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Roles of uptake, biotransformation, and target site sensitivity in determining the differential toxicity of chlorpyrifos to second to fourth instar *Chironomous riparius* (Meigen)

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Abstract

Early life stages of aquatic organisms tend to be more sensitive to various chemical contaminants than later life stages. This research attempted to identify the key biological factors that determined sensitivity differences among life stages of the aquatic insect *Chironomous riparius*. Specifically, second to fourth instar larvae were exposed in vivo to both low and high waterborne concentrations of chlorpyrifos to examine differences in accumulation rates, chlorpyrifos biotransformation, and overall sensitivity among instars. In vitro acetylcholinesterase (AChE) assays were performed with chlorpyrifos and the metabolite, chlorpyrifos-oxon, to investigate potential target site sensitivity differences among instars. Earlier instars accumulated chlorpyrifos more rapidly than later instars. There were no major differences among instars in the biotransformation rates of chlorpyrifos to the more polar metabolites, chlorpyrifos-oxon, and chlorpyridinol (TCP). Homogenate AChE activities from second to fourth instar larvae were refractory to chlorpyrifos, even at high concentrations. In contrast, homogenate AChE activities were responsive in a dose-dependent manner to chlorpyrifos-oxon. In general, it appeared that chlorpyrifos sensitivity differences among second to fourth instar *C. riparius* were largely determined by differences in uptake rates. In terms of AChE depression, fourth instar homogenates were more sensitive to chlorpyrifos and chlorpyrifos-oxon than earlier instars. However, basal AChE activity in fourth instar larvae was significantly higher than basal AChE activity in second to third instar larvae, which could potentially offset the apparent increased sensitivity to the oxon.

Keywords: Chlorpyrifos; Acetylcholinesterase; Biotransformation; Life stages; Chironomous

1. Introduction

Many organisms, such as aquatic insects have sensitive life stages, during which they are more susceptible to environmental insults. Often, these sensitive periods occur during earlier life stages. For example, 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 median lethality (LC50) values for the organophosphate insecticide, diazinon across

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aquatic insect taxa and developmental stages. Similarly, Kiffney and Clements (1994, 1996) found an inverse relationship between survivorship and body size in metals-exposed aquatic insects.

The basis for contaminant toxicity differences between developmental stages of an individual taxon and across a variety of species 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 excretion. It would be useful to better understand the relative importance of these biological parameters in determining contaminant sensitivity differences across species used extensively as ecological indicators, as well as during development within a single species.

Previous work in our lab showed that both body size and respiratory strategy were important biological attributes determining chlorpyrifos (an organophosphate insecticide) accumulation differences among 10 aquatic insect taxa. In addition, third and fourth instar *Chironomous riparius* differed considerably in their accumulation rates of ^{14}C chlorpyrifos in water-only exposures (Buchwalter et al., 2002). While it is tempting to conclude that insects that rapidly accumulate contaminants from the environment are likely more susceptible than slower accumulators, this is sometimes not the case. The relatively high rate of chlorpyrifos accumulation in *C. riparius* was surprising given their relative tolerance to organophosphate insecticides.

Organophosphate insecticides have been widely studied and their mode of action well characterized. The toxicity of chlorpyrifos is believed to result primarily from metabolic activation of the parent compound to the oxon metabolite. The oxon metabolite deactivates acetylcholinesterase (AChE) at neural junctions, resulting in over-stimulation of the peripheral and central nervous system (Matsumura, 1985). Due to the specific mode of action of this compound, AChE inhibition is a widely used biomarker of exposure and effect. In the studies presented here, AChE activity was used to evaluate target site sensitivity differences among developmental stages of *C. riparius*.

This paper examines the mechanistic basis for chlorpyrifos sensitivity differences in second to fourth instar *C. riparius* larvae. Specifically, it assesses the relative importance of uptake, metabolic processes, and target site sensitivity differences among instars. High exposure concentrations characterized biotransformation processes and sensitivity differences among instars. Low exposure concentrations compared uptake rates among instars. AChE measurements assessed in vitro differences in target site sensitivity.

2. Materials and methods

2.1. Insect culture and handling

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. A Sartorius R-200D-RS analytical balance was used to weigh insects to ±0.05 mg. For the biotransformation studies, larvae days were obtained from Environmental Consulting and Testing, Superior, WI. and held in the lab for acclimation for 3 days. Larvae were 7, 12, and 17 days old when this experiment was conducted.

