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Sublethal Effects of Insecticide Exposure on *Megacopta cribraria* (Fabricius) Nymphs: Key Biological Traits and Acetylcholinesterase Activity

Jin Miao,[1,2] Dominic D. Reisig,[2,3] Guoping Li,[1,2] and Yuqing Wu[1]

[1]Institute of Plant Protection, Henan Academy of Agricultural Sciences, Zhengzhou 450002, P.R. China (miaojin1977@163.com), [2]Department of Entomology, North Carolina State University, The Vernon James Research & Extension Center, 207 Research Station Road, Plymouth, NC 27962 and [3]Corresponding author, e-mail: ddreisig@ncsu.edu

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Abstract

Megacopta cribraria F. (Hemiptera: Plataspidae), the kudzu bug, is an invasive insect pest of U.S. soybean. At present, insecticide application is the primary and most effective control option for *M. cribraria*. In this study, the potential effects of sublethal and low-lethal concentrations (LC_{10} and LC_{40}) of three common insecticides on key biological traits and acetylcholinesterase (AChE) activity of the treated nymphal stage of insect were assessed. The results show that the sublethal concentration of imidacloprid significantly reduced adult emergence rate of *M. cribraria*. A low-lethal concentration of imidacloprid significantly increased nymphal development time, but significantly decreased adult emergence rate and adult longevity. Both sublethal and low-lethal concentrations of acephate caused an increase in nymphal development time and a reduction in adult emergence rate and adult longevity. Fecundity of females was significantly reduced only by exposure to low-lethal concentrations of acephate. Sublethal and low-lethal concentrations of bifenthrin increased nymphal development time, but significantly decreased adult emergence rate. In addition, we found that the AChE activity of *M. cribraria* was significantly increased only by LC_{40} imidacloprid, but strongly inhibited by acephate.

Key words: insecticide, nymphal developmental time, longevity, hormesis, fecundity

Megacopta cribraria F. (Hemiptera: Plataspidae), commonly referred to as kudzu bug, is a piercing–sucking insect that likely feeds on phloem in stems and foliage, intercepting nutrients and moisture from leguminous plants such as soybean, *Glycine max* Merrill and kudzu, *Pueraria montana* Loureiro (Zhang et al. 2012). *Megacopta cribraria* is known to be a pest of soybeans in its native range, with yield losses ranging from 10 to 50% in central and southern China (Xing et al. 2006, Eger et al. 2010). Although *M. cribraria* is native to Asia (Hosokawa et al. 2007, Eger et al. 2010), aggregations of this insect were discovered on kudzu in northeast Georgia during October 2009 (Suiter et al. 2010). This pest has spread quickly in the southeast and midsouth United States (Del Pozo-Valdivia and Reisig 2013); the infested acreage of soybeans in Virginia, North Carolina, Tennessee, and Alabama increased 24-fold, from 15,000 acres in 2011 to 366,600 acres in 2012 (Seiter et al. 2013). Two generations per year of this pest occur in the southeastern U.S. states (Eger et al. 2010, Suiter et al. 2010, Zhang et al. 2012, Gardner et al. 2013). In the spring, the overwintered adults fly to wild-legume hosts such as kudzu and wisteria to feed, mate, and oviposit. The first-generation nymphs develop on kudzu or early-planted soybean during May and June, and the first field-generation adults can re-invade later-planted soybeans during July (Zhang et al. 2012, Del Pozo-Valdivia and Reisig 2013, Seiter et al. 2013).

At present, insecticide application is the primary and most effective control option for *M. cribraria* in soybean fields. In China, up to 85% of *M. cribraria* in soybeans are controlled with pyrethroid and organophosphate class insecticides (Wang et al. 2004, Zhang and Yu 2005). Insecticide efficacy trials conducted during 2010–2012 in Georgia, South Carolina, and North Carolina have shown that several pyrethroids, organophosphates, and neonicotinoids provide excellent, but not total control. For example, many of these insecticides provided >90% control, defined as mortality, 6–14 days after being applied, alone or in tank-mixes (Greene, Reisig, and Roberts, unpublished data). Insects that survive exposure to insecticides can be behaviorally or physiologically modified, as a result (Desneux et al. 2007). The specific sublethal effects can include changes in life span, developmental rates, fecundity, changes in behavior, and changes in enzymatic activity (Stark and Banks 2003, Desneux et al. 2007, Miao et al. 2014).

