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Risk assessment of various insecticides used for management of Asian citrus psyllid, *Diaphorina citri* in Florida citrus, against honey bee, *Apis mellifera*

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Abstract The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), is a major pest of citrus trees worldwide. A wide variety of insecticides are used to manage *D. citri* populations within citrus groves in Florida. However, in areas shared by citrus growers and beekeepers the use of insecticides may increase the risks of *Apis mellifera* L. (Hymenoptera: Apidae) loss and contaminated honey. The objective of this research was to determine the environmental toxicity of insecticides, spanning five different modes of action used to control *D. citri*, to *A. mellifera*. The insecticides investigated were imidacloprid, fenpropathrin, dimethoate, spinetoram and diflubenzuron. In laboratory experiments, LD_{50} values were determined and ranged from 0.10 to 0.53 ng/µl for imidacloprid, fenpropathrin, dimethoate and spinetoram. LD_{50} values for diflubenzuron were >1000 ng/µl. Also, a hazard quotient was determined and ranged from 1130.43 to 10893.27 for imidacloprid, fenpropathrin, dimethoate, and spinetoram. This quotient was <0.447 for diflubenzuron. In field experiments, residual activity of fenpropathrin and dimethoate applied to citrus caused significant mortality of *A. mellifera* 3 and 7 days after application. Spinetoram and imidacloprid were moderately toxic to *A. mellifera* at the recommended rates for *D. citri*. Diflubenzuron was not toxic to *A. mellifera* in the field as compared with untreated control plots. Phenoloxidase (PO) activity of *A. mellifera* was higher than in untreated controls when *A. mellifera* were exposed to 14 days old residues. The results indicate that diflubenzuron may be safe to apply in citrus when *A. mellifera* are foraging, while most insecticides used for management of *D. citri* in citrus are likely hazardous under various exposure scenarios.

Keyword *Apis mellifera* · *Diaphorina citri* · Insecticide mode of action · Phenoloxidase activity · Acute toxicity · Chronic toxicity

Introduction

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), is the vector of a phloem restricted, gram negative bacterium, *Candidatus* Liberibacter asiaticus (Las), which causes citrus greening disease or huanglongbing (HLB) (Pelz-Stelinski et al. 2010; Halbert and Manjunath 2004; Boina and Bloomquist 2015). HLB is one of the most economically important diseases of citrus and is present in most citrus growing regions of the world (Halbert and Manjunath 2004; Manjunath et al. 2008). Citrus trees infected by this disease may live only 5–8 years, during which, they produce oranges that are small and distorted, with bitter taste (Halbert and Manjunath 2004). In Florida, it is estimated that HLB has caused over 1.3 billion dollars in lost revenue to the citrus industry over the last 5 years and overall losses to the Florida economy total $3.63 billion (Hodges and Spreen 2012). Currently, there is no cure for HLB (Morris et al. 2009; Tiwari et al. 2010). Vigorous vector control programs that rely heavily on conventional insecticides are a main component of HLB management. Excessive use of insecticides increases chances of lethally affecting non-target and/or beneficial insects in citrus

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groves. *Apis mellifera* L. (Hymenoptera: Apidae) are essential beneficial insects that could be affected by insecticide use in citrus (Grafton-Cardwell et al. 2013; Qureshi and Stansly 2010). Insecticides used in citrus for management of *D. citri* may kill *A. mellifera* by direct contact and residues may indirectly affect cognitive function, behavior, physiology, immune system function and contaminate honey (Guedes et al. 2016).

Concerns have been raised over the effect of sublethal doses of insecticides rendering non-target beneficial insects more susceptible to disease, particularly with regard to bees (James and Xu 2012). Low doses of insecticides often affect insect behavior by reducing activity and influencing feeding habits of *A. mellifera* (Guedes et al. 2016). This may impact behaviors that can increase disease susceptibility (Johnson 2015; Guedes et al. 2016). The phenoloxidase (PO) cascade is probably more vulnerable post application because uptake into the hemolymph occurs following direct insecticide contact with the cuticle, which are locations where the majority of PO activity occurs (James and Xu 2012). Also, this complex system involves detoxification mechanisms that degrade environmental toxins, which may explain interactions between insecticide contact and effects on immune function (Ishaaya and Casida 1974).

