AN ABSTRACT OF THE DISSERTATION OF

David B. Buchwalter for the degree of Doctor of Philosophy in Toxicology presented on August 5, 2002.

Title: Respiratory Strategies and Associated Exchange Epithelia as Determinants of Contaminant Uptake in Aquatic Insects.

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Larwence R. Curtis / Jeffery T. Jenkins

Aquatic insects are used extensively to evaluate water quality. Despite their widespread use as indicator organisms, relatively little is known about the organismal characteristics that determine sensitivity differences to individual and multiple stressors. Insects have evolved several respiratory strategies that range from breathing atmospheric air to utilizing dissolved oxygen in water via exchange epithelial surfaces. This dissertation examines the role of respiratory attributes in determining differential accumulation of the insecticide chlorpyrifos, and further examines how accumulation rates are affected by temperature shifts. In addition, the relative roles of uptake rates and target site sensitivity differences are examined among developmental stages of the aquatic midge, *C. riparius*. Major findings:

- Smaller, gill-bearing insects accumulate chlorpyrifos and water at higher rates than larger, air-breathing insects.
- Chlorpyrifos and water accumulation rates are highly covariant in aquatic insects.

- Temperature increases affect chlorpyrifos accumulation rates in dissolved oxygen breathers more so than in air-breathers.
- Earlier instars of *C. riparius* are more sensitive to chlorpyrifos than later instars.
- Sensitivity differences among 2^{nd}-4^{th} instar *C. riparius* are largely due to differences in chlorpyrifos accumulation rates.

Respiratory Strategies and Associated Exchange Epithelia as Determinants of Contaminant Uptake in Aquatic Insects.

by

David B. Buchwalter

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David B. Buchwalter, Author

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CONTRIBUTION OF AUTHORS

Each of these manuscripts has been co-authored by Dr. Lawrence R. Curtis and Dr. Jeffrey J. Jenkins. In addition, Chapter four was co-authored by Jason Sandahl.

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Respiratory Strategies and Associated Exchange Epithelia as Determinants of Contaminant Uptake in Aquatic Insects

INTRODUCTION

Aquatic insects play important ecological roles in freshwater ecosystems and are used extensively to evaluate water quality. Freshwater ecosystems may be subjected to a variety of natural and anthropogenic stressors. That the biotic components of disturbed systems may be differentially affected by a stressor is the basis for the use of ecological indicators and bioassessment efforts. Ecological communities retain information about events in their history (Matthews et al, 1996). The attempt to use biological indices in the assessment of water quality has been continually developing for the past 150 years (Hynes 1960; Woodiwiss 1964;; Plafkin *et al.* 1989; Davis 1995). Bioassessment efforts using macroinvertebrates have resulted in cost-effective and increasingly useful techniques for regulatory agencies across the Unites States and Europe (Plafkin *et al.* 1989). In the United States, bioassessment is an important feature of sections 303(c), 303(d), 305(b), and 402 of the Clean Water Act.

Traditionally, water quality monitoring has involved taking water samples, measuring various physical and chemical characteristics, and comparing these measurements to standards. This approach, while providing important information, has proven to be expensive, and does not take into consideration "beneficial uses" such as the health of the resident biota. Despite its widespread use, there is

substantial room for improvement in bioassessment. The current bioassessment techniques using aquatic macroinvertebrates are not stressor-specific. The same techniques or metrics are used for all types of situations, and tend to treat all stressor types equally (i.e. temperature, sedimentation, pH, soluble contaminants, sediment bound contaminants, flow regimes, physical habitat alterations). While current bioassessment approaches may be appropriate for making generalizations about ecosystem integrity, they lack the mechanistic specificity required to determine causal relationships, which are ultimately important for management.

The existing methodologies for bioassessment are based on ecological survey data. Observations made by numerous ecologists in tremendously varied aquatic ecosystems have essentially been codified into the practices that are in place today. The end result of this process is that current techniques are not built upon experimental data and/or studies designed to test hypotheses. For example, the Environmental Protection Agency (EPA) advocates the use of tolerance values for individual species. These tolerance values are not stressor specific. A single value is given to a taxon, which is intended to reflect its tolerance to a wide variety of stressors, such as sedimentation, chemical contaminants, altered flow regimes, and dissolved oxygen, to name several. This simplistic approach is, in part, due to our limited understanding of the organismal characteristics that mediate aquatic insect responses to individual stressors. As bioassessment advances increasingly into the regulatory arena in several states, there is clearly a need to further develop

and refine bioassessment techniques such that they are more scientifically defensible.

There are over 1,000 species of aquatic insects that live in Oregon's waters (Norm Anderson, personal communication). There is very little known about the physical, chemical and habitat requirements for most of these species. There are very few toxicologists and physiologists working with aquatic insects today. Much of the toxicological work has historically focused on generating No Observable Effect Concentrations (NOEC) and Lethal Concentrations (LC 50) values for relatively few aquatic insect species. These data are largely ineffective in dealing with environmentally realistic contamination scenarios. The limitations of toxicology tests when extrapolated to the field are well documented (Cairns, 1983; Kimball and Levin, 1985; Cairns 1986). An alternative approach is to test hypotheses generated from ecological observations and examine physiological and morphological characteristics that may be important determinants of species susceptibilities to different classes of environmental stressors.

Aquatic insects are believed to be secondarily aquatic. In other words, existing aquatic insect species are believed to be derived from terrestrial ancestors via numerous independent invasions of aquatic habitats (Kristensen, 1981). Such invasions potentially happened more than once within some aquatic insect orders. Terrestrial insects breath atmospheric air through trachea, with gas exchange being mediated through spiracles. Their integuments are waterproofed with waxes and cuticular materials to prevent dessication. Respiratory systems in aquatic insects

range from breathing air through trachea (similar to their terrestrial counterparts) to utilizing dissolved oxygen via gills and other epithelial surfaces (Ericksen et. al, 1996). Additionally, chloride cells and chloride epithelia exist on the external surfaces of most aquatic insects for osmoregulation (Konmick, 1977). As secondarily aquatic organisms, insects have evolved diverse strategies in response to the demands of aquatic life, particularly with respect to respiratory and associated osmoregulatory systems. "In a broad general way, the degree to which the functional spiracles are reduced is an indication of the degree to which the larva is adapted to life in the water" (Hinton, 1947). Thus, the invasion of aquatic habitats is accompanied by significant changes in the nature of the integument, in terms of exposed epithelial exchange (or cellular) surfaces.

From a toxicological and physiological perspective, these exchange epithelial surfaces are likely to be very important in determining relative susceptibilities to stressors such as pH, contaminant exposure, and dissolved oxygen. Given the water permeability of epithelial surfaces and the potential for contaminants to be accumulated through exposed cellular surfaces, there should be marked differences in species' susceptibilities to water column-associated stressors. It is not surprising that insects that have relatively large epithelial exchange surfaces (gills for example on ephemeropterans, plecopterans, trichopterans) are those that ecologists typically generalize as being sensitive, while insects that have retained open tracheal systems and a water-proof cuticle (many

Dipterans:Culicidae, Ephydridae, Tipulidae, etc; many Coleopterans: Dysticidae, Hydrophilidae etc.) tend to be tolerant to poor water quality.

The studies presented in this dissertation attempt to: 1). Characterize aquatic insect taxa with respect to their water permeabilities and relative exchange epithelial surface areas, 2). Assess the role of respiratory strategy and associated epithelia in determining different exposure susceptibility to the insecticide chlorpyrifos, 3). Assess the effects of temperature in modulating chlorpyrifos uptake rates in insects that differ with respect to respiratory strategy, and 4). Characterize and account for differential sensitivity across developmental stages of the aquatic midge *Chironomous riparius*. The following are abstracts for the chapters 2-4.

Chapter 1. Abstract

Despite the extensive use of aquatic insects to evaluate freshwater ecosystem health, little is known about the underlying factors that result in sensitivity differences between taxa. Organismal characteristics (respiratory strategy and body size) were used to explore the rates of [^{3}H]-H_2O and [^{14}C]-chlorpyrifos accumulation in aquatic insects. Ten aquatic insect taxa including ephemeropteran, trichopteran, dipteran, hemipteran, and coleopteran species were exposed to [^{14}C]-chlorpyrifos (240 ng $\bullet$ L^{-1}) and [^{3}H]-H_2O for up to 12 hours. Because exchange epithelial surfaces on the integument are permeable to water,

[^{3}H]-H_2O was used as a quantitative surrogate for exposed cellular surface area. [^{14}C]-Chlorpyrifos uptake rates were highly correlated with water permeability in all 10 taxa tested and largely covaried with body size and respiratory strategy. Rates were highest among smaller organisms on a per-weight basis and in taxa with relatively large external cellular surfaces such as gills. Air-breathing taxa were significantly less permeable to both [^{3}H]-H_20 and to [^{14}C]-chlorpyrifos. A method for labeling exposed epithelial surfaces with a fluorescent dye was developed. This technique allowed discrimination between exchange epithelium and barrier tissue on the integument. Fluorescent dye distributions on the body surface provided a rapid method for estimating exposed epithelium consistent with [^{3}H]-H_20 and [^{14}C]-chlorpyrifos accumulation.

Chapter 2. Abstract

Aquatic insects have evolved diverse respiratory strategies that range from breathing atmospheric air to breathing dissolved oxygen. These studies examine the role of respiratory strategy in determining water and chlorpyrifos influx rates under different thermal regimes. We obtained ^3H_2O influx rates for larval plecopteran *Pteronarcys californica* (dissolved oxygen breathers) and hemipteran *Notonecta kirvyi* (air breathers) under different thermal regimes. *P. californica* larvae were highly water-permeable and showed marked increases in water influx when subjected to temperature increases relative to *N. kirvyi.* In other studies,

temperature shifts of 4.5 °C increased ^{14}C-chlorpyrifos accumulation rates in the gill-bearing mayfly *Cinygma sp.* and in the air-breathing hemipteran *Sigara washingtonensis.* However, the temperature-induced increase in ^{14}C-chlorpyrifos uptake after 8 hours of exposure was 2.75 fold higher in *Cinygma* than in *Sigara.* ^{14}C-Chlorpyrifos uptake was uniformly higher in *Cinygma* than in *Sigara* in all experiments. These findings suggest that organisms with relatively large exchange epithelial surface areas are more vulnerable to both osmoregulatory distress as well as contaminant accumulation. Temperature increases are more likely to impact organisms that have relatively large exchange epithelial surface areas, both as an individual stressor and in combination with additional stressors such as contaminants.

Chapter 3. Abstract

Several researchers have observed that earlier life stages of aquatic organisms tend to be more sensitive to various chemical contaminants than later life stages. This research was conducted to assess the sensitivity differences among life stages of the aquatic midge *Chironomous riparius* and attempts to identify the key biological factors that determine these differences. Specifically, 2nd – 4th instar larvae were exposed to both high and low doses of chlorpyrifos to examine sensitivity differences as well as uptake rate differences among instars. Earlier instars were significantly more sensitive to chlorpyrifos than later instars. In

addition, earlier instars had higher chlorpyrifos accumulation rates than later instars. In vitro acetylcholinesterase (AChE) assays were performed with both chlorpyrifos and the metabolite, chlorpyrifos-oxon to investigate potential target site sensitivity differences among instars. Homogenates derived from 2^{nd}-4^{th} instar larvae were relatively unresponsive to chlorpyrifos, even at high concentrations. In contrast, homogenates were responsive in a dose dependent manner to chlorpyrifos-oxon. When normalized for basal AChE activity, 4^{th} instar homogenates appeared to be more sensitive than earlier instars. In general, it appears that chlorpyrifos sensitivity differences among 2^{nd}-4^{th} instar *C. riparius* are largely driven by differences in uptake rates.

Chapter 2

Respiratory Strategy is a Major Determinant of Temperature-Modulated $^{3}H_2O$ Flux and ^{14}C-Chlorpyrifos Uptake in Aquatic Insects

Introduction

Aquatic insects play fundamental roles in freshwater ecosystems. They are an important food source for fish and birds and play a significant role in nutrient cycling and organic materials processing. Due to their ecological importance and diversity, aquatic insects are used extensively to evaluate ecosystem health and water quality through both field biomonitoring and laboratory bioassays. Ecologists have observed that certain taxa tend to be extirpated from systems with degraded water quality. Similarly, toxicologists have observed marked sensitivity differences among aquatic insect species. However, despite this widespread attention to insects, the mechanistic bases for differences in species sensitivity to individual and multiple stressors remain unclear. As a result, few diagnostic tools exist to evaluate insect community-level responses to specific stressors such as temperature or chemical contamination. This lack of a conceptual framework for understanding the underlying factors that determine interspecies differences in sensitivity or tolerance to individual and multiple stressors remains a major obstacle in interpreting both ecological and toxicological data.

The small size of aquatic insects results in an extremely high surface-to-volume ratio, which in turn requires that the integument provide a substantial

barrier to water and ions. Osmotic gradients favor the passive loss of ions as well as the influx of water (Komnick and Stockem, 1973; Komnick, 1977; Frisbie and Dunson, 1988, Kirschner, 1991). This osmoregulatory situation is generally countered by barriers of waterproofing lipids, waxes, and proteins on the integument, and/or by compensatory activity of chloride cells on the body surface (Figure 1). Insects are extremely heterogeneous with respect to the integument both in terms of structure and function. Integument differences are largely due to a wide variety of respiratory strategies, which range from breathing atmospheric air to breathing dissolved oxygen in water through exchange epithelia. In this paper, we use the term exchange epithelium specifically to describe a thin layer of cells that are covered by porous or ultra-thin cuticle such that they are effectively in immediate physiological contact with the surrounding water.

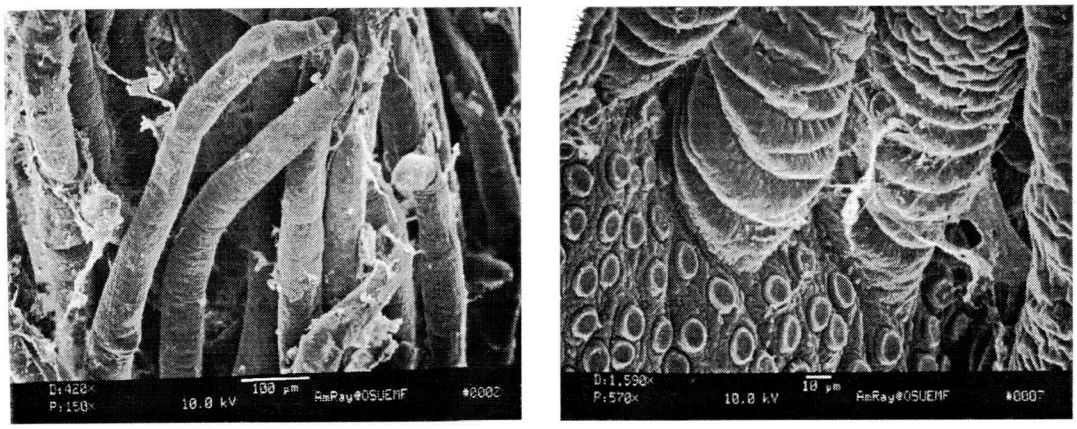

Figure 1. Electron micrographs of a single *Pteronarcys californica* gill tuft. Panel 1 shows the extensive epithelial surface area of the gills. Panel 2 shows chloride cells, which densely populate the basal structure of the gill.