The enzyme acetylcholinesterase (AChE, EC 3.1.1.7) hydrolyzes the neurotransmitter acetylcholine (Ach) to generate post-synaptic potentials. Within the field of ecotoxicology, AChE is used as a biochemical marker, since AChE is the primary target of inhibition by organophosphate and carbamate pesticides. Insects can be killed by these pesticides, because when AChE is inhibited, the post-synaptic neuron will continue to be stimulated. Studies using aquatic organisms have tested the inhibition of AChE activity by various toxicants and have found changes in behavior, feeding rate, and emergence of larvae (Domingues et al. 2007).

Our main objective of this study was to assess the sublethal effect of a single insecticide in each of three insecticide classes, a neonicotinoid (imidacloprid), a pyrethroid (bifenthrin), and an organophosphate (acephate), on *M. cribraria* nymphs. Biological traits and AChE activity of *M. cribraria* were observed under laboratory conditions. The results of this study can inform growers concerning the most effective products to control *M. cribraria*.

Materials and Methods

Insecticides. Commercial formulations were tested of the following insecticides registered for the use on soybean in the southeastern United States: imidacloprid (Admire Pro 42WPS, Bayer CropScience, Research Triangle Park, NC), bifenthrin (Brigade 25 EC, FMC Corporation, Philadelphia, PA), and acephate (Orthene 97WT, AMVAC Chemical Corporation, Los Angeles, CA). Distilled water was used as untreated control in all treatments.

Insects and experimental conditions. Soybean seedlings were grown in 5 by 10 cm plastic cups with a soil mixture, and in a greenhouse at 27 ± 1 °C, 70 ± 5% RH, and a photoperiod of 16:8 (L:D) h. Second-instar nymphs of *M. cribraria* were obtained from wild-kudzu plants in Plymouth, NC. These nymphs were maintained on soybean plants to allow them to develop to the third instar. Third-instar nymphs were used for all bioassay experiments. Environmental conditions in all experiments were 25 ± 1 °C, 60 ± 10% RH, and a photoperiod of 16:8 (L:D) h.

Concentration–mortality response. Preliminary experiments using leaf dip bioassays were carried out to determine the range of concentrations to be tested for each insecticide considered in the study. Concentrations tested resulted in 0–100% mortality. Preliminary experiments were conducted to select five concentrations of each insecticide so that concentration–mortality regression lines could be calculated. All assays were performed in 30 ml plastic cups (RT32W, Bio-Serv, Flemington, NJ). Seven-day-old greenhouse-grown soybean plants were cut at the soil line and placed in the appropriate insecticide concentration for a time period of 5 s; controls were treated with water only. Treated soybean plants were dried in ambient laboratory conditions, then placed upright on tissue paper that was moistened and placed inside plastic assay cups. Ten third-instar *M. cribraria* nymphs were carefully moved into the plastic cup bioassays using a paintbrush, and then sealed with a paper lid. For all bioassays, three replicates were conducted. All treatments were placed in a growth chamber at 25 ± 1 °C, 70 ± 5% RH, and a photoperiod of 16:8 (L:D) h. Mortality was assessed after 24 h. A nymph was recorded as moribund if there was no coordinated movement when touched with a fine-haired paintbrush.

Sublethal and low-lethal effects on the biological traits. We exposed *M. cribraria* nymphs to sublethal and low-lethal concentrations of the three insecticides using the leaf dip bioassay protocol described above. Concentrations were chosen at the LD_{10} and LD_{40} values, which were determined in the previously described section. For each treatment and control, 100 third-instar *M. cribraria* nymphs were exposed to imidacloprid, bifenthrin, and acephate. After exposure 24 h, the survivors were placed into the plastic assay cups containing insecticide-free soybean plants as food. Five nymphs were placed in each plastic cup sealed with a paper lid. The assay cups were placed into growth chambers (same conditions as before) and were checked daily to record mortality and to replace old soybean plants with new ones. The duration of nymph to adult and adult emergence rate was determined by daily recording the stadia present in each treatment. Adults that completed development were then removed from the previous assay and placed as mating pairs (one male and one female) into new plastic cups covered with a paper top and containing insecticide-free soybean plants (provided as food and as oviposition substrate). These assay cups were placed into growth chambers (same conditions as before) and were checked daily to record mortality, eggs and to replace old soybean leaves with new ones.

