Acute toxicity of five pesticides to *Apis mellifera* larvae reared in *vitro*

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Abstract

BACKGROUND: The reported high loss rates of managed honey bee colonies has been attributed to diverse stressors including pesticides. Honey bee larvae can be exposed to pesticides in contaminated nectar, pollen and wax. Due to the difficulties of rearing larvae *in* vitro, research focusing on adult bee exposure to pesticides is more common than that on larvae exposure to pesticides. Herein, we aimed to assess the acute toxicity of five insecticides to honey bee larvae using an improved *in vitro* rearing method.

RESULTS: The LC_{50} and LD_{50} were calculated for larvae at 72 hours following a single diet exposure administered when the larvae were 84 ± 12 h old. Solvent control

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larval mortalities were less than 15% at 72 h. The LC_{50} values for each tested pesticide were as follows: amitraz - 494.27 mg/L, chlorpyrifos - 15.39 mg/L, coumaphos - 90.01 mg/L, fluvalinate - 27.69 mg/L, and imidacloprid - 138.84 mg/L. The LD_{50} values were 14.83 (amitraz), 0.46 (chlorpyrifos), 2.70 (coumaphos), 0.83 (fluvalinate) and 4.17 (imidacloprid) µg/larva.

CONCLUSION: The toxicity of the test pesticides to honey bee larvae from most to least toxic was chlorpyrifos > fluvalinate > coumaphos = imidacloprid > amitraz.

Keywords: *Apis mellifera*; larvae; bioassay; LC_{50}; LD_{50}

1 INTRODUCTION

The western honey bee (*Apis mellifera* L.) provides pollination services to a diverse array of agricultural crops[1] and can forage a distance of 3 – 8 km from the hive (or an area of ~28 – 200 km^2), sometimes even longer.[2] Therefore, a honey bee hive can serve as a reservoir for many of the toxic compounds that exist in its environment due to adult bee exposure while foraging in diverse environments. Although honey bees are non-target organisms for most pesticide applications, they nevertheless are exposed to pesticides while collecting pollen, nectar, saps/resins, and water.[3,4] There have been several studies that report pesticide residues in hive matrices.[4-10] Honey bees often are simultaneously exposed to natural toxins from plants and microorganisms, pesticides, and environmental contaminants, as well as apicultural drugs applied by the beekeeper.[11] Some list pesticide exposure as a possible contributor to high yearly loss rates in managed bee populations.[12–16]

Environmental risk assessments conducted to determine the likelihood a given pesticide will impact honey bees primarily consider the survival of adult bees exposed to pesticides.[12,17] However, larvae are also exposed to pesticides as their diets can contain contaminated nectar and pollen collected by foragers.[18] Those effects likely vary according to the nature of the compound and its concentration in pollen and nectar.[19] The effects of pesticides on brood should be considered, especially given that colony survival depends on robust adult populations that, in turn, are directly tied to brood health.[17]

Testing the effects of pesticides on honey bee brood within a nest *in vivo* is not

easily done due to environmental variation, existing pesticide residues in the matrices composing the nest, etc. Thus, an *in vitro* method for rearing bee larvae has been developed[19–21] and is recommended for regulatory trials assessing pesticide toxicity to larvae.[19] This method has been used to test the potential effects of pesticides on honey bee larvae transcriptional levels[22] and midgut cell death.[23] Recently, a larval rearing method was adapted to assess the chronic toxicity of fluvalinate (3 mg/L), coumaphos (8 mg/L), chlorothalonil (34 mg/L) and chlorpyrifos (1.5 mg/L) to honey bee larvae tested alone and in all combinations.[24]

Amitraz (amidine), coumaphos (organophosphate) and fluvalinate (pyrethroid) are used often by beekeepers to control major honey bee pests like *Varroa destructor* Anderson & Trueman and/or small hive beetles, *Aethina tumida* Murray.[25] There are known impacts of these compounds on bees. For example, the presence of fluvalinate and amitraz in beeswax adversely affected the survival of *Apis mellifera* colonies in one study.[26] Thus, it seems prudent to determine the potential impacts these compounds may have on immature honey bees.