These differences in respiratory strategy have received surprisingly little attention, particularly since insects are used so frequently to assess water quality (Cooper, 1994). Studies have demonstrated the importance of biological barriers in terms of contaminant uptake mechanisms and fluxes in aquatic insects (Boudou et al. 1991, Saouter et al. 1991). Recent findings in our lab demonstrate substantial differences in water permeability and chlorpyrifos uptake rates among aquatic insect taxa. We hypothesize that these differences are based on vast differences in physiological interaction or "connectivity" with the surrounding water via exchange epithelial surfaces. Dissolved oxygen-breathing insects with larger exchange epithelial surfaces have significantly higher water and chlorpyrifos uptake rates than air-breathing taxa with smaller exchange epithelial surfaces (Buchwalter et al.2002). It would be useful to further evaluate the importance of exchange epithelial surfaces in determining temperature-modulated changes in both water influx and contaminant accumulation.

Elevated temperatures pose major problems to aquatic organisms. For example, a significant number of Oregon water bodies that are designated as "water quality limited" under section 303d of the Clean Water Act are listed because of elevated temperatures. Research with fish suggests that elevated temperatures may result in morphological changes in gill epithelia (Jacobs, et al., 1981), and osmoregulatory stress (Fry, 1967; Garside and Chin-Yuen Kee, 1972). Elevated temperatures have also been shown to reduce aquatic insect growth and fecundity in mayflies (Sweeny, 1978; Vannote and Sweeny, 1980) and stoneflies

(Buchwalter, unpublished data). However, few physiological studies with insects assess the role that respiratory system morphology plays in determining temperature tolerance and sensitivity across species. The decrease in oxygen solubility at warmer temperatures would suggest that water breathing might be disadvantageous in warming conditions. We suggest that water permeability, which is mediated by body size and exchange epithelial surface area, could also be an important determinant of temperature sensitivity differences among aquatic insect taxa.

This paper examines temperature modulation of $^{3}H_2O$ permeability and ^{14}C-chlorpyrifos uptake rates in aquatic insect taxa that have different respiratory strategies. The influence of temperature on the water influx rates is examined in larvae of the plecopteran *Pteronarcys californica* (Nelson). For comparative purposes, $^{3}H_2O$ influxes in the hemipteran *Notonecta kirvyi* (Hungerford) and trichopteran *Dicosmoecus gilvipes* (McLachlan) are also examined. A second study examines temperature effects on chlorpyrifos uptake in the ephemeropteran *Cinygma sp.* and hemipteran *Sigara washingtonensis* (Hungerford).

Materials and Methods

Insects – collecting and handling

All larvae were collected with a D-frame kick-net and transported in wet moss in ice-filled coolers. Larvae of the stonefly *Pteronarcys californica* were collected from the Deschutes River near Warm Springs, OR. Larvae of the caddis fly *Dicosmoecus gilvipes* were collected from the North Fork of the Alsea River near Alsea, OR. Larvae of the hemipteran *Notonecta kirvyi* were collected from the E.E. Wilson Wildlife Refuge in Adair, OR. Larvae of the mayfly *Cinygma sp.* were collected from the South Santiam River at Cascadia State Park, OR. Larvae of the hemipteran *Sigara washingtonensis* were collected from the Middle Fork of the Willamette River directly below the Hills Creek Dam near Oakridge, OR.

All insects were held in Instant Ocean® re-circulating aquaria containing approximately 75 gallons of soft well water collected from U.S. EPA's Willamette Research Station in Corvallis, OR. Insects were acclimatized for at least 7 days prior to initiating experiments in temperature-controlled environmental chambers maintained at +/- 0.5 ^{0}C. Conditions included a 16:8 light:dark photoperiod with indirect full spectrum lighting. With the exception of one set of experiments, all insects were acclimated to 8.5 ^{0}C. Insects were fed *ad libitum* a diet consisting of a mixture of wheat, alfalfa, yeast, and TetraMin® "Baby fish food L" enriched fish food.

Study 1. Water permeability in *Pteronarcys californica*

Reconstituted water (Fisher Scientific® 0.67 mM $CaCl_2$, 0.3 mM $MgSO_4$, 1.2 mM $NaHCO_3$, and 0.5 mM KH_2PO_4) was used for all experiments. Twenty ml of water were added to a 50-ml Erlenmeyer flask that contained 20 µl ^3H_2O to achieve a final activity of approximately 0.45 µCi/ml. Each bath was well stirred and two 20 µl samples were delivered directly into scintillation vials containing 3.5 ml ScintiSafe Econo2® cocktail. The average of the two replicates was taken to be the initial activity of the exposure media.

Individual larvae were placed into flasks for a set duration, removed, rinsed with de-ionized water, blotted dry, and weighed. After weighing, the larvae were rinsed again with de-ionized water and blotted dry. Hemolymph was extracted with 10 µl-calibrated capillary tubes after a small incision was made at the base of the most caudal gill tuft in the case of the stonefly and caddisfly larvae. For the Notonectids (which have no gills), hemolymph was extracted from an incision at the base of the rostrum with 5-µl calibrated capillary tubes. A Beckman LS6800 liquid scintillation counter with 43% ^3H counting efficiency was used for all ^3H_2O measurements. Hemolymph was expelled directly into scintillation vials containing 3.5-ml ScintiSafe Econo2® cocktail.

Preliminary experiments were conducted with 8.5 °C-acclimated *Pteronarcys* to determine the overall shape of the time-^3H_2O accumulation curves and allow us to focus on time points where efflux and changes in the external ^3H_2O activity were negligible (the linear portion of the uptake curves) (Figure 2).

Subsequent experiments with 8.5 °C-acclimated *Pteronarcys* were performed to examine the effects that temperature increases of 4.5 °C and 10.5 °C had on water uptake rates (Figure 3). In other experiments, *Pteronarcys* were acclimated at 13 ^{0}C and exposed to tritiated water at acclimation temperature (Figure 3). In all experiments, three to six individuals were exposed to each of several time points to obtain water influx rates. A total of 166 *Pteronarcys* larvae were used in these experiments, ranging in wet weight from 0.45 to 1.89 g, with an average wet weight of 1.02 g. To convert hemolymph $^{3}H_2O$ activities to flux rates, it was essential to determine hemolymph volume.

Determination of hemolymph volume

Hemolymph volume was determined by the dilution of ^{14}C- inulin carboxy (Moravek® Brea, California), as described by Levenbrook (1958) and Wharton et al. (1965). Inulin carboxy (50 μCi) was obtained as a dry solid and dissolved in 10 ml of deionized water. Individual insects were blotted dry, weighed, and anesthetized with CO_2. They were then injected with 5.8 µl (29 *n*Ci) of inulin in the dorsal aorta with calibrated, flame-pulled 10 µl capillary tube. Upon removal of the pipette, any fluid emerging from the injection point was swiped with a Kim-wipe®, which was placed in an LSC vial to ensure that no inulin loss occurred. Immediately after injection, the insects were placed into aluminum weighing dishes with just enough water to keep the gills moist but not enough water to disturb the injection site. Insects typically recovered from the anesthetic 5-8 minutes after

injection with inulin. Following a 30-minute mixing period, at least four 5 μl hemolymph samples were taken every 3 minutes to ensure that the inulin was thoroughly mixed. These results (Figure 4) were used to convert hemolymph ^3H_2O counts to water influx rates with the following equation:

$$V_h = (V_s (C_o)/C_b) - V_I$$

Where V_h = hemolymph volume

V_s = volume of hemolymph sample

C_o = count of original injection

V_I = μl of solution injected

C_b = count of hemolymph sample

Water permeability in *Notonecta kirvyi* and *Dicosmoecus gilvipes*

For comparative purposes, ^3H_2O uptake was examined via time course studies with 8.5 °C-acclimated *N. kirvyi.* These studies were performed over short-term durations (up to 9 minutes), similar to *Pteronarcys.* A total of 22 larvae ranging in wet weight from 0.09 – 0.12 g, with an average wet weight of 0.11g were used to obtain ^3H_2O uptake rates. In a separate experiment, three larvae each were exposed to ^3H_2O for 15 minutes at 12.5 °C, and three larvae each were exposed to ^3H_2O for 15 minutes at 21 °C (Figure 5). *D. gilvipes* larvae averaging 0.14 g wet weight (range 0.09-0.19g) were acclimated at 8.5 °C. Four larvae each were exposed to ^3H_2O for 15 minutes at acclimation temperature and four larvae were exposed to ^3H_2O for 15 minutes at 12.5 °C (Figure 5). In a separate

experiment, *Pteronarcys* larvae that had been acclimated at 8.5° were exposed for 15 minutes at 8.5, 12.5, and 21 °C (Figure 4).

Electron microscopy

A single gill tuft was dissected at the base from a *Pteronarcys californica* larva and fixed in 75% ethanol/25% water for 24 hours. The gills were then held through two changes of 100% ethanol for two hours each. The sample was critically point dried from carbon dioxide in a Balzer CPD-020 dryer following the method of Anderson (1951). The dried specimen was mounted on an aluminum planchette (Pelco # 16262) with DUCO cement (DEVCON CORP, Wood Dale, Il). The mounted specimen was coated with ~10nm of 60/40 wt % Au/Pd using an Edwards S150B sputter coater operating at 1 x 10^{-2} Torr, 5 mbar Argon pressure, 1.5kV, 20 milliampere plasma current for 60 seconds. Examinations were made using the AmRay 3300FE scanning electron micrograph in the Electron Microscope Facility, Department of Botany and Plant Pathology, Oregon State University. Images were recorded on Polaroid Type 55 P/N positive/negative 4 X 5" format film (Figure 1a,b).

Study 2. Chlorpyrifos Uptake

In this study, ^{14}C- chlorpyrifos uptake rates were compared under two thermal regimes in both the mayfly *Cinygma sp* and hemipteran *S. washingtonensis*. All insects were acclimated to 8.5 °C for at least 5 days prior to

experimentation. Assays were run at both acclimation temperature and 13 °C for both taxa. Exposure chambers were 50-ml Erlenmeyer flasks containing artificial water and 0.023 μCi * L^{-1} ^{14}C- chlorpyrifos. This ^{14}C- chlorpyrifos activity corresponds to a concentration of 240 *n*g * L^{-1}. Individual insects were placed in each flask. Individuals were held for 0, 1, 2, 4, or 8 hours. Five insects per time point were used. Following exposure, insects were removed from exposure flasks, rinsed profusely with water, weighed, and digested in Amersham NCS II® tissue solubilizer in large scintillation cocktail vials. The digests were held at 50 °C overnight and neutralized with glacial acetic acid to obtain pH 7. Eighteen mL of Amersham BCS-NA® non-aqueous scintillation cocktail were added, and samples were held in a refrigerator in the dark for at least 4 days to minimize chemiluminescence. These samples were mixed well and counted with a Beckman LS 6500 liquid scintillation counter. Five control insects of each taxa were digested as described above. The average of control ^{14}C activities was taken to be background for each species tested and subtracted from the counts of subsequent time points (Figure 6).

Results

^3H_2O flux

Pteronarcys californica

For organisms acclimated at 8.5 °C, accumulation of ^3H_2O was characterized by an initial linear response, which decayed over time (Figure 2). Acute temperature elevation increased the water uptake rate relative to uptake at acclimation temperature at each time point tested. In order to obtain initial rates of water influx, subsequent studies focused on water uptake within relatively short ^3H_2O exposure durations.

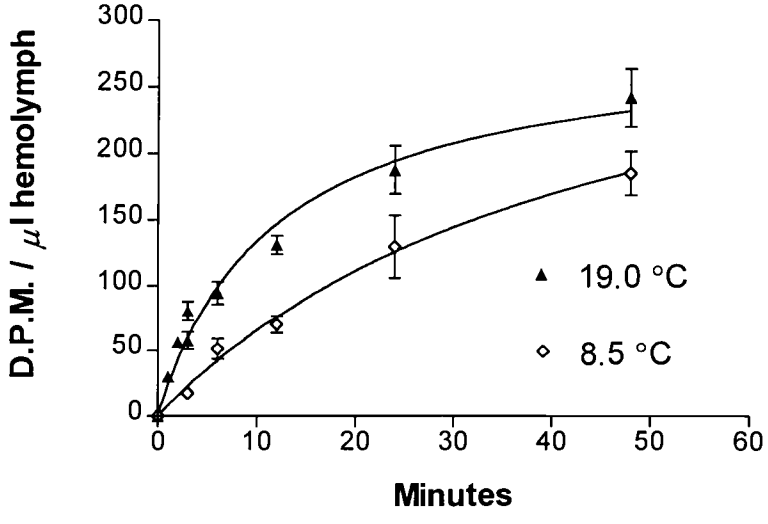

Figure 2. The influence of temperature on $^3H_20$ accumulation in the hemolymph of *Pteronarcys californica*. Based on these initial experiments, subsequent experiments focused on time points in the initial linear portions of these curves. Error bars represent the standard errors of the means for each time point.

Initial water influx rates for *Pteronarcys* are shown in Figure 3. A total of 46 organisms acclimated and exposed to 8.5 °C for 3, 4.5, 6, 7.5 and 12 minutes, respectively, had water influx rates of 1.78 +/- 0.18 µl/g/minute. A total of 20 organisms acclimated and exposed to 13 °C for 1.5, 2.5, 3.5, and 4.5 minutes, respectively, had water influx rates of 1.75 +/- 0.20 µl/g/minute. These rates are not statistically different and suggest that within a particular temperature range, acclimated *Pteronarcys californica* maintain a constant permeability. When 48 organisms acclimated to 8.5 °C were shifted to 13 °C for 0.5, 0.75 1.5, 2.5, 3.5, and 4.5 minutes respectively, water influx rates increased by 45% to 2.57 +/- 0.27 µl/g/minute. When 18 organisms acclimated to 8.5 °C were shifted to 19 °C for 1, 2, and 3 minutes, water influx rates increased 3.6 fold to 6.41 +/- 1.24 µl/g/minute. Increased ^3H_2O uptake was presumably related to temperature-induced changes in the physical state (cell membrane fluidity and/or cell-to-cell tight junctions) of the respiratory epithelium as well as increases in metabolic rates.

