Title

Evaluating toxicokinetic responses in two lepidopteran species exposed to a pyrethroid insecticide.

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Abstract

Soybean loopers (*Chrysodeixis includens*) and corn earworms (*Helicoverpa zea*) are moth pests of soybean and corn fields across the United States, including Missouri. In the previous year, we assessed their toxic and metabolic responses to cypermethrin, a pyrethroid insecticide registered for foliar use in agriculture. We found that soybean loopers were more susceptible to cypermethrin and, in both species, cytochrome P450 and esterase enzymes played a critical role in cypermethrin breakdown.

To help explain species differences in toxicity, we undertook in vitro studies to better understand each species' rates of metabolism and excretion. In the first study, we dosed caterpillars of both species with cypermethrin and analyzed cypermethrin concentrations in the body over time. This analysis was done through gas chromatography-mass spectrometry, and we generated standard curves using control larvae. To determine the activity of the critical metabolizing enzymes and their potential to break down cypermethrin, we conducted several in enzymatic assays including, bicinchoninic acid (BCA), mixed function oxidase (MFO), esterase (EST), and glutathione s-transferase (GST).

Introduction

A common topic amidst current events has been the conversation surrounding pest control on U.S. agricultural lands. Without supervision or interference, insect pest species can devastate ecosystems, which results in both crop and biodiversity loss and negatively impacts the livelihoods of farmers. Thus, it is imperative to manage native and non-native insect pests. One of the most popular forms of regulation is insecticide usage, but its widespread use can negatively impact human health and the environment. Additionally, many insect species have become resistant to insecticides. Resistance can occur due to several reasons, including indiscriminate use of insecticides or application of incorrect insecticide concentrations [1]. For example, if an insecticide is not toxic enough to kill a large population of a pest species, it is possible for the species to adapt and evolve resistance. This could make it difficult to manage its population in the future. It is therefore important to ensure the insecticides being applied at the label rate are effective in killing a large percentage of the pest population.

In the U.S., a variety of insecticides are being employed to manage insect pest populations. One of the insecticide classes is pyrethroids, which prevents the closure of voltage-gated sodium channels in the neurons. This results in the mass depolarization of neurons, which can lead to paralysis and/or death [2]. Pyrethroids are commonly employed against pest species such as soybean loopers (*Chrysodeixis includens*) and corn earworms (*Helicoverpa zea*). Both species are moths that belong to the Noctuidae family of Lepidoptera and, as the names suggests, are common pests of soybean and corn plants, respectively. Both species are found in

Missouri and can be managed by foliar applications of cypermethrin, a commonly used pyrethroid, However, as observed in a few literature studies and from our own experiments last year, the two species show different susceptibilities to cypermethrin. Soybean loopers are significantly more susceptible to cypermethrin, i.e., at the same cypermethrin doses, greater mortality was observed in soybean loopers compared to corn earworms. However, in the field, corn earworms are exposed to greater concentrations of cypermethrin as they are larger. Predictably, when we undertook risk assessment (i.e., compared the toxicity and exposure data), we found that labelled rates of cypermethrin were effective in controlling both species, despite their significant differences in toxicity. However, it is unclear why the two species show differences in susceptibility when they are closely related on a phylogenetic scale. This difference in susceptibility holds true even when we account for the body weight differences between the two species.

Consequently, the overall goal of our project is to elucidate the differences in cypermethrin toxicity between soybean loopers and corn earworms. Specifically, we wanted to determine the metabolic enzymes that contribute to differences in toxicity and evaluate if corn earworms metabolized and excreted cypermethrin quicker, thus making them less susceptible. To accomplish this, we undertook *in vivo* and *in vitro* enzymatic studies. In the *in vivo* studies, we treated larvae of both species with three inhibitors, PBO (piperonyl butoxide), TPP (triphenyl phosphate), and DEM (diethyl maleate), which inhibited the major metabolizing enzymes, cytochrome P450s, esterases, and glutathione-S-transferases, respectively [3]. The results were presented in last year's conference: we found that in both species, cytochrome P450s and esterases played a critical role in metabolizing cypermethrin. This year, we undertook *in vitro* studies where we measured the levels of the three metabolizing enzymes in the two species using the following assays: bicinchoninic acid (BCA), mixed function oxidase (MFO), esterase (EST), and glutathione s-transferase (GST). Finally, we dosed larvae of both species with cypermethrin and analyzed cypermethrin concentrations in their body over time. This analysis was done through gas chromatography-mass spectrometry (GC-MS), and standard curves were generated using control larvae.