Sublethal and low-lethal effects on AchE activity. Twenty third-instar *M. cribraria* nymphs were exposed to LC_{10} and LC_{40} concentrations of imidacloprid, bifenthrin, and acephate for 24 h following the method of above, The AChE activity of the survivors was tested using an enzyme-linked immunosorbent assay kit (Sigma-Aldrich Corporation, St. Louis, MO). A single nymph was homogenized for each replicate, and three replications were performed. Nymph homogenates were placed in 100 µl ice-cold 1× PBS buffer (Sigma-Aldrich Corporation). The homogenates were centrifuged at 4 °C, 10,000g (Eppendorf centrifuge 5417R, Hamburg, Germany) for 30 min, and the supernatant was used for the AChE activity assay. Following the instructions of the AchE activity assay kit, 200 µL water and 200 µL calibrator were pipetted into wells of a 96-well plate. Homogenate samples were added in 10 µL aliquots to separate wells; directly following this, 190 µL of working reagent was added to all sample wells, and the plate was lightly and briefly tapped to facilitate mixing. Reads were conducted at OD_{412} nm for 2 min and for 10 min in a plate reader (Spectra-max Plus 384, Molecular Devices, Sunnyvale, CA). AChE activity was calculated using the equation:

$$\text{AChE activity} = \frac{OD_{10} - OD_2}{OD_{CAL} - OD_{H20}} \times n \times 200\ (\text{U/L})$$

where OD_{10} and OD_2 are the OD_{412nm} values of the sample at 10 and 2 min, respectively. OD_{CAL} and OD_{H2O} are the OD_{412nm} values of the calibrator and water at 10 min and n is the dilution factor ($n = 1$). The number "200" is the equivalent activity of the calibrator under the assay conditions.

Data analysis. The median lethal concentration (LC_{50}) and the concentration–mortality response for third-instar *M. cribraria* nymphs were estimated using probit analysis (POLO-PC; LeOra Software 1987). One-way analysis of variance models were used to test the effects of the sublethal and low-lethal concentrations of three insecticides on the biological traits and AchE activity of *M. cribraria*, and means were separated using Tukey's honestly significant difference (HSD) test ($P < 0.05$). The SPSS 10.0 (SPSS, Chicago, IL) software was used for all statistical analyses.

Results

Determination of the LC_{10} and the LC_{40} insecticides. The mortality of *M. cribraria* increased with increasing concentrations of each insecticide. The LC_{50} and LC_{90} values for different insecticides against *M. cribraria* at 24 h after exposure are listed in Table 1. Toxicities varied among insecticides, with the highest acute toxicity measured

Table 1. Log-dose probit mortality data of imidacloprid, bifenthrin, and acephate for *M. cribraria*.

Insecticides	N^a	LC_{50} (ppm)	95% FL	LC_{90} (ppm)	95% FL	Slope	SE	LD-P line	χ^{2a}	df	*P*
Imidacloprid	180	15.56	12.70–18.79	70.7	52.17–107.17	1.96	0.21	y = 1.96x + 2.66	9.64	9	0.47
Bifenthrin	180	0.48	0.37–0.57	1.83	1.40–2.70	2.16	0.25	y = 2.16x + 5.68	9.49	9	0.39
Acephate	180	115.17	97.35–135.82	400.71	313.32–561.96	2.37	0.23	y = 2.37x + 0.12	9.26	9	0.27

(*N*) number of *M. cribraria* tested, (FL) fiducial limits..

[a] Chi-square linear test of dose–mortality response.