In any given experiment, body weight was not a statistically-significant explanatory variable. However, when a multiple linear regression model was fit using SAS for all experiments in which *P. californica* were acclimated to 8.5 °C, body weight was statistically significant ($p < 0.01$) and negatively related to ^3H_2O activity in the hemolymph. This was expected for a variety of reasons. Smaller organisms have larger surface area to volume ratios than larger organisms. In addition, metabolic demands of earlier life stages are typically higher than they are in more mature stages since growth rates tend to be considerably higher. These

findings agree with earlier studies with *Chironomous riparius,* which demonstrated that 3rd instar individuals had larger water influx rates than 4th instar individuals.

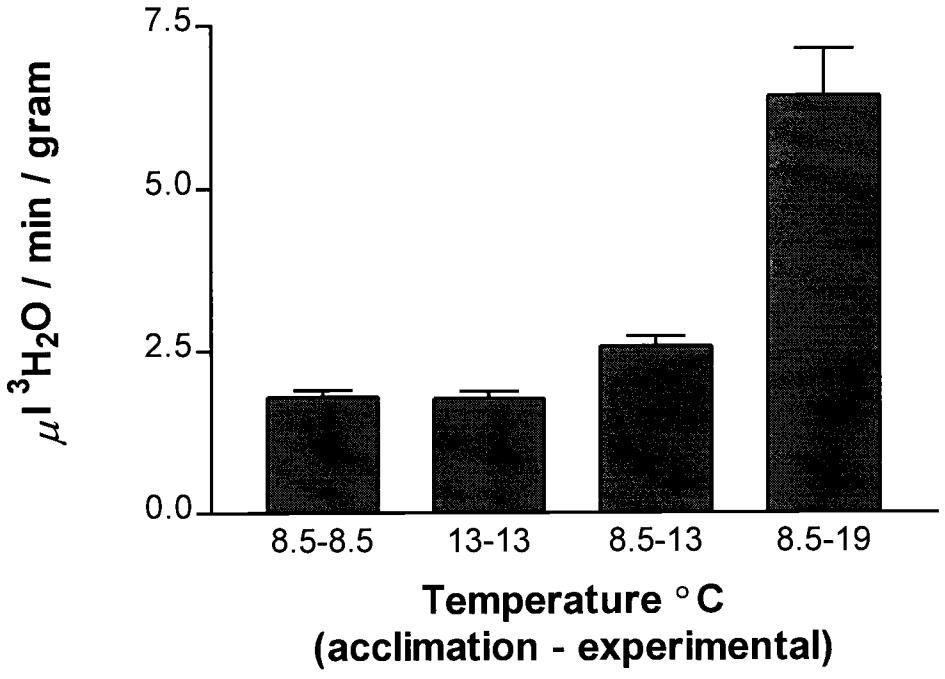

Figure 3. Effects of temperature shifts and acclimation on water influx. There is no difference in influx rates for animals exposed to $^3H_20$ at acclimation temperatures of 8.5 °C and 13 °C (p<0.01). A 4.5 °C shift (8.5-13) results in a 45% increase in water influx rate. A 10.5 °C shift (8.5-19) results in a 3.6 fold increase in initial water influx rates. Error bars represent standard errors of the slopes of uptake curves.

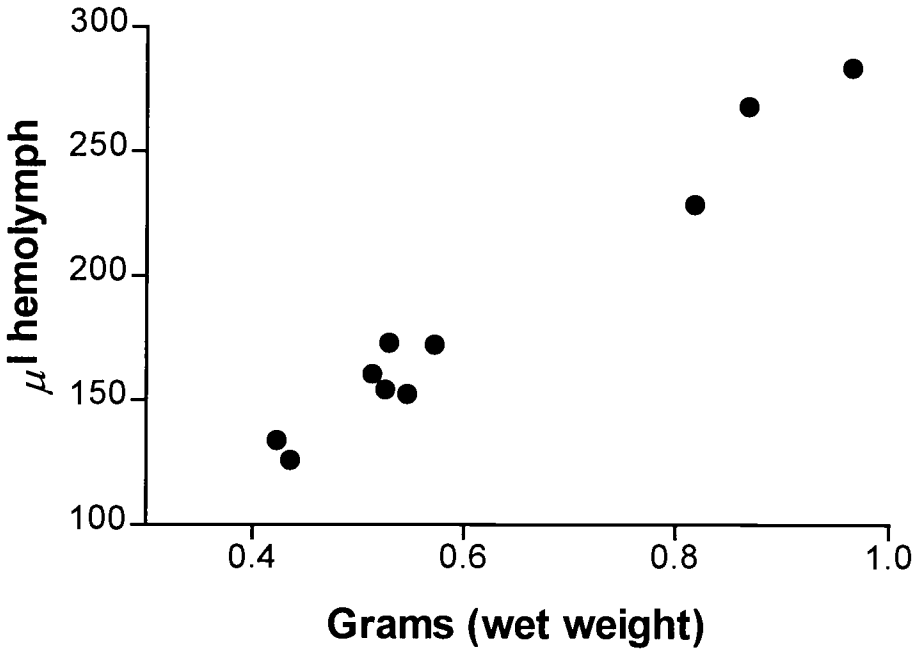

Figure 4. ^{14}C-Inulin carboxyl was used to determine hemolymph volumes in *P. californica.* Individual insects were injected with 5.8 μl (29 *n*Ci) of inulin in the dorsal aorta with calibrated, flame-pulled, 10-μl capillary tube. Inulin carboxyl is too large to cross cell membranes and remains in circulation for hours before it begins to be cleared by the excretory system. Each point represents the average of at least four hemolymph samples taken between 5-minute intervals after a 30-minute mixing interval. These data were used to convert hemolymph $^{3}H_20$ data to water influx rates.

Notonecta kirvyi

There was no significant accumulation of ^3H_2O into hemolymph of the air breather *Notonecta kirvyi* that had been acclimated and exposed to ^3H_2O at 8.5 °C for up to 9 minutes (data not shown). A total of 23 individuals averaging 0.11g were assayed for ^3H_2O in hemolymph after exposure durations ranging from 1.5 minutes to 9 minutes. The hemolymph ^3H_2O activity did not significantly increase over time and ranged from 4-7 d.p.m./μl. This contrasted significantly with *P. californica,* which under indentical acclimation and exposure regimes, accumulated hemolymph ^3H_2O activities that approached 50 d.p.m./μl. Considering that *N. kirvyi* weighed, on average, one-ninth of *P. californica,* the relative amounts of water influx required to obtain measurable concentrations in the hemolymph were considerably smaller. We concluded that the absence of respiratory epithelium resulted in a significantly less-permeable cuticle.

When three individuals acclimated at 8.5 °C were shifted to 13 °C for 15 minutes, the average hemolymph ^3H_2O activity was 18.8 +/- 2.3 d.p.m./ul. A shift from 8.5 °C to 21 °C significantly increased hemolymph ^3H_2O activity to 47.8 +/- 2.7 d.p.m./μ l (Figure 4). Due to the limited availability of *N. kirvyi,* we were unable to measure hemolymph volumes in this species, making absolute comparisons between these two species difficult, especially since they are so markedly different in size. However, it is evident that they are significantly less permeable than *P. californica.* To address the issue of size and further explore the relationship between respiratory surface and water permeability, we collected

larvae of the trichopteran *Dicosmoecus gilvipes* that were only slightly larger than *N. kirvyi.* Insects that averaged 0.14g were acclimated to 8.5 °C and exposed to ^3H_2O at acclimation temperature or 13 °C for 15 minutes (Figure 4). The accumulation of ^3H_2O in the hemolymph was significantly higher than both *N. kirvyi* and *P. californica* (Figure 4), suggesting that external respiratory surfaces are significantly more permeable to water than other integument.

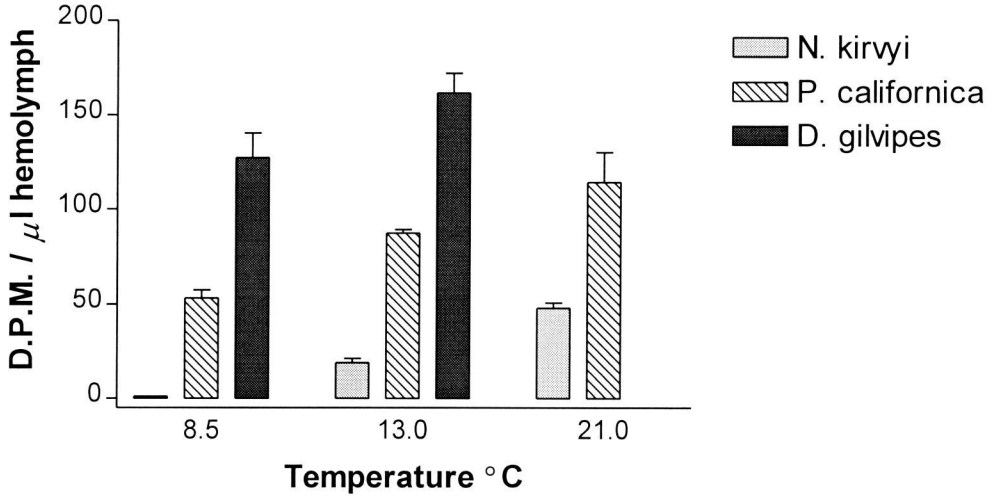

Figure 5. The influence of respiratory strategy, temperature and size on $^3H_20$ accumulation in three species of aquatic insects. The air-breathing *N. kirvyi* was essentially impermeable to water in time course studies to 9 minutes at 8.5 °C (data not shown). *N. kirvyi* accumulated significantly less $^3H_20$ at 13 °C than gill-bearing species *P. californica* and *D. gilvipes*. Because of the relative size differences between the large *P. californica* (1.02 g) and the smaller *D. gilvipes* (0.14 g) and *N. kirvyi* (0.11 g), exact comparisons cannot be made without hemolymph volume data. We can conclude, however, that gill-bearing species are more water permeable than air-breathing species.

^{14}C-chlorpyrifos uptake

Accumulation of ^{14}C-chlorpyrifos was consistently higher in the gill-bearing mayfly *Cinygma sp.* than in the air-breathing hemipteran *Sigara washingtonensis* in all experiments (Figure 6). A temperature increase of 4.5 °C increased uptake rates in both organisms. However, the increases were substantially greater in *Cinygma sp.* After 8 hours of exposure, the temperature induced increase in chlorpyrifos uptake was 2.75 fold higher in *Callibaetis sp.* than in *Sigara washingtonensis.*

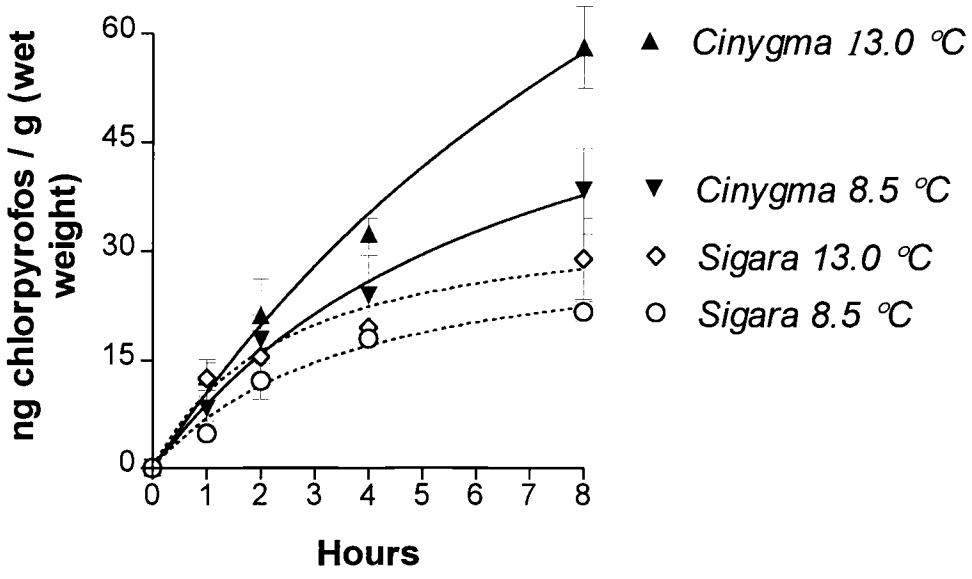

Figure 6. Temperature influences the rates of chlorpyrifos uptake in the air-breathing hemipteran *Sigara washingtonensis* and the gill-bearing ephemeropteran *Cinygma (sp).* All organisms were acclimated to 8.5 °C. At acclimation temperature, chlorpyrifos uptake rates are uniformly higher in *Cinygma* than in *Sigara.* Temperature shifts of 4.5 °C resulted in increases in chlorpyrifos uptake in both species. This temperature-induced increase in chlorpyrifos uptake is substantially higher in the gill breathing ephemeropteran. Error bars represent standard errors.

Discussion

Early permeability studies of aquatic insects employed various indirect techniques that included determining the output of rectal fluid (Staddon, 1969, 1973); weight increases after death (Holdgate, 1956; Staddon, 1969, 1973); and uptake rates of D_2O (Shaw, 1955; Staddon, 1966, 1973). Drinking rates were estimated using amaranth dye (Staddon, 1969) and indigo carmine dye (Frick and Sauer, 1974). Experiments by Frick and Sauer (1974), Arlian and Staiger (1979), and Frisbee and Dunson (1988) measured ^3H_2O uptake in whole insect bodies and calculated water influx across the cuticle. These experiments were typically conducted over the course of hours. Findings from these early studies indicate that drinking in aquatic insects was minimal to negligible, relative to water influx across the cuticle. The excretory system removed excess water. It also appeared that the cuticle of aquatic insects was more water permeable than the cuticles of terrestrial insects.

Early studies were typically conducted with air breathers, with emphasis on the Hemiptera, and resulted in water influx estimates that are substantially lower than later studies using tritiated water. We could not find direct comparisons of water influx between air breathing and water breathing species until Frisbee and Dunson (1988) compared water influx in an air-breathing beetle *Dytiscus verticalis* to water influx in the rectal gill-bearing odonate *Anax junius*. They found that not only were the gill breathers twice as permeable to water, but that they also lost

sodium to the surrounding water under low pH conditions. The air breathing *Dytiscus* was relatively impermeable to water (influx) and sodium (efflux) and tolerant to low pH. Our recent work with nine taxa showed that dissolved oxygen-breathing insects with larger exchange epithelial surfaces have significantly higher water uptake rates than air-breathing taxa with smaller exchange epithelial surfaces (Buchwalter et al. 2002).