The findings from our research will be incorporated into a larger project with the goal of identifying surrogate (pest and non-target) species for testing and elucidating differences in susceptibilities across and within insect orders. This is necessary to accurately estimate toxic responses in insect species that cannot be tested.

Materials and Methods

Corn earworm and soybean looper eggs were purchased from Benzon Research, Carlisle, PA. Once the eggs hatched, the larvae were plated on an artificial diet (Stonefly *Heliothis* Diet, Ward's Science) and were reared in an incubator maintained at 26 °C and 14:10 light: dark cycle. analytical grade cypermethrin (mixture of isomers; IUPAC name: [cyano-(3-phenoxyphenyl) methyl] 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate; CAS number: 52315-07-8; percentage purity: 99.8%) was purchased from Sigma-Aldrich, St. Louis, MO. Certified ACS reagent grade acetone was purchased from Fisher Scientific (Hampton, NH).

Larval Homogenate for Enzymatic Assays

The *in vitro* enzymatic assays required us to prepare larval homogenates. Sixth-instar larvae from both species were starved for two

hours. During this time, a 10 mL phosphate-buffered saline (PBS) solution containing 80 µL ethylenediaminetetraacetic acid (EDTA) and 80 µL phenylmethylsulfonyl fluoride (PMSF) was prepared. EDTA is an aminopolycarboxylic acid that binds to metal ions and prevents clotting, while the PMSF is a common serine protease inhibitor. The solution was kept on ice. Following the two-hour starvation period, we added 5 mL of the freshly prepared chilled-PBS buffer solution to a 30 mL tube and placed three (corn earworms) or five (soybean loopers) larvae in the tube. Using an electrical drill, we crushed the larvae until it was completely homogenized. The tubes were then centrifuged in ice-cold conditions for 4 hours and the resulting pellets were discarded. If the solutions remained cloudy, the centrifugation step was repeated until we obtained a clear supernatant. The supernatants were utilized over a period of two months; between experiments, they were stored in the freezer and on ice.

Bicinchoninic Acid (BCA) Assay

This assay was conducted to estimate protein concentrations in the larval homogenates [4]. Proteins in the homogenate reduce Cu^{+2} to Cu^{+1} in an alkaline medium. Chelation of two molecules of bicinchoninic acid with one molecule of Cu^{+1} produces a purple color reaction product.

The stock solution consisted of 10 mg/mL bovine serum albumin (BSA). It was diluted to lower concentrations (up to 0.5 mg/mL) using ice-cold PBS buffer containing EDTA and PMSF (the same buffer that was used to homogenize the larvae). The BCA working reagent was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B. We then pipetted 25 µL of the BSA standard samples or 25 µL of the insect homogenates (prepared previously) into a 96-well plate. Then 200 µL of the working reagent was added to each well. Each sample had three replicates. The well components were mixed by gentle shaking of the plate for 30 seconds. The plate was then covered and incubated at 37^0C for 30 minutes. Following cool down to room temperature, a spectrophotometer was utilized to measure the absorbance of the samples at 562 nm.

Mixed Function Oxidase (MFO) Assay

This assay was used to estimate cytochrome P540 levels in the insect homogenate [4]. Peroxidation of tetramethylbenzidine (TMBZ) is catalyzed by microsomal proteins with hydrogen peroxide as co-substrate. This assay does not measure the monooxygenase activity but measures heme content in the insect homogenate, majority of which are associated with cytochrome P450s. Since heme is present in the active site of monooxygenases, major changes in the quantity of (available) monooxygenases produces measurable changes in heme.

First, we prepared the following solutions: 50 mM PBO, 0.625 M potassium phosphate buffer, 0.05% TMBZ dissolved in methanol and 0.25 M sodium acetate buffer, and 3% H_2O_2. Then, to the microplate wells, we included the following: two control groups, one consisting of 20 µL of the buffer solution and acetone and the other consisting of 20 µL of the buffer solution and PBO, and two experimental groups, where we replaced the buffer with 20 µL of the insect homogenate and provided half with PBO and the other half with acetone. We had six replicates for each control and experimental group. The microplate was incubated for 30 minutes and 40 µL of potassium phosphate buffer, 100 µL of TMBZ stock, and 12.5 µL H_2O_2 stock were added. Following a one-hour incubation at room temperature, the absorbance of the

samples was measured with the help of a spectrophotometer at 650 nm.