Amidines, such as amitraz, block the regular neuromodulating octopamine receptor[27] and can lead to behavioral changes in honey bees.[28] The mechanism of tolerance of bees to amitraz still is not well understood, but it does not appear to be due to detoxification.[29,30] The voltage gated sodium channel is the major target site for fluvalinate,[31,32] but bees are more tolerant of some pyrethroids than other insects because of rapid detoxification by cytochrome P450s.[33,34]

Chlorpyrifos, coumaphos, and imidacloprid are used commonly in agricultural

settings and their residues have been found in honey bee colonies.[4] Chlorpyrifos and coumaphos are both organophosphate insecticides. Organophosphate insecticides act on the insect nervous system by inhibiting acetylcholinesterase (AChE), the enzyme that inactivates the neurotransmitter acetylcholine in the synapses of the insect central nervous system.[27] Neonicotinoids like imidacloprid are nicotinic acetylcholine receptor agonists that interfere with insect neurotransmission and are often used in agriculture due their high selectivity to insects.[35] Imidacloprid is metabolized slowly, through P450 activity, [36] with a half-life of 4 h.[37]

Many studies have focused on the lethal and sublethal effects of these and other pesticides on adult honey bees.[11] However, the toxicity of pesticides to larvae also should be examined to increase the rigor of risk assessments concerning pesticide impacts on honey bee health and survival.[17] In this study, we determined the 72 hour acute lethal concentration (72-h LC_{50}) of five pesticides (amitraz, chlorpyrifos, coumaphos, fluvalinate, and imidacloprid) for honey bee larvae in a single, 24 hour larval exposure event. Our data provide a better basis for determining lethal and sublethal effects of these pesticides on honey bee larvae.

2 MATERIALS AND METHODS

All experiments were conducted at the Honey Bee Research and Extension Laboratory at the University of Florida's Department of Entomology and Nematology (Gainesville, FL, USA). Honey bee larvae were reared *in vitro* according to Schmehl et al.[21] Three *in vitro* larval diet compositions (A, B, C) were used in the study and administered on different days (D): Diet A (D1 - D2) - royal jelly 44.25%, glucose

5.3%, fructose 5.3%, yeast extract 0.9% and water 44.25%; Diet B (D3) - royal jelly 42.95%, glucose 6.4%, fructose 6.4%, yeast extract 1.3 % and water 42.95%; Diet C (D4 - D6) - royal jelly 50%, glucose 9%, fructose 9%, yeast extract 2% and water 30%.

Mixed-race, European-derived honey bee queens were caged on a wax comb for 24 hours to lay eggs. The resulting larvae (t = 87 ± 12 h [75 h after the queens were released]) were transported to a sterile lab environment for grafting. The larvae were transferred from the comb to sterile tissue culture plates (STCPs) with 20 µL of diet A in each cell cup (D1). The STCPs then were placed horizontally in a larval growth chamber maintained at 94% R.H. using a K_2SO_4 salt solution (90 g K_2SO_4, 500 ml H_2O) and within an incubator maintained at 35°C. On D3, each larva was fed 20 µL of diet B. On D4, 5 and 6, each larva was fed 30 µL, 40 µL and 50 µL of diet C respectively.[21]

The five pesticides (amitraz: N-11068-250MG, purity 98.7%, exp 6/30/2018; chlorpyrifos: N-11459-250MG, purity 99.5%, exp 8/31/2018; coumaphos: N-11507-100MG, purity 99.3%, exp 10/31/2019; fluvalinate: N-13263-100MG, purity 95.0%, exp 10/31/2020; and imidacloprid: N-12206-500MG, purity: 99.5%, exp: 2/28/2020) used in this study were purchased from Chem Service, Inc, 660 Tower Lane West Chester, PA, US 19380. Pilot studies were conducted to determine a suitable concentration range to use for each compound. On this basis, the following concentrations were used to determine the LC_{50}: amitraz: 62.5, 125, 250, 500 and 1000 µg/ml; chlorpyrifos: 6.25, 12.5, 25, 50 and 100 µg/ml; coumaphos: 25, 50, 100,

200 and 400 μg/ml; fluvalinate: 6.7，13.3，26.7，53.3 and 106.7 μg/ml; and imidacloprid: 33.3, 66.7, 133.3, 266.7 and 533.3 μg/ml. The following doses were used to determine the LD_{50}: amitraz: 1.875, 3.75, 7.5, 15 and 30 μg/larva; chlorpyrifos: 0.1875, 0.375, 0.75, 1.5 and 3 μg/larva; coumaphos: 0.75, 1.5, 3, 6 and 12 μg/larva; fluvalinate: 0.2, 0.4, 0.8, 1.6 and 3.2 μg/larva; and imidacloprid: 1, 2, 4, 8 and 16 μg/larva. Amitraz, chlorpyrifos, fluvalinate, and imidacloprid were dissolved in methanol to prepare stock solutions, and the rate of the tested solution (solvent + active ingredient) in the diet was 5% of the final volume. Coumaphos was dissolved in acetone, and the volume of the tested solution in the diet was 2% of the final volume.

