted using a Perkin-Elmer (Waltham, MA, USA) Analyst 800 Atomic Absorption Spectrophotometer, with a Perkin-Elmer FIMS 400 flow injection mercury system coupled with an As-90 autosampler. The minimum detection limit of the HGAAS was previously determined as 0.050 µg/l for arsenic (Mogren et al., 2012). Data were analyzed using one way ANOVA in SAS v.9.2 (SAS Institute, Cary, NC).

#### *Larvicidal Activity*

In order to evaluate the physiological effects of sublethal exposure to arsenic, both mosquito species reared in these toxicants were then exposed to either Bti or Ls. Stock solutions of the pathogens were mixed using technical powder for Bti (lot 122-267-W5-02, ABG 6164 biological larvicide, 100% wt/wt, Valent Biosciences) and VectoLex technical powder for Ls (lot 117-140-N5-01, 1483 ITU/mg, Valent Biosciences) in Milli-Q HPLC-grade water. *Culex tarsalis* was tested at 0.01, 0.015, 0.02, and 0.05 mg/l for Bti and 0.001, 0.002, 0.005, and 0.01 mg/l for Ls. *Culex quinquefasciatus* was tested at 0.05, 0.08, 0.10, and 0.20 mg/l for Bti and 0.005, 0.01, 0.02, 0.05, and 0.08 mg/l for Ls. All of these doses were based on the estimated LC<sub>50</sub> for each species and pathogen combination as determined in preliminary trials.

Ten fourth instars of *Cx. quinquefasciatus* and *Cx. tarsalis* were transferred to 100 ml glass jars containing 100 ml of deionized water and the appropriate concentration of arsenate or arsenite in which the larvae were reared. The Bti and Ls stock solutions were further diluted such that a 1 ml addition to the test jars resulted in the desired test

concentration. Controls received 1 ml of HPLC-grade water only. Mortality was assessed after 24 h for Bti assays and 48 h for Ls assays (Sorensen et al. 2007, Zahiri et al. 2004). Larvae were fed as described for the mosquito growth assays at the start of the Bti and Ls trials and again after 24 h for the Ls trials. The assays were conducted at four different times using different batches of eggs, with two replicates at a time, giving a total of 8 replicates for each dose for both pathogens that were tested with each toxicant and each species.

Control mortality never exceeded 10%; however, Abbott's formula was applied to correct for any control mortality that did occur (Abbott 1925). In order to determine the Bti and Ls LC<sub>50</sub>s and LC<sub>90</sub>s for the treatments, corrected data were analyzed using Probit analysis (Finney 1971) in SAS (Proc Probit). Differences between arsenic treatments for the LC<sub>50</sub>s and LC<sub>90</sub>s were considered significant if the 95% fiducial limits did not overlap. In order to detect whether arsenic treatment significantly altered the effects of Bti or Ls across their respective concentrations, the proportions of dead larvae for each replicate were arcsine(sqrt(y)) transformed to achieve normality and homogeneous variances and analyzed using general linear modeling (Proc GLM, SAS). Post-hoc comparisons were made using Tukey's test.

## **Results**

### *Mosquito Growth Assays*

For *Cx. quinquefasciatus*, the RGI increased rapidly to day 6, after which point it leveled off until all individuals had either emerged or were dead by day 10 (Figure 3.1a). A similar pattern was observed for *Cx. tarsalis*, where RGI increased until day 6 before

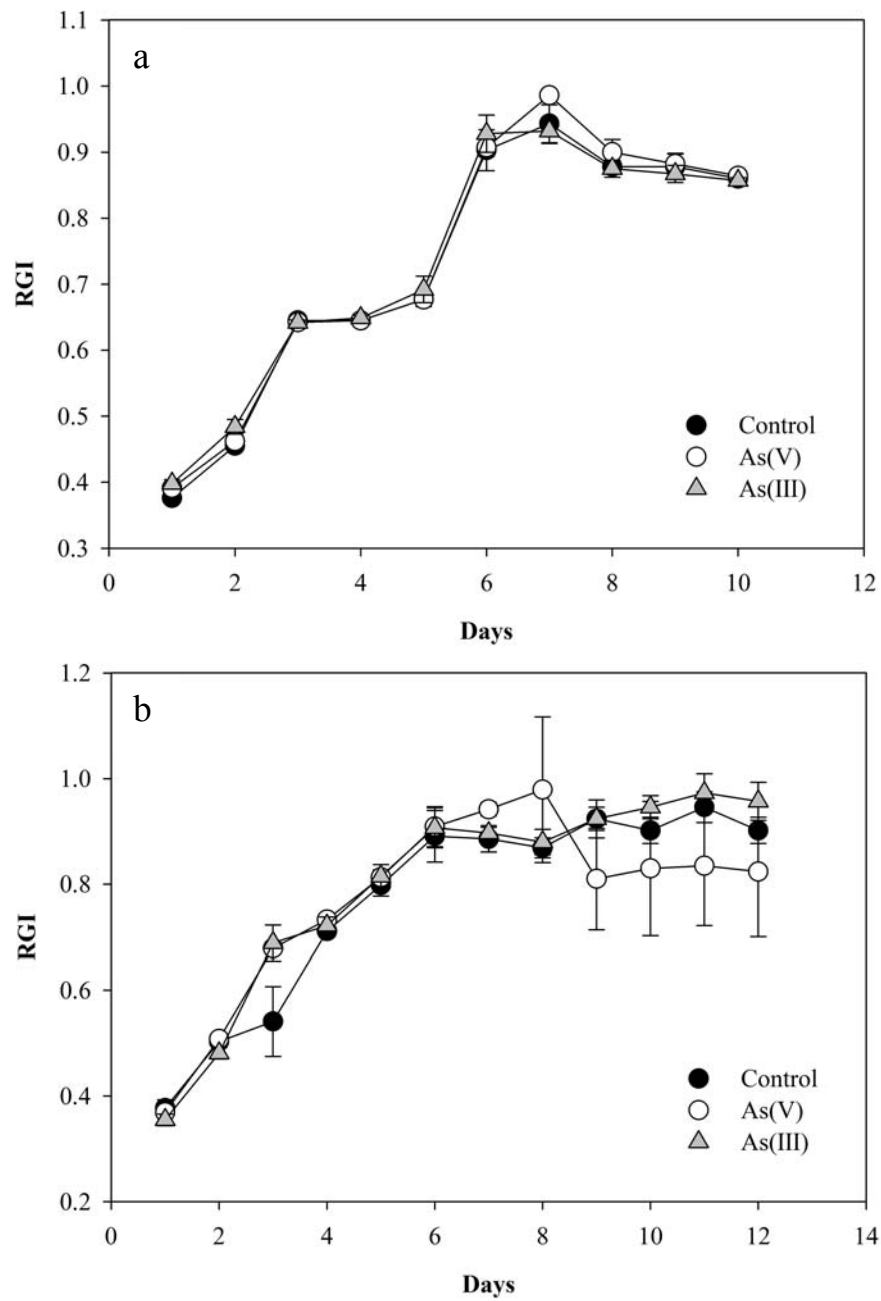
leveling off until day 12 (Figure 3.1b). There were no significant differences in RGI values between controls, As(V), and As(III) treatments for *Cx. quinquefasciatus* ( $\chi^2=0.1643$ , df=2, p=0.921) or *Cx. tarsalis* ( $\chi^2=0.5530$ , df=2, p=0.758). In this case, it did not appear that a simple measure of growth provided any indication of a sublethal effect of arsenic exposure in the mosquito larvae.

#### *Arsenic Accumulation*

The HGAAS analysis of digested mosquito larvae revealed significant differences between controls, As(V), and As(III) for both mosquito species (*Cx. quinquefasciatus*: F=224.09, df=2, 10, p<0.001; *Cx. tarsalis*: F=55.75, df=2, 10, p<0.001) (Figure 3.2). For both species, accumulation in As(V) and As(III) treatments was significantly greater than controls (p<0.001), though there was no difference in As accumulation for *Cx. tarsalis* between the As(III) and As(V) treatments (p=0.119). There were no significant differences between the species in their arsenic accumulating abilities (F=0.01, df=1, 20, p=0.912).

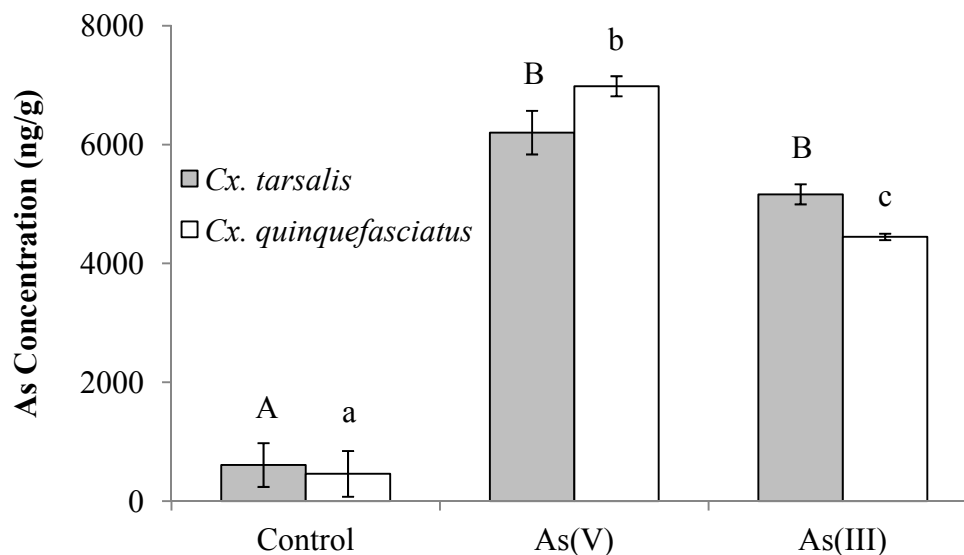
#### *Larvicidal Activity*

An examination of LC<sub>90</sub> values revealed that for *Cx. tarsalis* 39% less Bti was needed to achieve 90% mortality in the arsenite treatment as compared to the control. There was no difference between arsenate and arsenite treatments. For Ls exposure, mortality was greater in arsenite exposure than arsenate exposure, with only 33% of the Ls concentration from the arsenate treatment needed to cause 90% mortality in the arsenite treatment. Within both species and across arsenic treatments, there were no significant differences in LC<sub>50</sub> values (Table 3.1). There was also no significant



**Figure 3.1.** The mean Relative Growth Index (RGI) for *Cx. quinquefasciatus* (a) and *Cx. tarsalis* (b) calculated daily, starting from the second instar. Error bars represent the SE calculated for each treatment on each day (n=3 for each treatment).





**Figure 3.2.** Mean arsenic accumulation  $\pm$  SE for *Cx. quinquefasciatus* and *Cx. tarsalis*.

Capital letters indicate the significant differences between treatments for *Cx. tarsalis* and lower case letters indicate the significant differences between treatments for *Cx. quinquefasciatus* ( $\alpha < 0.05$ ).

difference in  $LC_{90s}$  for *Cx. quinquefasciatus* for Bti or Ls exposure. In all cases,  $LC_{50s}$  and  $LC_{90s}$  for *Cx. quinquefasciatus* were significantly greater than those for *Cx. tarsalis*.

There was a significant difference in Bti efficacy between *Cx. quinquefasciatus* and *Cx. tarsalis* ( $F=70.84$ ,  $df=1,190$ ,  $p<0.001$ ) and between doses of Bti ( $F=51.65$ ,  $df=2,190$ ,  $p<0.001$ ), though the interaction between the two was also significant ( $F=77.66$ ,  $df=1,190$ ,  $p<0.001$ ). This indicates that the overall effect of Bti at a particular dose was dependent upon the mosquito species being tested, particularly at higher doses of Bti. However, the efficacy of Bti was not found to be significantly different between arsenic treatments ( $F=0.26$ ,  $df=2,190$ ,  $p=0.774$ ) (Figure 3.3). Arsenic treatment was again

**Table 3.1.** LC<sub>50</sub>s and LC<sub>90</sub>s for joint arsenic exposure with Bti and Ls.

	LC <sub>50</sub> (µg/ml), FL <sup>†</sup>		LC <sub>90</sub> (µg/ml), FL	
	Bti	Ls	Bti	Ls
<i>Cx. quinquefasciatus</i>				
Control	0.140, <sup>a‡</sup>	0.019, <sup>a</sup>	0.417, <sup>a</sup>	0.088, <sup>a</sup>
	0.123 – 0.168	0.016 – 0.022	0.305 – 0.710	0.067 – 0.127
1000 µg As(V)/l	0.124, <sup>a</sup>	0.023, <sup>a</sup>	0.332, <sup>a</sup>	0.125, <sup>a</sup>
	0.111 – 0.0.143	0.019 – 0.027	0.256 – 0.505	0.091 – 0.198
1000 µg As(III)/l	0.138, <sup>a</sup>	0.021, <sup>a</sup>	0.352, <sup>a</sup>	0.254, <sup>a</sup>
	0.090 – 0.899	0.016 – 0.031	0.187 – 441	0.122 – 1.06
<i>Cx. tarsalis</i>				
Control	0.021, <sup>a</sup>	0.0043, <sup>a</sup>	0.046, <sup>a</sup>	0.012, <sup>ab</sup>
	0.019 – 0.024	0.0038 – 0.0050	0.037 – 0.064	0.010 – 0.017
1000 µg As(V)/l	0.019, <sup>a</sup>	0.0046, <sup>a</sup>	0.034, <sup>ab</sup>	0.013, <sup>a</sup>
	0.017 – 0.021	0.0040 – 0.0054	0.029 – 0.046	0.011 – 0.019
1000 µg As(III)/l	0.018, <sup>a</sup>	0.0043, <sup>a</sup>	0.028, <sup>b</sup>	0.0087, <sup>b</sup>
	0.017 – 0.019	0.0037 – 0.0049	0.024 – 0.035	0.0076 – 0.0105

<sup>†</sup>FL, 95% fiducial limits

<sup>‡</sup>Letters indicate significant differences between treatments for a mosquito species, based on overlapping fiducial limits.

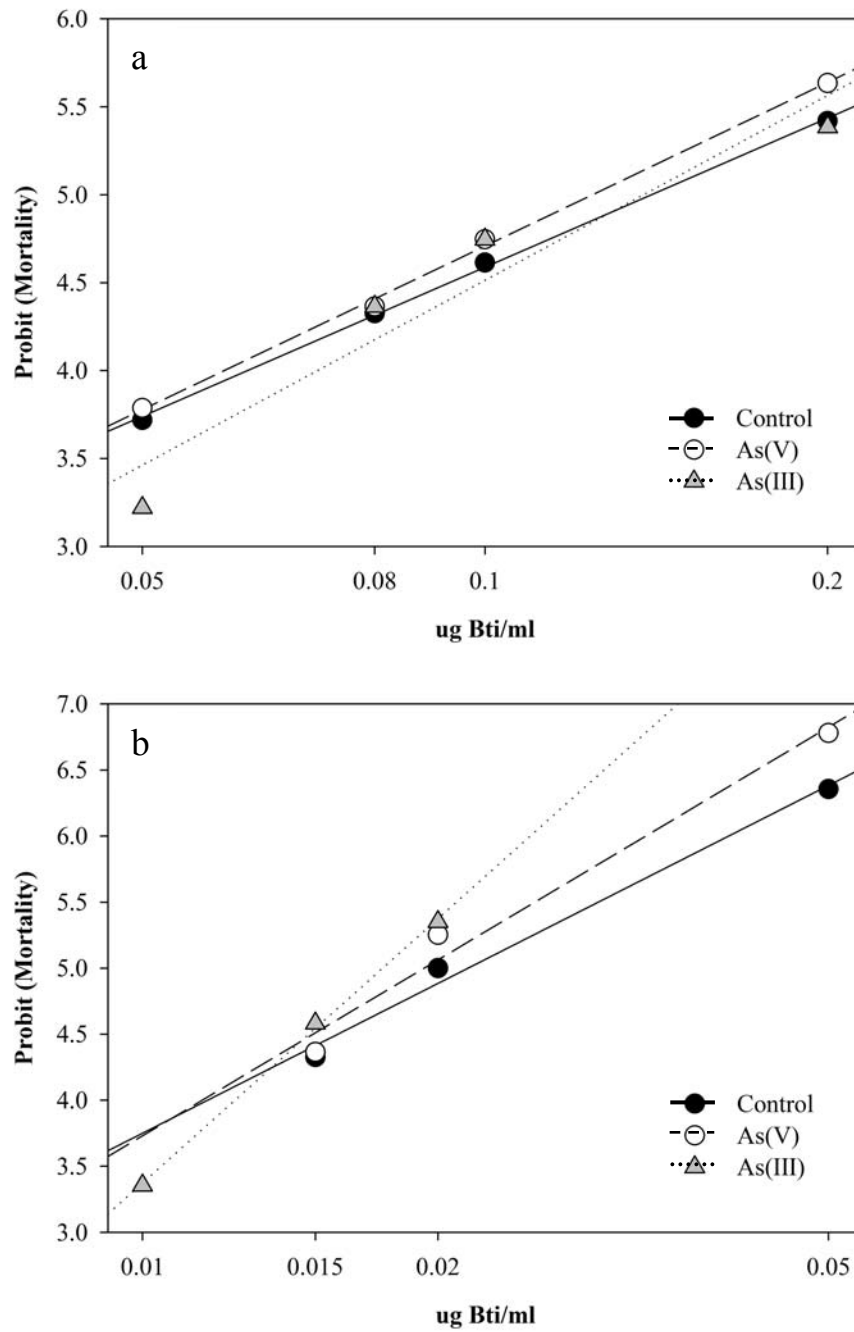
not significant when tested with Ls (F=0.17, df=2,238, p=0.842) (Figure 3.4), though mosquito species and dose were (species: F=17.47, df=1,238, p<0.001; dose: F=418.17, df=1,238, p<0.001). There was also a significant species x dose interaction (F=276.83, df=1,238, p<0.001), indicating the overall effect of Ls at a particular dose was also

dependent upon whether *Cx. quinquefasciatus* or *Cx. tarsalis* was being tested, particularly at higher doses.

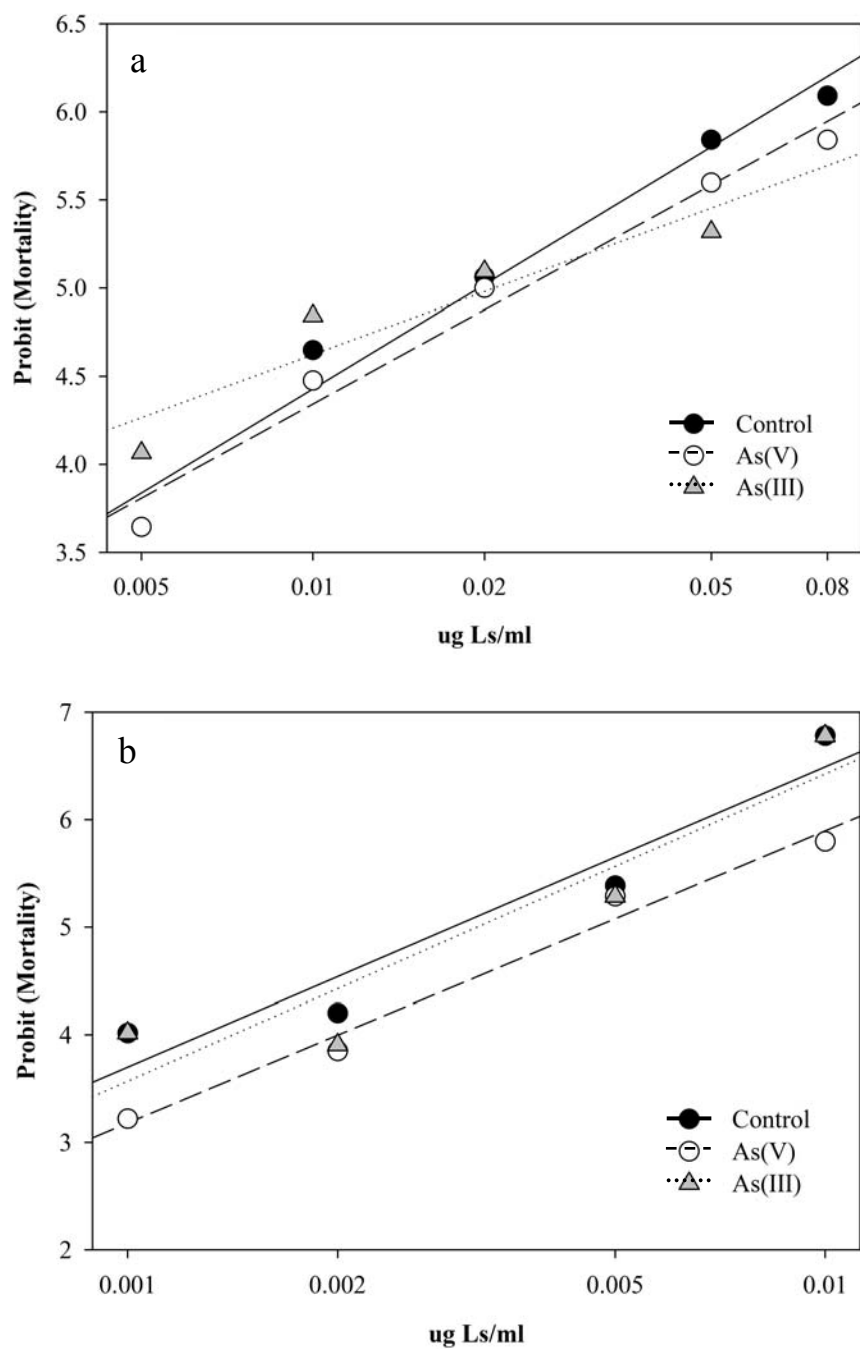
## **Discussion**

The presence of arsenic in surface and ground waters has been documented as a worldwide phenomenon (National Research Council 1999, Ravenscroft et al. 2009, Smedley & Kinniburgh 2002), and recent discoveries of the presence of arsenic in rice and apples in the United States have further prompted national discussions about food safety (Associated Press 2012, Melnick 2011). Arsenic in rice may result from irrigation with contaminated water (Williams et al. 2006), while arsenic in apples often results from lead-arsenate fertilizer residues persisting in soils decades after the last applications (Creger & Peryea 1994). In both cases, runoff into streams and lakes exposes aquatic life to concentrated and potentially harmful concentrations of arsenic, in addition to what they are already exposed to naturally (de Guzman et al. 2012). In this study, we wanted to determine if arsenic that results naturally or from runoff events exerts a physiological effect on aquatic dipterans.

When larvae of *Cx. quinquefasciatus* and *Cx. tarsalis* were exposed to 1000 µg As/l in the form of arsenate or arsenite, there was no significant effect of exposure on survival and growth in either species. However, there was a significant amount of arsenic accumulated in both mosquito species when exposed to either arsenate or arsenite. There was also significantly more arsenic accumulated in *Cx. quinquefasciatus* when exposed to arsenate than arsenite, which may be due to arsenate being taken up preferentially via phosphate transporters as a result of its chemical similarity to phosphate (Nriagu 1994,



**Figure 3.3.** The probit transformed mortality of *Cx. quinquefasciatus* (a) and *Cx. tarsalis* (b) exposed jointly to Bti and either control, 1000  $\mu\text{g/l}$  As(V), or 1000  $\mu\text{g/l}$  As(III). Bti dose has been log transformed on the x-axis.



**Figure 3.4.** The probit transformed mortality of *Cx. quinquefasciatus* (a) and *Cx. tarsalis* (b) exposed jointly to Ls and either control, 1000 µg/l As(V), or 1000 µg/l As(III). Ls dose has been log transformed on the x-axis.

Oremland & Stolz 2003). Given that there was a significant amount of arsenic accumulated in the larvae of these mosquitoes, we wanted to test whether it could be exerting a sublethal physiological stress.

Exposure to metals has been previously shown to increase susceptibility to pathogens. In their study examining susceptibility to the fungus *Beauveria bassiana* in the greater wax moth, *Galleria mellonella* (Lepidoptera: Pyralidae), Dubovskiy et al. (2011) found that sublethal nickel exposure reduced resistance to the fungus when compared to controls, despite heightened glutathione-S-transferase activity. In a different study using nickel accumulating insects, mortality of *Lygus hesperus* and the Ni accumulator *Melanotrichus boydi* (Hemiptera: Miridae) was also greater when simultaneously exposed to *B. bassiana* (Boyd 2002).