Esterase (EST) Assay

This assay was used to estimate esterase enzyme levels in the insect homogenate [4]. Esterases split simple esters in biological systems and such activity can be estimated in terms of the product formed by using various substrates. Here, α-naphthyl acetate is used as a substrate and the formation of α-naphthol is monitored. α-naphthol couples with Fast Blue salt to produce a diazo dye complex. Esterase activity is measured by monitoring absorbance changes due to formation of diazo dye complex.

First, we prepared the following solutions: 50 mM TPP, 0.3M α-naphthol in acetone, and 5 mg of Fast Blue dissolved in 5mL of 0.4M phosphate buffer. Then, to the microplate wells, we included the following: two control groups, one consisting of 20 µL of the buffer solution and acetone and the other consisting of 20 µL of the buffer solution and TPP, and two experimental groups, where we replaced the buffer with 20 µL of the insect homogenate and provided half with TPP and the other half with acetone. We had six replicates for each control and experimental group. The microplate was incubated for 30 minutes at room temperature. Then, 135 µL of the prepared α-naphthol stock was added to each well, and the plate was incubated for 30 minutes at 37^0C. Following that, 50 µL of freshly prepared fast blue was added. This was followed by a 15-minute incubation at room temperature to allow color development (i.e., for the chemical reaction to occur). The change in absorbance was measured on the spectrophotometer at 600 nm.

Glutathione S-Transferase (GST) Assay

This assay was used to estimate GST enzyme levels in the insect homogenate [4]. The glutathione transferases catalyze the conjunction of 2,4-dinitrochlorobenzene (CDNB) with reduced glutathione (GSH) to produce a yellow-colored product.

As with the earlier assays, the solutions were prepared first. This included 50 mM DEM, 10 mM glutathione (8.1 mg GSH in 2.5 ml 0.1 M phosphate buffer (pH 6.5)), 63 mM CDNB (128 mg of CDNB in 10 ml methanol), and a working solution of 125 µL of CDNB added to 2.5 ml GSH solution. Then, to the microplate wells, we included the following: two control groups, one consisting of 20 µL of the buffer solution and acetone and the other consisting of 20 µL of the buffer solution and DEM, and two experimental groups, where we replaced the buffer with 20 µL of the insect homogenate and provided half with DEM and the other half with acetone. We had six replicates for each control and experimental group. The microplate was incubated for 30 minutes and 200 µL of the working solution was added. This was followed by a 20-minute incubation at room temperature to allow color development (i.e., for the chemical reaction to occur). The change in absorbance was measured on the spectrophotometer at 340 nm.

GC-MS Larval Extraction

The GC-MS is an analytical method that combines the features of gas-chromatography and mass spectrometry to identify different substances and different concentrations within a test sample. We employed GC-MS to measure cypermethrin

concentrations over time in both larval species. We also measured cypermethrin concentrations within a stock solution.

First, we treated both corn earworms and soybean loopers with 5 ng/µL cypermethrin. The larvae were collected at the following time points after treatment: 0h, 6h, 24h, and after pupation. Five larvae were collected at each time point. We also collected over a dozen untreated (control) larvae for each species. All collected larvae were drowned in liquid nitrogen before being homogenized with a mortar and pestle lined with aluminum foil. A 0.2 g of each sample was extracted with 2 mL of acetonitrile, before being vortexed for 30 seconds and centrifuged for 5 minutes at 3000 rpm to remove solid particles. We then cleaned the liquid extract by transferring 1.5 mL to a dispersive solid phase extraction (dSPE) tube, after which the sample was vortexed for 1 minute and centrifuged at 6000 rpm. One mL of the supernatant was transferred to a vial with 100 µL of ethyl acetate. Then 500 µL of this solution was transferred to an autosampler vial for GC-MS analysis.