2.2. High dose sensitivity study

High exposure concentrations assessed the relative chlorpyrifos sensitivities of developing *C. riparius*. Larvae were exposed to a single, extremely high dose of ^{14}C chlorpyrifos. ^{14}C chlorpyrifos (4.26 mg, 25.2 mCi/mmol, >99% radiopurity) was obtained from Dow AgroSciences. 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 1 ml 50:50 acetone water stock solution of ^{14}C chlorpyrifos to yield 0.535 mg/l (1.53×10^{-6} M). Two 1 ml aliquots were taken from each exposure chamber to verify chlorpyrifos concentrations. The average of the two aliquots was taken to be the exposure concentration

for each flask. Exposure chamber concentrations were normalized for analysis.

Three, two and one larva each were placed into exposure chambers for second, third, and fourth instar larvae, respectively, in order to minimize differences in biomass to chlorpyrifos concentration ratios. Five replicates were used per instar. The larvae were checked hourly for the first 12 h of exposure and every 2 h thereafter. Larvae were removed when they were unresponsive to physical stimuli and exhibited no signs of life. Dead larvae 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 and fit to a linear regression model, and exponential curves, respectively. Control treatments consisted of five flasks per instar. Three, two and one larvae were placed into exposure chambers for second, third, and fourth instar larvae, respectively. Exposure media for controls consisted of 50 ml artificial water containing the carrier solution (1 ml of 50:50 acetone:deionized water). There was no control mortality. One fourth instar control larvae molted to its pupal stage during the experiment.

2.3. Biotransformation study

Three replicate exposure chambers containing 250 ml water and 270 ug/l ^{14}C chlorpyrifos were set up for second to fourth instar larvae, respectively. Following 5.5 h of exposure, insects were removed from exposure flasks, weighed, and homogenized in 80:20 acetonitrile:acetone (10 μl/mg tissue) to extract the chlorpyrifos and polar metabolites. Homogenates were centrifuged at 1000 rpm for 5 min, and the supernatant was transferred to a 1.5 ml eppendorf tube. The nine residual pellets were each extracted a second time with acetonitrile:acetone, and the supernatants from the two extractions were pooled. The extracts were then concentrated to a volume of approximately 100 μl. The residual pellets were assayed for radioactivity to assess extraction efficiencies, which ranged from 88 to 95%, and averaged 91.2%. Extracts (50 μl) were loaded onto 5 cm × 20 cm, thin layer chromatography (TLC) plates (Merck #1.05724, Darmstadt, Germany) with a 10 μl syringe. Thin layer chromatography procedures were based on Cheng et al. (1989). The TLC plates were scraped with a razor blade at 1 cm increments from the origin and assayed for radioactivity. This interval was based on the retention factors (r_f) described in Cheng et al. and in preliminary experiments with chlorpyrifos, chlorpyrifos-oxon, and TCP standards (Chem Service, West Chester, PA) run on fluorescent-backed TLC plates (Merck #1.05808, Darmstadt, Germany). This method resolves chlorpyrifos from the oxon and TCP, but does not resolve the metabolites TCP and the oxon from each other. The ratio of radioactivity found at r_f for the parent (0.52) and metabolites (0.19–0.26) was taken as a measure of the relative differences in biotransformation rates among the instars. As this experiment was conducted 12 months after the other experiments described in this paper, the radiopurity of the ^{14}C chlorpyrifos stock was assessed by TLC, and determined to have 7% oxidation products.

2.4. Low dose uptake study

Low concentration exposures were conducted for 7 h to compare relative ^{14}C chlorpyrifos uptake differences among second to fourth instars of *C. riparius*. This 7 h exposure duration was chosen because it is within the linear portion of the uptake curves for this species, while allowing for substantial chlorpyrifos accumulation. Artificial water (described above) was used for all experiments. Fifty milliliter 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} (6.85×10^{-10} M). Two 1 ml aliquots were taken from each exposure chamber to verify chlorpyrifos concentrations. The average of the two aliquots was taken to be the exposure concentration for each flask.