The efficacy of pathogens against insects in aquatic systems when the insect is jointly exposed to an environmental toxicant has also been evaluated. Sorensen et al. (2007) showed that Bti and Ls were more effective against *Cx. quinquefasciatus* when there was joint exposure with hexavalent chromium; this exposure led to a significant increase in the efficacy of both pathogens, and decreased resistance of the mosquito. The Bti and Ls formulations used in this experiment were technical powders, and thus do not contain active bacterial cells, though they do contain active toxins and spores. It has been previously shown that insects launch an immune response against spore-crystal formulations (Ericsson et al. 2009), with a significant response being measured as soon as 3 hours after ingestion (Huang et al., 2009). Therefore, the possibility existed that arsenic could alter susceptibility of the mosquitoes to Bti or Ls spore-crystals. In the present

study, we found that chronic exposure to arsenate and arsenite did not decrease the efficacy of Bti and Ls when exposed jointly with the pathogens, and the reported LC<sub>50</sub> values for Bti and Ls are consistent with what have been reported elsewhere for *Cx. quinquefasciatus* (Wirth et al. 2004, 2007), indicating arsenic tolerance in these species.

When determining the efficacy of Bti and Ls across a range of concentrations for the arsenic treatments, the efficacy of the pathogens was found to interact significantly with the mosquito species and the dose of the pathogen. *Culex tarsalis* reared in arsenite showed a significant reduction in their Bti LC<sub>90</sub> compared to the control. Therefore, we can only conditionally accept our hypothesis that exposure to arsenic induces sublethal physiological stress, with the caveat that in our experiment we were only able to show significance at a single Bti concentration for one species, as opposed to across a range of concentrations for both species. To date, only a few other studies have evaluated the sublethal effects of arsenic on aquatic insects. Mogren et al. (2012) found significant reductions in the reproductive capacity of *Chironomus riparius* (Diptera: Chironomidae) females, as well as an increase in the time between male and female emergence. Martinez et al. (2006) reported mentum deformities in *Chironomus dilutus* that were exposed to arsenite spiked soils. The mayfly *Baetis tricaudatus* (Ephemeroptera: Baetidae) experienced reduced nymph growth and development when exposed to 1000 µg/l of arsenate and arsenite (Irving et al., 2008). The mechanisms by which arsenic induces toxicity are varied (Kumagai & Sumi 2007) and are not well understood in insects, although research suggests glutathione synthetase may play a role in detoxification (Andrahennadi & Pickering 2008, Muniz-Ortiz et al. 2007).

Our second hypothesis tested whether arsenite is more toxic to *Culex* mosquitoes than arsenate. In this case, we can accept that this is true at the concentration tested (1000 µg As/l) in *Cx. tarsalis* when exposed jointly with Ls. Interestingly, there was no significant difference between arsenate and arsenite in *Cx. tarsalis* when exposed to Bti, indicating that the different arsenic forms may interact with the toxins produced by the different pathogen species before a significant effect is observed. The proteinaceous Cry and Cyt toxins in Bti bind to the midgut epithelia and cause cell lysis, while BinA and BinB are the predominant toxins responsible for the same effect in Ls (reviewed in Lacey 2007). Both Bti and Ls must be ingested and activated by midgut enzymes that cleave the protoxins into the toxic forms. This potentially puts them into direct contact with arsenic, which has been shown to accumulate and biotransform in the midgut of immature insects (Andrahennadi & Pickering 2008, Mogren unpublished data) and thus an interaction between arsenic form and Bti or Ls toxins is not unexpected, particularly as there is evidence for Ls entering target cells (Davidson et al. 1987). Unfortunately, there is no detailed understanding of the mode of action of Ls at the molecular level (Berry 2012). Just how the toxins of Bti and Ls interact with arsenic and other environmental pollutants at the molecular level specifically requires further investigation. Furthermore, because larvae are shown to accumulate As, future research on trophic transfer is warranted.



## **CHAPTER 4**

**Micro x-ray absorption spectroscopic analysis of arsenic localization and  
biotransformation in *Chironomus riparius* Meigen (Diptera: Chironomidae) and  
*Culex tarsalis* Coquillett (Culicidae)**

## **Abstract**

The distribution and speciation of arsenic (As) were analyzed in individuals of various life stages of a midge, *Chironomus riparius*, and the mosquito *Culex tarsalis* exposed to 1000 µg/l arsenate. X-ray absorption spectroscopy (XAS) revealed that *C. riparius* larvae accumulate As in their midgut, with inorganic arsenate [As(V)] being the predominant form, followed by arsenite [As(III)] and an As-thiol. Reduced concentrations of As in pupal and adult stages of *C. riparius* indicate excretion of As between the larval and pupal stages. In adults, As was limited to the thorax, and the predominant form was an As-thiol. In *Cx. tarsalis*, As was not found in high enough concentrations to determine As speciation, but the element was distributed throughout the larva. In adults, As was concentrated in the thorax and eyes of adults. These results have implications for understanding the biotransformation of As and its movement from aquatic to terrestrial environments.

## Introduction

X-ray spectroscopy is a useful method for toxicology studies with insects because of the non destructive manner in which samples can be analyzed (Parsons et al., 2002). Micro x-ray fluorescence ( $\mu$ XRF) allows visualization of the spatial distributions of elements within target organs of the insect with micron resolution. X-ray absorption spectroscopy (XAS) may be further utilized to determine speciation and oxidative states of a target element through x-ray absorption near-edge structure (XANES) analysis. This technique is particularly amenable to insect systems given the small size of insects, which allows for the entire animal to be scanned and compartmentalization of a target element to be determined. While XAS has been used in the past to evaluate metal and metalloid accumulation and speciation within insects (e.g. Andrahennadi and Pickering, 2008; Moriarty et al., 2009), it has not been applied to aquatic insects or other invertebrates whose lives are spent in direct contact with arsenic contaminated substrates.

Within biological tissues, the arsenic species most often encountered in XAS analysis are (in order of lowest to highest white line energies): arsenic glutathione [ $\text{As}(\text{Glu})_3$ ], monomethylarsenic DMPS ( $\text{MeAsDMPS}$ ), dimethylarsenic 2,3-dimercapto-1-propane sulfonic acid sodium salt ( $\text{Me}_2\text{AsDMPS}$ ), monomethylarsonous acid [ $\text{MMA}(\text{III})$ ], arsenite [ $\text{As}(\text{III})$ ], arsenobetaine (AB), arsenobetaine 2 (C2-AB), arsenobetaine 3 (C3-AB), arsenocholine (AC), tetramethylarsonium iodide (Tetra), trimethylarsine oxide (TMAO), (*R*)-2,3-dihydroxypropyl-5-deoxy-5-dimethylarsinyl- $\beta$ -D-ribose sugar, dimethylarsinic acid [ $\text{DMA}(\text{V})$ ], monomethylarsonic acid [ $\text{MMA}(\text{V})$ ], and arsenate [ $\text{As}(\text{V})$ ] (Smith et al., 2005). Distinguishing between arsenic species is

crucial in environmental analyses due to the different modes of toxic action between inorganic forms, such as arsenate [As(V)] and arsenite [As(III)], and organic forms. Inorganic arsenate, the thermodynamically favored form in freshwater systems (Rahman and Hasegawa, 2012), is a chemical analogue for phosphate and is taken into cells via phosphate transporters (Zangi and Filella, 2012). Thus, arsenate disrupts glycolysis by replacing phosphate in biochemical reactions, altering the structures of molecular intermediates and disrupting ATP synthesis (Hughes, 2002). Arsenite interacts with the sulfhydryl bonds of proteins, disrupting tertiary structures and enzymatic function as a result (Hughes, 2002). Arsenic may also exist in many organic forms in the environment (Reimer et al., 2010), which may result from microbial activity in aquatic environments (Lloyd and Oremland, 2006). Methylated forms are shown to be genotoxic to *Drosophila melanogaster* (Diptera: Drosophilidae), though *D. melanogaster* is not capable of methylating arsenic (Rizki et al., 2006).

Invertebrates possess a variety of methods by which they are generally able to eliminate toxic compounds from their cells, including 1) regulatory mechanisms that balance rates of metal uptake from the environment with excretion rates, 2) intracellular sequestration using metallothioneins and subsequent elimination through the lysosomal endomembrane system, and 3) intracellular sequestration processes involving vacuoles that produce solid metallic phosphorous or sulfur granules that then undergo exocytosis for elimination (Ahearn et al., 2004). Extra cellular sequestration via lipid particles containing iron has also been proposed (Rahman et al., 2009), as well as molting as a means for depuration (Bergey and Weis, 2007). We are not aware of any invertebrates

that have been shown to possess all of these mechanisms. There is some evidence for the formation of spherocrystals to regulate excess arsenic ions in *Formica polyctena* (Hymenoptera: Formicidae) (Jeantet et al., 1977). More recently, arsenic susceptibility has been shown to be mediated by the presence of glutathione in insects (Muñiz-Ortiz et al., 2007). Reduction of arsenate and subsequent coordination with sulfur [As(III)-S] has been found in terrestrial insects and other invertebrates (Andrahennadi and Pickering, 2008; Langdon et al., 2002, 2005; Moriarty et al., 2009). There is the possibility that thiols play an important role in mediating this reduction (Thomas, 2010). However, the current body of knowledge regarding potential mechanisms of arsenic reduction and excretion in insects and terrestrial invertebrates is limited to these examples. How invertebrates that spend the majority of their lives immersed in a toxicant enriched environment, particularly aquatics and soil dwelling insects, is of particular interest from a toxicological standpoint given the worldwide nature of arsenic contamination (Ravenscroft et al., 2009) and its status as a priority toxic pollutant (US EPA, 2006). Further, past research has shown that the larvae of aquatic Diptera are able to withstand chronic exposure to relatively high concentrations of arsenic (Mogren et al., 2012; Mogren, unpublished data), though the mechanisms by which they are able to do so are unknown.

In this study, we investigated potential modes of arsenic transformation and excretion in *Chironomus riparius* Meigen (Diptera: Chironomidae) and *Culex tarsalis* Coquillett (Diptera: Culicidae), whose larvae are aquatic and ubiquitous in North America. While *C. riparius* is a benthic detritivore, *Cx. tarsalis* is a filter feeder at the

water surface, and both species serve as important food sources for higher trophic levels (Merritt et al., 2008). Because *C. riparius* larvae reside in lake benthos, they often come into contact with contaminants in soil and water, including arsenic (Croisetière et al., 2006). *Culex tarsalis* larvae are found in surface water pools and tolerate a wide range of conditions, including organic enrichment and exposure to industrial effluent (Reisen, 1993). In order to understand the organs responsible for biotransformation and absorption of arsenic in these insects, we used XAS imaging with micro X-ray fluorescence imaging ( $\mu$ XRF) to determine the microscopic distribution of arsenic within intact specimens, in addition to speciation analysis of the same specimens with XANES analysis.

## **Methods**

### *Insect Rearing and Sample Preparation*

#### *Chironomus riparius*

Individuals of *C. riparius* were reared following the protocol established in Mogren et al. (2012). Briefly, egg masses were purchased from Environmental Consulting and Testing, Inc. (Superior, WI, USA). A thin layer of pre-rinsed quartz sand (Repti Sand, Zoo Med Laboratories, Inc., San Luis Obispo, CA, USA) was provided as a burrowing substrate for the larvae in 600 ml beakers containing 300 ml of reconstituted water and either 0 or 1000  $\mu\text{g As/l}$  as arsenate in the form of sodium hydrogenarsenate heptahydrate 99.99% (Sigma-Aldrich, St. Louis, MO, USA). The arsenic treatment concentration was chosen because it represents a high yet still ecologically relevant concentration of arsenic that would be found in a contaminated water body (Ravenscroft et al., 2009; Smedley and Kinniburgh, 2002), and to increase the likelihood of detecting

reduced or transformed species of arsenic in the samples. According to Ravenscroft et al. (2009) over 45 regions around the world have As levels occurring naturally in surface and ground waters that reach or exceed 1000 µg/l (with many regions exceeding 1500 µg/l).

Ten first instars were transferred to each beaker. Water loss through evaporation was accounted for daily through the addition of Milli-Q HPLC-grade water to maintain the 300 ml volume and one third of the water was replaced daily starting on day five to minimize injury to early instars. Larvae were fed a slurry of TetraMin® Tropical Fish Flakes (United Pet Group, Inc., Cincinnati, OH, USA), made by adding 1 g of flakes to 10 ml of deionized water. Beakers were aerated constantly in an environmental rearing chamber at 23°C under a 16L:8D cycle.

As the larvae grew, individuals were sacrificed at the second instar, fourth instar, pupal, and adult stages (females only) for XAS analysis. Egg masses were also collected from female adults that were allowed to oviposit. Samples were rinsed in deionized water and then frozen at -60°C prior to being freeze dried.

#### *Culex tarsalis*

Egg rafts of *Culex tarsalis* were obtained from colonies maintained at the University of California, Riverside. The use and care of animal hosts was done under Protocol A2010006 approved by the Institutional Animal Care and Use Committee of the University of California, Riverside. Egg rafts were hatched in white enamel pans containing 3 L of tap water (after Van Den Heuvel, 1962) and either 0 or 1000 µg As/l. Pans were maintained in an environmental rearing chamber at 28°C and 16L:8D light

cycle. Larvae were fed a ground mouse chow (mouse/rat diet, Harlan/Teklad, Madison, WI, USA) and brewer's yeast (MP Biochemicals, Aurora, OH, USA) 3:1 (wt:wt) mixture as a 10% suspension in deionized water. Three fourth instar larvae and adults were rinsed in deionized water and sacrificed by freezing at -60°C prior to being freeze dried.

#### *μXRF Mapping and μXANES*

Detailed explanations of the mechanisms of XAS may be found in Parsons et al. (2002, 2009) and Smith et al. (2005). Synchrotron-based hard X-ray microprobe measurements of element distributions were conducted at Beamline 2-3 at the Stanford Synchrotron Radiation Lightsource (SSRL), using procedures described by Mayhew et al. (2011). Experiments were conducted with the SPEAR accelerator ring containing ~350mA in constant top-off mode. A Si (111) double crystal monochromator fully tuned at 12 keV was used to select the incident energy. Harmonic rejection was accomplished via the micro-focusing mirrors, with an energy cutoff of ~22keV. The spot size was focused to 2.5 x 2.5 μm using Pt-coated Kirkpatrick-Baez mirrors (Xradia, Inc.). The sample was rastered across the micro-focused X-ray beam at a 45° incident angle, using a pixel step size of 2.5 μm and a dwell time of 100 ms per pixel. A continuous raster scanning mode using single-element Si drift Vortex detector (SII NanoTechnology USA Inc.) was used to generate element maps of As, Ca, Cl, Cu, Fe, K, Mn, P, S, and Zn. Windowed counts for each element extracted from the full x-ray fluorescence spectrum were normalized to the intensity of the incident x-ray beam ( $I_0$ ). Regions of the sample area of particular interest for arsenic speciation mapping at the arsenic K-edge were identified from an initial 12 keV map. Arsenic K-edge speciation mapping was conducted



at 4 discrete energies (11870, 11873, 11875, and 11880 eV) to determine changes in the arsenic oxidation state as a function of location on the sample. These energies were chosen as they are at energies of unique intensities for arsenic species likely to be encountered in insects ( $\text{As}(\text{Glu})_3$ ,  $\text{As}[\text{III}]$ ,  $\text{As}[\text{V}]$ , and a total arsenic energy, respectively) (Andrahennadi and Pickering, 2008).

Multiple energy maps were achieved by raster mapping a single line at each incident monochromator energy and repeating this process at each successive line. The maps were dead time corrected and underwent principal component analysis (PCA) using the MicroAnalysis Toolkit (Webb, 2011) prior to collection of  $\mu\text{XANES}$  data. Maps of unique components were used to guide the selection of spots for  $\mu\text{XANES}$  investigation, ensuring that the  $\mu\text{XANES}$  collected would represent the variety of arsenic phases present in each of the samples. Each  $\mu\text{XANES}$  spectrum was collected from approximately 240 eV below the arsenic K-edge to 700 eV above the edge. All  $\mu\text{XANES}$  data were dead time corrected, background subtracted, and normalized to unit step edge using standard methods available in the SIXPACK software package (Webb, 2005). The monochromator was calibrated using the first inflection of an arsenate sodium salt as the reference material at 11880 eV. The short dwell times for  $\mu\text{XRF}$  mapping (50-100  $\mu\text{s}$ ) and  $\mu\text{XANES}$  analyses (8 min) minimized potential radiation damage to the samples.

#### *Data Analysis*

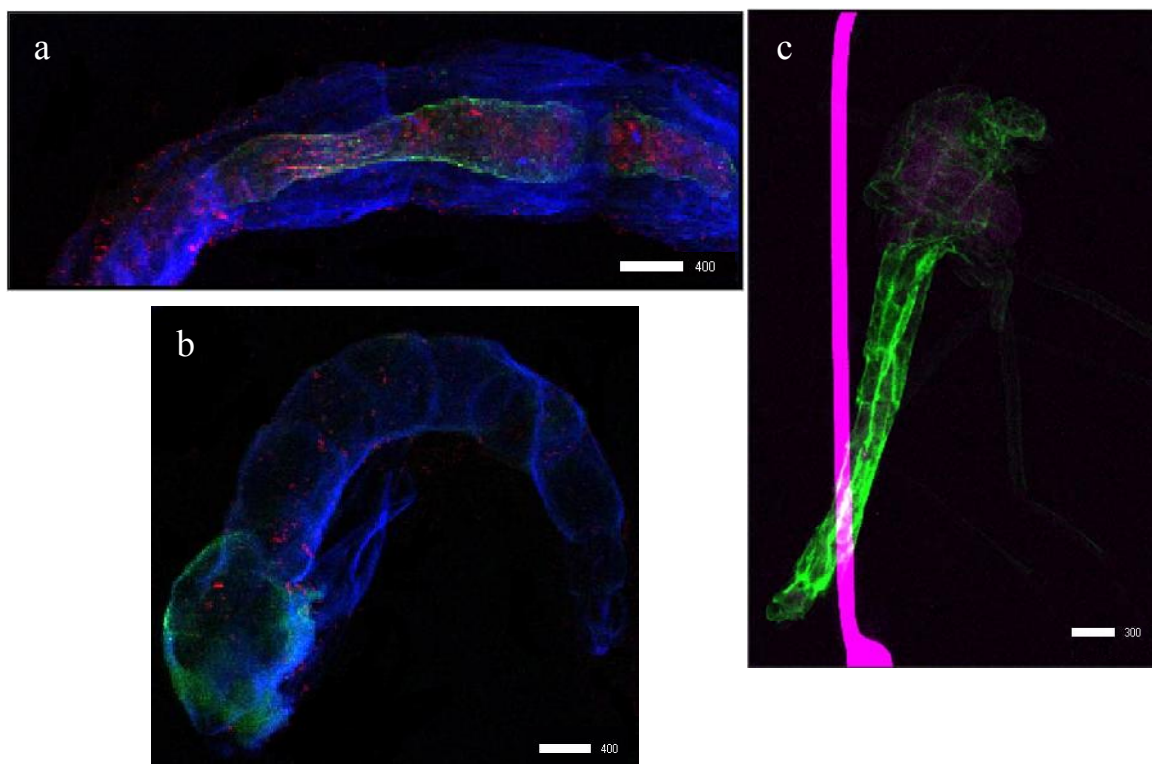
Arsenic speciation was determined by non-negative linear least squares fitting of the data. With XANES data, this was performed in the fitting section of SIXPACK, using model compounds for arsenate, arsenite, and  $\text{As}^{\text{III}}\text{-tris-glutathione}$ . For XRF image data,

the normalized intensities of each of the model compounds was determined at each of the image map energy points (11870, 11873, 11875, and 11880 eV). A non-negative linear least squares fitting was performed at each image pixel with each of these four energies to the model compounds intensities, giving the overall speciation at each pixel of the image map. Single point XANES spectra were measured to confirm the speciation as determined by the image map analysis. Results typically agreed within the nominal margin of error ( $\pm 5\%$ ).

## Results

### *Chironomus riparius*

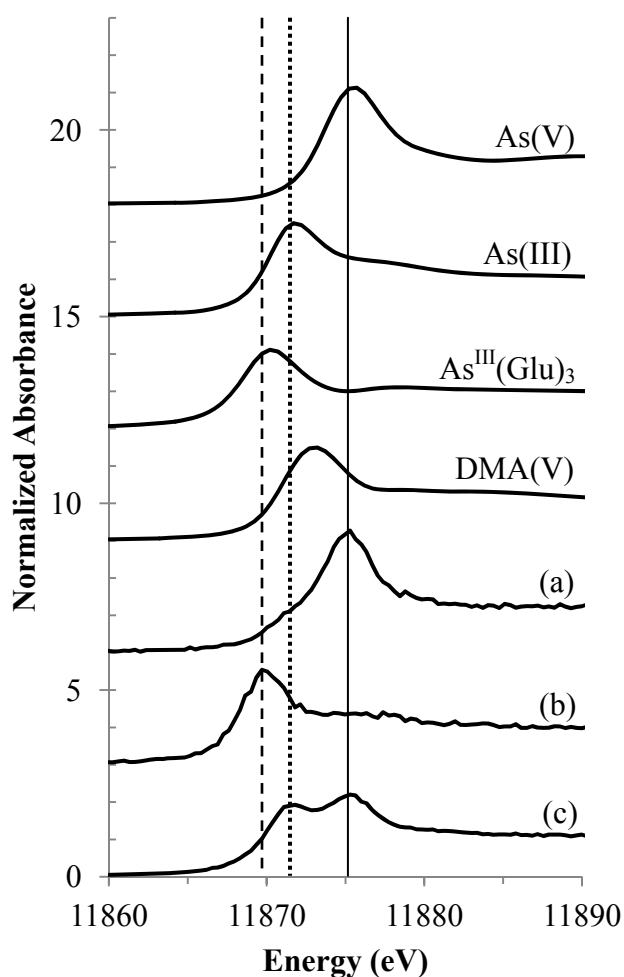
Arsenic was present in all scans of the different life stages of *C. riparius* in the x-ray fluorescence images (Figure 4.1). Although larvae were only exposed to arsenate, which is stable in solution (Al-Sibaa and Fogg, 1973; Liu et al., 2006), x-ray absorption across the arsenic K-edge revealed three species of arsenic in the samples: arsenate, arsenite, and an As-thiol, likely  $\text{As}^{\text{III}}$ -*tris*-glutathione, based on the energy absorbance (Andrahennadi and Pickering, 2008) (Figure 4.2). In second and fourth instar larvae, all three forms of arsenic were concentrated in the midgut, with none present in the fore- or hindgut. In the fourth instar larva (Figure 4.1a), XANES analysis revealed that 43.4% of whole body arsenic was present as arsenate, 29.2% was present as arsenite, and 27.4% was present as the As-thiol. There is some evidence as well for arsenate and As-thiol assimilation in the anterior region of the body, possibly in the cuticle. In addition to arsenic in the fourth instar larva, Fe, Mn, and Cu were also associated with the alimentary canal of the insect. Sulfur, P, Cl, K, and Zn were found to be constituents of the cuticle.



**Figure 4.1.** X-ray fluorescence imaging of *Chironomus riparius*. a) Fourth instar larva. Blue = potassium, Green = copper, Red = arsenic. b) Pupa. Blue = potassium, Green = copper, Red = arsenic. c) Adult female. Green = zinc, Pink = arsenic. The bright purple 'bar' running top to bottom in the picture shows the As in the glass capillary tube upon which the specimen is mounted. Scale bar units are  $\mu\text{m}$ .

Calcium and Mn were found in the Malpighian tubules (MT). The element pattern distribution was the same for second and fourth instar larvae, but the signal was stronger in fourth instar larvae.