For our experiment, a total of 79 larval samples were prepared. For both species, standard curves were prepared with untreated larvae. This involved spiking 0.2 g of the homogenized insect with 0.5, 1, 2.5, 5, 10, 25, and 50 ng/µL of cypermethrin. Blank samples, which contained no cypermethrin, were also prepared. Quality control (QC) larval samples were prepared with 5, 10, and 25 ng/µL of newly made cypermethrin stocks. Percent recovery of cypermethrin was determined by comparing two spiking methods, pre- and post-spike. Pre-spiking consisted of spiking one concentration from the standard curve on blank homogenized larval tissues and doing extractions. Post-spiking consisted of spiking the pre-spiking concentration on the blank extraction solvent just prior to GC-MS injection.

Additionally, we prepared standard curves using the following cypermethrin-acetone solution concentrations: 1, 2.5, 5, 10, 25, 50, 100, and 250 ng/µL. We used the standard curve to estimate the cypermethrin concentration within our stock solution, which had a nominal concentration of 50 ng/µL. To all solution vials, 100 µL of ethyl acetate was added prior to transfer to autosampler vials and GC-MS analysis.

Results

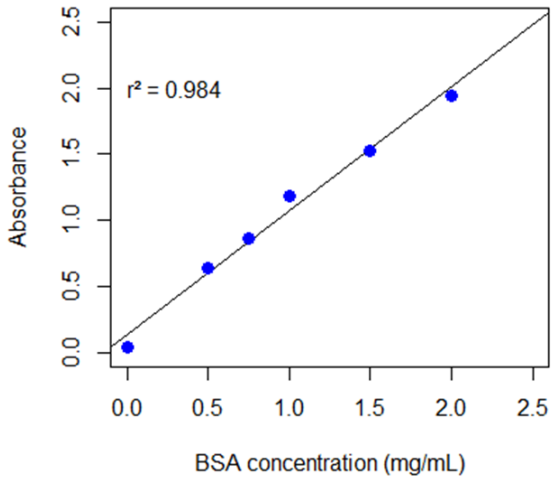

Figure 1: Standard curve obtained from BCA assay.

The standard curve prepared using BSA showed a very high R-squared (or coefficient of determination) value of 0.984 (Figure 1). By comparing the standard curve absorbance values with our larval homogenate absorbance values, we determined that the protein concentration within corn earworm was 1.4 mg/mL and the protein concentration within soybean looper was 2.0 mg/mL.

An *in vitro* assessment of enzyme levels found that in both corn earworms and soybean loopers, PBO significantly inhibited P450 levels ($p < 0.01$ and $p < 0.001$, respectively) while DEM did not

significantly inhibit GST levels ($p > 0.05$; Figure 2). Additionally, TPP slightly inhibited esterase levels in corn earworms ($p < 0.05$) but not soybean loopers ($p > 0.05$). In these *in vitro* analyses, we did not obtain the enzyme concentrations but rather used absorbance as a measure of concentration or level of enzyme.

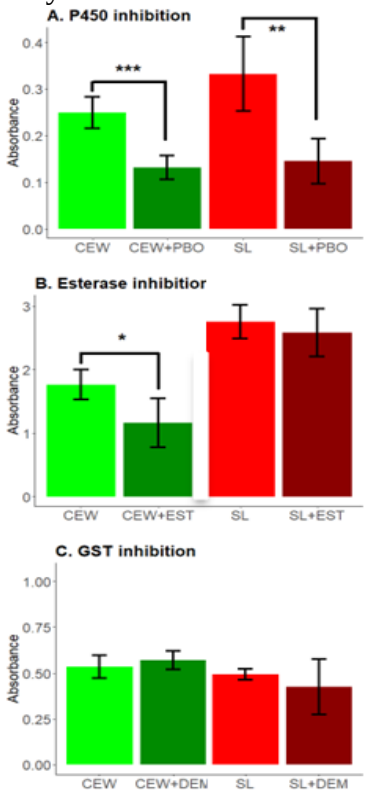

Figure 2: Metabolizing enzyme levels in both species in the presence and absence of inhibitors