The number of individuals placed in each flask varied with instar to reduce differences in the biomass to chlorpyrifos concentration ratios. Three, two and one larva each were placed into exposure chambers for second, third, and fourth instar larvae, respectively. Average chamber biomasses were 4.5, 5.2, and 6.9 mg for second, third, and fourth instars, respectively. Five replicates were performed for each instar tested. The second instar larvae were pooled for digestion and liquid scintillation counting (LSC). Third

and fourth instar larvae were individually digested for LSC analysis.

Following exposure, insects were removed from exposure flasks, rinsed with water, weighed, and digested in 20 ml scintillation cocktail vials. 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 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 earlier. The average of control [^{14}C] activities was taken to be background for each instar tested and subtracted from the counts of exposure groups.

2.5. Acetylcholinesterase analysis

In vitro AChE assays assessed the target site sensitivity differences among second to fourth larval instars of *C. riparius*. *C. riparius* homogenates were normalized for body weight, total protein content, and basal AChE activity. Acetylcholinesterase analysis followed the method of Ellman et al. (1961), with modifications described by Fisher et al. (2000) specific to *C. riparius*, and Sandahl and Jenkins (2002). 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 determines enzymatic activity by the rate that the substrate, acetylthiocholine (AtChI), is hydrolyzed, producing acetate and thiocholine. The thiocholine, in turn, combines with the chromogen 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), forming a colored product. Change in absorbance at 412 nm was measured at 12 s 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 pesticide chlorpyrifos (tested at 10^{-4} to 10^{-8} M) and the metabolite chlorpyrifos-oxon (tested at 10^{-6} to 10^{-10} M) 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), iso-OMPA (iso-tetramono-iso-propylpyrophosphortetramide), and eserine [1′-methylpyrrolidino (2′:3′:2:3)1,3-dimethylindolin-5-yl *N*-methylcarbamate], considered specific AChE, butyryl-cholinesterase (BuChE), and total cholinesterase inhibitors, respectively, were prepared at 10^{-4} M in deionized H_2O and tested at 10^{-5} M. Controls received blank treatments of deionized H_2O only.

Chironomous riparius larvae were separated by instar, homogenized in ice-cold 0.1 M sodium phosphate buffer (PB), pH 8.0, containing 0.1% Triton X-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 h, because initial studies showed very little response (AChE depression) when homogenates were incubated for up to 3 h. 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 and 3 mM, respectively.

2.6. 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 of varying size. 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 versus body weight explored allometric relationships during development.

3. Results

3.1. High dose sensitivity study

There was a marked increase in time to death as larvae matured. Earlier instars died more quickly than

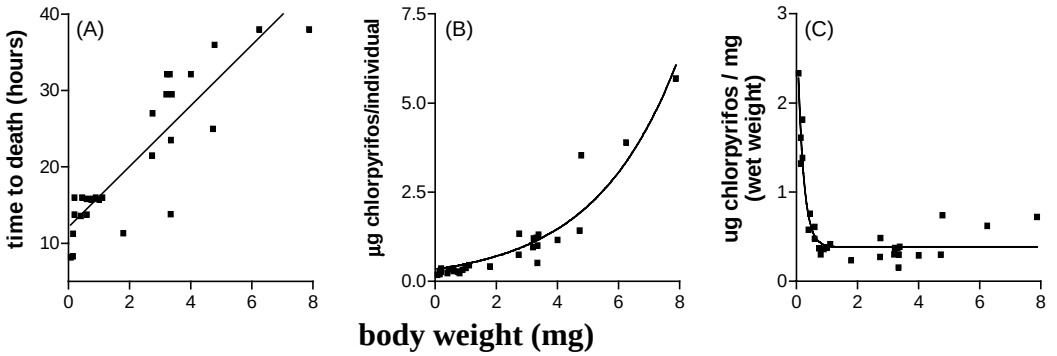

Fig. 1. Time to death (panel A) for *C. riparius* larvae exposed to 0.535 mg/l (1.53×10^{-6} M) ^{14}C chlorpyrifos and the body burdens (panels B and C) associated with lethality.

later instars, although there was considerable variation in survival times among individuals with similar body weights (Fig. 1A). Time to death data were described by a simple linear relationship with a slope of 3.97 ± 0.42 (95% CI = 3.11–4.83, $r^2 = 0.78$). The body burdens associated with lethality fit an exponential curve (Fig. 1B) with the form