In the pupa, there was less arsenic relative to what was found in the larva, indicating much of the larval body burdens are excreted during the molt to the pupal



**Figure 4.2.** XANES spectra from arsenic standards, and example spectra from insect tissues. a) Sample spectra of  $\text{As(V)}$  recovered from adult female chironomid. b) Sample spectra of an  $\text{As}$ -thiol from chironomid larvae. c) Sample spectra of  $\text{As(III)}$  and  $\text{As(V)}$  recovered from chironomid larvae. The solid line indicates the white line for  $\text{As(V)}$  (11875.3 eV); the dotted line indicates the white line for  $\text{As(III)}$  (11871.7 eV); the dashed line indicates the white line for  $\text{As}^{\text{III}}(\text{Glu})_3$  (11870.0).

stage (Figure 4.1b). The arsenic appeared in clusters throughout the thorax and abdomen. XANES analysis revealed that 55.1% of whole body arsenic was present as arsenate, 26.2% was present as the As-thiol, and 18.7% was present as arsenite. Arsenic, Fe, S, P, Cl, K, Ca, Mn, and Zn are associated with the cuticle of the pupa. Iron, S, P, Cl, K, along with Cu, are also associated with the head and thoracic region of the pupa, with Fe being especially concentrated in the region developing into the head.

Within the adult female, the majority of arsenic was concentrated in the thorax, likely in the exoskeleton or muscles (Figure 4.1c). In contrast to the 4<sup>th</sup> instar larva, the majority composition of arsenic shifts to As-thiol, constituting 53.3% of the total arsenic, with 23.7% as arsenate and 23.0% as arsenite. Copper was again associated with the alimentary canal. Iron, S, P, Cl, K, Mn, and Zn were also associated with the exoskeleton. Further, there was a strong signal of P, Cl, and K in the head of the adult female, with K, Ca, and Zn exhibiting a strong signal in the abdomen, either in the alimentary canal or reproductive organs.

The egg mass contained very low, yet still detectable, amounts of arsenic present in the gelatinous sheath of the egg mass (not pictured). The signal of arsenic in the sample was not strong enough to differentiate between arsenic species; however, this does provide some evidence for maternal transfer of arsenic. Though arsenic is not present within the eggs themselves, first instar larvae have been observed to feed on the gelatinous sheath upon hatching (C. M., personal observation), and could therefore be exposed to minute quantities of arsenic early on. Phosphorus, Cl, Ca, Mn, and Cu were also found in the gelatinous sheath, while Fe, S, P, K, and Zn were found within the eggs.

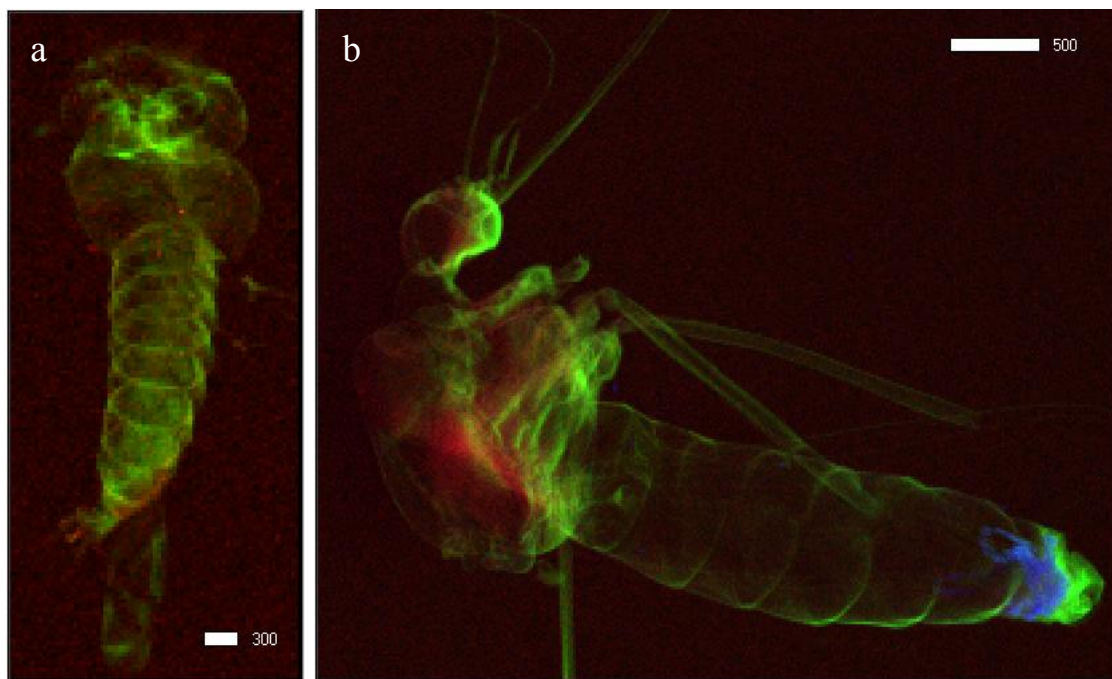
### *Culex tarsalis*

In contrast to *C. riparius*, the larva of *Cx. tarsalis* did not contain a high degree of differentiation with regards to organ structure that was apparent in the XAS images. Arsenic was found to be distributed throughout the body of the fourth instar larva, as opposed to being concentrated in the midgut (Figure 4.3a). In the adult female, arsenic was concentrated in the thorax, likely in the exoskeleton, as in *C. riparius* (Figure 4.3b). Arsenic fluorescence was also seen in the adult eye. For both the larva and the adult, although arsenic was detected in the samples, the fluorescence signal strength was not strong enough to distinguish between different oxidation states.

In immature *Cx. tarsalis*, Fe was found in the alimentary canal; Cu, Ca, K, Cl, S, and P were found in the cuticle, and Cu and Zn were concentrated in the posterior portion of the abdomen, possibly in the MT. In the adult female, Fe, Cu, Ca, K, Cl, S, and P were associated with the cuticle, while Zn, Mn, Ca, and P were associated with the MT.

## **4. Discussion**

Many invertebrates are capable of storing and detoxifying metals and other toxicants they encounter in the environment using a variety of mechanisms (Hopkin, 1989). The ability of insects to detoxify arsenic and the mechanisms by which they do so specifically has not received much attention in the literature. With this study we sought to generate initial information to help elucidate arsenic reduction and excretion pathways in aquatic dipterans. Although XAS analysis cannot provide direct information on molecular detoxification, it can provide corollary information with regards to biotransformation and localization of arsenic within whole insect samples.



**Figure 4.3.** X-ray fluorescence imaging of *Culex tarsalis*. a) Fourth instar larva. Green = potassium, Red = arsenic. b) Adult female. Blue = manganese, Green = potassium, Red = arsenic. Scale bar units are  $\mu\text{m}$ .

In a previous study, *C. riparius* was shown to excrete 72% of total body burdens of arsenic between the fourth instar and adult stages (Mogren et al., 2012). Elimination of other toxicants between larval and adult stages has also been demonstrated (e.g. Cd, Groenendijk et al., 1999), and as much as 75% of total body burdens were excreted during molts of fiddler crabs (Bergey and Weis, 2007). In the present study, we demonstrate that the loss of arsenic in *C. riparius* between the larval and adult stages occurs specifically between the larval and pupal stages, as indicated by the relatively low levels of arsenic recovered in the pupa. However, because very little arsenic was found to

accumulate in the exoskeleton of the larvae, we hypothesize that arsenic is not excreted via the exoskeleton, but could be lost in a meconium (defecation between fourth instar and pupal stages). This is evidence of limited movement of arsenic from the aquatic to the terrestrial environment via insects, but additional research is needed to document actual transfer rates.

Specific organs have been proposed as being crucial to detoxification of toxic compounds in insects, such as the Malpighian tubules (MT) and midgut (Hopkin, 1989). In insects, the MT, located at the anterior portion of the hindgut, function in filtering wastes and solutes from the hemolymph; this pre-urine passes into the hindgut and is excreted from the anus (Klowden, 2007). Their ability to filter soluble ions from the hemolymph and form concretions has marked them as detoxification organs. However, in *C. riparius* larvae, Mn is the chief element present in the MT (resulting from Mn exposures in the food), and no arsenic was detected. Therefore, it can be concluded that in this species, the MT do not play a significant role in arsenic detoxification or excretion. The lack of arsenic in the MT of *Cx. tarsalis* further supports the notion that this organ does not play a significant role in arsenic detoxification and excretion in these aquatic Diptera. The absence of As in the MT may be the result of ionic differences between metals (cations) and metalloids (anions).

Although there was no arsenic to be found in the midgut of *Cx. tarsalis*, in *C. riparius* the high concentration of arsenic in the midgut indicates this organ may be important in detoxification. Hare et al. (1991) found that the distribution patterns of trace element contaminants in freshwater insect tissues varied between taxa, but contrary to the



present study, reported the majority of arsenic incorporated into the body versus the midgut in *Hexagenia* mayflies. This difference may be due to the longer lifespan of mayflies, which in turn allows for arsenic incorporation into fat bodies. Arsenic being found in the midgut of *C. riparius* may be the result of cellular mineral inclusions, or concretions, sequestering excess arsenic ions in spherocrystals (Ballan-Dufrançais, 2002). Spherocrystals typically trap unusual cations but there is some evidence for limited uptake of arsenic in cells containing mineral inclusions. However, in the case of *Formica polyctena* (Hymenoptera: Formicidae), arsenic was mostly concentrated in organs devoid of spherocrystals (Jeantet et al., 1977). Arsenic in the eye of adult *Cx. tarsalis* may result from incorporation in ommochrome pigment granules, which has been observed in Orthoptera (White and Michaud, 1980).

Because larvae were exposed to inorganic arsenate, which is stable in solution (Al-Sibaai and Fogg, 1973; Liu et al., 2006), it can be concluded that the reduction of arsenate to arsenite in the larval midgut, in addition to the presence of As-thiol species, is the result of biotransformation and/or detoxification pathways in the larvae. Although the reduction of arsenate to arsenite results in a more toxic form of arsenic within the larva, As-thiols are indicative of arsenic binding cysteine residues in either glutathione or metallothionein (Andrahennadi and Pickering, 2008). In their study of *Formica* ants from an old arsenic smelter site, Kuehnelt et al. (1997) reported arsenate and arsenite as the major arsenical compounds recovered from the ants, with dimethylarsinic acid and traces of methylarsonic acid and arsenobetaine as well. More recently, XAS analysis has revealed that *Formica* ants store mainly inorganic arsenate and arsenite, though the

concentration of arsenic in the midgut is not apparent (Moriarty et al., 2009). In contrast, midgut differentiation is observed in larvae of the bertha armyworm, *Mamestra configurata* (Andrahennadi and Pickering, 2008). Further, *M. configurata* biotransforms inorganic arsenate to an As-thiol, modeled as As<sup>III</sup>-tris-glutathione (Andrahennadi and Pickering, 2008; Parsons et al., 2009). Sulfur coordination in arsenic metabolism was also documented to occur in the earthworm *Lumbricus rubellus* (Langdon, 2002, 2005). In *Lumbricus terrestris*, arsenobetaine, methylarsonate, dimethylarsinate, trimethylarsineoxide, and arsenosugars 1 and 2 were recovered (Button et al., 2011), indicating that even within the same genus, arsenic metabolism may differ. There is also the possibility that the observed thiolation of arsenite in *C. riparius* results from endosymbiont-mediated biotransformations (Basu et al., 2010), though further research is needed to explore this in depth.

XAS analysis did not reveal compartmentalization of arsenic in *Cx. tarsalis* as is observed in *C. riparius*. The greatly reduced concentrations of arsenic in this mosquito species may therefore be due to efficient excretion mechanisms not present in *C. riparius*. Other elements are also accumulated differently (e.g. Cu is not concentrated in the larval midgut), indicating possible physiological differences in the overall handling of metals and arsenic in *Cx. tarsalis*.

With regards to where the other measured elements accumulated within *C. riparius* and *Cx. tarsalis*, a summary is provided in Table 4.1. There are interesting differences between these two species and other insects that have been evaluated for metals accumulation. In *Formica* ant workers, Zn was largely associated with the MT and

**Table 4.1.** Element distributions for the a) fourth instar and pupa of *C. riparius* and the fourth instar of *Cx. tarsalis*, and b) the adult females of *C. riparius* and *Cx. tarsalis*.

a.	Alimentary Canal		Cuticle		Malpighian Tubules	Head		Abdomen	
	<i>C. riparius</i>	<i>Cx. tarsalis</i>	<i>C. riparius</i>	<i>Cx. tarsalis</i>	<i>C. riparius</i>	<i>C. riparius</i>		<i>C. riparius</i>	<i>Cx. tarsalis</i>
4th instar larva	As, Cu, Fe, Mn	Fe	Cl, K, P, S, Zn	Ca, Cl, Cu, K, P, S	Ca, Mn				As, Cu, Zn
Pupa			As, Ca, Cl, Fe, K, Mn, P, S, Zn			As, Cl, Cu, Fe, K, P, S		As, Cl, Cu, Fe, K, P, S	

b.	Alimentary Canal	Cuticle		Head		Thorax		Abdomen	
	<i>C. riparius</i>	<i>C. riparius</i>	<i>Cx. tarsalis</i>	<i>C. riparius</i>	<i>Cx. tarsalis</i>	<i>C. riparius</i>	<i>Cx. tarsalis</i>	<i>C. riparius</i>	<i>Cx. tarsalis</i> <sup>a</sup>
Adult female	Cu	Cl, Fe, K, Mn, P, S, Zn	Ca, Cl, Cu, Fe, K, P, S	Cl, K, P	As	As	As	Ca, K, Zn	Ca, Mn, P, Zn

<sup>a</sup>The Ca, Mn, P, and Zn found in the abdomen of *Cx. tarsalis* were associated with the Malpighian tubules specifically.

cuticle; Cu was associated with the midgut, MT, and cuticle; Fe was associated with the midgut and MT, and Mn was associated with the midgut and MT (Rabitsch, 1997). In *Gammarus pulex*, Mn, Fe, Cu and arsenic were associated with the gut, while Ca and Zn are associated with the cuticle (Schaller et al., 2011; Khan et al., 2012). In the collembolan *Tomocerus minor*, Ca, K, Mn, S, Cl, and P were associated with the midgut (Humbert, 1978). Zinc, Mg, P, Ca, and K were significantly enriched in the MT of the termite *Tumulitermes tumuli* (Stewart et al., 2011). Ballan-Dufrançais (2002) reviews the distributions of metals in the organs of other insects. Whereas Andrahennadi and Pickering (2008) found Mn in the cuticle of *M. configurata*, in this study we found Mn in the MT of *C. riparius* and adult *Cx. tarsalis*. Variations between these different species in metal accumulation and localization may result from differing element makeup in their respective diets, or reflect differences in physiological processing of metals.

With regards to arsenic localization and biotransformation in aquatic Diptera, it appears that in these two species there are marked differences in how the insects are able to mediate toxicity of arsenic. These differences are also reflected in differing distributions of other elements in the midgut and MT. Though the MT do not appear to serve an important role in detoxification of arsenic as it does with other toxicants, the midgut appears to be of particular importance for *C. riparius*. More research is needed to determine if the insect is mediating its own toxicity to environmental toxicants, or if endosymbionts play a role in helping insects to adapt to stressful environments.

## **CHAPTER 5**

**An introduction to the effects of contaminants on insect behavior: The impacts of  
metals and metalloids on insect behavior**

## **Abstract**

In toxicology studies, the use of death as an endpoint often fails to capture the effects a pollutant has on disruptions to ecosystem services by changing an animal's behavior. Many toxicants can cause population extinctions of insect species at concentrations well below the EC<sub>25</sub>, EC<sub>50</sub>, or EC<sub>90</sub> concentrations traditionally reported from short-term bioassays. A surprising number of species cannot detect metal and metalloid contamination, and do not always avoid food with significant metal concentrations. This frequently leads to modified ingestion, locomotor, and reproductive behaviors. For example, some species show a tendency to increase locomotor behaviors to escape from locations with elevated metal pollution, whereas other insects greatly decrease all movements unrelated to feeding. Still others exhibit behaviors resulting in increased susceptibility to predation, including a positive phototaxis causing immatures to move to exposed positions. For purposes of reproduction, the inability to avoid even moderately polluted sites when ovipositing can lead to egg loss and reduced fitness of offspring. Ultimately, impaired behaviors result in a general reduction in population sizes and species diversity at contaminated sites, the exceptions being those species tolerating contamination that become dominant. Regardless, ecosystem services, such as herbivory, detritus reduction, or food production for higher trophic levels, are disrupted. This review evaluates the effects of metal and metalloid pollution on insect behaviors in both terrestrial and aquatic systems reported in a diverse literature scattered across many scientific disciplines. Behaviors are grouped by ingestion, taxis, and oviposition. We

conclude that understanding how insect behavior is modified is necessary to assess the full scope and importance of metal and metalloid contamination.

## **Introduction**

Despite the importance of insects in most ecosystems, and the worldwide pollution of systems by heavy metals, surprisingly little information is available on the effects of metal and metalloid pollution on the behaviors of insects. Stark and Banks (2003) reviewed the available literature on toxicant effects on insects and determined that 95% of the reports used the lethal concentration,  $LC_{50}$  (50% of insects die), or simple mortality as a toxicological endpoint. Although short-term assays are efficient and allow for comparisons between compounds and insect species, they may not always provide a clear ecological picture on the potential effects of contaminants. For example, toxicants can affect populations enough to cause extinction at levels well below the LC values reported in the literature (Bechmann, 1994). Because insect behaviors are key contributors to the ecology of insect interactions with other plant and animal species, as well as with their abiotic environments, these behaviors are critical to the stability and diversity of ecosystems (Fisher, 1998). Thus, our review focuses on ecologically important behaviors related to ingestion, taxis, and reproduction as affected by natural and anthropogenic sources of a widespread class of pollutants: the heavy metals and metalloids.

Although there are many natural sources of elevated concentrations of metals (Boyd, 2004), anthropogenic activities such as mining, smelting, and industrial use have created both localized and regional pollution problems in nearly every country in the world (Nriagu, 1996). In some cases the pollution has been extensive enough to lead to environmental disasters and ecosystem shutdown (Hopkin, 1989; Sainz et al., 2004).



Insects may be exposed by direct contact with dissolved elements in aquatic systems, via contact with contaminated soils, through airborne pollution or atmospheric deposition, and through herbivory on plants that have sequestered these materials. Predators and parasites are also exposed when feeding on insects that contain elevated concentrations of these elements (Vickerman and Trumble, 2003). A few of the more common metal and metalloid pollutants that are discussed in this paper are briefly characterized below. A detailed description of every metal is beyond the scope of this paper, instead a few key references have been included.

Zinc (Zn) is a common metal in the earth's crust, averaging about 75 mg/kg soil (Emsley, 2001). For animals, Zn is an essential element; however, levels of 100-250 mg/d can cause significant health effects (OhioEPA, 2002). Zinc is commonly used in manufacturing of paints, dyes, wood preservatives, and rubber (Emsley, 2001). Zinc compounds found at industrial sites, at mines and nearby watersheds, and in sludge spread on agricultural fields include zinc chloride, zinc oxide, zinc sulfate, and zinc sulfide. In field conditions, hyperaccumulator plants may accumulate in excess of 12,000 mg/Zn kg dry weight (Deng et al., 2006).

Copper (Cu), which has been mined throughout the world for thousands of years, has many industrial uses. Widespread pollution has resulted from mining and smelting, brake dust from automobiles, uses as a marine antifoulant, the spreading of sewage sludge on agricultural lands, and the application of Cu as a fungicide in agriculture (Hutchinson and Whitby, 1974). Soil levels can exceed 2,890 mg Cu/kg, whereas concentrations in Cu-accumulating plants have been reported above 1,300 mg/kg

(Fernandes and Henriques, 1991). Because of the ubiquitous use in both developing and developed countries, and a long history of smelters releasing copper as an air pollutant, Cu pollution occurs on nearly all continents and in most countries (Nriagu, 1996). As a result, insects frequently interact with elevated concentrations of Cu either through atmospheric deposition or by uptake and sequestration by plants.

Dissolved inorganic selenium (Se), in the form of sodium selenate, is sequestered by many plants. This material is available directly to aquatic insects, or may be modified into a form bioavailable to herbivores which can be either inorganic (sodium selenite) or organic (selenomethionine and selenocystine). For most plants the total Se concentration rarely exceeds 50 mg/g, but some hyperaccumulators may have total Se levels exceeding 5,000 mg/g (Galeas et al., 2007). Selenium is a common pollutant in most Pacific Rim countries, and this metalloid is a major pollutant in the western United States where large deposits are leached by rainfall and irrigation practices (McNeal and Balistrieri, 1989). In water collection sites without outlets, such as the Kesterson Reservoir in central California, concentrations can exceed 1,400 µg/l (Wu, 2004). However, most available studies examine the effects of Se at much lower concentrations. Selenium can also reach high concentrations in vegetation found near coal burning power plants and some industrial sites (Huggins et al., 2007; McNeal and Balistrieri, 1989).

Arsenic (As), commonly found as arsenate, is an important pollutant of groundwater that is often used for drinking and irrigation. Arsenic contamination has become a significant problem in Southeast Asia, where concentrations in well water may exceed 3,000 µg As/l, and levels in soils can exceed 30 µg/g (Berg et al., 2007). Arsenic

contamination results from natural and anthropogenic disturbance of rock, resulting in oxidation and release of inorganic forms of As (arsenate [As(V)] and arsenite [As(III)]), which are available to plants. These form arsenobetaine and arseno-sugars (among other compounds), which may be complexed with phytochelatins, which are important in heavy metal detoxification (Meharg and Hartley-Whitacker, 2002). Some plants reduce arsenate to arsenite, and then further transform it into several methylated forms (Zaman and Pardini, 1996). Thus, insects are likely exposed to a range of As species. Extreme As concentrations in surface waters, soils, and plants have been reported to result from mining effluent (particularly at gold mines) (Eisler, 2004) and as a result of burning coal high in As (Huggins et al., 2007). At industrial sites, As levels can reach up to 38,000 µg/l in water that is available to plants (Cappuyns et al., 2002). Arsenic tolerant vegetation can sequester concentrations of 500 to nearly 3,500 µg/g (Porter and Peterson, 1975). However, most reports on crops describe concentrations that are 75 µg/g or less (see references in Meharg and Hartley-Whitacker, 2002).

Another widespread pollutant is cadmium (Cd), with contamination resulting from the application of sludge or urban composts, pesticides, fertilizers, emissions from waste incinerators, waste water irrigation, and residues from metalliferous mining and metal smelting (McGrath et al., 2001). Though unlikely to affect plant growth, Cd negatively influences enzymatic systems of cells in higher organisms as a result of transfer up the food chain (Sanità and Gabbrielli, 1999) and has a very long soil residency time. The US Environmental Protection Agency (USEPA) considers Cd a priority toxic pollutant, with an acute exposure limit in freshwater of 2.0 mg/l for up to 10 days and a

chronic exposure limit of 0.25 mg/l (USEPA, 2001). In soils intended for agricultural use, acceptable limits range from 1 to 8 mg Cd/kg soil dry weight, depending on pH (Environment Agency, 2002).

Although understanding the individual effects of metals and metalloids is important, most metals occur in combination, and joint effects must be evaluated. Yang (1994) reviewed the literature on the toxicology of metals to all classes of organisms and determined that >95% of all journal articles reported the effects of individual compounds or elements. In combination, effects may not simply be additive, but possibly potentiating or antagonistic. For example, the joint toxicity of mercury and selenium to an insect detritivore, the phorid fly *Megasilia scalaris* Loew, was strongly potentiating, with just 5% of the LC<sub>50</sub>'s of the two elements combined producing significantly increased developmental time and significantly greater mortality than the LC<sub>50</sub> of either element alone (Jensen et al., 2006). Where available, we have included the literature that provides information on the joint effects of metals on insect behaviors. However, we recognize that in some cases the concentrations/mixtures will be difficult to replicate exactly (particularly in field studies).

## **Methods**

ISI Web of Knowledge databases were searched using terminology including metals, metalloids, behavior, insects, and pollution. In order to be considered, papers were required to meet the following criteria: report quantifiable data regarding insect behavioral responses to metal pollutants, include a control or reference concentration, and include statistical analyses comparing test data. A substantial proportion of papers

(>50%) did not include control or reference concentrations, or described behaviors without including data or analyses to verify that behaviors changed as a result of the contaminant. Once suitable papers were obtained, their references were examined, and ISI Web of Knowledge searches conducted for any recent papers citing those already obtained. A total of 75 papers meeting the above criteria were found. Papers from both terrestrial and aquatic systems were considered, evaluating the behavioral responses of first instars through adults. Behaviors were further divided into three categories: ingestion, taxis (locomotion), and reproductive (oviposition) behaviors. See Tables 5.1 and 5.2 for a summary of metals, species, and behavioral outcomes for these categories.