These experiments indicate that water influx rates in aquatic insects are dependent on a number of factors including respiratory strategy, temperature, acclimation history, and body size. Based on our experiments with *Pteronarcys,* we suggest that focusing on early time points results in more accurate water influx rate estimates for a number of reasons. Water efflux rates become significant when the blood or hemolymph $^{3}H_2O$ concentrations approach 4-7% of the external medium (Leonard B. Kirschner, personal communication). Therefore, hemolymph samples taken beyond the initial linear portion of the time/$^{3}H_2O$ accumulation curves (Figure 1) would result in underestimating influx rates. Because $^{3}H_2O$ distributes to total body water via diffusion from the hemolymph, analysis of hemolymph is advantageous for instantaneous uptake rates.

In order to calculate water influx rates in *P. californica*, it was essential to measure hemolymph volume. This was accomplished via the injection of ^{14}C-inulin carboxy (Figure 3). This large sugar remains in circulation for hours before levels decrease through excretion (Wharton et al., 1965). Inulin is useful for the determination of hemolymph volume as it distributes to extracellular space only.

Acute temperature changes, but not acclimation temperatures, were important factors in water influx rates in *P. californica* (Figure 2). Insects that were acclimated and exposed to ^3H_2O at 8.5 °C and 13 °C, respectively, did not differ with respect to influx rates. This suggests that *P. californica* effectively acclimated to these temperatures. The 45% increase in water influx rate observed when organisms were shifted from 8.5 °C to 13 °C may be due to increased metabolic and circulatory rates, increased fluidity of the gill plasma membrane, and/or decreased tightness of cell-to-cell junctions in epithelial surfaces.

We observed that populations of Deschutes River *Pteronarcys* dramatically decline along a thermal gradient and are rare towards the mouth of the river where summer high temperatures reach 19 °C. We were interested in water influx in organisms approaching their thermal limits. We observed a 3.6-fold increase in water influx rate under these conditions. While a 10.5 °C acute temperature increase is not environmentally realistic, we speculate that the observed flux increase is due to more than simply an increase in metabolic rate. We did not observe the characteristic "push-up" ventillatory behavior in *Pteronarcys* until nearly 5 minutes of exposure to this extreme temperature increase and can rule out increased ventillation rates as a possible explanatory factor. We speculate that extreme temperature changes cause physical changes in the gill epithelium, resulting in increased permeability.

Insect species that breathe through external epithelia and gills are significantly more water permeable than air-breathing insects (Figure 4).

Furthermore, we demonstrate that temperature increases result in increased water influx rates. Sufficient temperature elevations result in increased metabolic rate in both air breathers and water breathers. However, water breathers are at a distinct disadvantage in these situations, because oxygen availability decreases and water fluxes increase substantially. Based on the metabolic costs associated with removing excess water and maintaining homeostatic processes, we suggest that insects with large epithelial surfaces may be more susceptible to temperature changes. Figure 1 shows the extensive gill surface area of *P. Californica* (1a) as well as the chloride cell-rich epithelial tissue at the base of each gill tuft (1b) These chloride cells are presumably necessary to acquire necessary cations from the water column and compensate for diffusive ion losses.

Accumulation of ^{14}C-chlorpyrifos was uniformly greater in the mayfly *Cinygma sp.* than in the hemipteran *Sigara washingtonensis* on a per-weight basis (Figure 6). This is notable because *Cinygma* was on average 45% heavier than *Sigara*. These results agree with previous findings that dissolved oxygen-breathing organisms generally have higher ^{14}C-chlorpyrifos uptake rates than air-breathing taxa (Buchwalter et al, 2002). As expected, an acute temperature increase of 4.5 °C increased ^{14}C-chlorpyrifos accumulation rates in both the ephemeropteran *Cinygma sp.* and in the hemipteran *Sigara washingtonensis.* However, the increases were more pronounced in the gill bearing *Cinygma* than in the air-breathing *Sigara* (Figure 6). This suggests that epithelial surfaces play a key role in determining uptake of chlorpyrifos, and that organisms with larger epithelial exchange surface

areas are potentially more susceptible to temperature increases in contaminated environments.

We suggest that differences in respiratory and osmoregulatory strategies may help explain the marked differences in sensitivities to water quality problems that have been observed in insect species. In polluted environments, organisms with relatively large exchange epithelial surfaces are likely more sensitive to a number of environmental stressors (temperature, dissolved oxygen, pH changes, and contaminant exposure) that interact via different mechanisms through these surfaces. Understanding the mechanistic bases for differences in species sensitivities to individual and multiple stressors is an essential step in assessing water quality impacts in nature. Further experimental work that helps establish causal relationships between stressors and organisms would greatly assist biologists who are interested in community level responses.

Acknowledgements- The authors acknowledge Dr. Darlene Judd and Dr. John Lattin in the Department of Entomology at Oregon State University for their assistance in identifying insect species. We also thank Dow chemical for providing [^{14}C]-chlorpyrifos. Al Soeldner, Electron Microscopy Facility at Oregon State provided the electron micrographs.

Chapter 3

Respiratory Strategy is a Major Determinant of [^{3}H]-Water and [^{14}C]-Chlorpyrifos Uptake in Aquatic Insects.

Introduction

Ecologists have developed a variety of indices, metrics, and other tools that attempt to assess freshwater ecosystem health via surveys of resident insect taxa (e.g., Hilsenhoff 1988; Plafkin et al. 1989). Remarkably, none of the current survey-based bioassessment techniques are based on understanding how insect physiological and morphological attributes affect responses to specific environmental stressors. Rather, they are based upon a wide assortment of observational studies that, by nature, cannot adequately define causal relationships. This paper examines organismal characteristics that are important in determining uptake of environmental pollutants.

Aquatic insects arose from numerous invasions of aquatic habitats by air-breathing terrestrial ancestors (Kristensen 1981). The radiation of aquatic insect species is accompanied by significant developments and modifications in respiratory and associated osmoregulatory systems. Several aquatic respiratory strategies have emerged from the basic open tracheal system, including epithelial gas exchange systems utilizing body walls and gills (Erickson et al. 1996). In some orders such as Diptera, more than one strategy is seen including air breathing and dissolved oxygen breathing. All taxa in the orders Ephemeroptera, Plecoptera, and

Trichoptera (EPT) have closed tracheal systems and are dependent on aqueous gas exchange. The integuments of aquatic insects are covered by protective surfaces of waxes, chitin, and scleretin that are relatively impermeable to gasses and water. However, insects that breathe dissolved oxygen have exposed epithelial surfaces that exchange gasses and salts including respiratory tissue, chemosensory cells, and chloride cells. Insects are extremely heterogeneous with respect to the relative epithelial surface area used for gas and salt exchange, which is determined in part by age, sex, size, life history, and water chemistry.

Our working hypothesis is that insects with relatively large areas of exchange epithelium are potentially more vulnerable to poor water chemistry conditions than those with smaller areas of exchange epithelium. Because organisms with larger exchange epithelial surface areas are more physiologically connected to the water column, they may be more sensitive to a variety of water column-associated stressors relative to organisms that do not have large exchange epithelial surfaces. For example, in low dissolved oxygen scenarios, air-breathing insects would largely be unaffected while dissolved oxygen-breathing insects would be stressed. The experiments presented in this paper examine the importance of respiratory strategy and associated exchange epithelium in determining accumulation of the insecticide [^{14}C]-chlorpyrifos. Water permeability is used as a quantitative surrogate for estimating relative differences in exchange epithelium that we predict are important in determining exposure to contaminants. In addition, we report a technique for characterizing taxa based on exchange epithelium via the

use of the fluorescent membrane dye, DPH (diphenylhexatriene). The goal of this technique is to aid in our predictive capacity to determine differences in species/instar sensitivity to stressors that exert their negative influences via exposed cellular surfaces.

Materials and Methods

All insects used in this study, with the exception of *C. riparius*, were field collected from sites in Oregon, using a D-frame kick net (Table 1). Insects were transported in damp moss in ice-filled coolers. *C. riparius* egg masses were obtained from Environmental Toxicology and Testing, Inc. Superior, Wisconsin. No collecting was done from obviously impacted sites. Prior to experiments, insects were held for a minimum of five days in a temperature-controlled chamber at 8.5 °C with a 16:8 light:dark photoperiod of indirect full-spectrum lighting. All insects were held in Instant Ocean® re-circulating aquaria containing approximately 75 gal of soft well water collected from U.S. Environmental Protection Agency's (EPA) Willamette Research Station in Corvallis, Oregon. Insects were fed a diet consisting of wheat, alfalfa, and TetraMin® "Baby fish food L" *ad libitum* prior to experimentation.

Artificial water (Fisher Scientific® 0.67 mM $CaCl_2$, 0.3 mM $MgSO_4$, 1.2 mM $NaHCO_3$, and 0.5 mM KH_2PO_4) was used for all experiments. Fifty mL of artificial water, 20 μL [^{3}H]-H_2O and 20 μL of [^{14}C]-chlorpyrifos (in 50:50 acetone:water) were added to Erlenmeyer flasks. This yielded final specific activities for [^{3}H]-H_2O and [^{14}C]-chlorpyrifos of approximately 6.77 μCi • L^{-1} and 0.023 μCi • L^{-1}, respectively. This [^{14}C]- chlorpyrifos activity corresponds to a concentration of 240 ng • L^{-1}. Individual insects were placed in each flask, with the exception of experiments with the organisms *C. riparius* and *Psectrotanypus (sp)*,

in which two individuals were placed in each flask. Individuals were held for 0, 1, 2, 4, 8, and, in some cases, 12 h. When available, five insects per timepoint were used. Following exposure, insects were removed from exposure flasks, rinsed profusely with water, weighed, and placed in 20 mL scintillation cocktail vials. Amersham NCS II® tissue solubilizer was added to each vial and digests were held at 50 °C overnight and neutralized with glacial acetic acid to obtain pH 7. Eighteen mL of Amersham BCS-NA® non-aqueous scintillation cocktail were added, and samples were held in a refrigerator in the dark for at least four days to minimize chemiluminescence. These samples were well mixed and counted with a Beckman LS 6500 liquid scintillation counter. Five control insects of each taxon were placed in water containing the acetone carrier for 1 h, removed, digested, and analyzed as described above. The average of control [^{3}H] and [^{14}C] activities was taken to be background for each species tested and subtracted from the counts of subsequent time points.

Five individuals per taxon were blotted dry, weighed, and transferred to pre-weighed aluminum weigh boats to be dried at 50 °C for 72 h to determine average percent body water composition. Average percent body water was used to estimate body water volumes for each taxon exposed to [^{3}H]-H_2O and [^{14}C]- chlorpyrifos. The accumulation of [^{3}H]-H_2O is expressed in terms of percent apparent steady-state. We define percent apparent steady-state as the percentage of the organisms' body water that has been exchanged with external [^{3}H]-H_2O. At 100% apparent steady-state, the internal and external concentration of [^{3}H]-H_2O is equivalent.

To fluorescently label external cellular surfaces on *C. riparius* and *Psectrotanypus (sp)*, the following procedure was used: Diphenylhexatriene (DPH) was obtained from Molecular Probes, Eugene, Oregon. A 43 mM stock solution was prepared in 100% dimethylformamide (DMF). DPH stock (0.25 mL) was added to 10 mL of a 250 mM mannitol, 10 mM HEPES buffer solution at pH 7.4. This aqueous suspension was sonicated and mixed prior to the addition of live larvae. One larva of each species was jointly incubated in this mixture as it was stirred slowly. The larvae were removed after a 15-min incubation, rinsed thoroughly with tap water, and anesthetized with CO_2. The larvae were then photographed with a Diagnostics Instruments SPOT 2® camera through a Leica MZFL111® stereoscope equipped with a 100-watt mercury vapor lamp and ultraviolet fluorescent filter.

Statistical methods employed include linear regression, non-linear regression, and Spearman Rank Correlation Coefficients (Sokal and Roth, 1995).

Results

Water permeability

There were large differences in the accumulation of water between taxa (Figure 7 a, b). Accumulation of [^{3}H]-H_2O was described in terms of the percentage of the individual's body water that was incorporated from the external media. As an arbitrary descriptor of water permeability, the 50% steady-state point was compared among taxa (Figure 8). Both body size and respiratory strategy appeared to be important determinants of water uptake rates. Smaller taxa generally had higher water turnover rates than larger organisms, while dissolved oxygen breathers had higher water turnover rates than air breathers. Body weights and times to 50% water steady-state were ranked. For all taxa, a negative correlation between body weight and time to 50% steady-state was observed (Spearman Rank Correlation Coefficient = -0.75; $p = 0.01$). All air-breathing taxa had lower permeability than expected based on body weight alone (Table 1). The only dissolved oxygen breather that had lower permeability than expected based on body weight was the chironomid *Psectrotanypus sp.* (Table 1)

Chlorpyrifos uptake

[^{14}C]-chlorpyrifos accumulation increased with time in all taxa (Figure 9 a, b). The decision to include 12 h as a time point was solely based on organism availability. As was the case with water permeability, body size was an important determinant of [^{14}C]-chlorpyrifos accumulation. For all taxa, a negative correlation

between ranked body weights and chlorpyrifos uptake rates was observed (Spearman Rank Correlation Coefficient = -0.86; p = 0.005). All air breathing taxa had relatively low chlorpyrifos uptake rates based solely on body size (Table 1). The only dissolved oxygen breather that had a lower than expected chlorpyrifos uptake rate was the chironomid *Psectrotanypus sp* (Table 1).

Water turnover rates were jointly determined by body size and respiratory strategy and were strong predictors of [^{14}C]-chlorpyrifos uptake. A Spearman Rank Correlation Coefficient of 0.98 (p = 0.001) was observed between chlorpyrifos uptake ranks and ranked times to 50% water steady-state across all taxa. The relationship between the magnitudes of [^{14}C]-chlorpyrifos uptake rates and water permeability was described by simple exponential decay with an r-square of 0.98 (Figure 10).