$Y = \text{start}\, e^{(kx)}$	($r^2 = 0.91$)
where start $= 0.34 \pm 0.05$	(95% CI = 0.23–0.45)
$k = 0.37 \pm 0.02$	(95% CI = 0.32–0.41)

Body burdens associated with lethality were higher on a per weight basis in earlier instars than later instars (Fig. 1C). A one-phase exponential decay model was fit to these data with the form:

$Y = \text{span}\, e^{(-kx)}$	($r^2 = 0.90$)
where span $= 2.87 \pm 0.3825$	(95% CI = 2.082–3.658)
$k = 5.23 \pm 0.91$	(95% CI = 3.360–7.096)

The signs of chlorpyrifos toxicity were similar across instars, with the onset of signs occurring only a few minutes sooner in earlier instars than later instars. Signs of toxicity included initial agitation, followed by "knock down" period in which all of the larvae remained relatively still on the bottom of the exposure flask. Tremors followed within 1 h of exposure, and continued through time, becoming weaker until there was no discernable movement or response to physical stimuli.

3.2. Biotransformation study

The ratios of metabolites to parent compound were not statistically different between instars (One way ANOVA, $P = 0.55$). The average ratios (±S.E.) from three replicates for second to fourth instars were 0.15± 0.025, 0.23 ± 0.018, and 0.207 ± 0.010, respectively. Unpaired t-tests compared each combination of instars, and were not significantly different in any cases. Signs of chlorpyrifos toxicity were similar to those described above.

3.3. Low dose uptake study

There were significant differences in the accumulation of ^{14}C chlorpyrifos among *C. riparius* instars 2–4 after 7 h of exposure (one-way ANOVA, $P < 0.01$) (Fig. 2). Second instar *C. riparius* accumulated approximately 1.5, and 2.4 times more chlorpyrifos on a per weight basis than third and fourth instars, respectively. These results were consistent with prior time course studies where differences in the accumulation rates of third and fourth instar *C. riparius* were observed (Buchwalter et al., 2002). No signs of chlorpyrifos toxicity were observed in these studies.

3.4. Acetylcholinesterase analysis

Esterase specific inhibitors assessed the relative activities of AChE, BuChE, and total esterase in homogenates of fourth instar larvae. Iso-OMPA, a BuChE specific inhibitor, did not reduce

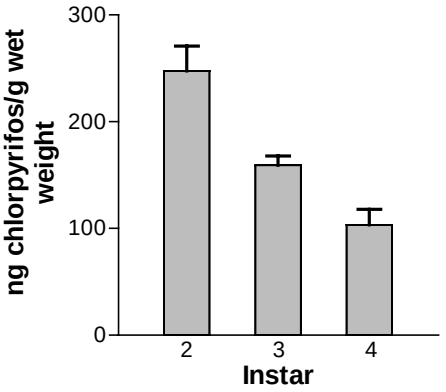

Fig. 2. Chlorpyrifos accumulation in second to fourth instar *C. riparius* larvae. Larvae were exposed for 7 h to ^{14}C chlorpyrifos and 240 ng/l (6.85×10^{-10} M). Average body weights for instars second to fourth were 1.5, 2.9, and 7.1 mg, respectively. Error bars represent the standard errors of the means.

cholinesterase activity. as compared to control treatments. In contrast, BW, an AChE specific inhibitor, resulted in a significant depression of 87% (one-way ANOVA, Bonferroni multiple comparison; $P < 0.001$). Eserine, a total cholinesterase inhibitor resulted in a similar degree of depression as BW (88% reduction, $P < 0.001$), suggesting that AChE is the predominant esterase in *C. riparius*.

In vitro AChE assays examined basal AChE activities and AChE sensitivities of second to fourth instar larvae to both chlorpyrifos and chlorpyrifos-oxon. 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, $P < 0.01$). Basal activities for second, third and fourth instars were 395.0±5.4, 321.3±31.9, and 1051.0±57.9 nmol AtChI hydrolyzed/min/g, respectively (Fig. 3). Total protein contents were similar among developmental stages.