## **Results**

### *Terrestrial systems*

#### Ingestion behavior

The majority of research concerning feeding behaviors of terrestrial insects has investigated the effects of metals on feeding preference when individuals were exposed to various concentrations and combinations of metals. For herbivorous insects, this has focused mostly on antifeedant properties of metals on agricultural pests. Zinc, Cu, Ni, Se, and As have been evaluated individually, whereas several other studies examined combinations of metals.

Zinc sulfate has been extensively studied, particularly in experiments involving first-instar lepidopteran pests. Zinc salts are known to cause toxicity in insects and their

**Table 5.1** Summary of contaminants and the resultant behavioral outcome observed for insect species in terrestrial habitats. Only individual metals/metalloids are considered for behavioral outcomes because mixtures may lead to synergistic or antagonistic interactions otherwise unaccounted for in the behavioral response. Positive and negative outcomes correspond to stimulation or suppression of the particular behavior as a result of metal presence, respectively, and ‘no effect’ means the organism was unaffected at the experimental conditions. \* indicates the behavioral outcome was only observed at high concentrations and the organism was unaffected at lower concentrations. \*\* indicates that there was an initial positive response to low concentrations vs. controls that then became negative as concentrations increased. In choice assays, aversion results in a negative behavioral outcome. See text for the measured concentrations.

Metal / metalloid	Form	Species	Behavioral Outcome	Reference
A. Ingestion behavior				
Arsenic	Na <sub>2</sub> HAsO <sub>4</sub>	<i>Schistocerca americana</i>	Negative	Rathinasabapathi et al., 2007
Cadmium	Cd	<i>Chorthippus spec.</i>	Negative	Migula & Binkowska, 1993
		<i>Lochmaea capreae</i>	No effect	Rokytořová et al., 2004
		<i>Neochetina bruchi</i>	Negative	Jamil et al., 1989a,b
	CdCl <sub>2</sub>	<i>Neochetina eichhorniae</i>	No effect	Kay & Haller, 1986
		<i>Frankliniella occidentalis</i>	Negative	Jiang et al., 2005
	Cd(NO <sub>3</sub> ) <sub>2</sub>	<i>Agasicles hygrophila</i>	Negative	Quimby et al., 1979
		<i>Bactra verutana</i>	No effect	Quimby et al., 1979
		<i>Folsomia candida</i>	Negative	Fountain & Hopkin, 2001
	3CdSO <sub>4</sub> ·8H <sub>2</sub> O	<i>Drosophila melanogaster</i>	Negative	Bahadorani & Hilliker, 2009

Copper	Cu	<i>Folsomia candida</i>	Negative	Filser & Hölscher, 1997
		<i>Neochetina eichhorniae</i>	No effect	Kay & Haller, 1986
	CuCl <sub>2</sub> ·3Cu(OH) <sub>2</sub>	<i>Folsomia manolachei</i>	Positive	Filser et al., 2000
		<i>Folsomia quadrioculata</i>	Positive	Filser et al., 2000
		<i>Isotomurus palustris</i>	No effect	Filser et al., 2000
		<i>Onychiurus armatus</i>	Negative	Filser et al., 2000
	Cu(NO <sub>3</sub> ) <sub>2</sub>	<i>Folsomia candida</i>	Negative	Fountain & Hopkin, 2001
	CuSO <sub>4</sub>	<i>Drosophila melanogaster</i>	Negative	Bahadorani & Hilliker, 2009
		<i>Leptinotarsa decemlineata</i>	Negative	El-Bassiouny, 1991
		<i>Mamestra brassicae</i>	Negative*	El-Bassiouny, 1991
		<i>Mamestra oleracea</i>	Negative*	El-Bassiouny, 1991
		<i>Pieris brassicae</i>	Negative	El-Bassiouny, 1991
		<i>Pieris napi</i>	Negative	El-Bassiouny, 1991
		<i>Orchesella cincta</i>	Negative	Nottrot et al., 1987
		<i>Drosophila melanogaster</i>	Negative	Bahadorani & Hilliker, 2009
Iron	Fe	<i>Orchesella cincta</i>	Negative	Nottrot et al., 1987
	FeSO <sub>4</sub> ·7H <sub>2</sub> O	<i>Drosophila melanogaster</i>	Negative	Bahadorani & Hilliker, 2009
		<i>Drosophila melanogaster</i>	Negative	Bahadorani & Hilliker, 2009
Lead	Pb	<i>Chorthippus spec.</i>	Negative	Migula & Binkowska, 1993
		<i>Folsomia candida</i>	Negative*	Fountain & Hopkin, 2001
		<i>Lochmaea capreae</i>	No effect	Rokyťová et al., 2004
		<i>Neochetina eichhorniae</i>	No effect	Kay & Haller, 1986
	Pb(NO <sub>3</sub> ) <sub>2</sub>	<i>Leptinotarsa decemlineata</i>	Negative	Kwartirnikov et al., 1999
		<i>Orchesella cincta</i>	No effect	van Capelleveen et al., 1986
		<i>Lochmaea capreae</i>	Negative	Rokyťová et al., 2004
		<i>Orchesella cincta</i>	No effect	Nottrot et al., 1987
Manganese	Mn	<i>Aphididae</i>	No effect	Boyd & Martens, 1999; Jhee et al., 2005
Nickel	Ni	<i>Delia radicum</i>	Negative	Jhee et al., 2005
		<i>Evergestis rimosalis</i>	Negative	Jhee et al., 2005

Selenium	Se	<i>Melanoplus femurrubrum</i>	Negative	Jhee et al., 2005
		<i>Pieris rapae</i>	Negative	Martens & Boyd, 1994
		<i>Plutella xylostella</i> G88	Negative	Freeman et al., 2006
		<i>Plutella xylostella</i> Stanleyi	No effect	Freeman et al., 2006
		<i>Pieris rapae</i>	Negative	Freeman et al., 2006
	Na <sub>2</sub> SeO <sub>3</sub>	<i>Spodoptera exigua</i>	Negative	Vickerman & Trumble, 1999
	Na <sub>2</sub> SeO <sub>4</sub>	<i>Acheta domestica</i>	Negative	Freeman et al., 2007
		<i>Myzus persicae</i>	Negative	Hanson et al., 2004
		<i>Spodoptera exigua</i>	Negative	Vickerman & Trumble, 1999; Vickerman et al., 2002b
	Seleno-cystine	<i>Spodoptera exigua</i>	No effect	Vickerman & Trumble, 1999
	Seleno-methionine	<i>Spodoptera exigua</i>	No effect	Vickerman & Trumble, 1999
Zinc	Zn	<i>Lochmaea capreae</i>	Negative	Rokyťová et al., 2004
		<i>Neochetina bruchi</i>	Negative	Jamil et al., 1989a,b
	ZnCl <sub>2</sub>	<i>Drosophila melanogaster</i>	Negative	Bahadorani & Hilliker, 2009
	Zn(NO <sub>3</sub> ) <sub>2</sub>	<i>Folsomia candida</i>	Negative	Fountain & Hopkin, 2001
	ZnSO <sub>4</sub>	<i>Heliothis virescens</i>	Negative	Sell & Bodznick, 1970
		<i>Ostrinia nubialis</i>	Negative	Gahukar, 1975
		<i>Pieris brassicae</i>	Negative	Pollard & Baker, 1997
		<i>Schistocerca gregaria</i>	Negative	Pollard & Baker, 1997
		<i>Schistocerca gregaria</i>	Negative	Behmer et al., 2005
		<i>Spodoptera littoralis</i>	Negative	Salama & El-Sharaby, 1972

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#### B. Taxis behavior

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Copper	CuCl <sub>2</sub> x	<i>Folsomia manolachei</i>	Negative	Filser & Hölscher, 1997
	3Cu(OH) <sub>2</sub>	<i>Mesophorura macrochaeta</i>	Negative	Filser & Hölscher, 1997
		Other Collembola	No effect	Filser & Hölscher, 1997; Filser et al., 2000



		<i>Pseudosinella alba</i>	Negative	Filser et al., 2000
		<i>Pterostichus cupreus</i>	Negative	Bayley et al., 1995
	CuSO <sub>4</sub>	<i>Drosophila melanogaster</i>	Negative	Bahadorani & Hilliker, 2009
Iron	FeSO <sub>4</sub> ·7H <sub>2</sub> O	<i>Drosophila melanogaster</i>	Positive	Bahadorani & Hilliker, 2009
Zinc	ZnCl <sub>2</sub>	<i>Drosophila melanogaster</i>	No effect	Bahadorani & Hilliker, 2009
<hr/> C. Oviposition <hr/>				
Cadmium	3CdSO <sub>4</sub> ·8H <sub>2</sub> O	<i>Drosophila melanogaster</i>	Negative	Bahadorani & Hilliker, 2009
Chromium	Cr(VI)	<i>Megaselia scalaris</i>	No effect	Trumble & Jensen, 2004
Copper	CuSO <sub>4</sub>	<i>Drosophila melanogaster</i>	Negative	Bahadorani & Hilliker, 2009
Iron	FeSO <sub>4</sub> ·7H <sub>2</sub> O	<i>Drosophila melanogaster</i>	Negative**	Bahadorani & Hilliker, 2009
	Fe <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub> ·xH <sub>2</sub> O	<i>Drosophila melanogaster</i>	Negative**	Bahadorani & Hilliker, 2009
Selenium	Se	<i>Plutella xylostella</i> G88	Negative	Freeman et al., 2006
		<i>Plutella xylostella</i> Stanleyi	No effect	Freeman et al., 2006
		<i>Pieris rapae</i>	Negative	Freeman et al., 2006
	Na <sub>2</sub> SeO <sub>4</sub>	<i>Spodoptera exigua</i>	Positive	Vickerman et al., 2002b
		<i>Spodoptera exigua</i>	No effect	Vickerman et al., 2002a
Zinc	ZnCl <sub>2</sub>	<i>Drosophila melanogaster</i>	Negative**	Bahadorani & Hilliker, 2009

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abilities as feeding deterrents for agricultural pests were quantified. *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) responded to Zn sulfate treatments in choice experiments by avoiding meridic diet containing concentrations  $\geq 0.1\%$  ZnSO<sub>4</sub> (Gahukar, 1975). Although variability in individual responses was high and not always significant,

aversion increased with increasing ZnSO<sub>4</sub> in the diet. These results are consistent with the results obtained by Sell and Bodznick (1971) for *Heliothis virescens* Fabricius (Lepidoptera: Noctuidae), which was deterred by concentrations  $\geq 0.2\%$  ZnSO<sub>4</sub>, and *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae), which was deterred by concentrations  $\geq 0.1$  M ZnSO<sub>4</sub> (Salama & El-Sharaby, 1972). Similarly, Pollard and Baker (1997) demonstrated significant preference of *Schistocerca gregaria* (Forskål) (Orthoptera: Acrididae) and *Pieris brassicae* (L.) (Lepidoptera: Pieridae) for low Zn treatments over high Zn treatments in choice experiments. Behmer et al. (2005) came to the same conclusion in choice experiments, and further showed that *S. gregaria* learned associatively to avoid Zn-treated foods. Behavioral responses of insect predators and parasitoids to zinc sulfate-contaminated prey have not been reported.

Gustatory perception assays with adult *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) showed that they also prefer low Zn treatments to high Zn treatments (Bahadorani and Hilliker, 2009). These authors observed the same pattern for Fe (II) and Fe (III), while adults preferred control diets to Cd- and Cu-contaminated diets significantly more often. Like adults, larvae also avoided feeding on high concentrations of heavy metals (Bahadorani and Hilliker, 2009).

Copper, as copper sulfate (CuSO<sub>4</sub>), has also been investigated as a feeding deterrent against agricultural pests. Mixing CuSO<sub>4</sub> with lime creates a Bordeaux mixture effective as a fungicide ([www.copper.org](http://www.copper.org)). El-Bassiouny (1991) investigated possible feeding deterrent properties for several lepidopteran species. These responded to CuSO<sub>4</sub> feeding deterrents with mixed responses, depending on species. Oligophagous species [*P.*

*brassicae* and *Pieris napi* (L.)] were deterred at lower concentrations (0.05-0.1 M CuSO<sub>4</sub>), whereas polyphagous species (*Mamestra brassicae* L. and *Mamestra oleracea* L.) were only inhibited by higher concentrations (0.2 M CuSO<sub>4</sub>) (El-Bassiouny, 1991). *Pieris brassicae* took shorter meals before feeding ceased, and experienced an increase in palpation frequency.

Some research has focused on hyperaccumulating plants and documented that high levels of metals in plant tissues may serve to deter herbivory. For example, herbivorous insects preferred *Streptanthus polygaloides* Gray (Brassicaceae) grown in low nickel (Ni) soils (15.6-76.5 µg/g) vs. high Ni soils (1 820-7 960 µg/g) (Jhee et al., 2005). These included the folivores *Melanoplus femurrubrum* (De Geer) (Orthoptera: Acrididae), *Evergestis rimosalis* Guenée (Lepidoptera: Pyralidae), and the rhizovore *Delia radicum* L. (Diptera: Anthomyiidae). *Pieris rapae* also preferred unamended to treated plants (180 mg Ni/kg soil and 7,400 mg Ni/kg soil, respectively) (Martens and Boyd, 1994). The feeding behaviors of aphids and other vascular feeding insects were not altered by Ni accumulation in plants (Boyd and Martens, 1999; Jhee et al., 2005).

The lepidopteran, *Spodoptera exigua* Hübner, exposed to different forms of Se were deterred from feeding by inorganic Se compounds (sodium selenate and sodium selenite) at LC<sub>30</sub> values and greater for first and third instars (Vickerman and Trumble, 1999). In contrast, this same study revealed that organic Se compounds did not serve as feeding deterrents for third-instar *S. exigua*, though first instars preferred controls to these compounds 50-75% of the time. Sodium selenate accumulated by *Brassica juncea* (L.) Czern. (Brassicaceae) also effectively prevented *Acheta domestica* (L.) (Orthoptera:

Gryllidae) feeding in choice experiments (Freeman et al., 2007) with 5× as many crickets preferring controls to treated leaves ( $546 \pm 38 \mu\text{g Se/g dry weight}$ ). At 10 mg/kg dry leaf weight, *B. juncea* with incorporated sodium selenate also successfully deterred *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) feeding and prevented colonization (Hanson et al., 2004). When fed alfalfa with incorporated Se, first-instar *S. exigua* were unable to distinguish between low and high ( $2.88 \pm 0.52$  vs.  $305.81 \pm 52.14 \mu\text{g/g plant dry weight}$ ) concentrations of Se compared to controls (Vickerman et al., 2002b). Fourth instars did not differentiate between low Se and controls, but avoided high Se plants. Alternatively, various polyphagous acridid grasshoppers chose low-Se *Stanleya pinnata* (Pursh) Britton significantly more often than high-concentration alternatives (1 vs. 230  $\mu\text{g/g dry weight}$ ) in choice experiments (Freeman et al., 2007). A recently discovered biotype of a lepidopteran, *Plutella xylostella* Stanleyi (Lepidoptera: Plutellidae), was shown to withstand accumulated concentrations of 2,000  $\mu\text{g Se/g dry weight}$  on *S. pinnata* and larvae showed no preference for low or high (47 vs. 792  $\mu\text{g/g dry weight}$ ) Se-treated plants in choice experiments, as opposed to *P. xylostella* G88 and *P. rapae* which avoided higher concentrations (Freeman et al., 2006).

Although there are many studies reporting the effects of arsenic on insects, relatively few report behavioral impacts. In terms of ingestion behaviors, only a single paper was found. Rathinasabapathi et al. (2007) reported avoidance by *Schistocerca americana* (Drury) of lettuce contaminated with As when given a choice with low-As treated plants ( $46.14 \pm 22$  vs.  $2.3 \pm 0.2 \text{ mg/kg}$ ). They showed adult *S. americana* took

taste bites before rejecting highly contaminated lettuce, indicating As is detected through gustation.

In polluted areas, metals often exist as simple or complex mixtures. Migula and Binkowska (1993) investigated the ability of populations of *Chorthippus* spp. (Orthoptera: Acrididae) from heavily and weakly polluted sites to distinguish between cadmium (Cd), lead (Pb), and Cd + Pb exposed diets. They found that grasshoppers locally adapted in weakly polluted sites did not have the ability to distinguish between leaves with different metal concentrations, whereas those from heavily polluted sites reduced their consumption rate with increasing Cd and Pb concentrations. This may indicate learned avoidance behavior in *Chorthippus* populations living in taxing environments. In a different experiment examining the effects of Cd alone, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) also experienced a significant decrease in feeding, as measured by the 'leaf feeding damage index' for treatment concentrations ranging from 0 to 300 mg/kg in *Thlaspi caerulescens* J. & C. Presl (Brassicaceae) varieties (Jiang et al., 2005).

A subset of research investigating metal impacts on ingestion behaviors examined plant biocontrol agents, with varying results. First-instar *Bactra verutana* Zeller (Lepidoptera: Tortricidae) exposed to purple nutsedge for up to 4 weeks were unaffected by Cd concentrations up to 18 µg/g (Quimby et al., 1979). *Agasicles hygrophila* Selman & Vogt (Coleoptera: Chrysomelidae) exposed to 8.7 µg Cd/g alligatorweed showed an inability to distinguish between Cd contaminated and uncontaminated plants, though they

did experience feeding depression when fed on Cd-contaminated leaves in choice experiments (Quimby et al., 1979).

*Neochetina bruchi* Hustache (Coleoptera: Curculionidae) is used in the control of water hyacinth, an emergent, metal-accumulating aquatic plant, and spends its life on the leaf surface. When separately exposed to Cd and Zn, Jamil et al. (1989a,b) found a significant decrease in the number of water hyacinth feeding lesions, reflecting a decrease in feeding activity with increasing exposure concentration for both metals. There was no significant difference between numbers of feeding lesions found in plants accumulating up to 89.5 and 165  $\mu\text{g Zn}/100\text{ g dry weight}$ ; however, lesions were significantly fewer when *N. bruchi* were fed on plants accumulating 232  $\mu\text{g Zn}/100\text{ g dry weight}$  (Jamil et al., 1989a,b). Cd exposure accumulating to levels of 3.78, 6.20, and 66.70  $\mu\text{g}/100\text{ g dry weight}$  showed the same pattern of feeding depression (Jamil et al., 1989a,b), with no effect of the lower concentrations on number of feeding lesions. This finding supports results by Quimby et al. (1979).

A different species of water hyacinth beetle, *Neochetina eichhorniae* Warner, had conflicting behavioral outcomes in the presence of Cd when compared to *N. bruchi* (Kay and Haller, 1986). Water hyacinth with 8.00 and 17.20  $\mu\text{g Cd/g}$  leaves did not experience decreased feeding activity of *N. eichhorniae* when compared to controls. *Neochetina eichhorniae* feeding activity when exposed to 21.62 and 44.77  $\mu\text{g/g Cu}$  and 5.89 and 9.84  $\mu\text{g/g Pb}$  was also not significantly different from controls. Kay and Haller (1986) exposed beetles to contaminated water hyacinth for 10 days, vs. Jamil et al. (1989a,b) who exposed beetles for 7 days, and did not report feeding depression at any point during their

assays. This implies that *N. eichhorniae* is more tolerant of metals uptake by water hyacinth than *N. bruchi*.

Other studies on the antifeedant effects of metals on beetles showed a consistent decrease in feeding activity as a result of dietary exposure. Third instars of *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) exposed to  $\text{CuSO}_4$  (El-Bassiouny, 1991) and  $\text{Pb}(\text{NO}_3)_2$  (Kwartirnikov et al., 1999) significantly decreased feeding activity compared to controls, which were more pronounced for increasing concentrations of each antifeedant. Adult *L. decemlineata* exposed to  $\text{Pb}(\text{NO}_3)_2$  also showed decreased feeding activity, though this result was less pronounced than for larvae (Kwartirnikov et al., 1999).

Complex preference experiments by Rokytová et al. (2004) revealed that adult *Lochmaea capreae* L. (Coleoptera : Chrysomelidae) did not alter feeding activity on birch leaves dipped in Cd (2-250  $\mu\text{g}/\text{ml}$ ) and Pb (4-500  $\mu\text{g}/\text{ml}$ ). For manganese (Mn) and Zn, feeding activity significantly decreased between low and high concentrations (100-500 and 10,000  $\mu\text{g}$  Mn/ml, and 80-400 and 8,000  $\mu\text{g}$  Zn/ml, respectively). They also avoided high concentrations of Mn and Zn more often than all concentrations of Cd and Pb. Another chrysomelid, *Melasoma lapponica* L., showed a preference for very high (273.3 mg Ni/kg and 95.4 mg Cu/kg) and very low (27.7 mg Ni/kg and 16.9 mg Cu/kg) concentrations of Cu and Ni in willow foliage along a distance gradient from a smelter (Zvereva & Kozlov, 1996). For this species, some feeding was necessary on undamaged leaves before rejection, though damaged leaves with metal exposures were identified and

rejected before feeding, possibly due to an increased release of deterrent substances (Zvereva and Kozlov, 1996).

Finally, a handful of studies have examined the effects of metals on soil-dwelling invertebrate feeding behavior. *Orchesella cincta* (L.) (Collembola: Entomobryidae) showed no significant preference for green algae diet contaminated with  $\text{Pb}(\text{NO}_3)_2$  up to 1,600  $\mu\text{g/g}$ , and the authors concluded that avoidance was not necessary due to efficient excretion mechanisms already in place (van Capelleveen et al., 1986). Fountain and Hopkin (2001) reported significant avoidance of Pb-contaminated diet at 2,170  $\mu\text{g/g}$  for *Folsomia candida* Willem (Collembola: Isotomidae), but no significant avoidance of diet contaminated with 406  $\mu\text{g/g}$  Pb, consistent with van Capelleveen et al. (1986). *Orchesella cincta* was similarly unaffected by Mn in the diet up to  $9.2 \pm 0.3 \mu\text{mol/g}$  dry mass, though iron caused a significant decrease in feeding activity, especially at higher concentrations (Nottrot et al., 1987). In a field study, *Sphaeridia pumilis* Krausbauer (Collembola: Sminthuridae), *Parisotoma notabilis* (Schäffer) (Collembola: Isotomidae), and *Mesaphorura macrochaeta* (Rusek) (Collembola: Onychiuridae) gut contents reflected preferential avoidance of the organic horizon where the majority of Cd, Pb, and Zn were concentrated (Gillet and Ponge, 2003).

Copper-contaminated diet significantly deterred *F. candida* at 1,500  $\mu\text{g/g}$  dry weight in the laboratory (Filser and Hölscher, 1997), and *Onychiurus armatus* (Tullberg) (Collembola: Onychiuridae) with a 13.5% Cu solution-soaked diet (Filser et al., 2000). In contrast, *Isotomurus palustris* (Müller) (Collembola: Isotomidae) fed on diet with Cu contamination as often as on uncontaminated diet, and *F. quadrioculata* and *F.*



*manolachei* preferred Cu-contaminated diet (Filser et al., 2000). *Folsomia candida* significantly avoided Cu-contaminated yeast at concentrations exceeding 10 µg/g (Fountain and Hopkin, 2001). *Folsomia candida* also avoided yeast contaminated with Cd at concentrations exceeding 28 µg/g, and always preferred controls to Zn-contaminated diet (Fountain and Hopkin, 2001).

#### Taxis behavior

Taxis is an oriented movement in response to a directional stimulus or a stimulus gradient. All of the available research investigating locomotory effects of pollution has focused on carabids and collembolans, with the exception of one study investigating pupation-site preference in *D. melanogaster*. Unfortunately, the studies appear to be contradictory, making deduction of patterns impossible. Bayley et al. (1995) found that *Pterostichus cupreus* L. (Coleoptera: Carabidae) larvae exposed to 500 µg Cu/g in soil and diet experienced severely impaired locomotion as adults. This resulted in decreased prey capture success as adults, despite the absence of antifeedant properties in the larval diet (Bayley et al., 1995). Though collembolans were shown to avoid Cu-contaminated diets, they tended to not avoid Cu-contaminated soils, the exceptions being *M. macrochaeta* and *Folsomia manolachei* Bagnall (Filser and Hölscher, 1997) and *Pseudosinella alba* (Packard) (Collembola: Entomobryidae) (Filser et al., 2000).