Information on the toxicity of insecticides used in citrus groves to *A. mellifera* is scarce despite the economic importance of this crop. However, this information is necessary for the implementation of integrated management programs for *D. citri*, which can assure the maintenance of *A. mellifera* in the field. Therefore, this study was undertaken to evaluate the environmental toxicity of insecticides of various modes of action that are commonly used for vector management in citrus groves in Florida on adult worker bees of *A. mellifera*. The aim of this study was to assess the acute, chronic, and residual toxicity of insecticides used in citrus against *A. mellifera.*

Material and methods

Honey bee cultures

A. mellifera were collected from a single colony at the University of Florida Citrus Research and Education Center (CREC) in Lake Alfred, Florida. The hive was placed in an area without any other *A. mellifera* colonies within a 3 km range to avoid the presence of foreign bees during the experiment. Before experiments, the hive was not exposed to any chemical treatments. Worker bees used in these experiments were of unknown ages. In order to collect bees, the hive was treated with smoke and then opened and a frame removed. The worker bees were then gently shaken into a plastic container for transport to the laboratory. For laboratory bioassays, we used cages made from 500 ml jars (8 × 14 cm) with modified lids containing a metal screen. These served as containers to transfer *A. mellifera* to the laboratory. *A. mellifera* were fed with aqueous solutions of 85% sugar and 15% honey, as well as, water alone applied to separate cotton balls (US Cotton LLC, 531 Cotton Blossom Circle, Gastonia, NC) 24 h before and after treatment.

Insecticides

Insecticides were of analytical grade and included dimethoate (99.8%), fenpropathrin (99.1%), imidacloprid (99.9%), diflubenzuron (97.9%) and spinetoram (76.2% J-21.0% L) representing several insecticide classes. Dimethoate, fenpropathrin and imidacloprid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Spinetoram and diflubenzuron were obtained from Chem Service Inc (West Chester, PA, USA). Each insecticide was stored according to the manufacturer's recommendation. For field application, the tested formulated insecticides were dimethoate 4E (dimethoate), micromite 80WG (diflubenzuron), admire Pro 4.6F (imidacloprid), delegate™ WG (spinetoram) and danitol 2.4EC (fenpropathrin). Insecticide active ingredient, trade name and formulation, insecticide classification, manufacturer, application rates and application method used in the citrus trial are listed in Table 1.

Adult topical bioassay for acute and chronic toxicity

For acute toxicity experiments, six to eight doses of each insecticide were tested with three replicates. All insecticide dilutions were performed using acetone. Each replicate was conducted on a group of 5 honey bees; 5 µl of insecticides were applied to the thorax of each honey bee using a micropipette. For the control treatment, 5 µl of acetone was applied to the thorax of each honey bee. Each group of 5 honey bees was kept in a plastic Petri dish (10 × 2 cm, Fisher Scientific, USA) with filter paper lining the bottom. Two plastic containers (35 mm diameter by 0.5 cm height) were placed into each Petri dish containing food (solutions of 85% sugar, 15% honey w/w) and distilled water on separate cotton balls until conclusion of the experiment 48 h later. All dishes were placed in a plastic box in a room at 25 ± 1 °C, 60 ± 5% relative humidity, and 14:10 L:D light cycle. The entire experiment was replicated twice.

For chronic toxicity experiments, *A. mellifera* were obtained from the CREC hive as described above. Chemical concentrations were prepared freshly by addition of a measured amount of chemical in defined volume of acetone. The tested lethal doses in this study were: LD_0, LD_{25}, LD_{50} and LD_{95} for each insecticide. For this experiment, three doses of each insecticide were tested in duplicate. Each

Table 1 Insecticides used for management of Asian citrus psyllid, *Diaphorina citri*, tested against adult honey bees, *Apis mellifera*

Insecticide trade name	Active ingredient	Chemical class	Rate/Acre (lb)	Target insect	Mode of action	Manufacture
Dimethoate 4E	Dimethoate	Organophosphate	1.00	*Diaphorina citri*	Acetylcholinesterase Nicotinic	Cheminova
Admire Pro 4.6F	Imidacloprid	Neonicotinoid	0.50	*Diaphorina citri*	Acetylcholine receptor	Bayer Crop Science
Micromite 80WGS	Diflubenzuron	Benzoylureas	0.39	*Diaphorina citri*	Inhibitors of chitin biosynthesis	Chemtura
Danitol 2.4EC	Fenpropathrin	Pyrethroid	0.40	*Diaphorina citri*	Voltage gate sodium ion channel	Valent Inc
Delegate™ WG	Spinetoram	Spinosyn	0.53	*Diaphorina citri*	GABA and nicotinic receptors	DowAgroScience

replicate was conducted on a group of five *A. mellifera* and 5 µl of insecticide was applied to the thorax of each honey bee using a micropipette. For the control treatment, the dose of insecticide was replaced with acetone. Each group of 5 honey bees was kept in a plastic Petri dish. Adult mortality was assessed 24, 48, 72, 96, 120 and 144 h after treatment.