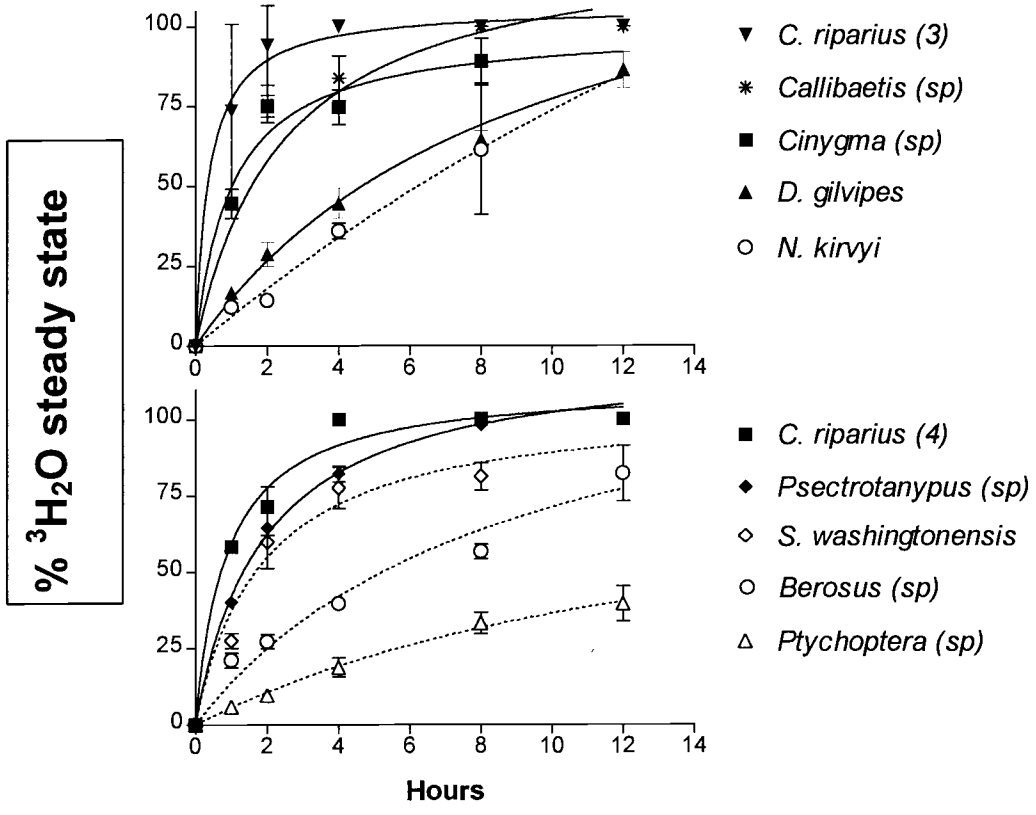

Figure 7. Water permeability in aquatic insects is expressed in terms of percent body water based on the accumulation of ^3H_2O relative to total body water composition. Dashed lines and open symbols represent air-breathing taxa. Solid lines and closed symbols represent dissolved oxygen breathing taxa. Error bars represent the standard errors of the means at each time point. Time courses were run to 8 h or 12 h, depending on the availability of organisms. The (3) and (4) following *C. riparius* denote larval instar.

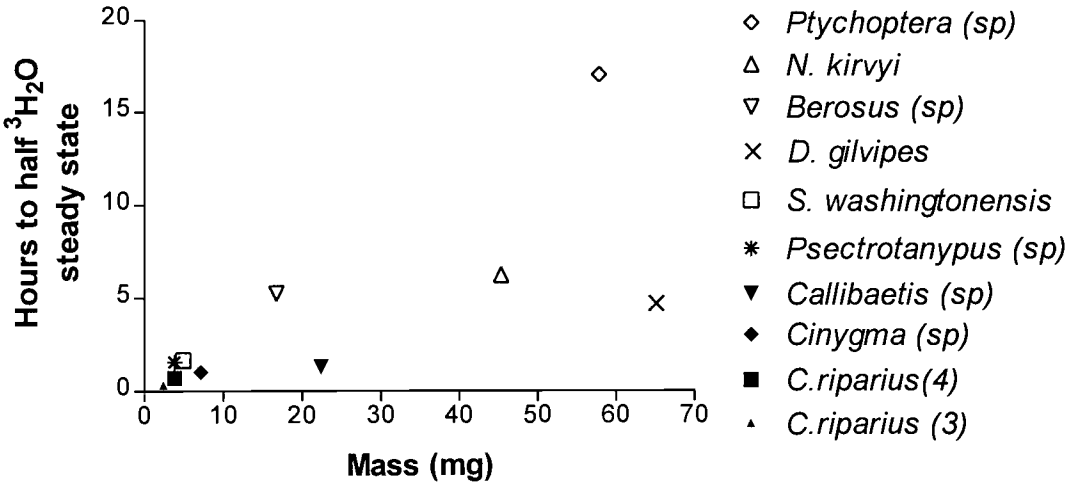

Figure 8. Water permeability in aquatic insects is a function of both body size and respiratory strategy. The open symbols represent organisms with open tracheal systems (air breathers). Closed symbols represent organisms with closed tracheal systems (dissolved oxygen breathers). The (3) and (4) following *C. riparius* denote larval instar.

Test organisms, respiratory strategies (air vs. dissolved oxygen (D.O.)), body weights, chlorpyrifos uptake (slopes, ranks, r^2), and water uptake (ranked times to ½ steady state).

Order	Family	Genus, species	Respiratory strategy	Mass (mg)	Chlorpyrifos, slope, rank, r^2	Water rank
Diptera	Chironomidae	Chironomus riparius(3)	D.O.	2.4	13.53, 1, 0.96	1
Diptera	Chironomidae	Psectrotanypus (sp)	D.O.	3.8	2.67, 4, 0.90	5
Diptera	Chironomidae	Chironomus riparius(4)	D.O.	3.8	7.04, 2, 0.85	2
Hemiptera	Corixidae	Sigara washingtonensis	Air	4.9	1.96, 6, 0.75	6
Ephemeroptera	Heptageniidae	Cinygma (sp)	D.O. (gills)	7.1	3.90, 3, 0.70	3
Coleoptera	Hydrophilidae	Berosus (sp)	Air	16.7	1.97, 7, 0.91	8
Ephemeroptera	Baetidae	Callibaetis (sp)	D.O. (gills)	22.4	2.45, 5, 0.91	4
Hempitera	Notonectidae	Notonecta kirvyi	Air	45.3	0.91, 9, 0.93	9
Diptera	Ptychopteridae	Ptychoptera (sp)	Air	57.8	0.47, 10, 0.88	10
Trichoptera	Limnephilidae	Dicosmoecus gilvipes	D.O. (gills)	65.1	1.84, 8, 0.83	7

Note: (3) and (4) following *C. riparius* denote instar.

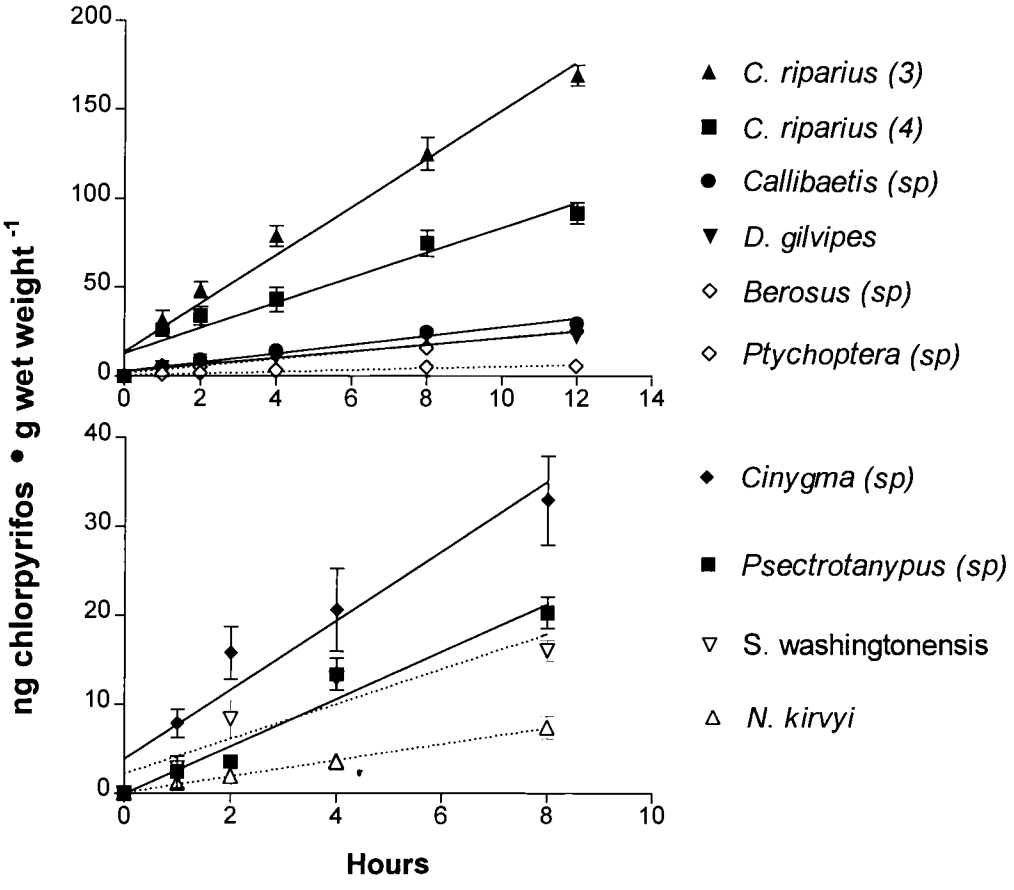

Figure 9. Chlorpyrifos uptake rates in aquatic insects are determined by body size and respiratory strategy. Dashed lines and open symbols represent air-breathing taxa. Solid lines and closed symbols represent dissolved oxygen breathing taxa. Error bars represent the standard errors of the means at each time point. Time courses were run to 8h or 12 h, depending on availability of organisms. The (3) and (4) following *C. riparius* denote larval instar.

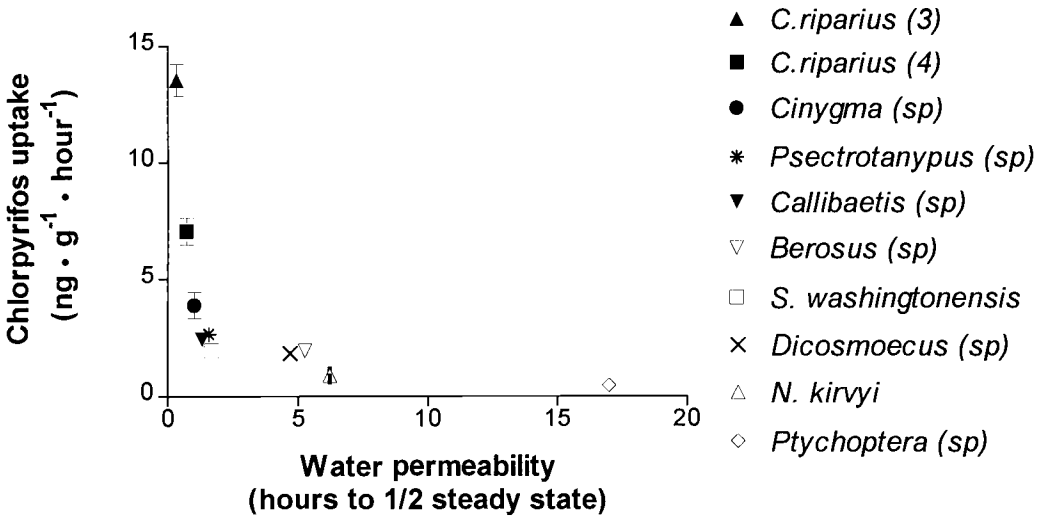

Figure 10. Chlorpyrifos uptake rates vs. water permeability in aquatic insect taxa. Water permeability is described in terms of the length of time required for an organism to accumulate 50% body water steady-state. The open symbols represent organisms with open tracheal systems (air breathers). Closed symbols represent organisms with closed tracheal systems (dissolved oxygen breathers). Error bars represent the chlorpyrifos uptake slope standard errors This exponential decay relationship has an r-square of 0.98.

Fluorescence characterization

Fluorescence of DPH was more broadly distributed on the body surface of *C. riparius* than *Psectrotanypus sp.* (Figure 11). These larvae were jointly incubated, rinsed, and photographed. This image was not adjusted or altered in any manner. The fluorescence distributions in these larvae indicated that *C. riparius* has a relatively large cellular interface with the water column as compared to *Psectrotanypus sp.* DPH has several characteristics that make it useful for examining insect body surfaces. DPH is a cylindrically-shaped molecule that is essentially non-fluorescent in water. However, fluorescence excitation and emission dipoles are oriented roughly parallel to the axis of the cylindrically shaped molecule, resulting in increased fluorescence when incorporated into the plasma membranes of epithelial cells. With this in mind, images of DPH-labeled insects can be compared based on overall brightness, relative size of fluorescing surfaces, and photographic attributes such as magnification and exposure time (Haugland 1996).

Figure 11. Diphenylhaxatriene (DPH) labeled *C. riparius* (below) and *Psectrotanypus sp.* (above). These larvae were jointly incubated for 15 m in DPH and photographed on the same slide at 12.5 x magnification. No additional modifications were performed on this image.

Discussion

Studying traits at the organismal level has advanced our understanding of how freshwater systems function. For example, functional feeding guild approaches (Cummins 1974) have helped scientists understand how communities of organisms change from stream headwaters to mouth (Vannote et al. 1980). To date, no such structural-functional approach has adequately determined which organismal characteristics are important in determining responses to specific water chemistry problems, although biological traits are beginning to be explored in this context (Charvet et al. 1998).

Water turnover

Based upon results reported here and basic physiological principles, we suggest that body size and respiratory strategy (including associated exchange epithelia) are primary determinants of water turnover rates in aquatic insects. Smaller individuals have larger surface area to volume ratios. Additionally, larger organisms typically contain more water than smaller organisms. However, size alone did not determine water turnover rates in these studies. Respiratory strategy also played a key role. All air-breathing taxa had lower water turnover rates than dissolved oxygen breathers within a given size range. This was expected, as there is more exchange epithelium in dissolved oxygen breathing taxa.

It is likely that highly permeable taxa are more vulnerable to osmoregulatory distress than other taxa. Based on the metabolic costs associated

with removing excess water and retaining salts, we suggest that there are some disadvantages in having a water-permeable integument, particularly in degraded water chemistry conditions. Exchange epithelia are directly involved in osmoregulation, as water (passively) and ions (passively and actively) pass in both directions through exchange (Frisbie and Dunson 1988; Kirschner 1991; Cooper 1994). These fluxes can be altered by environmental stressors in insects (Havas and Hutchinson 1983; Lechleitner et al. 1985; Peters et al. 1985) and crustaceans (Havas et al. 1984; Havas and Advokaat 1995).