In contrast, Lock et al. (2001) sampled seven sites within the vicinity of an abandoned Pb-Zn mine and found no significant relationship between relative activity of carabids and measureable metal concentrations in soils. Activity was measured using pitfall traps in combination with diversity sampling to determine whether certain species

were more active given particular soil metal concentrations. The lack of significance despite a gradient in total Pb, Cd, and Cu concentrations was attributed to these metals not being bioavailable to the predatory carabids (Lock et al., 2001).

*Parisotoma notabilis* exposed to a gradient of Cd, Pb, and Zn pollution in the field followed the distribution of its weakly polluted food source by shifting position in the soil from surface to deeper horizons, and thus avoided changing its feeding habits (Gillet and Ponge, 2003). Using methodology identical to that of Lock et al. (2001), Lock et al. (2003) determined that a location with similar contaminants as that of Gillet and Ponge (2003) showed no significant relationship between activity of collembolans and metal concentrations in soils. This difference in collembolan activity may have been caused by the high concentrations of Cd, Pb, and Zn reported in Gillet and Ponge (2003), compared to those in Lock et al. (2001). Other studies examining mixtures of metals in contaminated soils revealed that *F. candida* consistently avoided heavily contaminated soils, though there was high variability in individual response (Natal da Luz et al., 2004). Reporting an overall response of a conglomerate soil insect fauna, Gongalsky et al. (2009) found consistent avoidance of heavily contaminated soils. Collectively, the broadly contradictory results from the available research suggest there are many environmental factors that may influence movement. Thus, substantial opportunities exist for additional research on this topic.

In choice trials, Bahadorani and Hilliker (2009) found no significant difference in pupation-site preference in late-instar *D. melanogaster* when presented with normal food and food with a concentration of 70 mmol Zn/l. Larvae did, however, significantly prefer

normal food to Cu-contaminated food (20 mmol/l). They also significantly preferred pupating in Fe (II)-contaminated food (70 mmol/l) to non-contaminated food.

### Oviposition

To date, only five publications reported the effects of metals on ovipositional response in terrestrial insects. When given a choice between concentrations of ovipositional substrate exposed to hexavalent chromium (Cr VI) (50, 500, 1000 µg/g), Trumble and Jensen (2004) found that females of *Megaselia scalaris* Loew (Diptera: Phoridae) did not discriminate between control, low, and high concentrations. This occurred despite the observation that the highest level was toxic to the larvae. In a different study on a dipteran, Bahadorani and Hilliker (2009) reported that mated female *D. melanogaster* significantly decreased egg laying at relatively high concentrations of heavy metals (2 mmol/l Cd, 10 mmol/l Cu, 40 mmol/l Fe (II), 20 mmol/l Fe (III), and 30 mmol/l Zn). Interestingly, for both Fe and Zn, oviposition increased significantly relative to controls (0 mmol/l for each) at lower concentrations. This indicates that the female not only senses metals in the environment, but also knows which concentrations will maximize the fitness of her offspring.

Two studies were available that examined oviposition by *S. exigua* in response to selenium (Se). Females preferred to oviposit on low concentrations of Se-treated alfalfa ( $2.88 \pm 0.52$  µg Se/g dry weight) over controls (Vickerman et al., 2002b). However, adult females were unable to distinguish between low and high ( $305.81 \pm 52.14$  µg Se/g dry weight) concentrations of Se for oviposition, despite the fact that the high level was toxic. In a second study examining oviposition on *Atriplex* spp. plants which accumulate Se, *S.*

*exigua* did not distinguish between plants that contained concentrations of Se that were toxic to their offspring (Vickerman et al., 2002a). For both *M. scalaris* and *S. exigua*, the inability to distinguish between lethal concentrations of Cr VI and Se, respectively, puts eggs and larvae at risk at exposed oviposition sites.

Finally, the newly discovered *P. xylostella* Stanleyi biotype does not differentiate between high and low concentrations of Se in *S. pinnata* when ovipositing. This is in direct contrast to a different ecotype, *P. xylostella* G88, and *P. rapae*, which avoided ovipositing on highly Se-contaminated plants (Freeman et al., 2006).

#### *Aquatic systems*

##### Ingestion behavior

As was the case with CuSO<sub>4</sub> and oligophagous terrestrial herbivores, Hatakeyama and Yasumo (1981) found that aquatic systems with Cu available in concentrations from 0.01 to 0.64 mg Cu/l resulted in reduced food uptake in first-instar *Paratanytarsus parthenogeneticus* Freeman (Diptera: Chironomidae), as measured by the area of deposited feces. Lowered egestion rates were similarly observed for *Chironomus riparius* Meigen (Diptera: Chironomidae) exposed to Zn, Cd, and iron (Fe) contaminated sediments (Leppänen et al., 1998). However, this was only the case at one of their treatment locations (2 356.4 µg Cd/g sediment, 38 mg Zn/g, and 17 mg Fe/g). One of the reference locations with minimal contamination also contained chironomids with decreased egestion (7.2 µg Cd/g sediment, 0.9 mg Zn/g, and 42.4 mg Fe/g) when compared to the other reference location (9.0 µg Cd/g sediment, 1.1 mg Zn/g, and 17.4 mg Fe/g). Because of this discrepancy, the authors were unable to conclude whether

**Table 5.2** Summary of contaminants and the resultant behavioral outcome observed for insect species in aquatic habitats. Only individual metals/metalloids are considered for behavioral outcomes because mixtures may lead to synergistic or antagonistic interactions otherwise unaccounted for in the behavioral response. Positive and negative outcomes correspond to stimulation or suppression of the particular behavior as a result of metal presence, respectively, and ‘no effect’ means the organism was unaffected at the experimental conditions. In choice assays, aversion results in a negative behavioral outcome. See text for the measured concentrations.

Metal	Form	Species	Behavioral outcome	Reference
A. Ingestion behavior				
Copper	CuSO <sub>4</sub> ·5H <sub>2</sub> O	<i>Paratanytarsus parthenogeneticus</i>	Negative	Hatakeyama & Yasumo, 1981
Cadmium	Cd CdCl <sub>2</sub>	<i>Hydropsyche slossonae</i>	Negative	Tessier et al., 2000
		<i>Baetis tricaudatus</i>	Negative	Irving et al., 2003; Riddell et al., 2005
		<i>Glyptotendipes pallens</i>	Negative	Heinis et al., 1990
		<i>Kogotus nonus</i>	Negative	Riddell et al., 2005
Selenium	Na <sub>2</sub> SeO <sub>4</sub>	<i>Culex quinquefasciatus</i>	Negative	Jensen, 2006
		<i>Sympetrum corruptum</i>	Positive	Jensen, 2006
Zinc	ZnSO <sub>4</sub> ·7H <sub>2</sub> O	<i>Hydropsyche betteni</i>	Negative	Balch et al., 2000
B. Taxis behavior				
Aluminum	AlCl <sub>3</sub>	Chironomidae	Positive	Bernard et al., 1990
		Ephemeroptera	Positive	Bernard et al., 1990; Bernard, 1985
		Plecoptera	No effect	Bernard et al., 1990; Bernard, 1985
		<i>Simulium</i> spp.	Negative	Bernard, 1985
		<i>Simulium</i> spp.	No effect	Bernard et al., 1990
		Trichoptera	Positive	Bernard et al., 1990

Cadmium	Al <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub>	<i>Baetis rhodani</i>	Positive	Ormerod et al., 1987
		<i>Dicranota</i> spp.	Positive	Ormerod et al., 1987
		<i>Dixa puberula</i>	Positive	Ormerod et al., 1987
		<i>Elmis aenea</i>	No effect	Ormerod et al., 1987
		<i>Ephemerella danica</i>	Positive	Herrmann & Andersson, 1986
		<i>Ephemerella ignita</i>	Positive	Ormerod et al., 1987
		<i>Heptagenia fuscogrisea</i>	Positive	Herrmann & Andersson, 1986
		<i>Heptagenia sulphurea</i>	Positive	Herrmann & Andersson, 1986
		<i>Leuctra</i> spp.	No effect	Ormerod et al., 1987
		<i>Protonemura meyeri</i>	Negative	Ormerod et al., 1987
		Simuliidae	Negative	Ormerod et al., 1987
	Cd	<i>Chironomus salinarius</i>	No effect	Hare & Shooner, 1995
		<i>Procladius</i> spp.	No effect	Hare & Shooner, 1995
		<i>Sergentia coracina</i>	No effect	Hare & Shooner, 1995
	CdCl <sub>2</sub>	<i>Baetis tricaudatus</i>	Negative	Riddell et al., 2005
		<i>Glyptotendipes pallens</i>	Negative	Heinis et al., 1990
		<i>Hexagenia limbata</i>	No effect	Gosselin & Hare, 2004
		<i>Hexagenia rigida</i>	Positive	Odin et al., 1995
	3CdSO <sub>4</sub> ·8H <sub>2</sub> O	<i>Baetis rhodani</i>	Negative	Gerhardt, 1990
		Hydropsychidae	Positive	Vuori, 1994
		<i>Leptophlebia marginata</i>	No effect	Gerhardt, 1990
Copper	Cu	<i>Adenophlebia auriculata</i>	Positive	Gerhardt & Palmer, 1998
		<i>Chimarra</i> spp.	Negative	Clements et al., 1989
		<i>Cinygmula</i> spp.	No effect	Stitt et al., 2006
		<i>Hydropsyche morosa</i>	Negative	Clements et al., 1989
		<i>Paragnetina media</i>	No effect	Clements et al., 1989
		<i>Hydropsyche angustipennis</i>	Negative	van der Geest et al., 1999
	CuSO <sub>4</sub>	<i>Baetis</i> spp.	Positive	Leland, 1985
		Chironomidae	Positive	Leland, 1985
		<i>Chironomus riparius</i>	No effect	Dornfeld et al., 2009
		<i>Cleptelmis addenda</i>	No effect	Leland, 1985
		<i>Lepidostoma</i> spp.	Positive	Leland, 1985
		<i>Optioservus divergens</i>	Positive	Leland, 1985
		<i>Paraleptophlebia</i>	Positive	Leland, 1985

		<i>pallipes</i>		
		<i>Simulium</i> spp.	No effect	Leland, 1985
		<i>Symphitopsyche oslari</i>	Negative	Leland, 1985
Iron	FeSO <sub>4</sub> ·7H <sub>2</sub> O	<i>Leptophlebia marginata</i>	Negative	Gerhardt, 1992, 1994
Lead	PbCl <sub>2</sub>	<i>Leptophlebia marginata</i>	Negative	Gerhardt, 1994
Mercury	CH <sub>3</sub> HgCl	<i>Hexagenia rigida</i>	Positive	Odin et al., 1995
C. Oviposition				
Cadmium	CdCl <sub>2</sub> ·2½H <sub>2</sub> O	<i>Chironomus riparius</i>	Negative	Williams et al., 1987
Copper	CuSO <sub>4</sub> ·5H <sub>2</sub> O	<i>Chironomus riparius</i>	No effect	Dornfeld et al., 2009
Mercury	CH <sub>3</sub> ClHg	<i>Culex quinquefasciatus</i>	No effect	Jensen et al., 2007
Selenium	Na <sub>2</sub> SeO <sub>4</sub>	<i>Culex quinquefasciatus</i>	No effect	Jensen et al., 2007

metal-contaminated sediments had a significant impact on feeding rates of *C. riparius* (Leppänen et al., 1998). A separate field study examining post-exposure feeding depression of *C. riparius* revealed no significant difference between reference, low contamination, and highly contaminated field locations (Faria et al., 2006). Contaminated sites contained a mixture of metals, including As, Cd, Cr, Cu, Fe, Pb, Mn, Ni, and Zn. Post-exposure feeding depression was measured as the amount of algae consumed in an uncontaminated environment after exposure for 6 days, in order to evaluate the effect of acute pollution pulses on long term population viability of *C. riparius* (Faria et al., 2006).

Irving et al. (2003) reported significantly decreased feeding rates for *Baetis tricaudatus* Dodds (Ephemeroptera: Baetidae) exposed to dietary Cd, though they did not preferentially avoid Cd-contaminated diatom mats. Feeding inhibition was apparent after 8 days for both Cd treatments (10 and 84 µg Cd/g diatoms). Cd concentrations of 0.5 to 1.0 mg/l were further shown to disrupt filter feeding behaviors in fourth-instar

*Glyptotendipes pallens* (Meigen) (Diptera: Chironomidae) and result in increased defecation rates, possibly in an attempt to regulate Cd uptake (Heinis et al., 1990).

Only two studies evaluated the impacts of heavy metal contamination on predator hunting behaviors. In a complex factorial design, *B. tricaudatus*, *Kogotus nonus* (Needham and Claassen) (Plecoptera: Perlodidae), and two fish species were placed in a mesocosm and the effects of dietary and waterborne Cd contamination evaluated (Riddell et al., 2005). Because *K. nonus* are predators, only waterborne Cd contamination (0.5 and 5 µg Cd/l) was relevant to their behaviors in this experiment. At both concentrations, locomotory activities were significantly reduced, resulting in impaired foraging abilities. Only 2 of 9 attacks on prey were successful, and both occurred at the 0.5 µg/l treatment, so further extrapolation about effects on predation behaviors could not be made (Riddell et al., 2005). Jensen (2006) evaluated the effects of Se and/or methyl-mercury (MeHg) on consumption rates of *Sympetrum corruptum* (Hagen) (Odonata: Libellulidae) when fed *Culex quinquefasciatus* Say (Diptera: Culicidae). He found that *S. corruptum* in Se treatment solutions consumed significantly more mosquito larvae per day than controls; however, predators eating prey contaminated with Se + MeHg consumed significantly fewer prey per day. Predators' consuming more in Se-treated water with non-treated prey was attributed to the mosquito larvae experiencing a reduction in avoidance behavior. Predators' consuming less in the Se + MeHg treatments was attributed to treatments making the prey unpalatable or suppressing the predator's appetite (Jensen, 2006).

Several studies have evaluated the impacts of metal exposure on the construction of capture nets by *Hydropsyche* spp. (Trichoptera: Hydropsychidae). These nets are used



to capture drifting plant and animal materials; therefore, construction anomalies have the potential to negatively impact the efficiency with which nymphs are able to recover food items. Fifth-instar *H. betteni* adapted to either polluted or unpolluted environments were collected from the field and exposed to waterborne (5.4 or 10.7 mg/l) and dietary (113 µg/g) Zn (Balch et al., 2000). After 5 and 7 weeks, nymphs from unpolluted and polluted populations, respectively, exhibited significantly looser net structures with such large openings between net strands that capture efficiency was negligible. Interestingly, larvae exposed to waterborne Zn at 42.1 and 21.7 mg/l showed no significant difference in net spinning capabilities (Balch et al., 2000). Third and fourth-instar *H. slossonae* exposed to Cd also exhibited an increase in net anomalies (Tessier et al., 2000). After 5 days, approximately 60% of nymphs exposed to 43.3 and 21.4 µg Cd/l experienced strand crossover anomalies, with 100% of nymphs showing anomalies after 10 and 20 days, respectively. After 10 days, approximately 60% of nymphs exposed to 11.6 µg Cd/l experienced strand crossover anomalies, with 100% of nymphs showing anomalies after 15 days. The lowest concentrations tested (1.2 and 0.37 µg Cd/l) exhibited approximately 50% of nymphs with net anomalies apparent after 15 and 20 days, respectively. Background anomalies in control treatments were found to be approximately 20% (Tessier et al., 2000).

In contrast, Petersen and Petersen (1983) pooled data for *Hydropsyche* spp. net anomalies after determining that the number of anomalies was independent of species. Specifically, they evaluated the possibility of increased strand crossover frequencies which can result in smaller mesh openings with less uniform strand arrangements. They

found that net strand crossover frequency at heavy metal-contaminated field locations was not significantly different from control sites. Metals present at different locations varied and though exact concentrations were not reported, exposure levels were always sublethal. However, the lack of information on metal concentrations in the Petersen and Petersen (1983) report makes direct comparisons with the studies by Balch et al. (2000) and Tessier et al. (2000) impossible. This highlights the critical need for reporting detailed information on metal concentrations which are necessary for documenting potential patterns in insect responses.

#### Taxis behavior

Drift was the most commonly measured response of invertebrates in aquatic systems to pollution, and is defined as an organism detaching from the substrate and swimming or floating downstream. This behavior is used to escape localized pollution, with the tradeoff being greater exposure to predation. It is easily measured in the field just downstream of point sources of pollution and allows for a community-wide assessment of the impacts of contaminants on downstream movements of insects.

For example, aluminum (Al) (0.95 mg/l) caused chironomids to enter the drift column 4-8 times more frequently after exposure for 6 h than controls (Bernard et al., 1990). In this same study, Ephemeroptera and Trichoptera spp. entered the drift column 10-15× and 3-5× more frequently, respectively, while *Simulium* spp. (Diptera: Simuliidae) and Plecoptera spp. experienced no significant increase in drift throughout the 12-h study duration. When exposed to Al with varying pH values, Plecoptera nymphs failed to respond by increasing drift when dissolved Al concentration was increased from

31.0 to 40.2  $\mu\text{g/l}$ , and the pH decreased from 7.0 to 5.9 (Bernard, 1985). This may be due to a lack of sensory capacity to detect Al increases within the range tested, or because the chemical was not physiologically stressful at the tested concentration. Trichoptera and *Simulium* spp. both experienced a delayed response at the above concentrations, possibly due to a disruption of physiological processes. Ephemeroptera, the most sensitive order evaluated, showed an immediate response to the Al influx, possibly because they are able to detect this ion in their environment through chemoreceptors (Bernard, 1985).

Chironomids became sensitive to Al only after pH had decreased in this experiment. In a different study on pH impacts on Al toxic response, Ormerod et al. (1987) examined a wider range of taxa. Both *Leuctra* spp. (Plecoptera: Leuctridae) and *Elmis aenea* (Müller) (Coleoptera: Elmidae) did not change drift patterns in response to an acid and Al pulse. *Ephemerella ignita* (Poda) (Ephemeroptera: Ephemerellidae) showed significantly increased drift during the episode, and *Baetis rhodani* Pictet (Ephemeroptera) drift density increased 8.4 $\times$  relative to the control. Simuliidae drift increased 3.6 $\times$ , and *Protonemura meyeri* Pictet (Plecoptera: Nemouridae) increased 1.6 $\times$  during treatment and remained high the following day. Both *Dixa puberula* Loew (Diptera: Dixidae) and *Dicranota* spp. (Diptera: Pediciidae) showed significantly increased drift, but only during the episode (Ormerod et al., 1987).

However, not all metals produce a significant drift response. There was no significant difference in drift rate of *Cinygmula* spp. (Ephemeroptera: Heptageniidae) at concentrations of 78 and 229  $\mu\text{g/l}$  Cu (Stitt et al., 2006), and rates of drift at 3  $\mu\text{g/l}$  Cu were not significantly different from the control. After dosing experimental stream

channels with Cu at three concentrations (2.5, 7, and 15 µg/l), Leland (1985) found that *Cleptelmis addenda* (Fall) (Coleoptera: Elmidae) showed no significant increase in drift compared to controls, whereas drift rate of *Optioservus divergens* (Leconte) (Coleoptera: Elmidae) increased slightly at 2.5 and 15 µg Cu/l. *Paraleptophlebia pallipes* (Eaton) (Ephemeroptera: Leptophlebiidae), *Baetis* spp., and *Lepidostoma* spp. (Trichoptera: Lepidostomatidae) increased drift at 7 and 15 µg Cu/l, whereas *Symphitopsyche oslari* Banks (Trichoptera: Hydropsychidae) decreases drift at these concentrations. Drift of chironomids decreased slightly at 7 µg Cu/l, and increased at 15 µg Cu/l, whereas *Simulium* spp. drift was unaffected by Cu (Leland, 1985).

One study documented a decreasing response as pollution levels increased. Riddell et al. (2005) demonstrated that *B. tricaudatus* drift decreased with increasing concentrations of Cd. However, they used a recirculating stream system, so drift behavior did not allow insects to escape to a lower concentration. They suggested that continued exposure could have reduced locomotory behavior, or that an increased energy demand due to contaminant acclimation or detoxification could have reduced the energy available for relocation.

When exposed to multiple metals (2.2 µg Cd/l, 24 µg Cu/l, and 200 µg Zn/l), various aquatic insects (Coleoptera: Elmidae; Diptera: Chironomidae; Ephemeroptera, Plecoptera, and Trichoptera: Hydropsychidae) from populations locally evolved in uncontaminated streams experienced significant increases in drift (Clements, 1999). Because all of the organisms collected in drift nets were still alive, the authors were able to conclude that drift was a behavioral response to avoid heavy metals in the water. In

choice experiments with contaminated sediments, first-instar *C. riparius* exposed to 2 mg Cu/l (Dornfeld et al., 2009) and fourth-instar *C. salinarius*, *Sergentia coracina* (Zetterstedt), (Diptera: Chironomidae) and *Procladius* spp. (Diptera: Chironomidae) exposed to 0.15 µg Cd/l (Hare and Shooner, 1995) were unable to distinguish between treated and control sediments. In choice experiments using sediments collected from five treatments and two reference locations, *C. tentans* preferred control over treatment sediments for the most highly contaminated locations only (774 mg/l Cd, 11,134 mg/l Zn, 1,393 mg/l Cr; 964 mg/l Cd, 16,397 mg/l Zn, 2,129 mg/l Cr; and 1,029 mg/l Cd, 17,262 mg/l Zn, 1 640 mg/l Cr) (Wentzel et al., 1977).

Evaluations of heavy metal impacts on other locomotory behaviors are also common. Cd concentrations ranging from 2.5 to 10 mg/l were shown to significantly increase time spent in inactive states for *G. pallens* (Heinis et al., 1990). Exposure to levels as low as 0.02 mg Cd/l were shown to reduce locomotion in *B. rhodani*, whereas *Leptophlebia marginata* (L.) (Ephemeroptera: Leptophlebiidae), exposed to 0.2 mg Cd/l, showed no difference in locomotory activities compared to controls (Gerhardt, 1990). Based on these results, Gerhardt (1990) concluded that locomotion was a good parameter to measure in cases of suspected subacute chemical stress. He reached the same conclusion when Fe-exposed *L. marginata* decreased motility in proportion to the concentration of dissolved Fe (10, 20, and 50 mg/l) (Gerhardt, 1992). At much lower levels of contamination, female *Hexagenia limbata* (Serville) (Ephemeroptera: Ephemeridae) exposed to 18.9 µg Cd/g in sediment and 5.8 µg Cd/l in water experienced no discernable effect on burrowing activities (Gosselin and Hare, 2004). Similarly,

concentrations of 0.05-0.1 mg Cd/l had relatively minor impacts on activity of *G. pallens* (Heinis et al., 1990).

Exposure at concentrations of 0.05-0.296 mg Cu/l led to increased escape behavior by *Adenophlebia auriculata* Eaton (Ephemeroptera: Leptophlebiidae): mayflies searched for stones away from areas of Cu input (Gerhardt and Palmer, 1998). There were more ventilation and abdominal undulations observed at these concentrations as well, possibly in an attempt to rid Cu ions bound in gill membranes. Finally, *A. auriculata* was more prone to climbing on top of rocks instead of maintaining negative phototactic behaviors observed in controls. In contrast, *L. marginata* showed a decrease in escape behavior correlated to increasing exposure time and Fe (10-500 mg/l) and Pb (0.1-5.0 mg/l) concentrations (Gerhardt, 1994). Odin et al. (1995) reported increased bioturbation activity of *Hexagenia rigida* McDunnough (Ephemeroptera: Ephemeridae), as measured by turbidity in the water column, when nymphs were exposed to sediment concentrations up to 10 mg Cd/kg and MeHg concentrations up to 2.98 mg/kg. Interestingly, nymphs were unaffected when the exposure route was water only (Odin et al., 1995).