For each test substance, the following treatments were used: control with solvent, and the five concentrations to be tested. In each experiment, three replicates were run, each replicate using larvae from a different colony. Additional plates of bees reared *in vitro* simultaneously were used at D4 to replace the larvae that died before they had started consuming the diet containing the insecticide (< 5% mortality). On D4 post grafting (larvae = 84 ± 12 h), 36 larvae per treatment (12 larvae × 3 colonies = 36 larvae) were selected and each larva was treated with 30 μL of the diet C containing the treatments for each test substance as described[38]. Following the chemical exposure on D4, mortalities were checked and recorded at the time of feeding on D5, 6 and D7 (project termination, Fig. S1). The 72-hr LC_{50} was calculated for larvae (cumulative mortality at day 7). Mortality was determined by viewing larval movement and the activity of the spiracles under a microscope. Immobile larvae or larvae failing to

respire (i.e. no spiracle movement) were considered dead.

Statistical analyses were performed using the Microsoft Excel Data Analysis package (ver. 11.5). The mortality ratio was corrected using solvent control mortality via the Abbott's formula. The log concentration–response curves allowed determination of the LC_{50} values for the larval bioassay according to probit analysis. The 95% confidence limits for the range of LC_{50}s were determined by least-square regression analyses of the relative growth rates (percentage of control) against the logarithm of the compound concentration. The log dose–response curves allowed determination of the LD_{50} values for the larval bioassay according to probit analysis.

3 RESULTS

Solvent control mortality was lower than 15% at 72 hours (D7) after feeding diet C on D4. The toxicities of the compounds tested are reported in Table 1 and shown graphically in Fig. S2. The LC_{50} values of amitraz, chlorpyrifos, coumaphos, fluvalinate and imidacloprid for honey bee larvae were 494.27, 15.39, 90.01, 27.69 and 138.84 mg/L, and the LD_{50} values were 14.83, 0.46, 2.70, 0.83 and 4.17 μg/larva respectively. The toxicity of the test pesticides to honey bee larvae from most to least toxic was chlorpyrifos > fluvalinate > coumaphos = imidacloprid > amitraz. The 95% confidence intervals for coumaphos and imidacloprid overlapped, suggesting no difference in the toxicities of these two compounds to larval honey bees. The honey bee larvae were most sensitive to chlorpyrifos and most tolerant of amitraz.

Table 1. Acute toxicity of amitraz, chlorpyrifos, coumaphos, fluvalinate and imidacloprid on honey bee (*Apis mellifera* L.) larvae reared *in vitro*

Compound	n	Slope	R^2	LC_{50}[a] (mg/L)	LD_{50}[b] (µg/larva)
amitraz	180	1.3771	0.9933	494.27 (348.68 - 700.65)	14.83 (10.13 – 21.71)
chlorpyrifos	180	1.6285	0.9872	15.39 (11.79 - 20.09)	0.46 (0.31 – 0.68)
coumaphos	180	1.9625	0.9379	90.01 (72.27 - 112.11)	2.70 (2.15 – 3.40)
fluvalinate	180	3.0806	0.9979	27.69 (23.13 – 33.15)	0.83 (0.70 – 0.99)
imidacloprid	180	1.3057	0.9629	138.84 (98.20 - 196.30)	4.17 (2.96 – 5.85)

[a]Lethal concentration and [b]lethal dose, with 95% confidence intervals in parentheses, causing 50% larval mortality at 72 h (D7) after feeding diet C on D4.

4 DISCUSSION

The success of bee colonies depends on health of developed larvae. The *in vitro* test, demonstrated its usefulness for testing toxicity of pesticide on immature bees by avoiding environmental variation and providing quantitative data with a high reproducibility.[13,20,21,39] There are significant physiological differences between honey bee adults and larvae, which affect their sensitivity to pesticides,[24] thus acute exposures of pesticides to honey bee larvae should become an important part of future pollinator risk assessments. The techniques described herein demonstrate the effectiveness of a reliable acute larval toxicity assay.

Larvae rearing protocols have significantly improved over time, although consistent survival of untreated adults has affected the reproducibility of such tests[21]. According to the OECD, total bee survival from 48 hours (2 days) must be 85% overall seven days after grafting. Otherwise, the data generated from those studies would not be considered valid.[38] Previously published studies have noted higher

control mortality rates than found in this study,[24,40-43] thus demonstrating the reliability that the Schmehl *et al. in vitro* rearing method offers.[21]

In this study, honey bee larvae were exposed to a single dose of pesticides at D4 (larvae age = 84 ± 12 h), varying from other recent chronic toxicity tests of pesticides to larvae.[24] The solvent control (2% acetone or 5% methanol) produced a cumulative larval mortality from D4 - D7 that was lower than 15%, thus validating the test protocol. The resulting data can be used to rate pesticides as highly toxic (LD50 0.001–1.99 μg/bee), moderately toxic (LD50 2.0–10.99 μg/bee) and relatively nontoxic (LD50 > 11 μg/bee).[44,45]