Chlorpyrifos accumulation

Several studies have indicated the importance of exchange epithelium as determinants of responses to environmental pollutants. For example, crude oil (Simpson 1980) and chlorine (Simpson 1980; Camargo 1991) were found to damage gills in freshwater insects. Respiratory and osmoregulatory epithelium is a critical target site in the toxicity of metals such as copper (Hare 1992), mercury (Boudou et al. 1991), and aluminum (Gunderson and Curtis 1995). However, direct uptake via the water column is not always the predominant exposure pathway in aquatic insects. (Hare 1992). Research with the air-breathing insect *Chaoborus* demonstrates that cadmium exposure via food is a more important exposure pathway than direct uptake via the water column (Munger and Hare 1997; Munger et al. 1999).

To date, we are aware of no other published studies that have examined the role of aquatic insect respiratory strategies in determining differences in uptake of

organic pollutants. Many organic contaminants are known to partition to the lipid-rich environments of cell membranes in aquatic organisms. In their extensive work with the lampricide TMF (3-trifluoromethyl-4-nitrophenol), Maki and Johnson (1977) observed that "macroinvertebrate species with soft, relatively permeable integuments accumulate significantly higher residue concentrations....than those species with hard chitinized or calcareous exoskeletons." Our results indicate that accumulation of [^{14}C]-chlorpyrifos is highly correlated with water turnover rates. However, we did not observe a relationship between the hardness of the integument and water permeability or [^{14}C]-chlorpyrifos accumulation. Soft-bodied taxa were both highly (*C. riparius*) and minimally (*Ptychoptera sp*) permeable. Rather, we observed that respiratory strategy and body size were more important factors in determining both [^{3}H]-H_2O and [^{14}C]-chlorpyrifos uptake.

Maki and Johnson (1975) also observed differences in TMF LC50 values between younger and older individuals of the same taxa, with younger instars being more sensitive than older instars. These results are consistent with Stuijfzand et al. (2000) who observed more than a 1000-fold difference in diazinon LC50 values for 1st and 4th instar *C. tentans*. Although we did not determine LC 50 values in our experiments with *C. riparius*, we did observed that third instar individuals had almost a 2-fold higher [^{14}C]-chlorpyrifos uptake rate than the fourth instar individuals. This could be attributed to larger surface area to volume ratios in earlier instars. Additionally, higher growth rates and metabolic demands can result in relatively larger areas of exchange epithelia in earlier instars. We are

currently exploring the extent to which chlorpyrifos sensitivity differences among *C. riparius* instars are driven by differences in target site sensitivity, metabolic processes, or simply a function of differences in uptake rates.

Uptake differences among taxa do not necessarily translate to differences in contaminant sensitivity, particularly when comparisons are being made across unrelated taxa. Differences in target site sensitivity, metabolic capabilities, and detoxification mechanisms can be expected to vary widely at the ranks of order and family and, in some cases, genus. Despite the fact that *C. riparius* had high chlorpyrifos accumulation rates, it is not particularly sensitive to this compound. We have determined that this lack of sensitivity is due to the relatively slow biotransformation of chlorpyrifos to the oxon metabolite, which is the more toxic form (unpublished data). However, within closely related organisms, differential sensitivity may be possibly be predictable based on exposure potential.

A major challenge in characterizing an organism's epithelial surface area is that these surfaces are not readily identifiable. We used water permeability as a quantitative surrogate to estimate the relative differences between taxa in terms of exposed cellular surface. Water permeability differences are driven by body size and exchange epithelial surfaces. We rule out the effect of drinking in these studies. It is generally thought that drinking is an osmoregulatory strategy limited to primitive forms (Komnick 1977). In addition, we suspect that large exchange epithelial surface areas are primarily driven by respiratory requirements and, to a lesser extent, osmoregulatory and chemosensory functions.

To facilitate the categorization of taxa based on exchange epithelial surface area, we offer the DPH fluorescence technique as a precurser to quantitative measures of exchange epithelia surface areas. The membrane dye DPH offers an inexpensive and rapid way of discriminating between taxa in terms of epithelial surface area differences, which may be used to predict differences in contaminant uptake rates. Two species of Chironomidae were chosen for comparison based on their phylogenetic relatedness, the simplicity, size, and similarities of the body plan, and the lack of gill coverings or complex three dimensional gill structures. The more extensive fluorescence seen on the body surface of *C. riparius* versus *Psectrotanypus sp.* was consistent with the higher water permeability and chlorpyrifos accumulation seen in *C. riparius* versus *Psectrotanypus sp.* A higher incidence of fluorescence was associated with higher flux rates. Due to the limited availability of *Psectrotanypus sp.,* we were unable to compare the sensitivities of these taxa.

The DPH fluorescence approach has the potential to add predictive power in assessing differences in species' exposure potential. The degree to which exposure potential and sensitivity are related should be examined further within a phylogenetic context to minimize confounding factors due to physiological differences among unrelated taxa. DPH is potentially useful in describing the attributes of resident biota and can be developed into diagnostic tools to discriminate between stressors that manifest their effects via epithelial surfaces and stressors that are based on physical habitat problems.

Aquatic insect organismal characteristics and life history attributes have not been adequately incorporated into either toxicological or ecological approaches to studying water pollution. In the results reported here, body size and respiratory strategy were important determinants of both water permeability and chlorpyrifos uptake. We suggest that these attributes could be incorporated into bioassessment protocols. For example, a percent air-breather metric could be particularly useful in wetland and other lentic systems to discriminate between water chemistry and physical habitat degradation. Additionally, in areas where pulses of contaminants are present, we suggest that smaller, dissolved oxygen-breathing organisms would be more likely to be impacted than larger and/or air-breathing taxa. In systems with dissolved oxygen problems, we suggest that larger dissolved oxygen-breathing organisms would be at a disadvantage relative to smaller and/or air-breathing taxa. Finally, the DPH technique may provide the basis for testing hypotheses regarding the potential sensitivity differences among taxonomically related organisms. This could refine techniques such as EPT-based metrics to evaluate stressor-specific responses that currently do not exist.

Chapter 4.

Roles of Uptake and Target Site Sensitivity in Determining the Differential Toxicity of Chlorpyrifos to 2nd- 4th Instar *Chironomous riparius.*

Introduction

Given the diversity of aquatic insect species, it is not surprising that they exhibit such a wide array of sensitivities and tolerances to water quality degradation. The basis for sensitivity differences to toxicants among insects in aquatic ecosystems remains largely unknown. However, these differences can most likely be segregated into three major biological attributes: (1) exposure/accumulation relationships (2) target site sensitivity, and (3) metabolism and elimination. It would be useful to better understand the relative importance of these biological parameters in determining contaminant sensitivity differences across aquatic insect taxa as well as during development within a given taxon. Such understanding could potentially be used to refine biomonitoring and bioassay techniques

For simplicity, we have chosen to work with a single toxicant-the organophosphate insecticide chlorpyrifos, and three instars of the widely studied Chironomid *Chironomous riparius* (Meagan). Organophosphate pesticides have been widely studied and their mode of action well characterized. The toxicity of chlorpyrifos is believed to result from metabolic activation of the parent compound to the oxon metabolite. The oxon metabolite deactivates acetylcholinesterase (AChE) at neural junctions, resulting in overstimulation of the peripheral nervous

system (Matsumura, 1985). Due to the specific mode of action of this compound, AChE is a widely used biomarker of exposure and effect. In these studies, AChE activity is used to evaluate target site sensitivity differences among developmental stages of *C. riparius*.

Toxicological comparisons among instars or taxa are often based on LC50 values. Earlier life stages are often considerably more sensitive than latter life stages. For example, Kiffney and Clements (1994, 1996) found an inverse relationship between survivorship and body size in metals-exposed aquatic insects. Similarly, McCahon et al (1989) found that earlier instars of the trichopteran *Agapetus fuscipes* were significantly more sensitive to cadmium than later instars. Stuijfzand et al. (2000) found significant differences in aquatic insect diazinon LC50 values across aquatic insect taxa and developmental stages.

Previous work in our lab showed that both body size and respiratory strategy were important biological attributes determining chlorpyrifos accumulation differences among 9 aquatic insect taxa. In addition, 3rd and 4th instar *C. riparius* differed considerably in their accumulation rates of ^{14}C-chlorpyrifos in water-only exposures (Buchwalter et al, 2002). The relatively high rate of chlorpyrifos accumulation in *C. riparius* was surprising given their relative tolerance to organophosphate insecticides

This paper examines the mechanistic basis for chlorpyrifos sensitivity differences in 2nd-4th instar *C. riparius* larvae. Specifically, it assesses the relative importance of uptake versus target site sensitivity differences among instars. High

dose studies were performed to characterize the sensitivity differences among instars. Low dose experiments were conducted to compare uptake rates among instars. Acetylcholinesterase assays were performed *in vitro* to assess differences in target site sensitivity.

Materials and Methods

C. riparius cultures were initiated from egg masses originally obtained from Environmental Consulting and Testing, Superior, WI. Mixed cohort groups were established in 10 gallon glass aquaria in an environmentally controlled chamber at 23+/-0.5 °C. A 16:8 light:dark photoperiod regime was used with indirect full spectrum lighting. Substrate and food consisted solely of vitamin fortified Nature's Café Bunny Buffet® alfalfa pellets. Artificial water (Fisher Scientific® 0.67 mM $CaCl_2$, 0.3 mM $MgSO_4$, 1.2 mM $NaHCO_3$, and 0.5 mM KH_2PO_4) was used throughout all culture and experimental procedures.

High dose experiments were conducted solely to assess the relative chlorpyrifos sensitivities of developing *C. riparius*. Larvae were exposed to a single, extremely high dose of ^{14}C-chlorpyrifos. Two endpoints were measured – time to death and the body residues associated with lethality. In these experiments, larvae were placed in Erlenmeyer flasks containing 50 mL of the artificial water described above, spiked with a 1ml 50:50 acetone water stock solution of ^{14}C chlorpyrifos to yield 0.535 mg/l ($1.53x10^{-6}$M). Two 1-ml alloquats were taken from each exposure chamber to verify chlorpyrifos concentrations. The average of the two alloquats was taken to be the exposure concentration for each flask. Exposure chambers were normalized for analysis.

Three, two and one larva each were placed into exposure chambers for 2nd, 3rd, and 4th instar larvae respectively. The larvae were checked hourly for the first

12 hours of exposure and every 2 hours thereafter. Larvae were removed when they were unresponsive to physical stimuli and exhibited no signs of life. Dead larvae they were rinsed, weighed, and digested individually using the procedures described by Buchwalter et al., (2002). Time to death was recorded and residues associated with lethality were measured (Figure 13) Control treatments consisted of five flasks per instar, with three, two and one larva each were placed into exposure chambers for 2^{nd}, 3^{rd}, and 4^{th} instar larvae respectively. Exposure media for controls consisted of artificial water that contained 1 ml of 50:50 acetone: deionized water. There was no control mortality. One 4^{th} instar control larvae molted to its pupal stage during the experiment.

Low dose experiments were conducted to compare relative ^{14}C-chlorpyrifos uptake differences among 2^{nd}-4^{th} instars of *C. riparius*. Previous studies in our lab showed marked differences in slopes of instantaneous uptake rates of 3^{rd} and 4^{th} instar *C. riparius* in time course studies conducted to 12 hours (Buchwalter et al., in press). These studies were conducted at a single timepoint (7 hours) within the linear portion of the uptake curves for this species. Artificial water (described above) was used for all experiments. Fifty mL of artificial water and 20 μL of ^{14}C-chlorpyrifos (in 50:50 acetone:water) were added to Erlenmeyer flasks. This yielded final specific activities for ^{14}C-chlorpyrifos of approximately 0.023 μCi • L^{-1}. This ^{14}C- chlorpyrifos activity corresponds to a concentration of 240 ng • L^{-1}($6.85x10^{-10}$M). Two 1-ml alloquats were taken from each exposure chamber to verify chlorpyrifos concentrations. The average of the two alloquats

was taken to be the exposure concentration for each flask. Exposure concentrations were normalized for analysis.

The number of individuals placed in each flask varied with instar to reduce differences in the biomass to contaminant ratios. Three, two and one larva each were placed into exposure chambers for 2^{nd}, 3^{rd}, and 4^{th} instar larvae respectively. Average chamber biomasses were 1.7, 2.8, and 6.9 mg for 2^{nd}, 3^{rd}, and 4^{th} instars respectively. Five replicates were performed for each instar tested. The 2^{nd} instar larvae were pooled for digestion and LSC analysis. Third and 4^{th} instar larvae were individually digested and LSC analysis.

Following exposure, insects were removed from exposure flasks, rinsed profusely with water, weighed, and placed in 20 mL scintillation cocktail vials. A Sartorius R-200D-RS analytical balance was used to weigh insects to +/- 0.00005 g. Amersham NCS II® tissue solubilizer was added to each vial and digests were held at 50 °C overnight and neutralized with glacial acetic acid to obtain pH 7. Eighteen mL of Amersham BCS-NA® non-aqueous scintillation cocktail were added, and samples were held in a refrigerator in the dark for at least four days to minimize chemiluminescence. These samples were well mixed and counted with a Beckman LS 6500 liquid scintillation counter. Five control insects of each instar were placed in water containing the acetone carrier for 7 h, removed, digested, and analyzed as described above. The average of control [^{14}C] activities was taken to be background for each instar tested and subtracted from the counts of exposure groups (Figure 12).

Acetylcholinesterase analysis

In vitro acetylcholinesterase (AchE) assays assessed the target site sensitivity differences among *C. riparius* larval instars 2-4. *C. riparius* homogenates were normalized for body weight, total protein content, and basal AChE activity. Acetylcholinesterase (AChE) analysis followed the method of Ellman et al. (1961), with modifications described by Fisher et. al. (2000) specific to C. *riparius*. Measurements were performed on a SpectraMax Plus Spectrophotometer (Molecular Devices Corp., Sunnyvale, CA) and all reagents purchased from Sigma Chemical Co. (St. Louis, MO). This assay determined enzymatic activity by the rate that the substrate, acetythiocholine (AtChI), was hydrolyzed, producing acetate and thiocholine. The thiocholine, in turn, combined with the chromogen DTNB [5.5'-dithio-bis(2-nitrobenzoic acid)], and formed a colored product. Change in absorbance at 412 nm was measured at 12 sec intervals for 10 min. Protein content was determined by the method of Bradford (1976) (BioRad, Richland, CA, USA), using bovine serum albumin as the standard.

The pesticides chlorpyrifos (tested at 10^{-4} to 10^{-8} M) and chlorpyrifos-oxon (tested at 10^{-6} to 10^{-10}) were prepared in ethanol by serial dilution of 10^{-2} M and 10^{-4} M stocks, respectively. BW1,5-bis-(4-allyldimethylammoniumphenyl) pentan-3-one dibromide (BW) and eserine [1'-methylpyrrolidino (2':3':2:3)1,3-dimethylindolin-5-yl *N*-methylcarbamate], considered specific AChE and total cholinesterase inhibitors, respectively, were both prepared at 10^{-4} M in deionized H_20 and tested at 10^{-5} M.