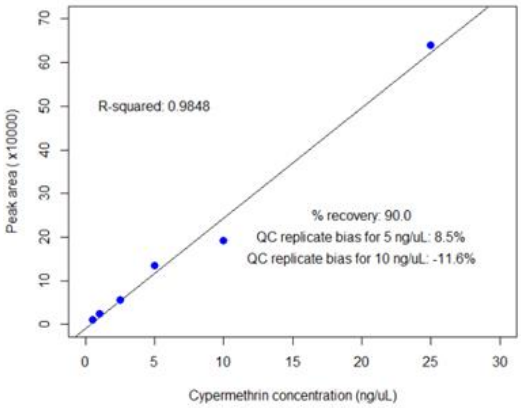

Figure 3: GC-MS standard curve for soybean loopers

With our GC-MS analysis, we successfully obtained cypermethrin standard curves for soybean loopers (Figure 3) and corn earworms (Figure 4). Both curves had high R-squared values of 0.985 and 0.999, respectively. The cypermethrin percent recovery was also high; 90% and 82%, respectively. The QC replicate bias was generally low (9 to 12% for soybean loopers and 5 to 21% for corn earworms).

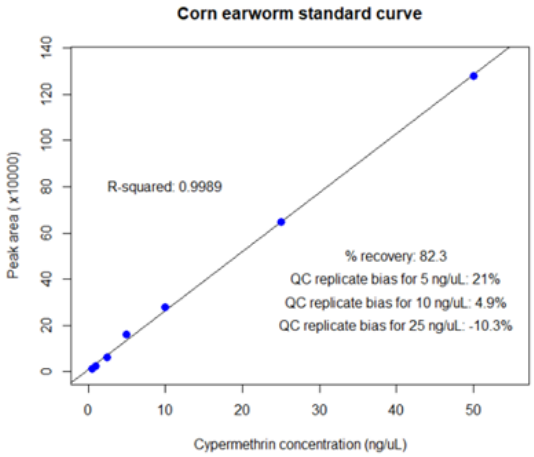

Figure 4: GC-MS standard curve for corn earworms

However, our analysis of cypermethrin-treated corn earworms and soybean loopers did not produce peak areas that were distinguishable from the background peaks. Therefore, we could not estimate the concentration of cypermethrin within the larval bodies over time.

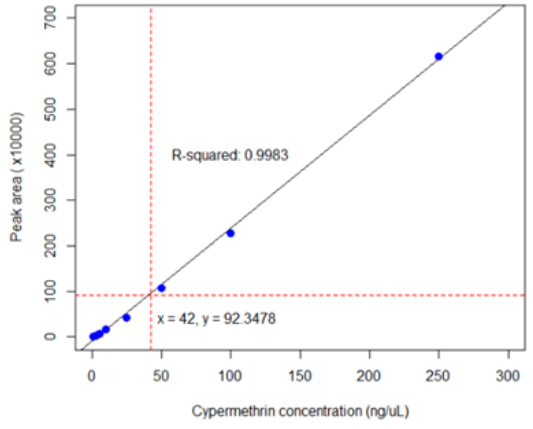

Figure 5: GC-MS standard curve for solutions

The standard curve generated for cypermethrin solutions also produced a high R-squared value (0.998). A comparison of the nominal 50 ng/µL stock solution with the standard curve indicated that the measured concentration of the stock solution (that was used in larval spiking) was 42 ng/µL.

Discussion

There are two factors that are responsible for species differences in chemical susceptibility: toxicokinetics and toxicodynamics. Toxicokinetics refers to the absorption, metabolism, and excretion of a chemical while toxicodynamics refers to the chemical binding site, usually a protein receptor or enzyme. In phylogenetically related species, we expect to see greater differences in toxicokinetics rather than toxicodynamics as receptor sequences are unlikely to change significantly between closely evolved species. Therefore, our study assessed toxicokinetic factors between two closely related species: corn earworms and soybean loopers.

As our previous results had shown that soybean loopers were more susceptible to cypermethrin, we undertook three kinds of toxicokinetic studies to elucidate the differences in susceptibility between soybean loopers and corn earworms. The results from the first study were presented in last year's conference: an *in vivo* bioassay with three enzyme inhibitors, PBO, TPP, and DEM, indicated that both PBO and TPP were effective in reducing the activity/levels of cytochrome P450s and esterases, respectively, as the larvae treated with the inhibitors showed greater susceptibility to cypermethrin. This was seen in both corn earworms and soybean loopers. This was encouraging as insecticides are often added with enzyme inhibitors to increase larval mortality in the field [5]. Also, GST (inhibited by DEM) is not very effective in breaking down cypermethrin [6], and our results unsurprisingly showed DEM had little effect on larval mortality.