The toxicity of a substance to bees can vary depending on age, genetic stock, nutritional status, disease state and concurrent chemical exposure of a colony or individuals.[11] In our study, the LD_{50} of imidacloprid for larvae was 4.17 μg/larva. Using the toxicity scale, our data suggest imidacloprid is moderately toxic to larval honey bees. We tested the acute oral toxicity of imidacloprid to 3D old adult honey bees of similar backgrounds (same genetic stock, same lab, similar experimental timeframe, and the same tester) and determined the LC_{50} to be 27.62 mg/L (95% CI 16.44 - 46.38 mg/L, data unpublished). The LD_{50} value of imidacloprid to adult honey bees was estimated as 0.17 μg/bee when delivering the compound to bees using syrup. The LC_{50} of imidacloprid was ~5-times higher for larvae than for adults while the LD_{50} was ~25-times higher. Therefore, our data suggest that honey bee larvae have a lower sensitivity to imidacloprid than adults. This finding is in line with results that the larvae were more tolerant to imidacloprid than the adults of honey bees.[46]

Similarly, larvae were more tolerant to thiamethoxam[47] and formetanate[48] compared with adults of honey bees. Different sensitivity between larvae and adults can be explained by the presence of the insect fat body which plays major role in intermediary metabolism.[48]

Chlorpyrifos is not used in apiculture, but this highly toxic organophosphate is one of the most ubiquitous chemicals found in hive matrices like honey bee wax, pollen, and adult honey bees.[4,49,50] Chlorpyrifos is of high acute toxicity (LD_{50} 0.06 - 0.11 μg/bee) to honey bee adults.[44] Recently, Zhu found that the accumulative 6-d percent mortality of larvae exposed repeatedly to chlorpyrifos at 1.5 mg/L was more than 50%.[24] We determined that the LD_{50} of chlorpyrifos to honey bee larvae is 0.46 μg/larva; therefore, chlorpyrifos has a high toxicity to both adults and larvae.

Coumaphos is used by beekeepers to control *Varroa*.[25] With repeated use, coumaphos accumulates in the wax of colonies to concentrations as high as 90 μg/g[4,51] and in pollen to levels of 5.828 μg/g.[4] In our study, the LC_{50} of coumaphos to honey bee larvae was 90.01 mg/L, about the maximum amount that was found in wax. The use of coumaphos in colonies has been associated with increased larval mortality of workers.[52,53] The accumulative mortality of larvae was more than 50% when larvae were fed the diet contained 8 mg/L coumaphos for 6 d.[24] Per our LD_{50} data, coumaphos is moderately toxic to honey bee larvae. Zhu et al. described an additive toxicity of chlorpyrifos and coumaphos to honey bee larvae,[24] as both chemicals are organophosphate inhibitors of acetylcholinesterase. There is potential risk for these pesticides to combine in honey bee colonies as coumaphos is commonly applied by

beekeepers and chlorpyrifos is frequently applied by farmers.

Fluvalinate is also frequently used by beekeepers to control *Varroa*, but with repeated application, it may contaminate wax to levels as high as 200 μg/g[4,44] and in pollen upwards to 2.67 μg/g.[4] Our results show the LC_{50} of fluvalinate to larvae is 27.69 mg/L. The LD_{50} seen in our study suggest fluvalinate is highly toxic to honey bee larvae. Larvae exposed to fluvalinate are less likely to survive at both the larval and the adult stages in colony trials.[24,49]

The final acaricide tested in this study was amitraz. Amitraz is not detected in wax after its use against *Varroa*, but its breakdown product, 2,4-dimethylphenyl formamide (DMPF), is present in wax at concentrations as high as 43 μg/g and in pollen 1.117 μg/g.[4] Our data suggest amitraz has a low LC_{50} toxicity to honey bee larvae at only 494.27 mg/L. The residue of amitraz in pollen is about 0.23% of LC_{50}. Varroacides are among the most abundantly detected chemical residues in honey bee colonies[4] and there is significant potential for synergistic interactions between these residues and other agricultural pesticides to further diminish honey bee health.[24]

Future studies should examine the chronic toxicity of these pesticides to honey bee brood with the low concentrations based on the LC_{50} values reported here as well as residue levels in brood food found in colonies. Sublethal effects should also be investigated in both brood and adult bees exposed to these pesticides during the larval stage. As honey bee brood are constantly being exposed to agricultural and apicultural pesticides, there is a great need to test the effects of these chemicals regularly as part of environmental risk assessments.

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