C. riparius larvae were separated by instar, homogenized in ice-cold 0.1 M sodium phosphate buffer (PB), pH 8.0, containing 0.1% TritonX-100, at 20 mg tissue per ml PB. The homogenate was then centrifuged at 1000 rpm for 5 min and the supernatant divided into four 1.5 ml Eppendorf tubes. Two tubes were used for in-vitro chlorpyrifos and chlorpyrifos-oxon tests as duplicate samples. One additional sample (from fourth instar larvae) was prepared for specific cholinesterase determination, and run in duplicate. To a microtiter plate well, 100 µl of the homogenate supernatant, 5 µl of 6 mM DTNB, and 10 µl of test chemical was transferred. Chlorpyrifos-oxon, BW and eserine mixtures were incubated for 30 min at 25 °C. Chlorpyrifos mixtures were incubated at room temperature for 24 hrs. Following the incubation period, 30 µl of 15 mM AtChI was added to the well to initiate enzymatic reaction. Final well concentrations of DTNB and AtChI were 0.2 mM and 3 mM, respectively.

Allometry

A Boeckeler Instruments® (Tuscon, Arizona) Digital Positioner and Microcode II were connected to a Leica MZ 95 stereoscope to measure length and width dimensions on 22 *C. riparius* larvae or varying sizes. Width measurements were taken at the center point of each larva. For simplicity, we assumed a cylindrical shape for the larvae, and calculated surface area to volume ratios for each larva. A plot of surface area: volume vs body weight was used to create a standard curve.

Results

There was a marked decrease in sensitivity as larvae mature in time to death experiments. Earlier instars reach lethal doses more quickly than later instars (Figure 12). In addition, the accumulated chlorpyrifos concentrations associated with lethality were significantly lower on a per-individual basis in smaller organisms than in larger organisms.

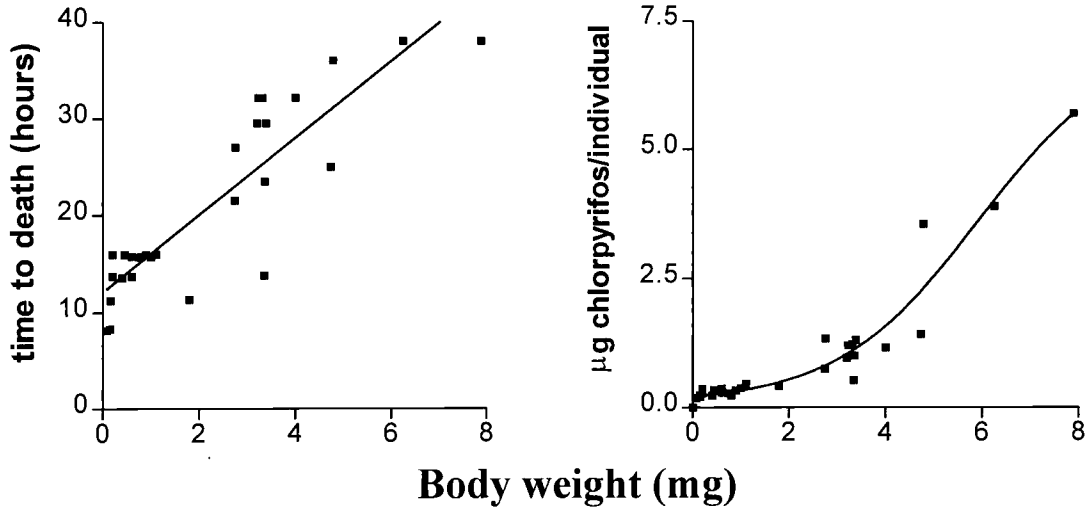

Figure 12. Time to death for *C.riparius* larvae exposed to 0.535 mg/l ($1.53x10^{-6}$M)^{14}C-chlorpyrifos and associated body burdens. Time to death data are described by a line with a slope of 3.97+/- 0.42 (r^2= 0.78). Residue data were fit to a sigmoidal curve (r^2= 0.93).

There were significant differences in the accumulation of ^{14}C-chlorpyrifos among *C. riparius* instars 2-4 after seven hours of exposure (1 way ANOVA, $p<.01$) (Figure 13). These results are consistent with prior time course studies where differences in the accumulation rates of 3rd and 4th instar *C. riparius* were observed (Buchwalter et al. 2002). Smaller organisms accumulate chlorpyrifos more rapidly on a per weight basis than larger organisms. Second instar C. riparius accumulated approximately 1.5, and 2.4 times more chlorpyrifos on a per weight basis than 3rd and 4th instars, respectively.

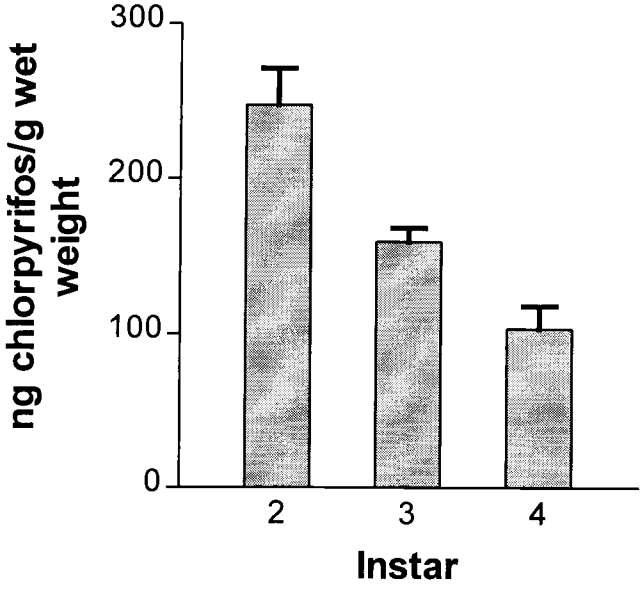

Figure 13. Chlorpyrifos accumulation in 2nd-4th instar *C. riparius* larvae. Larvae were exposed for 7 hours to ^{14}C-chlorpyrifos and 240 ng/l ($6.85x10^{-10}$M). Average body weights for instars 2-4 were 1.5, 2.9, and 7.1 mg respectively. Error bars represent the standard errors of the means.

Esterase specific inhibitors were used to assess the relative importance of AChE, butylcholinesterase (BuChE), and total esterase in homogenates of 4th instar larvae. Iso-OMPA, a BuChE specific inhibitor, did not reduce cholinesterase activity. In contrast, BW, an AChE specific inhibitor, resulted in significant depression. Eserine, a total cholinesterase inhibitor resulted in a similar degree of depression as BW, suggesting that AChE is the predominant esterase in *C. riparius*.

In vitro acetlycholinesterase assays were performed to examine basal AChE activities and AChE sensitivities of 2nd – 4th instar larvae to both chlorpyrifos-oxon and chlorpyrifos. There were significant differences in the basal AChE activities between instars on a per weight basis and on a per mg protein basis (1-way ANOVA $p<.01$). Basal activities for 2nd, 3rd and 4th instars were 395.0+/-5.4, 321.3+/-31.9, and 1051.0+/-57.9 nmoles AtChl hydrolyzed/min/gram, respectively (Figure 14). Total protein content was similar among developmental stages.

To characterize differences in target site sensitivity, homogenates were diluted such that basal AChE activities were similar in 2nd-4th instar-derived homogenates. The intent was for each well to hold similar AChE concentrations, such that does dependent changes in activity could be directly compared among developmental stages. In these studies, there was no age-specific trend in AChE depression associated with exposure to both chlorpyrifos-oxon and chlorpyrifos (Figure 14).

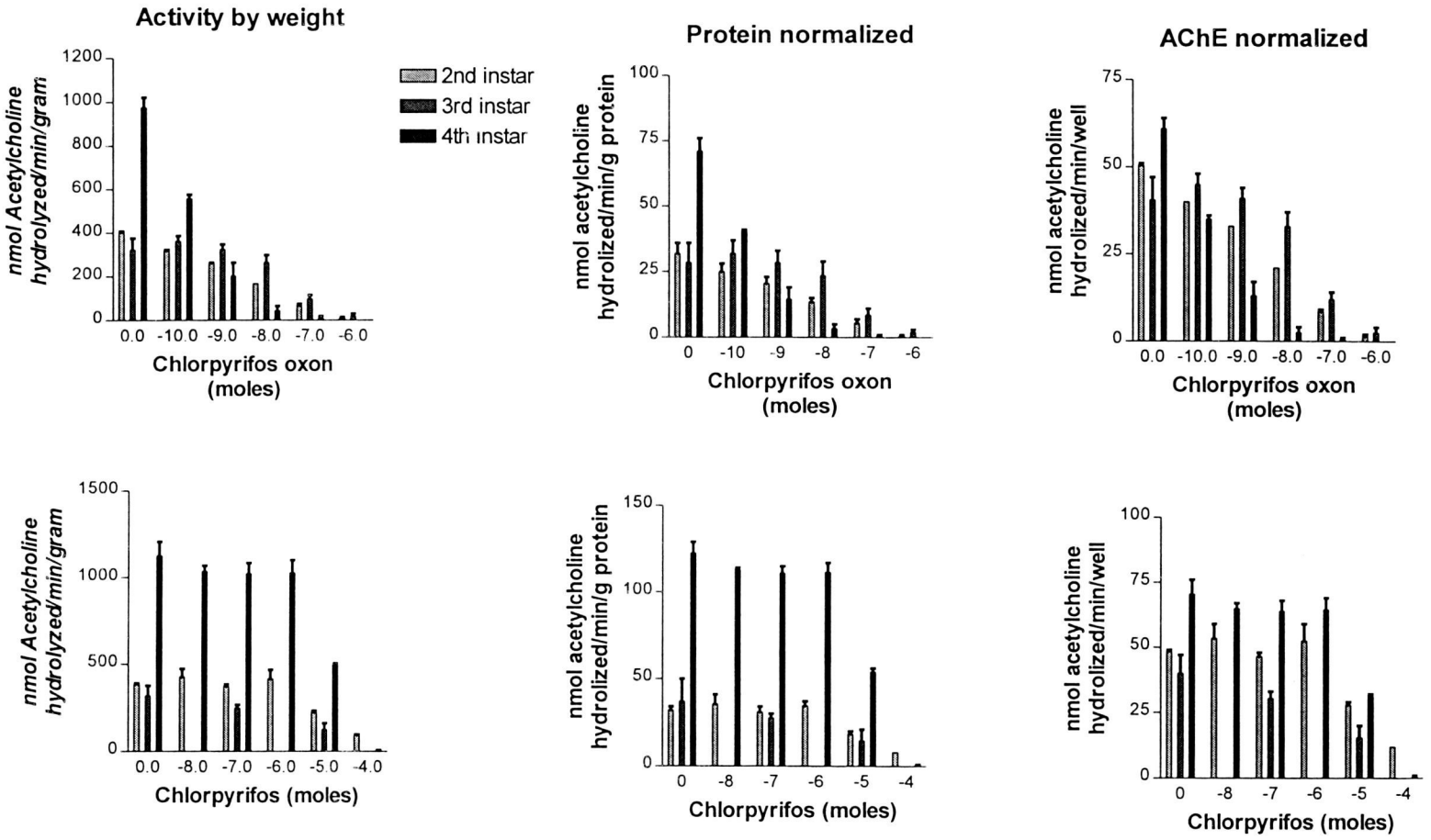

Figure 14. In vitro acetylcholinesterase activity associated with exposure to chlorpyrifos-oxon and chlorpyrifos in *C. riparius* homogenates derived from 2^{nd}-4^{th} instar larvae.

Allometry

Data for both ethanol-fixed and carbon dioxide anesthetized larvae fit similarly shaped exponential curves (Figure 15). Fixed larvae were slightly lighter than anesthetized larvae.

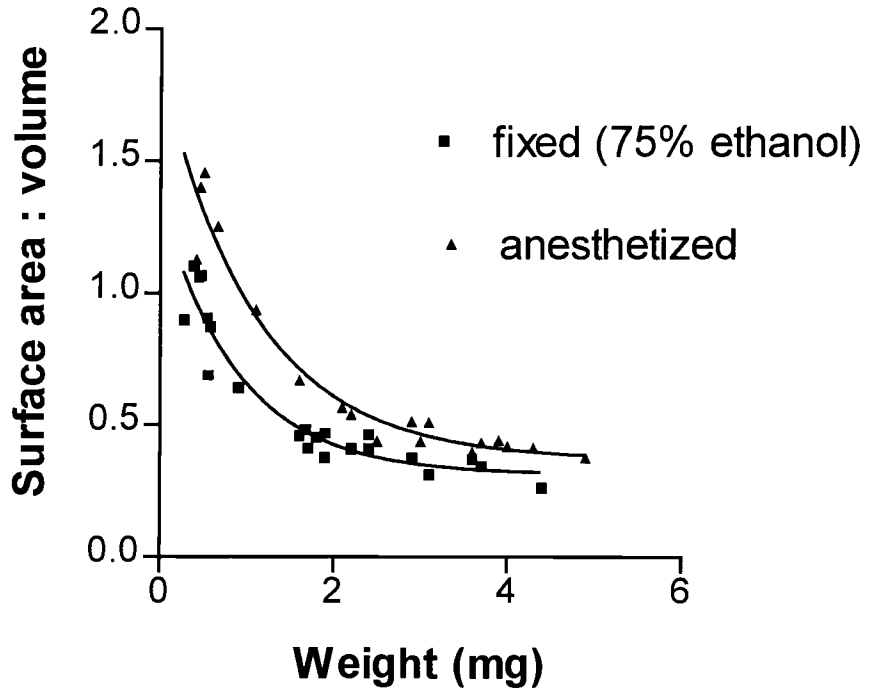

Figure 15. Surface area vs body weight in ethanol-fixed and CO_2 anesthetized *C. riparius* larvae. The exponential curve for anesthetized larvae is described as follows:
Surface area : volume = $1.479 \bullet e^{(-.91 \bullet \text{ weight})} + 0.3692$. This curve has an $r^2=0.95$.

Discussion

Biological attributes that affect the differential toxicity of contaminants to aquatic insects are poorly understood and are often not considered in bioassessment, modeling or other risk assessment procedures (Luoma and Fisher, 1997). Ecologists have observed that certain taxa tend to be extirpated from systems with degraded water quality. However, the mechanistic bases for differences in life stage and species sensitivity to individual stressors remain unclear. As a result, few tools exist to evaluate insect community responses to specific stressors such as pesticide contamination.