Third-instar nymphs were tested during the 2014 summer.

for bifenthrin, moderate toxicity measured for imidacloprid, and the least toxicity measured for acephate. The LC_{10} values for imidacloprid, bifenthrin, and acephate were 1.86 (1.41–2.29) ppm, 0.027 (0.024–0.032) ppm, and 32.36 (27.53–38.22) ppm, respectively; and the LC_{40} values were 6.31 (5.16–7.74) ppm, 0.065 (0.059–0.078) ppm, and 89.13 (71.22–105.63) ppm, respectively.

Sublethal and low-lethal effects on the biological traits. The developmental time of nymphs varied significantly among treatments (Fig.1; imidacloprid, $F2,223 = 3.27$, $P = 0.043$; bifenthrin, $F2,235 = 4.02$, $P = 0.035$; acephate, $F2,206 = 4.65$, $P = 0.022$). The development time of nymphs exposed to the LC_{40} concentration of imidacloprid, the LC_{10} and LC_{40} concentrations of bifenthrin, and the LC_{10} and LC_{40} concentrations of acephate were significantly longer than those in the control group.

Adult *M. cribraria* emergence rate was significantly reduced when nymphs were exposed to the LC_{10} and LC_{40} concentration of imidacloprid ($F2,223 = 4.16$, $P = 0.032$), bifenthrin ($F2,235 = 3.93$, $P = 0.038$), and acephate ($F2,206 = 4.59$, $P = 0.026$; Fig. 2). Furthermore, the exposure to the LC_{40} concentration of imidacloprid ($F2,235 = 3.02$, $P = 0.045$), and the LC_{10} and LC_{40} concentration of acephate ($F2,206 = 5.13$, $P = 0.017$) significantly reduced adult longevity. However, no significant difference was observed after exposure to bifenthrin ($F2,235 = 0.92$, $P = 0.36$; Fig. 3). Finally, fecundity of females (as measured by the number of eggs laid per female) was significantly reduced by exposure to the LC_{40} concentration of acephate, whereas there was no significant impact of other treatments on fecundity (Fig. 4).

Sublethal and low-lethal effects on AchE activity. The AchE activity of *M. cribraria* was significantly influenced by imidacloprid and acephate compared with their respective controls (Fig. 5; imidacloprid, $F2,8 = 6.23$, $P = 0.034$; acephate, $F2,8 = 20.386$, $P = 0.002$). AChE activity was increased significantly (28.57%) by LC40 imidacloprid, compared with the control (Fig. 5A). The AchE activity of *M. cribraria* exposed to the LC_{10} and LC_{40} concentration of acephate significantly decreased by 34.19 and 55.48%, respectively, compared with the control, (Fig. 5C). However, no significant difference was found in the AchE activity of *M. cribraria* exposed to the LC_{10} and LC_{40} concentration of bifenthrin compared the control (Fig. 5B; $F2,8 = 0.181$, $P = 0.739$).