For *Hydropsyche angustipennis* (Curtis) (Trichoptera: Hydropsychidae), there was a significant decrease in ventilation, or abdominal undulatory movements, at 20 µg Cu/l, resulting in a proportional increase in other locomotory behaviors and inactivity (van der Geest et al., 1999). Concentrations ranging from 100 to 600 µg Cu/l caused *H. angustipennis* to spend very little time ventilating, and individuals were, for the most part, inactive. When exposed to waterborne Al at 2.0 mg/l, nymphs of *Heptagenia*

*fuscogrisea* (Retzius), *Heptagenia sulphurea* (Müller) (Ephemeroptera: Heptageniidae), and *Ephemera danica* Müller (Ephemeroptera: Ephemeridae) showed significant increases in respiration (Herrmann and Andersson, 1986).

There are a few studies on combinations of metals and industrial effluents inhibiting locomotory behaviors of insects. Unfortunately, because of the variability, complexity, and unknown constituents of the industrial effluents, analysis of potential synergistic and antagonistic interactions with metals and other nonmetals are not possible. However, a few of these studies are included here and will allow the reader to access this literature. Nymphs of *H. angustipennis* exposed to effluent downstream of an industrial area exhibited decreased ventilation, but other locomotory activities increased in frequency (Gerhardt, 1996). Exposed *H. pellucidula* also increased activity relative to controls (Macedo-Sousa et al., 2008). *Choroterpes picteti* (Eaton) (Ephemeroptera: Leptophlebiidae) experienced an initial increase in locomotion, but by the end of the assay, individuals in the control treatment were more active than those exposed to the acid mine drainage (AMD) treatment (Macedo-Sousa et al., 2008). In a similar study, *C. picteti* in AMD treatments increased locomotion in response to heavy metals and lower pH, which had the potential to increase nocturnal drift behavior (Gerhardt et al., 2005).

Inter- and intraspecific behaviors have not been frequently reported, particularly with relation to impacts of metals. When exposed to 0.5 and 5 µg/l dissolved Cd in water and diet, *B. tricaudatus* were more vulnerable to predators as a result of decreased predator avoidance behaviors (Riddell et al., 2005). When crop and stomach contents from *Paragnetina media* (Walker) (Plecoptera: Perlidae) were assessed in Cu-exposed vs.

control individuals, Clements et al. (1989) found an increased amount of *Hydropsyche morosa* Hagen and *Chimarra* spp. (Trichoptera: Philopotamidae) remains, indicating increased susceptibility to predation in 5.5  $\mu\text{g Cu l}^{-1}$  contaminated waters for these species. However, various species of caddisflies exposed to a wide range of Pb, Zn, and Cd concentrations simultaneously in the field showed no difference from controls in amount of time taken to emerge from their cases after a predatory threat (Lefcort et al., 2000). In a factorial experiment, Kiffney (1996) showed that metal-contaminated water (0.7  $\mu\text{g Cd/l}$ , 6  $\mu\text{g Cu/l}$ , and 50.3  $\mu\text{g Zn/l}$ ) had no impact on *B. tricaudatus* and *Rhithrogena hageni* Eaton (Ephemeroptera: Heptageniidae) predator avoidance behaviors, while it increased predation risk to *Hydropsyche* spp. *Prostoia besametsa* (Ricker) (Plecoptera: Nemouridae) predation decreased in metals treatments compared to controls (Kiffney, 1996). Therefore, it would appear that the impact of metals on predator avoidance behavior is species dependent.

In an experiment investigating the impacts of Cd on competition behavior between conspecifics of hydropsychid larvae for optimal foraging habitat, Vuori (1994) found that exposed intruders performed shorter and less fierce attacks when paired with control residents. Exposed residents were surprisingly active during attacks against exposed intruders and fiercer than control residents, though the fights were still shorter. Attacks between control residents and intruders were longer than those involving exposed individuals. Vuori (1994) speculated this Cd-induced behavioral change might have been due to individuals weighing the personal risk involved in combat to the energy that had already been expended in spinning silk to construct a net. Though the animal may be



poisoned from Cd exposure, energetically a prime territory is worth defending when a net has already been spun. In contrast, an invading caddisfly may abandon a fight to construct a net in less suitable territory, particularly if they are outmatched.

### Oviposition

As was the case for terrestrial reproductive behaviors, only a few papers analyzed ovipositional responses of aquatic insects to metal-contaminated environments. Williams et al. (1987) found that when given a choice between water contaminated with different levels of Cd, *C. riparius* adult females distinguished between control and low concentrations (0, 0.3, and 30 mg/l) vs. high concentrations (100 and 300 mg/l). Significantly fewer eggs were laid in Petri dishes with high concentrations of Cd vs. dishes with low or control concentrations. Despite this, female aversion was only sensitive enough to avoid concentrations acutely toxic to eggs; although not toxic to eggs, these concentrations are acutely toxic to first instars (Williams et al., 1987). In another preference study, Dornfeld et al. (2009) found *C. riparius* females unable to distinguish between control media and treatment media with 1.3 mg Cu/l when ovipositing. Though egg hatchability was significantly reduced in Cu treatments, Cu did not affect larval survival (Dornfeld et al., 2009).

Similarly, *C. quinquefasciatus* did not discriminate between water contaminated with sodium selenate (30 mg/l), MeHg chloride (7 mg/l), or a mixture of sodium selenate and MeHg chloride (at the above concentrations) (Jensen et al., 2007). The authors concluded that females were either unable to detect these compounds at the tested concentrations, or did not prefer unpolluted to polluted water when ovipositing.

## Conclusions

A summary of these studies and the observed behavioral outcome for a particular contaminant can be found in Tables 5.1 and 5.2. These tables allow for a generic analysis of the broader impacts of metal and metalloid pollution in both terrestrial and aquatic systems, but are not comprehensive and not meant to serve as a quantitative meta-analysis. This qualitative classification is meant to offer the reader a quick summary of the published literature. Further, the designation of positive and negative outcomes does not necessarily confer a fitness advantage or disadvantage, and in some cases a positive behavioral outcome may have negative fitness impacts. For any given study, the outcomes may be dependent on the instar and concentrations tested, as well as exposure routes.

Over 95% of studies (53 of 55) on terrestrial ingestion behaviors reported either no effect or negative impacts as a result of individual pollutant exposure for the various behaviors quantified (Table 5.1A). In these studies there was some degree of repellency or feeding inhibition. Only 3.6% reported positive effects, equating to a stimulation of feeding behavior as a result of the metal being present. The substantial majority of these studies investigated impacts on lepidopteran pests, Collembola, and Orthoptera; absent or underrepresented orders merit future research. For aquatic taxis behaviors, 40% of studies (18 of 45) reported a positive behavioral stimulation of insects to some form of pollution, with the rest reporting suppressed behaviors or no effects.

Although many insect species were capable of distinguishing contaminated from uncontaminated locations, a surprising number of species evidently cannot detect the

presence of metal and metalloid contamination. For purposes of reproduction, an inability to avoid heavily polluted sites would lead to loss of eggs and reduced fitness. Although some species showed a tendency to increase locomotory behaviors to escape from locations with elevated metal pollution, other species remained and greatly decreased all movements unrelated to feeding. Still other species exhibited behaviors that would result in increased predation, including positive phototaxis that caused immatures to move to exposed positions. Ultimately, for some insects these behaviors result in reduced species fitness at contaminated sites, a general reduction in population sizes as well as species diversity, and a trend toward preponderance of those species that can tolerate pollution.

Due to the paucity of information regarding terrestrial taxis and reproductive behaviors, and aquatic ingestion and reproductive behaviors, further conclusions cannot be drawn about patterns of insect response to metals and metalloids. Analyses by feeding guild, environment (terrestrial vs. aquatic), and systematic classification did not provide evidence for a single dominant response. Additionally, the total number of species that have been investigated is relatively small. Patterns may not become evident until more research is published, particularly as many responses appear to be species-specific. A large number of papers also do not include a comparison with behaviors at uncontaminated sites, or document concentrations of the key pollutants. Although still valuable, these cannot be used as reliable evidence for behavioral changes that occur in response to metals and metalloids.

Because of the extent of the problem with metal and metalloid pollution worldwide, there is considerable opportunity for additional research. Knowledge of the

effects of these pollutants at the bottom of the food web will be critical to understanding the true impact of metal contamination and to the potential reconstruction of damaged ecosystems.

## **CHAPTER 6**

### **Changes to mosquito behavior resulting from arsenic exposure**

## **Abstract**

Behavioral assays performed in the presence of toxicants have the potential to provide valuable information with regards to how a particular toxicant exerts sublethal effects on target organisms. Larvae of *Culex tarsalis* (Diptera: Culicidae) were exposed to 0, 1000, or 5000 µg/l concentrations of arsenate and various taxis and aggregation behaviors evaluated. Larvae at 1000 µg/l spent significantly more time gliding than larvae in the 5000 µg/l treatment. In predator avoidance trials, larvae in the 5000 µg/l treatment showed a decreased response to a shadow stimulus, in addition to spending significantly more time away from the water surface than the other treatments, which could make them more susceptible to predation. Oviposition trials to determine if adult females that emerged from the control and 1000 µg/l treatments avoided arsenic contaminated waters revealed significant differences and opposite trends between the treatments, with control reared mosquitoes preferring the sodium phosphate control and arsenic reared mosquitoes preferring the deionized water control, followed by arsenate water.

## Introduction

Although not widely applied in toxicological studies (Mogren and Trumble, 2010; Stark and Banks, 2003), behavioral assays performed in the presence of toxicants can provide valuable information with regards to how a particular toxicant exerts sublethal effects on a target organism. In aquatic toxicology studies, widespread contaminants such as arsenic may have different effects on different taxa when only mortality is considered. For example, survival of *Chironomus riparius* exposed to arsenate was unaffected, even at the highest concentration tested (1000 µg/l, Mogren et al., 2012), whereas the more environmentally sensitive Ephemeroptera, Plecoptera, and Trichoptera (Barbour et al., 1999) were nearly eliminated from a stream affected heavily by arsenic contamination (Chaffin et al., 2005). In previous studies of other metal contaminants in aquatic systems, significant behavioral modification has been recorded at concentrations that did not affect survival (Mogren and Trumble, 2010, and references therein). By extension, disruptions to these behaviors can negatively affect the ecosystem services provided by these insects, in addition to indirectly affecting their survival by making them more prone to predation.

The metalloid arsenic is considered to be one of the world's greatest environmental hazards, and affects the lives of hundreds of millions of people (Ravenscroft et al., 2009). Although contamination may result from anthropogenic activities such as lead-arsenate insecticides, irrigation, and smelting activities (Nriagu, 1994), the majority results from natural sources that include parent materials and volcanic activity (Smedley and Kinniburgh, 2002). Arsenic has been classified as a priority toxic pollutant in the United States, and 150 µg/l is the concentration deemed safe for chronic

exposure for aquatic life (US EPA, 2006), though concentrations as high as 10,000 µg/l are known to occur (Smedley and Kinniburgh, 2002). However, the specific effects that arsenic has at environmentally relevant concentrations on aquatic insects are not well understood, particularly in the context of how exposures may exert sublethal effects on lower trophic levels when survival is unaffected (Chapter 3).

In this study, I evaluated the effects of prolonged exposure to arsenic on various taxis and reproductive behaviors of *Culex tarsalis* Coquillett (Diptera: Culicidae). In addition to serving as an important filter feeder and food source for higher trophic levels as larvae (Wallace and Walker, 2008), the adults of this species are of medical concern, and vector the causative agents of important diseases such as St. Louis encephalitis and West Nile Virus (Farajollahi et al., 2011). Understanding the behavioral changes that result from accumulated metals in this aquatic insect is important to understanding the broader effects a contaminant has on an ecosystem, particularly with regards to trophic interactions, and for gaining further insight into the sublethal impacts of exposure on aquatic insects.

## **Materials and Methods**

### *Mosquito Rearing*

Larvae of *Culex tarsalis* were reared as has been previously described (Chapter 3). Briefly, mosquito egg rafts were obtained from colonies maintained at the University of California, Riverside. Eggs were hatched in shallow white enamel pans (39 x 23 x 10 cm or 39 x 23 x 6 cm) containing 3 liters of tap water that were kept in an environmental rearing chamber under a 16:8 L:D cycle at 28.0±0.25°C. Treatments initially consisted of



controls and 1000 µg/l of sodium hydrogenarsenate heptahydrate, 99.998% (Sigma-Aldrich, St. Louis, MO, USA). The 1000 µg/l concentration represents a high yet still ecologically relevant concentration of arsenic (Ravenscroft et al., 2002; Smedley and Kinniburgh, 2002). Later, larvae were exposed to 5000 µg/l in order to quantify a measureable effect. Larvae were fed a mixture of a 3:1 (wt:wt) ground mouse chow (mouse/rat diet, Harlan/Teklad, Madison, WI, USA) and brewer's yeast (MP Biochemicals, Aurora, OH, USA) as a 10% suspension in deionized water daily.

#### *Behavior Assays*

Five- one to two day old fourth instar *Cx. tarsalis* larvae were chosen randomly and transferred to an observation aquarium (14.0 cm x 2.6 cm x 12.2 cm) with a clear front, white sides and back, and a black bottom (Workman and Walton, 2003). The aquarium was filled to a depth of 10 cm (300 ml) with deionized water that was changed between videotaping sessions. Either 1000 or 5000 µg/l of arsenic was added for the arsenic treatments. For all treatments, 1 ml of the mouse chow suspension was added to the observation aquarium.

The observation aquarium was placed inside a box turned on its side to minimize the effects of shadows and reflections on mosquito behavior. An external microscope lightsource (Fisher Scientific) was used to illuminate the observation aquarium. A Sony Handycam digital HD video camera recorder was used to record mosquito behaviors. Mosquitoes were allowed to acclimate to the test aquarium for 3 minutes before behaviors were recorded for 8 minutes. The camera operator stood out of sight of the mosquitoes to reduce effects that the observer may have had on the subjects. The clear

side of the aquarium filled the view of the camera, and all larvae were visible for the duration of the recording. The 8 minute behavior recording was followed immediately afterwards by a three minute recording where a paper towel was passed in front of the light source in order to create a shadow and to elicit a predator avoidance diving response. This was done at 30 second intervals, with the shadow cast over the aquarium lasting for two seconds. The dive frequency and duration in response to the shadow stimulus was recorded for all five larvae.

The occurrence and duration of behaviors was recorded using Noldus Observer. During the behavioral observation, the recorded behaviors were resting (characterized by movement of mouthparts with no vertical or horizontal movement), gliding (horizontal movement where the mosquito's siphon never broke contact with the water surface), swimming (defined as horizontal movement where the siphon was not in contact with the water surface), shallow diving (the siphon broke contact with the water surface and the mosquito descended to a depth no greater than 5 cm), deep diving (the mosquito descended to a depth greater than 5 cm), and ascending (the larva was swimming vertically towards the water surface). Occurrence and duration data was gathered for these behaviors for three of the five larvae in the test aquarium, which were chosen as the left-most, right-most, and middle larva after the 3 minute acclimation period.

Aggregation data were also recorded as the number of larvae grouped together (within 1 cm of nearest neighbors) for the three focal subjects, with aggregation values of 1, 2, 3, 4, or 5 being possible. The observations occurred on two days: the first was a control and

the 1000 µg/l treatment, and the second was a different control and the 5000 µg/l treatment.

### *Oviposition*

*Culex tarsalis* were reared as described above. Approximately one week after hatching, larvae began pupating. Pupae were removed using a mesh net and transferred to a bowl of deionized water. The bowl was placed inside a 30 cm x 30 cm x 30 cm plastic insect cage at ambient light and temperature for adult emergence, with one insect cage for control mosquitoes and one cage for mosquitoes reared in 1000 µg/l arsenic. Three petri dishes were provided for oviposition: the control contained only deionized water, the arsenic treatment contained 1000 µg/l arsenic as sodium arsenate, and the third contained a sodium control using sodium phosphate monobasic dihydrate (Fisher Scientific) with an equivalent molar amount of sodium as the sodium arsenate treatment ( $2.66 \times 10^{-5}$  M) to ensure the adult females were not preferentially ovipositing in the water with more dissolved sodium ions. These petri dishes were rotated within the insect cages every day that oviposition occurred to avoid a confounding effect of the petri dish location within the cage. Adults were provided with a 20% sucrose solution for feeding. The total number of autogenously produced (females were not blood fed) egg masses in each petri dish for each mosquito treatment group was recorded.

### *Data Analysis*

The frequency and duration data for the behaviors resting, gliding, swimming, shallow dives, deep dives, and ascending were exported from Noldus Observer to JMP v.10 statistical software. Data were checked for normality (Kolmogorov-Smirnov) and

homoscedastic (Bartlett's test) assumptions before being analyzed using a nested ANOVA, with replicate nested under treatment (control and 1000 µg/l or 5000 µg/l arsenic). In all cases, the nested variable was not significant ( $p>0.05$ ). For the aggregation data, the amount of time spent aggregated at different densities (1, 2, 3, or 4 larvae) did not uphold the assumptions of normality. Because the nested factor was not significant, data were analyzed using Friedman's nonparametric test with treatment and the number of larvae aggregated as the independent variables.

For the shadow stimulus trials, data were analyzed in SAS v.9.2. Dive frequency was analyzed by conducting 2x2 chi square contingency comparisons between treatment groups (control vs. 1000 µg/l As, control vs. 5000 µg/l As, 1000 µg/l As vs. 5000 µg/l As;  $n=25$  larvae for each treatment). Each shadow event (five total) was treated separately to account for potential acclimation of the mosquito larvae to the stimulus. Data for dive duration in response to the shadow stimulus did not follow a normal distribution and did not uphold normality assumptions after transformation, though variances were homogeneous (Bartlett's test,  $p=0.885$ ). Thus, they were analyzed using the nonparametric Kruskal-Wallis test. Because there was no significant difference between the two controls, they were pooled for analysis against the 1000 and 5000 µg/l treatments.

Percentage of egg masses laid in the different ovipositional substrates (control, sodium phosphate, and sodium arsenate water) for each of the larval exposure treatments (control and 1000 µg/l arsenate) were compared against expected percentages (33% for each if no preference) in order to determine if there was any preference for an ovipositional substrate within an exposure treatment using the Proc Freq procedure in

SAS v.9.2. Proc Freq was also used to evaluate any differences between larval exposure treatments in preference for ovipositional substrates.

## **Results**

### *Behavior Assays*

The duration data for resting and gliding were not normally distributed, and variances for resting behavior were not homogeneous. Thus, a box-cox power transformation was applied to each ( $\lambda=3.5$  for resting and  $\lambda=0.1$  for gliding) to uphold the assumptions. There were no significant differences between treatments for the duration (Table 6.1) or frequency (Table 6.2) of resting events. There was a significant treatment effect on the duration of gliding events ( $F=6.86$ ,  $df=2$ ,  $p=0.003$ ), with larvae in the 1000  $\mu\text{g/l}$  treatment gliding for significantly longer periods of time than larvae in 5000  $\mu\text{g/l}$  treatment ( $p=0.002$ ), though there was no difference from the control ( $p=0.072$ ).

Swimming duration data were reciprocal square root transformed to uphold normality and homoscedastic assumptions, but there was no significant effect of treatment on swimming duration (Table 6.1). Differences between treatments for the frequency of swimming events could not be evaluated due to a low number of occurrences. There was similarly no significant treatment effect on the duration (Table 6.1) or frequency (Table 6.2) of deep dives or on the duration of shallow dives, though differences between the frequencies of shallow diving events could not be evaluated due to a low number of occurrences. There was no significant effect of treatment on duration (Table 6.1) or frequency (Table 6.2) of ascents after dives as well.

**Table 6.1.** Duration (in seconds) of selected behaviors in response to arsenic exposure during eight minute observation periods.

	Control		1000 µg/l		5000 µg/l		F	p
	Mean	95% CL	Mean	95% CL	Mean	95% CL		
Resting	409	381-437	373	334-413	411	371-450	2.13	0.133
Gliding	55.8	33.7-77.8	105	74.5-136	40.5	9.94-71.0	6.86	0.003
Swimming	10.0	-43.6-63.6	4.6	-43.3-52.5	53.5	-0.078-107	2.71	0.213
Deep Dive	22.9	13.8-32.0	16.8	3.35-30.3	39.0	21.6-56.4	3.28	0.099
Shallow Dive	11.5	-3.48-26.5	13.8	-1.23-28.7	17.0	-13.0-47.0	0.156	0.865
Ascending	10.6	4.05-17.2	7.14	-1.11-15.4	11.3	-1.28-23.9	0.062	0.940

**Table 6.2.** Number of occurrences of selected behaviors in response to arsenic exposure during eight minute observation periods.

	Control		1000 µg/l		5000 µg/l		F	p
	Mean	SE	Mean	SE	Mean	SE		
Resting	6.97	0.776	6.47	0.919	6.33	1.45	0.116	0.891
Gliding	5.88	0.696	6.31	0.857	4.54	1.17	0.837	0.443
Deep Dive	1.55	0.247	1.40	0.400	1.67	0.258	0.068	0.935
Ascending	1.45	0.280	1.14	0.143	1.33	0.333	0.115	0.893

**Table 6.3.** Duration (in sec) of time spent aggregated at densities of 1, 2, 3, or 4 larvae during eight minute observation periods.

#	Control		1000 µg/l		5000 µg/l	
	Mean	95% CL	Mean	95% CL	Mean	95% CL
<b>Aggregated</b>						
1	296	231-360	238	173-302	286	162-409
2	188	111-265	169	106-233	303	191-415
3	142	75.9-208	108	57.7-158	24.2	3.96-44.4
4	115	78.3-153	38.0	-1.37-77.4	n/a	n/a

Friedman's test revealed no significant differences between treatments in aggregation behavior ( $F=1.57$ ,  $df=2$ ,  $p=0.457$ ) (Table 6.3). However, there is evidence that at the highest treatment level (5000 µg/l) larvae were more likely to spend larger amounts of time solitary or aggregated in twos as opposed to at higher densities.

For the shadow stimulus trials, results from the 2x2 contingency comparisons between shadow events are shown in Table 6.4. There were significantly more dives in the 1000 µg/l treatment vs. control in response to the first shadow stimulus. In the 5000 µg/l treatment, there were significantly fewer dives than controls in response to the second, third, and fifth shadow stimuli. When comparing the 1000 and 5000 µg/l treatments to each other, larvae would dive significantly more often in response to the second shadow stimulus in the 1000 µg/l treatment (Table 6.5).

Results of the Kruskal-Wallis test for differences in dive duration revealed a significant treatment effect ( $\chi^2=9.63$ ,  $df=2$ ,  $p=0.008$ ). Wilcoxon rank sum post hoc

comparisons revealed that dive time was significantly greater in the 5000 µg/l treatment than both the control ( $\chi^2=7.48$ ,  $df=1$ ,  $p=0.006$ ) and 1000 µg/l treatment ( $\chi^2=9.11$ ,  $df=1$ ,  $p=0.003$ ), though there was no difference between control and 1000 µg/l ( $\chi^2=0.003$ ,  $df=1$ ,  $p=0.953$ ) (Figure 6.1).

### *Oviposition*

Adult females that were exposed to control water as larvae collectively laid 4% of egg masses in the control water, 73% in the sodium phosphate water, and 23% in the sodium arsenate water, indicating a preference for sodium phosphate (sodium ion control) water. These values differed significantly from expected frequencies of 33% for each, which assumed no preference or aversion to any of the oviposition treatments ( $\chi^2=78.0$ ,  $df=2$ ,  $p<0.001$ ). The same was true for adult females reared in the 1000 µg/l treatment as larvae ( $\chi^2=42.1$ ,  $df=2$ ,  $p<0.001$ ), where females collectively laid 54%, 3%, and 43% of egg masses in control, sodium phosphate, and sodium arsenate water. However, in this case females preferred control and arsenic contaminated water. When compared to each other, there was a significant difference between the control and 1000 µg/l arsenic reared adult females with regards to which water treatments they selected to oviposit in ( $\chi^2=28.1$ ,  $df=2$ ,  $p<0.001$ ).