Since basal AChE activities varied with developmental stage, but total protein content by weight did not, protein normalized AChE assays did not adequately address target site sensitivity differences among instars. The ratios of substrate (the parent compound and the oxon metabolite) to AChE were dissimilar within the wells for each instar. To characterize

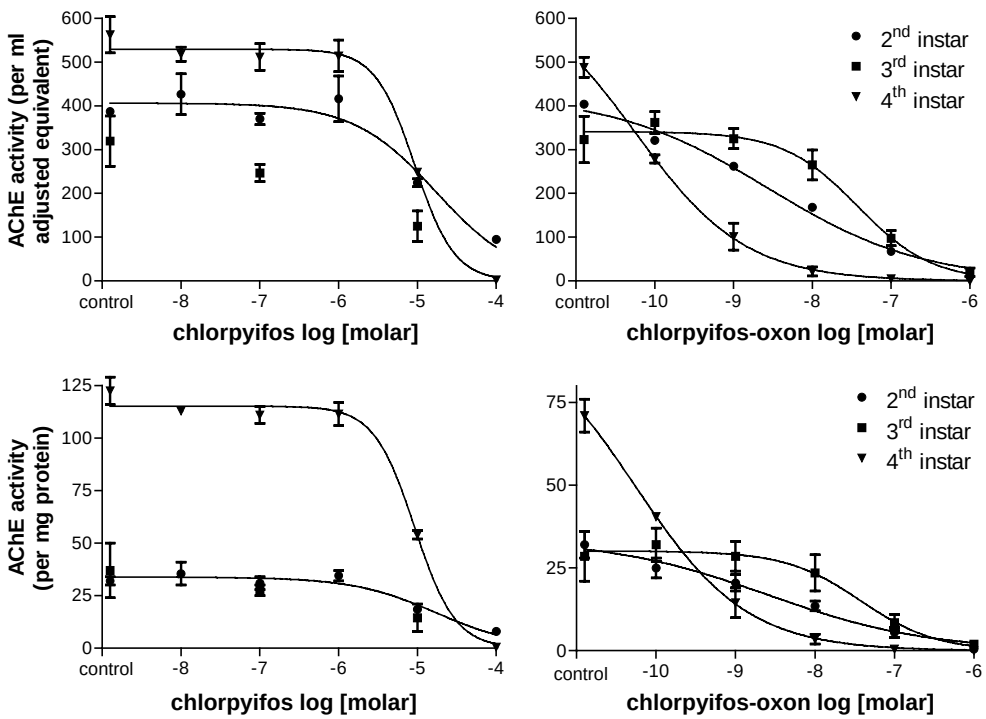

Fig. 3. In vitro AChE activity associated with exposure to chlorpyrifos and chlorpyrifos-oxon in *C. riparius* homogenates derived from second to fourth instar larvae.

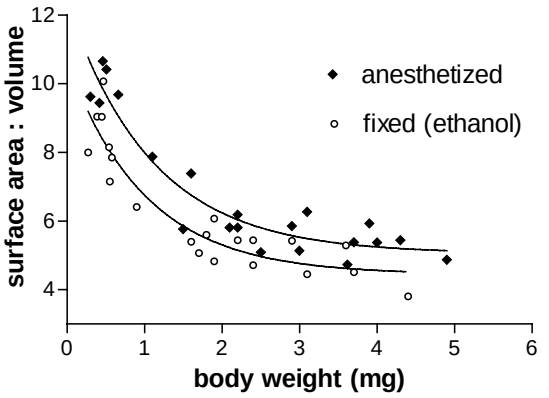

Fig. 4. Surface area vs. body weight in ethanol-fixed and CO_2 anesthetized *C. riparius* larvae. The exponential curve for anesthetized larvae is described as follows: surface area, volume $= 1.479\,e^{(-0.91\text{weight})} + 0.3692$. This curve has an $r^2 = 0.95$.

differences in target site sensitivity, homogenates were diluted such that basal AChE activities were similar in second to fourth instar-derived homogenates. The intent was for each well to hold similar AChE concentrations, such that concentration-dependent changes in activity could be directly compared among developmental stages. There was no age-specific trend in AChE depression associated with exposure to either chlorpyrifos-oxon or chlorpyrifos (Fig. 3).

3.5. Allometry

Data for both ethanol-fixed and carbon dioxide anesthetized larvae fit similarly shaped exponential curves (Fig. 4). Fixed larvae were slightly lighter than anesthetized larvae. While *C. riparius* larvae were not exactly cylindrically shaped these data were useful for estimating differences in surface area to volume relationships during development. Since chlorpyrifos is a hydrophobic compound (Koc = 31,000) (USEPA, 2000) it was expected to sorb onto the body surface of the larvae, making surface area to volume relationships important determinants of differential chlorpyrifos accumulation among developmental stages.