Hazard assessment

Hazard assessments were developed with the quotient method for pesticides (Chen et al. 2010; EPPO 2010). Hazard is determined by dividing the manufacturer recommended application rate stated on the formulated product label (g of product per ha converted to g of a.i. per ha) by the acute LD_{50}.

$$\text{Equation: Hazard quotient} = \text{Label rate}/LD_{50}.$$

Using this method, a hazard quotient of <50 suggests the compound is nonhazardous to *A. mellifera* for given exposure rate (EPPO 2010, 2003).

Field insecticide applications

A Valencia sweet orange (*Citrus sinensis* (L) Osb.) grove with trees between 6–7 years old in Polk County, FL (N: 28° 07′ 849; W: 81° 42′ 930) was utilized for field experiments in 2016. This grove is maintained according to standard grower practices; however, no insecticides were applied for at least 24 months prior to the experiments and during testing. We investigated the effect of field residues on *A. mellifera* based on the laboratory results for five insecticides with different modes of action. The experiment was arranged as a randomized complete block design with four blocks and each block was replicated four times. The foliar application experiment consisted of the previously stated five insecticides and one negative control, which was application of water only. All replicated blocks were separated by at least four rows of trees. Foliar applications we made on July 12, 2016 with 946 ml handheld sprayers (6C13, Rubbermaid Home Products, Woodter, OH, USA) to 35 cm branches per tree. Applications consisted of 25 ml of spray to each branch at recommended field rates. Two branches were sprayed per replicate. At 3, 7, 14 and 21 days after application, ten bees were exposed to each treatment within sleeve cages. Sleeve cages consisted of a 50 cm mesh fabric bag and (13 × 4 cm) plastic bottom. Each sleeve was supplied with sugar solutions of 85% sugar, 15% honey or water alone dispensed with separate 0.53 ± 0.01 g cotton balls. Mortality was recorded 24 h after bees were introduced for each residual period tested. Living bees were collected from the 14 and 21 days residual treatments to determine PO activity and total PO as described below. PO activity and total PO was not assessed for the 3 and 7 days

residual treatments due to high bee mortality occurring for these treatments (see results).

Hemolymph collection from bees

Twenty microliters of 1X PBS (Phosphate Buffered Saline) was placed on ice in 1.5 ml Eppendorf tubes. All bees were knocked down using CO_2 prior to hemolymph extraction. Slight pressure was applied to the posterior of the bee abdomen, while a micro-capillary pipette punctured the anterior portion of the abdomen. For each treatment, a minimum of three bees were collected from the field after exposure to the 14 and 21-day-old insecticide residues and bled into 1X BPS within 24 h of collection. Each treatment was replicated three times. All samples were spun down at 3000 rpms for 3 min at 4 °C. Two microliters of hemolymph was used for each well in the phenoloxidase activity assay, which was performed as described in Fedorka et al. (2013).

Phenoloxidase activity assay for bees

Active PO represents the enzyme ready to initiate the production of melanin. To estimate PO activity, 2 µl of bee hemolymph from each replicate were added to a flat bottom 96-well plate. Subsequently, 100 µl of 7 mmo/l L-Dopa (Sigma-Aldrich, St Louis, MO) was added to each well with hemolymph. Controls were 1X PBS and L-DOPA only. Plates were added to a microplate reader at 490 nM (A_{490}); absorbance was measured at 5 min intervals for 90 min. Data represents total change in absorbance from 0 to 30 min. Plates were read for 90 min because the assay inherently has background to allow absorbance reads beyond 30 min to assess the slope or rate of melanization increase by the rate darkening in controls. The units were obtained by dividing the change in absorbance by 0.01. Bradford reagent (Sigma–Aldrich) was utilized to quantify total protein content of hemolymph. Final PO activity was measured in units of activity per microgram of total protein.