### **Discussion**

Of the behaviors tested, there was only a significant treatment effect on the duration of the gliding behavior, with *Cx. tarsalis* larvae in the 1000 µg/l treatment gliding significantly longer than the 5000 µg/l treatment. This potentially paradoxical dose-response outcome has been previously documented to occur with fluoride

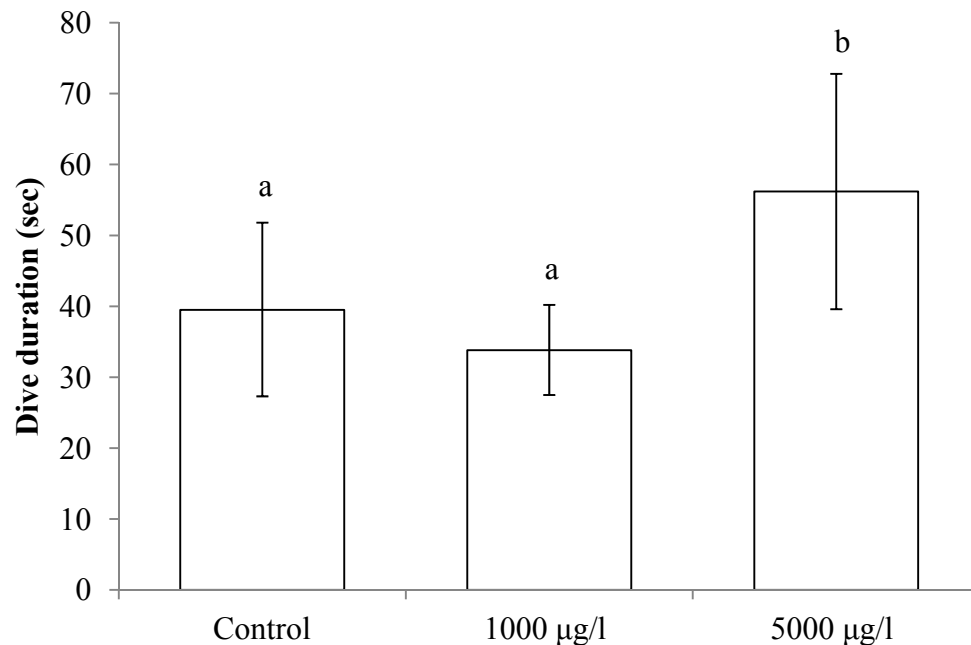


**Table 6.4.** 2x2 contingency comparisons for dive frequency in response to the shadow stimulus for controls vs. treatments.  $\chi^2$  analyses were conducted between the treatment and their respective controls. Values for N represent the number of larvae that responded to the shadow by diving (control, As treatment), out of a total of 25 larvae for each control and treatment.

	1000 $\mu\text{g/l}$ As					5000 $\mu\text{g/l}$ As				
	1	2	3	4	5	1	2	3	4	5
N	10,18	10,11	11,11	8,12	6,6	19,14	11,4	15,5	9,8	14,3
$\chi^2$	5.19	0.082	0.00	1.33	0.00	2.23	4.67	4.67	0.086	10.8
p	0.023	0.775	1.00	0.248	1.00	0.134	0.031	0.031	0.765	0.001

**Table 6.5.** 2x2 contingency comparisons for dive frequency in response to the shadow stimulus for 1000  $\mu\text{g/l}$  vs. 5000  $\mu\text{g/l}$  treatments.  $\chi^2$  analyses were conducted between the treatments. Values for N represent the number of larvae that responded to the shadow by diving (1000, 5000  $\mu\text{g/l}$ ), out of a total of 25 larvae for each control and treatment.

	5000 $\mu\text{g/l}$ As				
	1	2	3	4	5
N	18,14	11,4	11,5	12,8	6,3
$\chi^2$	1.39	4.67	3.31	1.33	1.22
p	0.239	0.031	0.069	0.248	0.270



**Figure 6.1.** Average overall duration spent underwater in response to shadow stimuli between treatments. Error bars represent 95% confidence limits.

(Burgstahler, 2002, and references therein), though has yet to be documented for arsenic in biological systems. The dose-response paradox occurs when the rate of a response increases with increasing concentration of a toxicant and then shows a decrease at even higher concentrations. These effects may not necessarily be statistically significant, though they are reproducible, and may be dismissed as unimportant or due to experimental error given the lack of attention of this affect in toxicological textbooks (Burgstahler, 2002). Further research would be needed to determine whether a dose-response paradox is indeed responsible for the higher effect of arsenic at the 1000 vs. 5000 µg/l treatment. Contrary to the results presented here, past studies have documented

decreases in taxis behaviors at all exposure concentrations in other aquatic insects (e.g. *Baetis rhodani* (Ephemeroptera: Baetidae) and cadmium, Heinis et al., 1990).

Significant differences were observed between treatments for the shadow stimulus trials. At the 5000 µg/l concentration, larvae dove significantly less than controls, though when they did, they dove for significantly longer periods of time. This indicates that arsenic may negatively affect predator avoidance behaviors in *Cx. tarsalis* and slow recovery times. Increased escape behaviors have been documented for *Adenophlebia auriculata* (Ephemeroptera: Leptophlebiidae), where the nymphs preferred locations away from point sources of copper contamination (Gerhardt and Palmer, 1998). More often, studies report a decrease in predator avoidance behaviors. *Leptophlebia marginata* (Ephemeroptera: Leptophlebiidae) decreased escape behaviors when exposed to iron and lead (Gerhardt, 1994). *Baetis tricaudatus* (Ephemeroptera: Baetidae) were more vulnerable to predation when exposed to cadmium (Riddell et al., 2005). When Clements et al. (1989) examined the gut contents of the predator *Paragnetina media* (Plecoptera: Perlidae) in copper-exposed environments, they found an increase in trichopteran remains, indicating an increased susceptibility of those insects to predation. There are also examples where there was no effect on predator avoidance (e.g. Kiffney, 1996; Lefcort et al., 2000), indicating that the impact of metals and metalloids on predator avoidance behaviors may be species specific. However, it should be noted that in all of the published studies, avoidance behaviors were being measured in response to metal cations in solution, as opposed to a metalloid anion.

Female *Cx. tarsalis* had significantly different preferences for oviposition water depending upon whether they were reared in control water or the 1000 µg/l arsenate treatment. However, whether these differences are due to a treatment effect or are an artifact of the experimental design is not clear, although the petri dishes were rotated to avoid preference for one location in the cage. Very few studies investigate the ovipositional responses of aquatic insects to metal or metalloid contaminated environments. *Chironomus riparius* (Diptera: Chironomidae) showed no aversion to water contaminated with copper (Dornfeld et al., 2009), and only avoided cadmium at the highest concentrations tested, which were acutely toxic to the eggs (Williams et al., 1987). In *Culex quinquefasciatus*, Jensen et al. (2007) found no effect of methyl mercury or sodium selenate on oviposition preference.

The results from this study indicate that there are minimal effects of chronic arsenic exposure, even at extreme concentrations, on *Cx. tarsalis* larval behaviors. However, the significant increase in the amount of time spent gliding by larvae in the 1000 µg/l treatment may result in increased visibility to predators, and therefore an increased predation risk. The diminished response to a shadow stimulus by larvae in the 5000 µg/l treatment further demonstrates that an increased risk of predation may occur to larvae exposed to high levels of arsenic. The results from the adult female oviposition trials indicate that individuals reared in control water mostly avoid arsenic contaminated substrates, while those reared in arsenic contaminated water prefer control and arsenic contaminated water. However, further studies using different mosquito and dipteran species would be useful in determining whether these effects for arsenic are unique to *Cx.*

*tarsalis* or are more broadly indicative of behavioral responses to chronic arsenic exposure.

## **CHAPTER 7**

### **Trophic transfer of arsenic from an aquatic insect to terrestrial insect predators**

**Abstract:**

The movement of energy and nutrients from aquatic to terrestrial ecosystems can be substantial, and emergent aquatic insects can serve as biovectors not only for nutrients, but also for contaminants present in the aquatic environment. The terrestrial predators *Tenodera aridifolia sinensis* (Mantodea: Mantidae) and *Tidarren haemorrhoidale* (Araneae: Theridiidae) and the aquatic predator *Buenoa scimitra* (Hemiptera: Notonectidae) were chosen to evaluate the efficacy of arsenic transfer between aquatic and terrestrial environments. *Culex tarsalis* larvae were reared in either control water or water containing 1000 µg/l arsenic. Adults that emerged from the control and arsenic treatments were fed to the terrestrial predators, and fourth instar larvae were fed to the aquatic predator reared in control or arsenic contaminated water. *Tenodera a. sinensis* fed arsenic-treated *Cx. tarsalis* accumulated 658±130 ng/g of arsenic. There was no significant difference between control and arsenic-fed *T. haemorrhoidale* (range 142-290 ng/g). *Buenoa scimitra* accumulated 5120±406 ng/g of arsenic when exposed to arsenic-fed *Cx. tarsalis* and reared in water containing 1000 µg/l arsenic. There was no significant difference between controls or arsenic-fed *B. scimitra* that were not exposed to water-borne arsenic, indicating that for this species environmental exposure was more important in accumulation than strictly dietary arsenic. These results indicate that transfer to terrestrial predators may play an important role in arsenic cycling, which would be particularly true during periods of mass emergence of potential insect biovectors. Trophic transfer within the aquatic environment may still occur with secondary predation, or in predators with different feeding strategies.

## Introduction

Numerous studies have evaluated the effects of runoff on nontarget aquatic life (DeLorenzo et al., 2001). Urban runoff (Zhang et al., 2012) and erosion (Nriagu, 1994; Ravenscroft et al., 2009) have been considered important routes for heavy metal and metalloid contaminants, such as arsenic, to reach aquatic systems. However, more recently, it has become apparent that the flow of nutrients and contaminants is not unidirectional, and that energetic pathways also link aquatic to terrestrial systems (Sullivan and Rodewald, 2012). In aquatic-to-terrestrial transport of contaminants, the movements of animals that have accumulated contaminants, either through space or through trophic transfer, allows for the transfer of potentially harmful substances to locations away from the contaminant origin (Blais et al., 2007). Aquatic insects, whose biomass can account for up to 190 kg/ha/d in very productive lake systems (Gratton et al., 2008), have been shown to effectively export contaminants such as anthropogenic nitrogen and polychlorinated biphenyls from aquatic systems to terrestrial predators (Akamatsu and Toda, 2011; Menzie, 1980; Walters et al., 2008).

Arsenic is a widespread surface and ground water contaminant worldwide (Smedley and Kinniburgh, 2002). The terrestrial bogong moth, *Agrotis infusa* (Lepidoptera: Noctuidae), has been shown to act as a biovector of arsenic when migrating individuals estivate gregariously (Green, 2008). In the environment, arsenic may result from both natural and anthropogenic sources. Natural sources of arsenic in soils are mainly the parent materials from which arsenic is derived and volcanic activity, while anthropogenic sources include lead-arsenate insecticides, irrigation, and atmospheric



deposition resulting from the burning of fossil fuels and copper smelting (Nriagu, 1994). Weather events and runoff then transfer arsenic into aquatic systems.

In the United States, the Environmental Protection Agency (EPA) regulates safe concentrations of arsenic in surface waters for aquatic life, given its designation as a priority toxic pollutant. The maximum safe concentration for chronic exposure is 150 µg/l (US EPA, 2006), although concentrations can exceed 1000 µg/l (Smedley and Kinniburgh, 2002). Concentrations in soils typically average 1 – 40 mg/kg and can exceed 600 mg/kg in contaminated locations (see Mandal and Suzuki, 2002, and references therein). The forms most often encountered in the environment are arsenate, which substitutes for phosphate in ATP synthesis, and arsenite, which has a high affinity for sulfhydryl bonds and disrupts protein folding (Hughes, 2002). While the abiotic processes linking terrestrial and aquatic arsenic cycles have been extensively studied (see Nriagu, 1994; Ravenscroft et al., 2002), the ecological interactions of biovectors and arsenic cycling pertaining to insects is not well understood. The trophic transfer of arsenic between an invertebrate prey and insect (Burghelea et al., 2011; Croisetière, 2006; Lavilla et al., 2010; Mason et al., 2000) or vertebrate (Culioli et al., 2009; Dutton and Fisher, 2011) predators has been documented for aquatic systems. However, in terrestrial systems, trophic transfer of arsenic has only been evaluated for movement to vertebrate predators (Albert et al., 2008; Hopkins et al., 2002; Morrissey et al., 2007; Morrissey et al., 2008; Torres and Johnson, 200122-26).

The goal of this research is to evaluate the potential for trophic transfer of arsenic from the aquatic to terrestrial environment via insects, and to evaluate efficiency of

transfer for three aquatic or terrestrial predatory species with different feeding strategies that prey on mosquitoes. For this purpose we chose the Chinese praying mantis, *Tenodera aridifolia sinensis* (Mantodea: Mantidae), which is a widespread mantid native to east Asia that is often used in behavior and physiology studies (Kral, 2012); the orb-weaving spider, *Tidarren haemorrhoidale* (Araneae: Theridiidae), a native to southern California and known to feed on small flying insects (Nyffler et al., 1988), and; the backswimmer, *Buenoa scimitra* (Hemiptera: Notonectidae), which flies nocturnally (thus occasionally leaving the aquatic system (Gäde et al., 2004)), and is an effective predator of mosquito larvae being evaluated for release as a biological control agent against mosquitoes (Rodriguez-Castro et al., 2006). The ease in rearing these predators combined with their accessibility makes them optimal laboratory assay organisms.

## **Methods**

### *Mosquito Rearing*

Mosquito egg rafts of *Cx. tarsalis* were obtained from colonies maintained at the University of California, Riverside. Eggs were hatched in shallow white enamel pans (39 × 23 × 10 cm or 39 × 23 × 6 cm) containing 3 liters of tap water. Arsenic-treated water contained 1000 µg/l of sodium hydrogen arsenate heptahydrate, 99.998% (Sigma-Aldrich, St. Louis, MO, USA). This concentration was chosen because it represents a high yet still ecologically relevant concentration of arsenic (Mogren et al., 2012; Smedley and Kinniburgh, 2002). Pans were kept in an environmental rearing chamber under a 16:8 L:D cycle at 28.0±0.25°C, and larvae were fed a mixture of a 3:1 (wt:wt) ground mouse

chow (mouse/rat diet, Harlan/Teklad, Madison, WI, USA) and brewer's yeast (MP Biochemicals, Aurora, OH, USA) as a 10% suspension in deionized water.

At approximately one week after hatching, pupae were removed using a mesh net and transferred to a bowl of deionized water. The bowl was placed inside a 30 cm × 30 cm × 30 cm plastic insect cage at ambient light and temperature for adult emergence. Adults were provided with a 20% sucrose solution for feeding (Müller and Schlein, 2006). Larvae of *Cx. tarsalis* accumulated 6200±397 ng/g of arsenic and adults retained 2450±242 ng/g.

#### *Arsenic Analysis*

All of the arsenic analyses were conducted on entire individual insects using a previously described method (Mogren et al., 2012; Ringmann et al., 2002). Briefly, samples underwent a two step microwave digestion process with sodium persulfate, sodium fluoride (Sigma-Aldrich, St. Louis, MO, USA), and nitric acid in HP-500 Teflon PFA digestion vessels (CEM Corporation, Matthews, NC, USA). Once cooled, the digestate was diluted and an aliquot pre-reduced using concentrated HCl and a 5%/5% w/w KI (potassium iodide, Sigma-Aldrich, St. Louis, MO, USA)/L-ascorbic acid (Fisher Scientific, Pittsburgh, PA, USA) solution. Arsenic concentrations were detected using a Perkin-Elmer (Waltham, MA, USA) Analyst 800 Atomic Absorption Spectrophotometer. The minimum detection limit of the HGAAS was previously determined to be 0.050 µg/l for arsenic (Mogren et al., 2012). Simultaneous digestions of oyster tissue standard reference material (NIST 1566b, Gaithersburg, MD, USA) and reagent blanks were conducted to validate the arsenic concentrations recovered in the unknown insect tissues.

*Tenodera aridifolia sinensis*

Egg masses of *Tenodera aridifolia sinensis* were purchased from Rincon-Vitova Insectaries (Ventura, CA) and hatched in 5-liter aquaria covered with cheesecloth in an environmental rearing chamber at 28°C and 16L:8D light cycle. A beaker containing water was placed inside the aquaria to increase humidity and induce hatching. A branch of *Photinia* sp. was also added to reduce cannibalism of newly hatched first instars. The eggs hatched two weeks after arriving. Once hatched, each of 40 individuals were transferred to a 600 ml beaker containing a moist cotton ball and covered with cheesecloth, similar to Kaltenpoth (2005). Twenty nymphs were randomly assigned to the control treatment (fed adult control mosquitoes) and the other 20 were assigned to the arsenic treatment (fed adult mosquitoes exposed to 1000 µg/l arsenate as larvae).

Mantids were provided with one mosquito from their respective treatment group after hatching, and within 2 hours, most of the first instars had caught and eaten the mosquitoes. While first instars, each individual *T. a. sinensis* was fed one mosquito daily. Second instars were fed two mosquitoes daily and third instars were fed three mosquitoes daily. If a mosquito died before being consumed, then it was replaced. Once individuals reached the fourth instar (approximately 31 days), they were fed three more mosquitoes and then allowed to depurate for 48 hours before being frozen and analyzed for arsenic. Instar, the number of mosquitoes fed, the number of mosquitoes consumed, and survival were monitored daily. Morphometric parameters were measured (head width, hind tibia length, body length, dry mass, longevity). Individuals were then oven dried, a final dried mass recorded, and digested and analyzed for arsenic accumulation.

Multiple regression analysis using backwards model selection (SAS v.9.2) was used to determine if any of the morphometric parameters, in addition to treatment and total number of mosquitoes consumed, were significant predictors of the final arsenic concentration. Although arsenic data were normally distributed, they did not uphold the assumption of homogeneous variances, and thus data were square root transformed. Beakers were blocked by location and a Relative Growth Index (RGI) calculated (Zhang et al., 1993, after Jensen et al., 2007). Abbott's formula (Abbott, 1925) was applied to correct for control mortality. Significant differences between treatments were determined using repeated measures ANOVA in R Statistical Software (v.2.15.0) with the lme4 package.

#### *Tidarren haemorrhoidale*

Forty individuals of *T. haemorrhoidale* were field collected from the University of California Riverside Botanic Garden in Riverside, California from a stand of *Opuntia littoralis* var. *vaseyi*, a prickly pear cactus native to southern California. Because *T. haemorrhoidale* were field-collected, a subset of individuals were sacrificed prior to the start of the experiment and analyzed for arsenic that may have been accumulated from the environment. Arsenic was not detected in *T. haemorrhoidale* using HGAAS.

A preliminary mass was taken before individuals were randomly assigned to a treatment group, 20 controls and 20 for the arsenic treatment. Individuals were placed in a 600 ml beaker and provided with a twisted piece of wire covered with masking tape as a web-building substrate. Beakers were covered with cheese cloth. Spiders were allowed to depurate for one week before being fed their respective treatment mosquitoes at a rate

of one every three days. This interval was deemed appropriate because in preliminary tests, individuals would not feed daily and mosquitoes would die before being consumed. The depuration period gave the spiders time to construct a web and once mosquitoes were caught in the web, the spiders were observed wrapping them in silk and feeding upon them.

The experiment was terminated after 30 days and the spiders preserved in ethanol. The species was verified using Levi (1957) and all specimens were determined to be adult females. A final mass was taken and the total number of consumed mosquitoes tallied (carcasses wrapped in silk were cast from the web after feeding). The total number of fecal spots, which measured 1-4 mm in diameter, was also recorded. Individuals were then oven dried, a final dried mass recorded, and then digested and analyzed for arsenic accumulation.

An analysis of covariance (ANCOVA) (SAS v.9.2) was used to determine if population means of the final mass (dependent variable) were the same across treatments (independent variable), while controlling for initial mass. Multiple regression analysis using backwards model selection (SAS v.9.2) was used to determine if any of the measured variables affected arsenic accumulation in *T. haemorrhoidale* adult females. A square root transformation was applied to achieve normality and homogeneous variances. Abbott's formula was applied to correct for control mortality (Abbott, 1925). Morphometric measurements were not possible for *T. haemorrhoidale* because all of the individuals used were adults, and they could not be reared from a standardized age.

*Buenoa scimitra*

Adults of *Buenoa scimitra* were collected from the Valley Sanitary District Treatment Wetland C in Indio, CA (access was authorized by the VSD for insect collection as part of an ongoing mosquito monitoring partnership with W.E.W.). The temperature in the wetland averages  $21.8 \pm 0.7^{\circ}\text{C}$ , pH averages  $7.8 \pm 0.1$ , and dissolved oxygen averages  $5.4 \pm 1.6$  mg/l. The average concentrations of ammonium-nitrogen and nitrate-nitrogen are  $41.5 \pm 4.30$  and  $5.1 \pm 1.3$  mg/l, respectively (Walton, unpublished data). Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) (Perkin-Elmer Optima 7300 DV, Waltham, MA, USA) analysis of the wetland water revealed that arsenic levels were not detectable. The notonectid species was verified and voucher specimens deposited in the UCR Entomology Research Museum collection (UCRC ENT 145417 and 145418). Because *B. scimitra* adults were field-collected, a subset of individuals were digested and analyzed using HGASS to determine if arsenic was present prior to the start of the experiment. While low levels of arsenic were detected in individuals from Wetland C ( $102 \pm 69.9$  ng/g), they were significantly lower than those in the control treatment, and thus did not affect experimental outcomes.

Individuals were transported back to the lab and transferred to a 37.9-liter aquarium containing 50% tap and 50% deionized water and reared following the procedures of Hazelrigg (1973). However, because *B. scimitra* is a free swimming notonectid, no substrate was provided. Eight individuals were transferred to each of fifteen 9.5-liter aquaria filled with 6 liters of tap water and pre-rinsed quartz sand (Repti Sand, Zoo Med Laboratories, Inc., San Luis Obispo, CA, USA). The aquaria were

randomly assigned to one of three treatments: control water with control mosquitoes (0/0), control water with arsenic reared mosquitoes (0/As), or arsenic water and arsenic reared mosquitoes (As/As), with five replicate aquaria per treatment. These aquaria were covered and aerated in a rearing room. Water temperature was 23.3°C through the duration of the experiment.

The notonectids were allowed to depurate for 48 hours before arsenic was added to the VV treatment (as sodium hydrogen arsenate heptahydrate, 99.998%, Sigma-Aldrich, St. Louis, MO, USA). After this point, each treatment received an input of approximately 40 mosquitoes daily for a month. This feeding rate was deemed appropriate because the following day, there would only be a couple mosquito larvae left. The notonectids are voracious mosquito predators (Rodriguez-Castro et al., 2006), and as soon as the mosquitoes were introduced, they were observed to strike at and feed on the larvae. Survival of notonectids was monitored daily. After 30 days, the notonectids from each treatment replicate were frozen and then oven dried. A dry mass was taken prior to digestion and arsenic analysis.

Multiple regression analysis using backwards model selection (SAS v.9.2) was used to determine if treatment, treatment replicate, or mass were good predictors of the total arsenic concentration in individuals. Treatment replicate was included as a variable to ensure there were no significant differences between replicates. A double square root transformation was applied to achieve normality and homogeneous variances. Abbott's formula was applied to correct for control mortality (Abbott, 1925). Morphometric



measurements were not possible for *B. scimitra* because all of the individuals used were adults, and they could not be reared from a standardized age.

## Results

### *Arsenic Analysis*

Digestion and prereduction blanks (containing only prereduction solutions and 6 ml Milli-Q HPLC-grade water) revealed no major sources of arsenic contamination from the reagents used or from analyst error, and any arsenic present was below detection limits (0.050 µg/l). Digestion and analysis of the NIST oyster tissue validated the protocol used to recover and analyze arsenic from the insect tissues with recoveries from the *T. a. sinensis*, *T. haemorrhoidale*, and *B. scimitra* digestions of  $102 \pm 3.67\%$ ,  $105 \pm 3.07\%$ , and  $110 \pm 1.92\%$ , respectively. These values are all in agreement with previously published recovery values (Mogren et al., 2012). ICP-OES analysis of the mosquito rearing water and the water *B. scimitra* were reared in revealed that actual arsenic concentrations were within 7% agreement of the target concentration of 1000 µg/l in the arsenic treatments.  $11 \pm 0.00$  µg/l of arsenic was detected in control water, but this is attributed to arsenic being present at safe drinking levels in the tap water in which insects were reared.