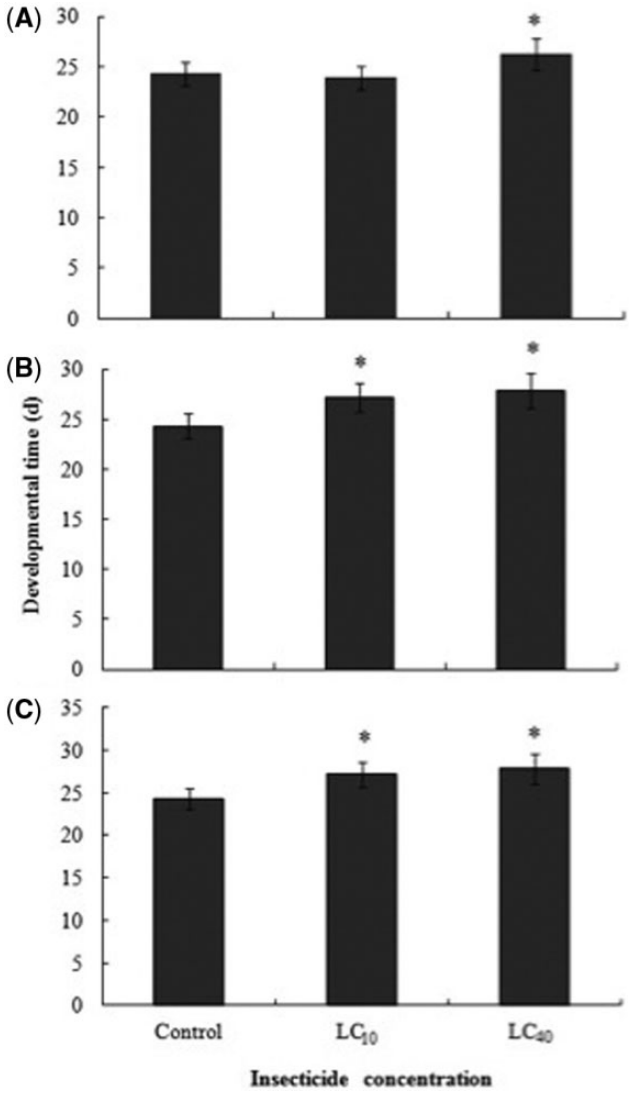

Fig. 1. Developmental time of third instar *M. cribraria* nymphs exposed to an LC_{10} and LC_{40} concentration of imidacloprid (A), bifenthrin (B), and acephate (C) solutions ($n = 30$). Time to adult emergence was used for estimating the nymph developmental time. Data represent mean ± SE. Asterisks on the bars of the histogram indicate significant differences between treatment and respective control at $P < 0.05$, one-way ANOVA followed by Tukey's HSD test.

Discussion

This study in demographic toxicity (Akçakaya and Stark 2008) can serve to provide a baseline for management of *M. cribraria* in the United States. In addition to direct mortality, low or sublethal concentrations of insecticides may also affect population dynamics of insects through altering the behavioral and physiological traits, such as life span, development rates, fertility, and fecundity (Tan et al. 2012, He et al. 2013, Miao et al. 2014, Xiao et al. 2015). Therefore, characterization and assessment of sublethal effects could be crucial for understanding the global effects of insecticides on the pest and non-target organisms, and then optimizing its management in the crops (He et al. 2013, Pan et al. 2014).

We demonstrated that imidacloprid and bifenthrin were more lethal than acephate for *M. cribraria*. Moreover, based on our results evaluating development, fecundity, and AChE activity, negative sublethal effects of these insecticides on *M. cribraria* were observed.

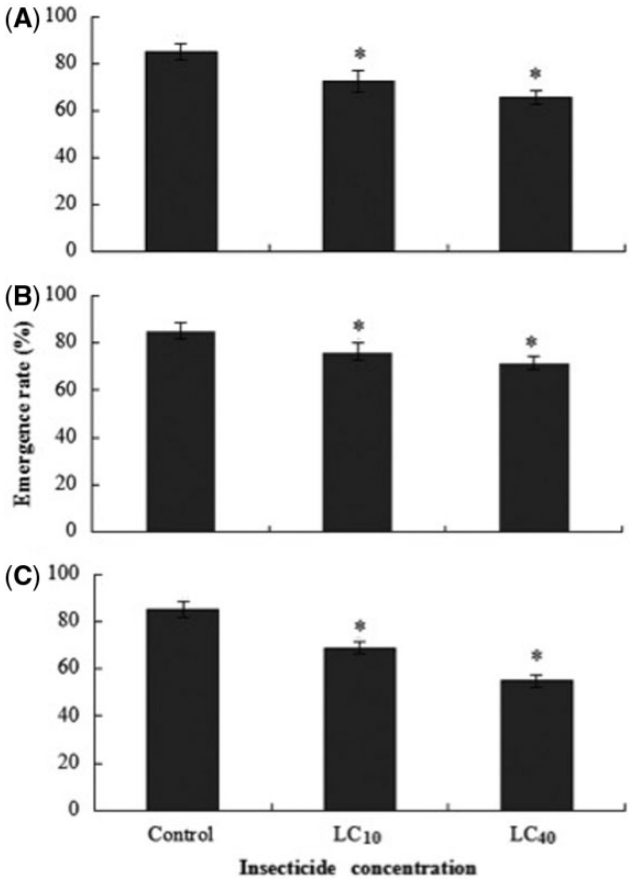

Fig. 2. Emergence rate of *M. cribraria* exposed to an LC_{10} and LC_{40} concentration of imidacloprid (A), bifenthrin (B), and acephate (C) solutions ($n = 30$). Data represent mean ± SE. Asterisks on the bars of the histogram indicate significant differences between treatment and respective control at $P < 0.05$, one-way ANOVA followed by Tukey's HSD test.