4. Discussion

Biological characteristics that determine the differential sensitivity of aquatic organisms to contaminants 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. Toxicologists have observed marked differences in the sensitivities of aquatic insect species and developmental stages. 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 body sizes and integument characteristics of aquatic insects result in potential differences in contaminant uptake rates. Simultaneously, differences in metabolic processes and target site sensitivities are likely to exist across species and developmental stages. This work investigated chlorpyrifos sensitivity differences in developing *C. riparius* by examining accumulation, biotransformation, and target site sensitivity differences among instars.

Time to death (Fig. 1A) and associated lethal body burdens after high concentration exposures (Fig. 1B) reveal a marked decrease in chlorpyrifos sensitivity with increasing age in *C. riparius* larvae. Size and age specific sensitivity differences have been observed in aquatic insects by several researchers including McCahon et al. (1989), Kiffney and Clements (1994, 1996) and Stuijfzand et al. (2000). While this trend of decreased sensitivity with age and body size seems to be relatively consistent, the mechanistic basis for age/size related sensitivity differences to different contaminants in aquatic insects remains poorly understood. Lethal body burdens were higher on a per weight basis in earlier instars than in later instars (Fig. 1C). While this may seem counter-intuitive, AChE data help provide a mechanistic explanation.

Chlorpyrifos metabolism rates were not significantly different among instars. This study could not quantitatively assess rate differences in biotransformation to the oxon among instars because TLC methods could not resolve chlorpyrifos oxon and TCP. Also, the presence of 7% oxidation products in the chlorpyrifos stock during this experiment precludes the direct measurement of conversion to the oxon. However, the ratios of metabolic products to parent

compound among instars clearly demonstrate that the increased sensitivity of earlier instars is not related to chlorpyrifos metabolism differences. Second instar larvae had the smallest ratios of metabolites to parent compound, but differences were not statistically significant. This smaller ratio could simply result from an increased uptake rate of the parent compound and saturation of metabolism.

Consistent with previous work with ^{14}C chlorpyrifos uptake rates in third and fourth instar *C. riparius* (Buchwalter et al., 2002), we observed marked differences in chlorpyrifos accumulation in second to fourth instar *C. riparius* in these studies (Fig. 2). Uptake rate differences were partially explained by differences in surface area to volume relationships among instars (Fig. 4). For example, in the low concentration exposures, total surface areas in exposure chambers for second instar larvae were, on average, approximately 1.1- and 1.7-fold higher than third and fourth instar larvae, respectively. The chlorpyrifos uptake rates were 1.5- and 2.3-fold higher in second instar larvae than third and fourth instar larvae, respectively, on a per-weight basis. Conceivably, the thickness of the cuticle may vary between developmental stages, as differences in the water permeability of third and fourth instar larvae have been observed (Buchwalter et al., 2002).

In vitro AChE assays revealed that homogenates from *C. riparius* larvae were refractive to chlorpyrifos, even at high concentrations. In contrast, homogenates from *C. riparius* larvae were inhibited by chlorpyrifos-oxon in a concentration-dependent manner (Fig. 3). Basal AChE activity was significantly higher in fourth instar larvae than in second or third 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 concentration-dependent decreases in AChE activities, fourth instar homogenates were more sensitive than earlier instars. This at least partially explained why body burdens associated with lethality were higher on a per weight basis in earlier instars. Different isoforms of AChE may exist over the course of development in *C. riparius*, but further examination of this was beyond the scope of these experiments.

Based on these results, we concluded that the relative chlorpyrifos sensitivity differences among *C. riparius* larval instars were primarily due to accumulation rate differences, and basal AChE activities. Accumulation rate differences were largely driven by surface area to volume ratios 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).

It is clear that developmental stages of aquatic insects can vary tremendously in their sensitivities to environmental stressors. For this reason, toxicity bioassays with insects should, if possible, focus on earlier life stages. In attempting to use the resident aquatic insect fauna to make inferences about water quality, it may be appropriate to consider developmental stages, body sizes and other life history attributes of insect communities.

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