In vivo results, however, have several shortcomings. For one, it might be difficult for the inhibitors to penetrate larval cuticle and enter the body. Also, both cypermethrin and inhibitors could be bound to other factors within the body, i.e., they might not be freely available to exert their actions. Therefore, we undertook *in vitro* assays to determine the true nature of interaction between the inhibitors and metabolizing enzymes in the two moth species.

Our *in vitro* assays indicated that, in both species, PBO was indeed effective in inhibiting P450s while DEM was ineffective in inhibiting GSTs. So even though GSTs don't play a big role in cypermethrin breakdown, our results indicate DEM can be an ineffective inhibitor of GST. Therefore, other GST inhibitors [7] should be explored for lepidopteran enzyme inhibition assays. Our results also showed that TPP was not an effective inhibitor of soybean looper esterases; however, it was effective in inhibiting corn earworm esterases. It is unclear why the esterase results for soybean loopers differed across *in vitro* and *in vivo* settings. Finally, our protein estimation assay, BCA, indicated that soybean loopers had approximately 40% more total proteins compared to corn earworms. This might explain why we generally obtained greater absorbance readings in soybean loopers (Figure 2).

We undertook GC-MS to see if corn earworms could metabolize and excrete cypermethrin quicker than soybean loopers as this might help explain why corn earworms were less susceptible to cypermethrin. While we obtained good GC-

MS standard curves for soybean loopers and corn earworms, we did not find detectable cypermethrin concentrations in the treated larvae of both species. In fact, detectable concentrations were not seen at any of the time points, including 0 hours, which is when we collected and froze the larvae following cypermethrin treatment. This would have prevented any cypermethrin metabolism.

We think our experimental setup could have been responsible for non-detects of cypermethrin in the treated larvae. Initially, we dipped the treated larvae in liquid nitrogen and then tried to crush it. However, this produced a sludge as the larvae did not completely freeze. We then poured the liquid nitrogen in the mortar and tried crushing the larvae within it. However, this resulted in the mortar breaking. Our third method proved successful. We got dry ice, placed the mortar and pestle on it for several minutes and then poured liquid nitrogen into the mortar. Larvae were then immediately added to the liquid nitrogen and crushed with the pestle. This method produced a finely crushed larval powder, which is needed for GC-MS analysis. However, the trial and error along the way had resulted in the treated larvae being outside for significant periods of time. This could have resulted in degradation of cypermethrin within the larval body. This was not an issue for the development of the standard curves as the larval samples were spiked with cypermethrin only after the samples were ground into a fine powder.

Finally, we analyzed the concentration of the cypermethrin stock solution that was used to spike all larval samples. A standard curve was prepared with a range of cypermethrin solutions, and our stock solution peak area was compared with the curve's peak areas. The measured concentration was found to be 84% of the nominal concentration. This is within an acceptable range (generally, nominal concentrations should be within 20% of measured concentrations).

Conclusion

Soybean loopers and corn earworms belong to the same order and family of insects. However, we found that they exhibited significant differences in susceptibility to cypermethrin, a commonly used insecticide. While labelled rates of cypermethrin can kill a large percentage of both species in the field, it is unknown why the two species differ in susceptibility. We undertook three toxicokinetic experiments to elucidate this difference. Our *in vivo* assay showed that, in both species, cytochrome P450 and esterase enzymes, the two major enzymes responsible for cypermethrin breakdown, can be successfully inhibited. Our *in vitro* assay showed that cytochrome P450 and esterase enzymes can be successfully inhibited in corn earworms; however, in soybean loopers, we could only inhibit cytochrome P450 enzymes. While it is unclear why we see an *in vitro* and *in vivo* difference with soybean loopers, our results indicate that in both species, cytochrome P450s are critical in breaking down cypermethrin and reducing its toxic effects. The addition of an inhibitor like PBO could make larvae of both species more susceptible to cypermethrin. We then undertook GC-MS analysis to see if the two species had different rates of cypermethrin metabolism and excretion. However, we could not detect cypermethrin concentrations in the treated larvae and were therefore unable to draw a conclusion on the rate of cypermethrin degradation in the two species.

Acknowledgement

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