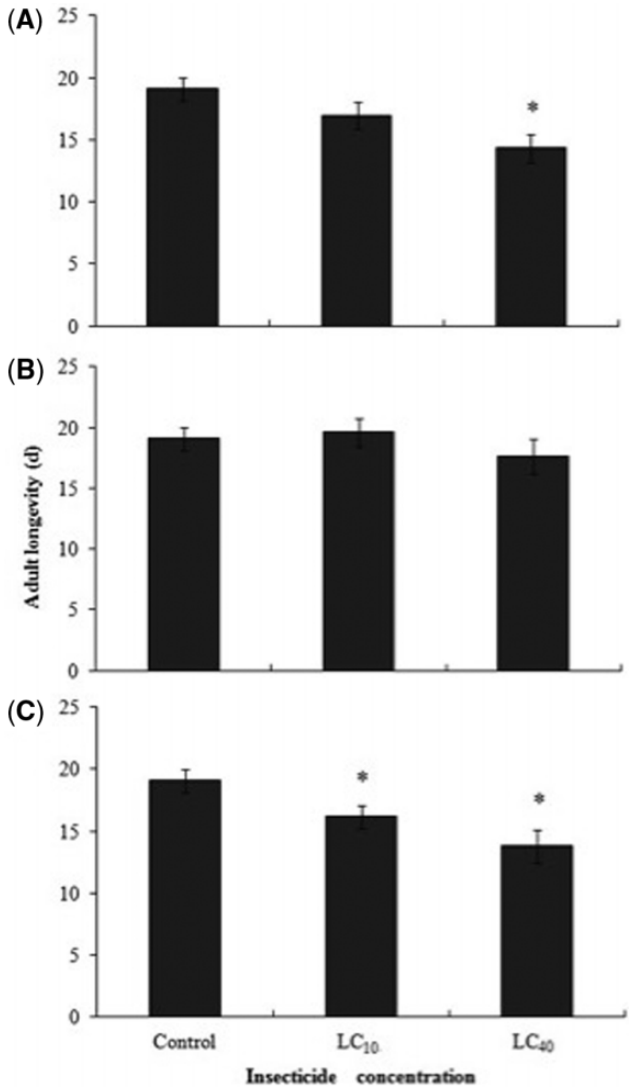

Fig. 3. Adult longevity of *M. cribraria* exposed to an LC_{10} and LC_{40} concentrations of imidacloprid (A), bifenthrin (B), and acephate (C) solutions ($n = 30$). Data represent mean ± SE. Asterisks on the bars of the histogram indicate significant differences between treatment and respective control at $P < 0.05$, one-way ANOVA followed by Tukey's HSD test.

Exposure to LC_{40} of imidacloprid significantly extended the development time of nymphs and reduced the emergence rate and longevity of adults, but an LC_{10} concentration of imidacloprid only reduced the emergence of adults. Furthermore, the fecundity of females was unaffected by exposure to either the LC_{10} or the LC_{40} concentration of imidacloprid. The differences in sublethal effects observed among insecticides and within insecticides by concentration should be expected, as it is known that these effects of insecticides on pests depend on the particular insecticide and its concentration, the pest species, and the pest stage (Holland and Chapman 1993, Pan et al. 2014). Similar results have been reported by Tan et al. (2012), where a sublethal concentration (LD_{25}) of imidacloprid reduced the longevity of *Apolygus lucorum* males, but the LD_5 and LD_{40} did not influence the longevity. Furthermore, He et al. (2011) did not find sublethal effects on fecundity of *B. tabaci* adults when they were exposed to LC_{20} and LC_{40} concentrations of imidacloprid for 24 h.

A number of studies have been conducted on the toxicity of acephate for different organisms (Rao et al. 2006). The results obtained from our study indicate that acephate had lower toxicity to *M. cribraria* than bifenthrin and imidacloprid, but the sublethal (LC_{10}) and low-lethal (LC_{40}) concentrations of acephate had a significant inhibitory effect on population development of *M. cribraria*. Similar effects were also observed in studies of greenhouse whitefly, *Trialeurocles zuporarioriim* (Omer and Leigh 1995) and cotton aphid, *Aphis gossypii* (Kerns and Stewart 2000).