### *Tenodera aridifolia sinensis*

Results from multiple regression analysis indicated that of the seven independent variables considered (treatment, instar, lifespan, headwidth, tibia length, body length, and number of mosquitoes consumed), treatment, instar, and lifespan were good predictors of final arsenic concentration in *T. a. sinensis* (AIC= 409), although the variable instar was

removed because of multicollinearity (VIF=4.23), and thus the analysis was conducted using only treatment and lifespan. There was no significant interaction between these two variables ( $df=1,33$ ,  $F=0.37$ ,  $p=0.547$ ). However, there was a significant effect of treatment on arsenic accumulation (Table 7.1). The amount of arsenic accumulated by individuals did not, however, significantly affect RGI ( $df=1$ ,  $\chi^2=0.064$ ,  $p=0.801$ ). There was also no significant treatment effect on lifespan or morphometric characters, and there was no significant difference between treatments for the number of mosquitoes consumed (Table 7.2).

Samples of mantid frass, liquid excrement, and exoskeletons were analyzed at the Stanford Synchrotron Radiation Lightsource (Menlo Park, CA) using x-ray atomic spectroscopy (XAS) (Mogren, unpublished data). Any arsenic that was being excreted through these mechanisms by *T. a. sinsensis* was below detection limits, indicating that these insects may not efficiently excrete arsenic and will therefore accumulate dietary arsenic in their bodies.

#### *Tidarren haemorrhoidale*

ANCOVA revealed no significant covariate effect of the initial mass of the spiders with the final mass of the spiders between treatments, and no effect of treatment on the final mass of *T. haemorrhoidale* (control:  $12.0 \pm 0.59$  mg; As:  $11.5 \pm 0.58$  mg,  $F=2.10$ ,  $df=2,30$ ,  $p=0.140$ ). Results from multiple regression analysis indicated that of the five independent variables (treatment, initial mass, final mass, cast prey, and number of fecal spots) examined, treatment, final mass, and number of fecal spots were good predictors of the final arsenic concentration (AIC=198). There were no significant

**Table 7.1.** Arsenic accumulation in the terrestrial predators. Units are ng/g dry weight.

The arsenic treatments were fed *Cx. tarsalis* adults that retained 2450±242 ng/g.

	Control			As(V)			t	P
	N	Mean	95% CI	N	Mean	95% CI		
<i>Tenodera aridifolia sinensis</i>	15	145	63.4 - 227	19	658	384 - 931	4.54	<0.001
<i>Tidarren haemorrhoidale</i>	13	142	20.7 - 264	17	291	104 - 478	1.46	0.158

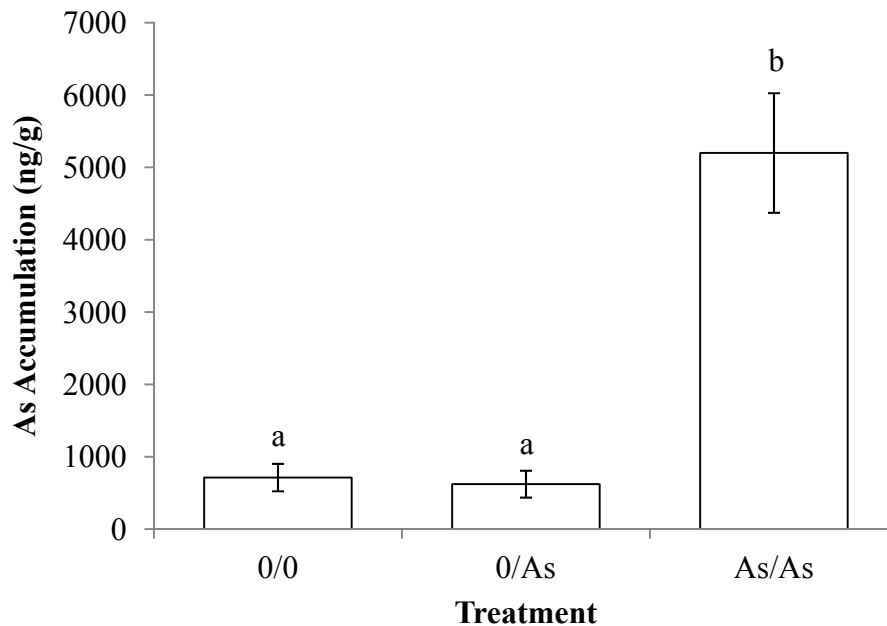
**Table 7.2.** Lifespan, morphometric characteristics, and number of mosquitoes consumed by *Tenodera aridifolia sinensis*. There were no significant differences between treatments.

	Control			As(V)			F	P
	N	Mean	SE	N	Mean	SE		
Lifespan (d)	17	35.5	1.02	20	35.3	1.33	0.02	0.899
Tibia length (mm)	17	7.76	0.16	20	7.55	0.22	0.59	0.447
Headwidth (mm)	17	3.44	0.09	20	3.43	0.08	0.00	0.966
Body length (cm)	17	2.50	0.07	20	2.40	0.08	0.78	0.382
Mosquitoes consumed	17	45.1	2.21	20	46.4	2.78	0.12	0.727

interactions between these variables ( $p > 0.05$  for all 2-way combinations), and no multicollinearity (average VIF=1.05). There was no significant difference in As accumulation between control-fed and arsenic-fed spiders (Table 7.1). Similarly, there was no significant effect of treatment on the number of fecal spots (a measure of egestion and potential detoxification) produced by individuals during the course of the experiment (control:  $14.4 \pm 1.46$  fecal spots; As:  $13.9 \pm 1.02$  fecal spots;  $df=1,33$ ,  $F=2.50$ ,  $p=0.126$ ). There was no significant treatment effect on the number of cast prey (control:  $13.4 \pm 1.82$  cast prey; As:  $13.0 \pm 2.18$ ;  $df=1,33$ ,  $F=1.10$ ,  $p=0.302$ ).

#### *Buenoa scimitra*

Results from multiple regression analysis indicated that only the variable treatment was a good predictor of the final arsenic concentration, thus differences between treatments were analyzed using a one-way ANOVA. There was a significant difference between the three treatments (0/0, 0/As, As/As) ( $df=2,94$ ,  $F=164$ ,  $p < 0.001$ ; Figure 7.1) and Tukey's post hoc comparisons showed the As/As treatment having significantly greater amounts of arsenic than both the 0/0 and 0/As treatments ( $p < 0.001$ ), which were not significantly different from each other ( $p=0.644$ ). This indicates that water contamination may play a more important role than prey ingestion in arsenic accumulation for these predators. There was a high degree of variability in the As/As treatment, and outliers as high as 40,000 ng/g were excluded from analysis (two outliers were removed based on studentized residuals greater than 2 (Chen et al., 2003)).



**Figure 7.1.** Arsenic accumulation in the aquatic predator, *Buenoa scimitra*. Mean  $\pm$  95% CI. N=35 for the 0/0 treatment (control water and control mosquitoes), N=28 for the 0/As treatment (control water and arsenic-fed mosquitoes), and N=34 for the As/As treatment (arsenic water and arsenic-fed mosquitoes). The arsenic treatments were fed fourth instar *Cx. tarsalis* prey that accumulated  $6200 \pm 397$  ng/g.

## Discussion

The terrestrial predators evaluated for trophic transfer in this experiment have different feeding strategies: spiders feed by injecting digestive fluids into their prey and ingesting the partially digested remains, while mantids consume prey whole. Therefore, any indigestible parts of the prey that may contain arsenic, such as the prey's exoskeleton, would not be biologically available to the spider. This may explain the

significant difference observed in arsenic accumulation for *T. a. sinensis* and *T. haemorrhoidale*, where *T. a. sinensis* accumulated twice as much arsenic as *T. haemorrhoidale* when fed adults of *Cx. tarsalis*. Previous work using X-ray Atomic Spectroscopy (XAS) has shown that in adults of *Cx. tarsalis*, arsenic is accumulated mostly in the exoskeleton of the thorax (Mogren, unpublished data), which would be largely unavailable to a piercing-sucking predator. However, *B. scimitra*, which also has a piercing-sucking feeding strategy, accumulated eight times more arsenic in the As/As treatment than *T. a. sinensis* and 18 times more arsenic than *T. haemorrhoidale*. Increased accumulation in this species may be due to the larvae of *Cx. tarsalis* having more than twice as much accumulated arsenic as adults, or because *B. scimitra* was also exposed to arsenic through the water. At least in this case, direct environmental exposures play a much more significant role in accumulation than dietary exposure.

Other aquatic predators have also been shown to accumulate arsenic through trophic exposure. In a field study, *Sialis velata* (Megaloptera: Sialidae) only accumulated arsenic from contaminated chironomid prey, and the presence of arsenic in water did not affect overall accumulation in *S. velata* (Croisetière et al., 2006), in contrast to our findings with *B. scimitra*. Burghilea et al. (2011) reported three species of predaceous dystiscids living in water containing 0.32 µg/l of arsenic accumulating 320±40 ng/g (*Hydroglyphus pusillus*), 410±100 ng/g (*Laccophilus minutus*), and 320±50 ng/g (*Rhantus suturalis*). However, Lavilla et al. (2010) found that 98% of total arsenic in three species of anisopterans was bound to the exoskeleton as opposed to being ingested and accumulated (see also Martin et al., 2008). The differences observed in accumulation

and trophic transfer of arsenic from prey to predators in these field experiments and the results from *B. scimitra* may be due to an interplay of biotic and abiotic factors (water chemistry, fluctuations in flow) in the field that cannot be replicated in the lab (Mason et al., 2000), and which may affect transfer through the food web. The ability of a specific aquatic predator to accumulate arsenic will likely be affected by the physiology of that predator and the accumulation and biotransformation abilities of the prey, in addition to abiotic conditions (Martin et al., 2008).

The trophic transfer literature with regards to terrestrial systems focuses mainly on the effects of arsenic on vertebrates. Banded water snakes fed fish from a contaminated location did not exhibit signs of physiological stress, though they did accumulate arsenic (Hopkins et al., 2002). However, potential effects of arsenic were confounded by the presence of many other elements, and how arsenic moved through the food chain can only be inferred because invertebrates were not sampled from the location. Arsenic accumulation was similarly negligible in mice living in a contaminated seasonal wetland, whose diet consisted of seeds, plants, and arthropods that accumulated significant concentrations of arsenic (up to 0.419 mg/kg in arthropod prey) (Torres and Johnson, 2001). In contrast, studies addressing the affects of organoarsenical pesticides on woodpeckers and their prey, the mountain pine beetle, found that the birds accumulated potentially toxic levels of arsenic from the beetles (Morrissey et al., 2007), and that these levels caused both lethal (Albert et al., 2008) and sublethal (Morrissey et al., 2008) effects. The much more toxic effects of arsenic moving up the food chain in these studies may be the result of the organic form of arsenic in which beetles and birds

were exposed versus inorganic forms in the Hopkins et al. (2002) and Torres and Johnson (2001) studies, as well as the concentrations accumulated in the mountain pine beetles. Differences between these vertebrate predators and the invertebrate predators *T. a. sinensis* and *T. haemorrhoidale* may be attributed to exposure levels and the form of arsenic in which the predators are ultimately exposed. The degree of arsenic accumulation may be further influenced by the detoxification and excretion pathways within the insect predators, which are thought to be mediated by glutathione (e.g. Andrahennadi and Pickering, 2008; Muñiz-Ortiz et al., 2009; Zaman et al., 1995).

Examples of arsenic trophic transfer with a terrestrial insect as the biovector have been shown for the bogong moth, *Agrotis infusa*. The gregarious estivations of this moth, which reach densities in the millions, result in the concentrating of arsenic to phytotoxic levels (Green, 2008). Although a direct correlation of trophic transfer to vertebrate predators has yet to be documented in this system, there is evidence for accumulation of arsenic in birds and small mammals in this system, with small predators accumulating more arsenic than herbivores (Green et al., 2001).

Within an aquatic environment, arsenic is not likely to biomagnify (Culioli et al., 2009; Dutton et al., 2011; Farag et al., 1998), though it is bioavailable and does accumulate in invertebrates, as seen in *B. scimitra*. With regards to the linkages between aquatic and terrestrial arsenic cycles, there are no published field studies investigating the movement of this contaminant via emergent insects, or the effects they have on terrestrial predators, although there is theoretical evidence that inputs could be substantial (Menzie, 1980; Sullivan and Rodewald, 2012). However, both mantids (Schmidt-Rhaesa and



Ehrmann, 2001) and spiders (Dreyer et al., 2012) are shown to ingest insects with aquatic life stages in the wild. Recent work suggests that energy flows from aquatic to terrestrial environments may positively affect productivity in adjacent habitats through the rapid incorporation of insect detritus (Baxter et al., 2005; Hoekman et al., 2012). However, there could also be negative consequences to these inputs when insects are exposed to persistent environmental contaminants, such as organic polychlorinated biphenyls (PCBs) (Walters et al., 2008) and heavy metals such as mercury (Runck, 2007). Even if individuals emerging from the aquatic environment do not contain heavy loads of arsenic, the potential for the biomagnification of arsenic from mass emergences to adjacent terrestrial habitats is high (Blais et al., 2011). Some aquatic insects, such as notonectids, are known to disperse en masse to different aquatic habitats, and could therefore vector contaminants from aquatic to aquatic systems as well (Lavilla et al., 2010; Stewart et al., 1969).

Further research is needed to evaluate the transfer potential of arsenic to terrestrial systems by emergent aquatic invertebrate biovectors, particularly in the context of climate change, as warmer temperatures may allow for more generations of consumers to be produced, increasing flow to terrestrial systems. Similarly, studies evaluating the movement of arsenic within aquatic food webs would provide valuable links in the biotic transfer potential of arsenic. Based on the results from this study, the generalist predator *T. a. sinensis* has the potential to be a good organism for tracking the movement of arsenic from aquatic to terrestrial predators, and other generalist terrestrial predators, such as ground beetles, should be evaluated as well.

## **CHAPTER 8**

### **Conclusions**

Arsenic is an important contaminant of surface and groundwaters worldwide. Contamination is largely the result of natural sources, such as geologic activity and oxidative processes that release arsenic from parent materials, though may also be introduced into the environment through anthropogenic activities like smelting, in industrial effluents, and from the legacy of lead-arsenate pesticides. Through irrigation and rain events, it then runs off into aquatic systems. But despite the increasing awareness of arsenic in the aquatic environment, the effects of arsenic on lower trophic levels of insects inhabiting contaminated ecosystems are not well understood. In toxicology studies, the use of death as an endpoint often fails to capture the effects a pollutant has on disruptions to ecosystem services of individual insects, and the community effects that may result from exposures. For my PhD research, I evaluated the sublethal effects of arsenic exposures on the ubiquitous aquatic midge, *Chironomus riparius*, and the mosquitos *Culex tarsalis* and *Culex quinquefasciatus* and the ecosystem services provided by these insects.

In the first study of this dissertation, I evaluated the effects of chronic arsenic exposure (as arsenate) at the environmentally relevant concentrations of 0, 10, 150, 400, and 1000  $\mu\text{g/l}$  on larvae of *C. riparius* in factorial combinations with phosphate at 0, 14, and 1400  $\mu\text{g/l}$ . Because arsenate is a chemical analogue for phosphate, we wanted to evaluate a potential interaction between these two contaminants. Larvae were exposed as first instars through pupal emergence to these contaminants. Though there was no significant effect on larval survival, arsenic exposure at 1000  $\mu\text{g/l}$  significantly increased the time between male and female emergence from  $1.8 \pm 0.17$  days to  $2.9 \pm 0.34$  days, by

delaying female emergence. In *C. riparius*, as is the case with other aquatic insects, the males emerge first and form mating swarms. By delaying female emergence, arsenic contamination could result in reductions in incidence of mating as males could experience predation before having the opportunity to mate. Females could similarly be more prone to predation by aquatic predators due to this delay. The highest arsenic exposure level also led to a reduction in the number of eggs per egg mass, which could affect population maintenance in this species. There was no significant effect of phosphate, and no interaction of arsenate and phosphate.

Another important component to this study was to determine total arsenic accumulation in larval and adult tissues, which was achieved by developing a novel protocol for digestion and analysis of arsenic in insect tissue using Hydride Generated Atomic Absorption Spectroscopy (HGAAS). Concentrations ranged from  $2.48 \pm 0.363 - 30.5 \pm 0.473$   $\mu\text{g/g}$  in fourth instar larvae and  $1.03 \pm 0.286 - 8.97 \pm 0.662$   $\mu\text{g/g}$  in adults, indicating that 72% of arsenic accumulated as larvae is eliminated before the adult stage. There was no effect of phosphate, indicating that phosphate does not alter uptake of arsenate in *C. riparius*.

Another approach that may be used to document sublethal effects of contaminants is to assay them in combination with microbial pathogens to evaluate shifts in survival curves of the test organisms. This can be used to test whether the contaminant exerts a physiological stress on the insect lead leads to increased susceptibility to another stresser. Larvae of *Culex quinquefasciatus* and *Culex tarsalis* were reared in water containing 0 or 1000  $\mu\text{g/l}$  of arsenate or arsenite, the two most common forms of arsenic in the

environment and exposed fourth instars to a range of doses of the technical powders of the biological control agents *Bacillus thuringiensis* subsp. *israelensis* (Bti) or *Lysinibacillus sphaericus* (Ls). Larvae of both species accumulated between  $4447 \pm 169$  and  $6983 \pm 367$  ng As/g, though values obtained from calculating a Relative Growth Index (RGI) indicated that accumulation did not affect growth and development of the larvae.

In all of the exposure combinations (control, arsenate, and arsenite for the contaminant against Bti and Ls), the  $LC_{50}$ s and  $LC_{90}$ s of *Cx. quinquefasciatus* were higher than *Cx. tarsalis*. *Culex tarsalis* reared in arsenite showed a significant reduction in their Bti  $LC_{90}$  values compared to the control, indicating a sublethal effect of Bti. When exposed jointly with Ls, arsenite was more toxic than arsenate in *Cx. tarsalis*. Overall, these results indicate tolerance of these *Culex* species to arsenic exposures.

Next, to determine where arsenic was accumulating in these aquatic Diptera and to elucidate potential routes of arsenic detoxification, I used X-ray absorption spectroscopy to map the distributions and oxidation states of arsenic in whole insect specimens of *C. riparius* and *Cx. tarsalis* at various life stages after exposure to 1000  $\mu\text{g/l}$  of arsenate. Larvae of *C. riparius* accumulated arsenic in their midgut, with inorganic arsenate being the predominant form, followed by arsenite, and an arsenic thiol, likely  $\text{As}^{\text{III}}$ -tris-glutathione. Reduced concentrations of arsenic in the pupal and adult stages indicates that the 72% reduction in arsenic body burdens seen between the larval and adult stages in *C. riparius* is excreted between the larval and pupal stages. In the adult females of *C. riparius*, arsenic was limited mostly to the thorax, in either the muscle tissue or exoskeleton, and the predominant form was the arsenic thiol.

Contrary to *C. riparius*, arsenic was not found in high enough concentrations in *Cx. tarsalis* for accurate determinations of arsenic speciation. There were also differences between *C. riparius* and *Cx. tarsalis* in where arsenic was accumulated: in *Cx. tarsalis* fourth instar larvae, there was no distinct accumulation of arsenic in the midgut of the insect, and arsenic appeared to be distributed throughout the larva. In adults, arsenic was concentrated in the thorax and eyes, possibly in ommochrome pigment granules. Combined, the XAS results from both of these species provide valuable information to the growing body of literature regarding arsenic excretion in insects, and lend further support that at least in the case of *C. riparius*, glutathione mediated reduction or excretion is occurring.

In Chapter 5 of this dissertation, I review the literature pertaining to the sublethal effects of contaminants, specifically metals and metalloids, on insect behavior. There was a wide range of behaviors analyzed, but these could be divided into three categories: ingestion behaviors, taxis behaviors, and reproductive behaviors. Different species responded differently to various contaminants. For example, some species showed a tendency to increase locomotor behaviors to escape from locations with elevated metal pollution, whereas other insects greatly decreased all movements unrelated to feeding. Still others exhibited behaviors resulting in increased susceptibility to predation. With regards to reproduction, females were often unable to detect and therefore avoid even moderately polluted sites when ovipositing, which leads to egg loss and reduced fitness of offspring. Ultimately, these impaired behaviors in individuals can result in reduced

population sizes and diversity at contaminated locations, with the exceptions being those insects able to tolerate and adapt to local conditions.

This review provided a solid background from which to further evaluate the sublethal effects of arsenic on *Cx. tarsalis*. Larvae were reared in 0, 1000, or 5000 µg/l concentrations of arsenate until they reached the fourth instar, when behavioral observations were conducted. Of all the behaviors evaluated (resting, gliding, swimming, shallow dives, deep dives, and ascending), significance was only noted for an increase in the amount of time spent gliding in the 5000 µg/l versus the 1000 µg/l treatment. In predator avoidance trials, larvae in the 5000 µg/l treatment showed a decreased response to a shadow stimulus, which could make them more susceptible to predation. In addition, they spent significantly more time away from the water surface than the control and 1000 µg/l treatments when they did dive, which could increase the potential for drowning, or predation from benthic predators.

Oviposition trials were conducted to determine if adult females that emerged from the control or 1000 µg/l treatments avoided arsenic contaminated waters. Females were provided with a control (deionized water), 1000 µg/l arsenate (as sodium hydrogen arsenate) in deionized water, and a sodium phosphate control with an equivalent molar amount of sodium as the sodium arsenate treatment ( $2.66 \times 10^{-5}$  M) to ensure the adult females were not preferentially ovipositing in the water with more dissolved sodium ions. The results from these trials were mixed, with females reared in control water showing a significant preference for the sodium phosphate water, and females reared in the arsenic

treated water preferring control water and ovipositing the least in the sodium phosphate water.

Finally, I wanted to ascertain whether the aquatic insects that can accumulate arsenic from contaminated sources were capable of serving as biovectors of arsenic from the aquatic to terrestrial environments. For this, I chose the terrestrial predators *Tenodera aridifolia sinensis* and *Tidarren haemorrhoidale* and the aquatic predator *Buenoa scimitra*. *Culex tarsalis* were reared in either control water or water containing 1000 µg/l arsenate fed to the various predators. *Tenodera a. sinensis* accumulated 658±130 ng/g of arsenic, but there was no significant difference between control and arsenic-fed *T. haemorrhoidale* (range 142-290 ng/g), indicating an interesting difference between these terrestrial generalist predators that have different feeding strategies.

Because *B. scimitra* is an aquatic predator, the opportunity existed to manipulate not only arsenic in the food, but arsenic in the environmental matrix as well. Thus, there were three treatments for this species: control water and control mosquitoes, control water and arsenic fed mosquitos, and arsenic water and (1000 µg/l) arsenic fed mosquitoes. They accumulated 5120±406 ng/g of arsenic in the last treatment, but there was no significant difference between the control or arsenic fed *B. scimitra* when they were not exposed to water-borne arsenic, indicating that for this species environmental exposure was more important in accumulation than strictly dietary arsenic.

Taken together, these results indicate that transfer of arsenic to terrestrial predators may play an important role in arsenic cycling, which would be particularly true during periods of mass emergence of potential insect biovectors, such as mosquitoes or



midges. Trophic transfer of arsenic may still occur in the aquatic environment with secondary predation, or in predators with different feeding strategies (engulfers versus piercer-suckers).

Exposure to arsenic results in varied outcomes in different insects. Arsenic accumulation is highest in the lowest trophic levels, particularly in the case of *C. riparius*, which lives in benthic sediments as larvae. Despite the fact that *Cx. tarsalis* and *Cx. quinquefasciatus* accumulate arsenic, there were few sublethal effects that were measured in joint exposure assays with microbial control agents and in behavioral assays of *Cx. tarsalis*, though *C. riparius* experienced reduced fecundity. Both *C. riparius* and *Cx. tarsalis* showed marked differences in how they accumulate and potentially excrete arsenic physiologically, though the arsenic retained in their bodies is still bioavailable to higher trophic levels. The results in this dissertation contribute to the growing body of literature with regards to the effects of arsenic contamination in aquatic systems.

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