The small size of insects results in an extremely high surface-to-volume ratio, which in turn requires that the integument play a critical role in maintaining homeostasis. Differences in the respiratory biology of aquatic insects result in potential differences in contaminant uptake rates. Recent findings demonstrate that dissolved oxygen-breathing insects with larger exchange epithelial surfaces have significantly higher water and chlorpyrifos uptake rates than air breathing taxa with smaller exchange epithelial surfaces (Buchwalter et al., 2002). While it is tempting to assume that organisms that rapidly accumulate contaminant residues are more likely to be sensitive than organisms that are relatively poor accumulators, this is not always the case. In previous studies, *C. riparius* accumulated chlorpyrifos residues at a higher rate than 8 other taxa (Buchwalter et al, 2002), yet is relatively

insensitive to the compound. This study was designed to investigate the factors that determine differences in sensitivity to developing *C. riparius* larvae.

High dose experiments measuring time to death and associated lethal body burdens reveal a marked decrease in chlorpyrifos sensitivity with increasing age in *C. riparius* larvae (Figure 12). Size and age specific sensitivity differences have been observed in aquatic insects by several researchers including McCahon (1989), Kiffney and Clements (1994, 1996) and Stuijfzand et al., (2000). While this trend of decreased sensitivity with age seems to be relatively consistent, the mechanistic bases for age/size related sensitivity differences to contaminants in aquatic insects remains poorly understood.

Consistent with previous work with ^{14}C-chlorpyrifos uptake rates in 3rd and 4th instar *C. riparius* (Buchwalter et al., 2002), we observed marked differences in chlorpyrifos accumulation in 2nd-4th instar C. riparius in these studies (Figure 13). Uptake rate differences are consistent with differences in surface area to volume ratios (Figure 15). For example, 2nd instar larvae have an average surface area to volume ratio that is 1.6, and 2.7 fold higher than 3rd and 4th instar larvae, respectively. The chlorpyrifos uptake rates we 1.5 and 2.3 fold higher in 2nd instar larvae than 3rd and 4th instar larvae respectively, on a per-weight basis.

In vitro acetylcholinesterase assays revealed several interesting results. First, homogenates from *C. riparius* larvae were refractive to chlorpyrifos, even at high doses. In contrast, homogenates from *C. riparius* larvae were highly responsive to chlorpyrifos-oxon in a dose-dependent manner (Figure 14). The

relative insensitivity of *C. riparius* to chlorpyrifos *in vivo*, may result from a relatively slow rate of conversion of the parent compound to the oxon metabolite, which is clearly much more biologically active. Second, basal AChE activity was significantly higher in 4th instar larvae than in 2nd or 3rd instar larvae. These results are consistent with other studies, which demonstrate increase in basal AChE activity with age in developing ticks (Wright, 1989). Third, it appears that in terms of dose-dependent decreases in AChE activities, 4th instar homogenates were slightly more sensitive than earlier instars.

Based on these results, we conclude that the relative chlorpyrifos sensitivity differences among *C. riparius* larval instars are primarily due to accumulation rate differences. These accumulation rate differences are largely driven by surface area to volume ratio differences during development. Body size (presumably surface area to volume ratio) was an important determinant of chlorpyrifos accumulation in aqueous-only exposures for 9 taxa in previous studies, including two instars *of C. riparius*. (Buchwalter et al., 2002).

Insects are highly variable with respect to their AChE sensitivities to inhibitors such as chlorpyrifos. Working with 20 species within the Order Homoptera, emphasizing aphids, Novozhilov et al (1989) demonstrated that AChE sensitivity to various inhibitors closely followed phylogenetic relationships. Future work that examines the variation in AChE sensitivity along phylogenetic lineages could improve our ability to predict organophosphate sensitivity differences among aquatic insects.

Chapter 5: Future Directions

Investigating the Role of Aquatic Insect Respiratory and Osmoregulatory Epithelia in Determining Heavy Metal Accumulation From Environmental Matrices.

Introduction

Aquatic insects play important ecological roles in freshwater ecosystems and are used extensively to evaluate water quality. Ecologists have observed that certain taxa tend to be extirpated from systems with degraded water quality. However, the mechanistic bases for differences in species sensitivity to individual stressors remain unclear. As a result, few tools exist to evaluate insect community responses to specific stressors such as heavy metal contamination. This proposal outlines a comparative toxicological approach to understanding the factors that determine heavy metal exposure and toxicity differences between aquatic insect species.

The small size of insects results in an extremely high surface-to-volume ratio, which in turn places uniquely heavy osmoregulatory demands on their exchange epithelia (Dow, 1994). This is particularly true in freshwater insects where osmotic gradients favor the passive loss of ions as well as the influx of water (Komnick, 1977). Insects utilize a variety of waterproofing lipids and waxes to reduce these fluxes. However, the array of respiratory strategies that aquatic insects have evolved result in dramatic differences in the relative surface areas of exchange

epithelium on the body surface. Waterproof barriers do not protect these functionally exposed cellular surfaces. Therefore, there are large differences in the potential for osmoregulatory distress among taxa. Recent findings demonstrate that dissolved oxygen-breathing insects with larger exchange epithelial surfaces have significantly higher water and chlorpyrifos uptake rates than air breathing taxa with smaller exchange epithelial surfaces (Buchwalter et al., in press, *Can. J. Fish. Aquat. Sci.*). These differences among species' osmoregulatory and respiratory situations prompt investigation into differences in heavy metal uptake and sensitivity among aquatic insect taxa.

Biological attributes that affect the bioavailability of contaminants are poorly understood and are often not considered in modeling or other risk assessment procedures (Luoma and Fisher, 1997). The studies outlined in this proposal are designed to answer the following questions: Do differences in aquatic insect respiratory and osmoregulatory physiology significantly affect accumulation rates of aqueous cadmium and zinc from the water column? (Study #1). What are the relative contributions of dietary and aqueous sources of cadmium and zinc in organisms with varied respiratory and osmoregulatory physiology? (Study #2). Answers to these questions have immediate applications to exposure modeling, risk assessment, and bioassessment of metals-contaminated systems.

Study 1: Comparative accumulation rates of aqueous Cd and Zn in aquatic insect taxa.

Rationale

Relatively few studies have investigated the role that insect respiratory and osmoregulatory systems play in the accumulation of, and sensitivity to, contaminants. This is partially due to the lack of available tools to characterize insect taxa based on differences in exchange epithelial surface area. Exchange epithelia such as gill surfaces have been studied extensively as important metal exposure pathways in fish (e.g., Hollis et al., 2000). An extensive biotic gill ligand modeling approach has been developed for fish (e.g., Alsop and Wood, 2000). It would be useful to understand the extent to which differences in respiratory and osmoregulatory exchange epithelial surface areas drive differences in metal uptake across insect species. Field evidence and toxicological studies show that insect species vary widely in their sensitivity to metals. For example, some mayfly species show great metal sensitivity and are among the first to disappear from mining-impacted streams (Clements, 2000). However, some caddisflies can be widespread in such streams and show higher tolerance in toxicity tests (Clements, 2000). Mechanistic explanations of the differential sensitivity of insect species are lacking. Understanding such mechanisms could help predict which species are likely to suffer adverse consequences from metal contamination in nature. Linking

exposure potential to an organismal characteristic (external exchange epithelial surface) can potentially provide such a mechanistic explanation for differences in taxa sensitivity within a phylogenetic framework. The diversity of the insect community can be impacted by metals (Clements, 2000). Insects play an important role in aquatic ecosystem food chains, which suggests that metal contamination can contribute to decreased diversity within food webs in general. These findings will be immediately useful in terms of providing heavy metal specific diagnostic tools for ecologists involved in bioassessment of metal contaminated areas.

Methods

Measuring dissolved cadmium and zinc uptake rates.

Insects that vary with respect to respiratory and osmoregulatory strategy will be collected from freshwater systems that are not metals contaminated. Radioisotopes of cadmium and zinc will enable the non-destructive measurement of metal uptake. Repeated measures on individual larvae over time course studies will measure the instantaneous uptake rates of individual metals and metal mixtures from the water column. Additionally, depuration rates will be determined by transferring larvae (post-exposure) to continually refreshed clean water for additional time course studies. Depuration rate studies will provide evidence for differences in metabolic capabilities among taxa. Reconstituted water will be used for all experiments to maintain consistency and a well-defined environmental

matrix. Organisms will be chosen based on respiratory strategy and phylogenetic relationships. Up to ten taxa will be examined. Uptake rates for each taxon will be compared to water permeability (section B), fluorescent membrane dye distribution (section C), and chloride cell characterization (section D).

Characterizing taxa water permeability

For each taxa tested, instantaneous $^{3}H_2O$ uptake rates will be measured via time course studies. I have used these techniques extensively in my Ph.D. studies and have found that organisms that breath dissolved oxygen are significantly more water permeable than air-breathing insects. Furthermore, dual label studies showed a high degree of co-variation between $^{3}H_2O$ and ^{14}C-chlorpyrifos accumulation. Differences in chlorpyrifos uptake rates are consistent with toxicity data for aquatic insects for other organophosphate pesticides, suggesting that differences in sensitivity may be directly related to differences in exposure levels. While chlorpyrifos uptake is presumably a passive process, we suspect that metal uptake will be actively mediated by V-ATPase activity. Water permeability is directly related to exchange epithelial surface area which, in turn, indicates osmoregulatory demands across insect taxa. We expect that highly water permeable organisms have more chloride cell and V-ATPase activity and, subsequently, higher metal uptake rates.

Fluorescent membrane dye (DPH)

I have developed the following approach to characterizing aquatic insects based on differences in external exchange epithelia (Buchwalter et al., 2002). The fluorescent dye DPH (diphenylhexatriene) is used to label cellular surfaces. DPH is a cylindrically shaped molecule that orients parallel to the fatty acyl chains of membranes. It has minimal fluorescence in water. Fluorescence excitation and emission dipoles are oriented roughly parallel to the axis of the molecule, resulting in increased fluorescence when incorporated into the plasma membranes of epithelial cells (Haugland, 1996). Images of DPH-labeled insects may be compared based on overall brightness, relative size of fluorescing surfaces, and photographic attributes such as magnification and exposure time. For each taxa tested, individuals will be incubated in DPH and photographed through a fluorescence stereoscope equipped with ultraviolet filters. The resulting images will be analyzed with image analysis software. Comparisons will be made between fluorescence data and metal uptake rates.

Identifying and quantifying chloride cells

Silver ($AgNO_3$) has been used to identify chloride cells in aquatic insects. Silver ions precipitate with chloride ions secreted by chloride cells and epithelia (Komnick, 1977). This technique can be utilized to compare differences in species' osmoregulatory demands, which, in turn, are linked to respiratory strategy. Individuals from each taxon will be labeled with silver and assayed for total silver

content. We expect to see a strong correlation between metal accumulation rates (section A) and silver precipitation on chloride cells. Of particular interest are the relationships among DPH distribution and intensity and silver accumulation. Because the DPH technique is rapid and inexpensive, its utility as a predictor of direct metal exposure via the water column will be compared with the silver precipitation method.

Insect osmoregulation relies extensively on V-ATPases. These pumps exist in chloride cells of aquatic insects, which are found on gill tissue as well as specialized osmoregulatory tissue in some taxa. Because diffusive ion loss and water influx occur at these epithelial surfaces, it is likely that organisms with larger epithelial surfaces will require more V-ATPase activity. Increased enzyme activity should be associated with increased metal uptake. Bafilomycin, a potent V-ATPase inhibitor, will be used to block enzyme activity and thereby reduce metal accumulation. A bafilomycin treatment will be used in all assays as an additional line of evidence for the role of epithelial exchange surfaces in determining exposure. Differences in chloride cell density (as measured by silver accumulation) and activity (inferred from differences in metal accumulation between bafilomycin and non-bafilomycin exposures) will be correlated with metal uptake rate differences between taxa.

Study 2: Determining the relative importance of dietary versus aqueous metal uptake.

Rationale

Little is known about the relative importance of aqueous versus dietary metal-exposure pathways in aquatic insects. Studies by Munger and Hare (1997) demonstrated that for the dipteran *Chaoborus punctipennis*, diet is the primary route of exposure for cadmium. This species is known to tolerate a wide range of chemical conditions, presumably due to its relatively impermeable integument. This air-breathing organism should not be expected to accumulate significant levels of cadmium directly from the water column because it breathes with a respiratory siphon. Another Diptera (*Ptychoptera sp.*) with a similar breathing strategy was found to be relatively impermeable to both water and chlorpyrifos (Buchwalter et al., in press). In other studies, aquatic organisms were found to accumulate the majority of cadmium from aqueous sources (Warren et al., 1998) or dietary sources (Roy and Hare, 1999). There is an obvious need to further evaluate the relative importance of these exposure routes in a variety of species, particularly since water quality criteria typically focus only on aqueous sources. Data from taxa exhibiting a variety of respiratory and osmoregulatory strategies will provide a means of evaluating the relative importance of dietary versus aqueous cadmium and zinc exposure pathways.

Methods

A subset of insects from Study #1 will be used in these experiments. Organisms tested will include air-breathing and dissolved oxygen-breathing taxa. Individuals will be acclimated to lab conditions for at least 5 days before the initiation of experiments and will be fed during this period. The procedures employed by Roy and Hare (1999) will be compared to those developed by Wang et al. (1996b) in which assimilation efficiencies are determined for specific food types. Metal uptake rates from food are calculated based upon ingestion rate, assimilation efficiency, and metal concentration. Comparisons with metal uptake rates from water (Study #1) can differentiate between the relative importance the two sources of metal in determining overall exposure (Reinfelder et al., 1999). A third treatment consisting of both contaminated food and water will be used to more accurately reflect situations faced by organisms in contaminated environments. The food and water exposures will be used to evaluate the importance of exchange epithelial surface area differences on overall metal exposure. As in the aqueous exposures discussed in Study #1, loss rates will also be determined using methods developed by Wang et al. (1996b).

For each species tested, water permeability, DPH fluorescence, and chloride cell (silver) characterizations will be conducted as outlined above. The organisms will be roughly the same size, both within and across species, to reduce

confounding factors since smaller organisms have been shown to accumulate contaminants at a higher rate than larger organisms on a per-weight basis

The comparative approach of these studies will be instrumental in elucidating key biological factors that determine the accumulation of cadmium and zinc from environmental matrices. Furthermore, they will help evaluate the relative importance of metal exposure pathways in aquatic insects. Finally, these studies have the potential to provide the mechanistic basis for assigning metals tolerance values for aquatic insects. The outcomes from this work will have relevance to exposure modelers, risk assessors, regulators, and ecologists involved in bioassessment.

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