Because of their rapid action and excellent contact toxicity against a broad-spectrum of arthropod pests, the pyrethroid bifenthrin is widely used against many insect and mite pests (Herron et al. 2001, Zhang et al. 2012). The effects of lethal and sublethal concentrations of some pyrethroid insecticides can induce resurgence of the pest populations; for example, increased fecundity can be observed for *A. gossypii* following bifenthrin treatments (Kerns and Stewart 2000; hormoligosis in this case). Furthermore, bifenthrin can reduce *Apis mellifera* Linnaeus fecundity and decrease the developmental rate of *Tetranychus urticae* Koch (Dai et al. 2010, Wang et al. 2014). Our current results indicate that the effects of bifenthrin on population development of *M. cribraria* are concentration-dependent. Both the LC_{10} and LC_{40} concentrations of bifenthrin significantly increased developmental time of the nymph, and significantly decreased adult emergence rate.

Our results demonstrate that the AChE activity of *M. cribraria* is significantly reduced after exposure to the LC_{10} and LC_{40} concentrations of acephate. Previous studies involving the effect of various

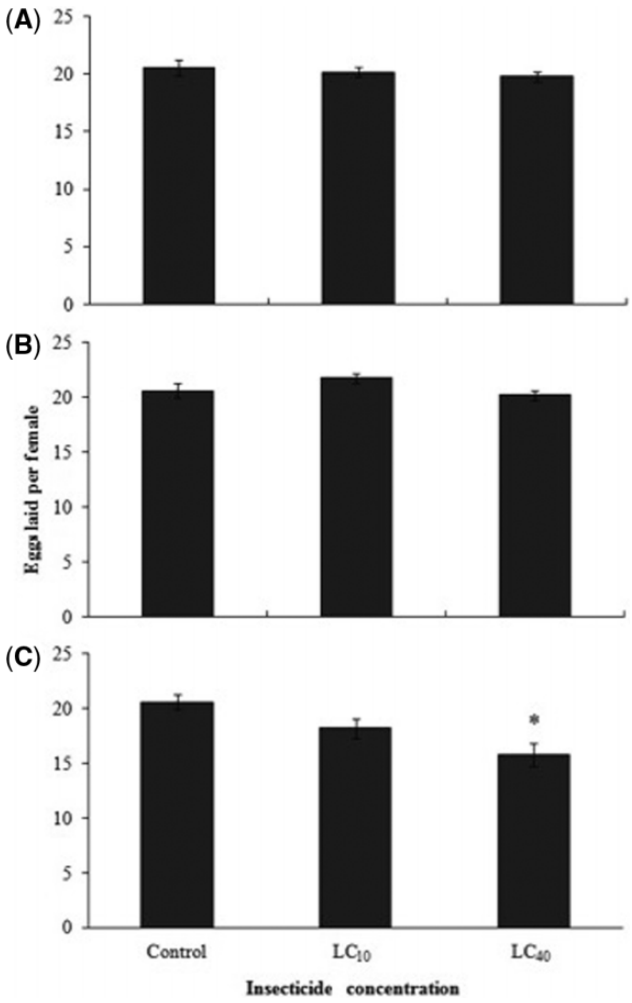

Fig. 4. Number of eggs laid per female of *M. cribraria* exposed to an LC_{10} and LC_{40} concentrations of imidacloprid (A), bifenthrin (B), and acephate (C) solutions ($n = 30$). Data represent mean ± SE. Asterisks on the bars of the histogram indicate significant differences between treatment and respective control at $P < 0.05$, one-way ANOVA followed by Tukey's HSD test.

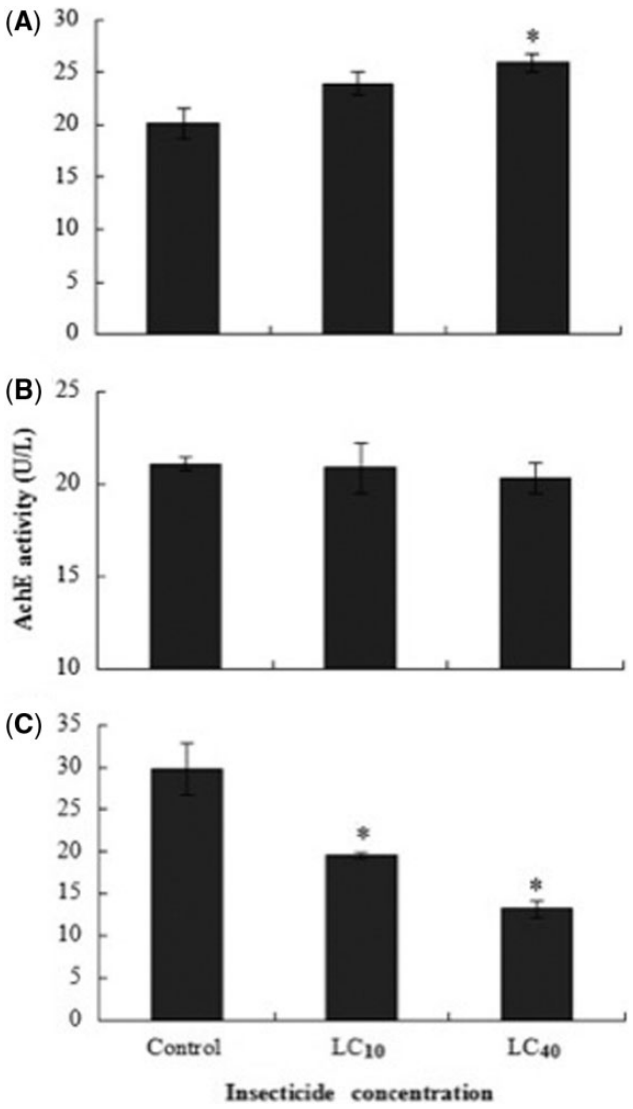

Fig. 5. AChE activity of *M. cribraria* exposed to an LC_{10} and LC_{40} concentrations of imidacloprid (A), bifenthrin (B), and acephate (C) solutions ($n = 30$). Data represent mean ± SE. Asterisks on the bars of the histogram indicate significant differences between treatment and respective control at $P < 0.05$, one-way ANOVA followed by Tukey's HSD test.

organophosphate insecticides on AcheE activity in *Micromus tasmaniae* (Hodge et al. 2000), *Helicoverpa armigera* (Liu et al. 2003), and *Rhopalosiphum padi* (Booth et al. 2007) have demonstrated similar effects. While the results also show that the AChE activity of *M. cribraria* was significantly increased by LC_{40} imidacloprid. Despite the fact that AChE is not a target for imidacloprid, our studies corroborated the finding that the AChE activity can be used as a biomarker of imidacloprid sensitivity (Jemec et al. 2007), adding to the growing body of literature with similar findings. For example, in one study, the AChE activity of chironomid larvae increased when they were exposed to relatively high concentration of imidacloprid and then transferred to clean water following exposure (Azevedo-Pereira et al. 2011). In another study, the AChE activity of *Myzus persicae* (Sulzer) decreased when they were exposed to sublethal concentrations of imidacloprid (Zeng and Wang 2007). Previous studies have demonstrated that the AChE activity of aquatic organisms were strongly inhibited by pyrethroids class insecticides (Mushigeri and David 2005, Kumar et al. 2009, Tu et al. 2009), but studies in a few terrestrial insects (*Blattella germanica* Linnaeus, and *A. mellifera* have confirmed that their AChE activity was affected by the pyrethroid class of insecticides (Ma et al. 2004, Badiou et al. 2008). In the present study, the AChE activity of *M. cribraria* was not affected after exposure to the LC_{10} and LC_{40} concentrations of bifenthrin. Consequently, a low concentration of imidacloprid, bifenthrin, and acephate can induce some negative effects on biological traits of *M. cribraria*. These results suggest that these three insecticides can be used as tools in pest management programs of *M. cribraria* in the field. Further experiments are warranted to link these findings to field settings, both involving insecticide choice and resistance management.

Acknowledgments

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