Total phenoloxidase activity assay for bees

The total PO is used to determine the amount of potential PO activity that could be induced due to pathogen challenge at a given time. Total PO represents the active PO, with the precursor (proPO), and represents the total potential active PO in the hemolymph. To estimate total PO, 2 µl of bee hemolymph from each replication was added to a flat bottom 96-well plate. Twenty microliters of a-chymotrypsin (Sigma-Aldrich; 1.3 mg/ml) were added to each hemolymph sample and the mixture was allowed to incubate at room temperature for 20 min. Subsequently, 100 µl of 7 mmol/l L-Dopa (Sigma-Aldrich, St Louis, MO) was added to each well with hemolymph. Controls consisted of 1X PBS and L-DOPA only. Plates were added to a microplate reader at 490 nM (A_{490}); absorbance was measured at 5 min intervals for 90 min. Data represents total changes in absorbance from 0 to 30 min; plates were read for 90 min as described above. It is important to perform chymotrypsin and L-DOPA controls due to background color change, which needs to be subtracted from total unit calculation. The units were obtained by dividing the change in absorbance by 0.01. Bradford reagent (Sigma-Aldrich) was utilized to quantify total protein content of hemolymph. Total PO was measured in units of activity per microgram of total protein.

Statistical analysis

SAS probit (SAS Institute Inc 2012-2013) was conducted to calculate LD_{50} values and 95% fiducial limits. Chi-square tests were applied to ensure the goodness of fit of the models. If a bioassay failed the goodness of fit chi square test, the experiment was repeated. If an insecticide exhibited low toxicity to honey bees, the 95% fiducial limits could not be calculated. In this case, the assays were repeated until mortality patterns were reached that were similar to chemicals for which 95% fiducial limits could be calculated over a similar dose range. For field mortality and PO data, statistical significance of difference among treatments was determined using analysis of variance (ANOVA) followed by Tukey's test for mean separation. Mortality data from residual contact and field assessments were arcsine transformed. In chronic toxicity experiments, data were analyzed using logistic regression analysis. Analyses were performed using the SAS Logistic procedure (SAS Institute Inc 2012-2013).

Results

Acute and chronic toxicity to honey bee

Six to eight doses of each insecticide were examined. LD_{50} values and statistical analysis for all five insecticides are summarized in Table 2. The LD_{50} value and 95% fiducial limits were 0.10 ng/µl (0.001–795) for dimethoate; 0.53 ng/µl (0.07–7.75) for imidacloprid; 0.11 (0.01–1.95) for spinetoram; 0.29 ng/µl (0.01–70.25) for fenpropathrin; and >1000 ng/µl for diflubenzuron.

Mortality of *A. mellifera* was significantly higher at lethal than and sublethal doses in the chronic toxicity experiment ($df = 3$; $p < 0.001$). At 144 h post treatment, we observed 65% mortality at LD_{25}, 78.30% mortality at LD_{50}, and 100% mortality at LD_{95} for dimethoate (Fig. 1a). Chronic mortality was higher than acute mortality toxicity at these same doses (Fig. 1a–d). Relatively similar results were

Table 2 Toxicity and hazard of various insecticides to honey bees, *Apis mellifera,* 48 h following contact application

Insecticide	n	Intercept (SE)	LD_{50} (ng/µl)	95% FL	χ^2	Label rate (ng/µl)	Hazard quotient
Imidacloprid	240	0.13 ± 0.18	0.53	0.07–7.75	0.58	599.13	1130.43
Spinetoram	240	0.55 ± 0.23	0.11	0.01–1.95	5.53	225.27	2047.90
Dimethoate	240	0.42 ± 0.27	0.10	0.001–795	2.47	1198.26	10893.27
Fenpropathrin	240	0.41 ± 0.28	0.29	0.01–70.25	2.19	479.30	1652.76
Diflubenzuron	240	–	>1000	–	–	467.70	>0.47

Based on application volumes of 935.4 l/ha. Where a range of recommended application rates was given on the formulated insecticide label, a mean rate was used: imidacloprid: 227 g a.i./acre; spinetoram: 85 g a.i./acre; dimethpoate: 453 g a.i./acre; fenpropathrin: 181 g a.i./acre. diflubenuron: 176 g a.i./acre. Hazard quotient = label rate divided by its LD_{50}. An insecticide having a hazard quotient of <50 is considered nonhazardous

FL fiducial limits

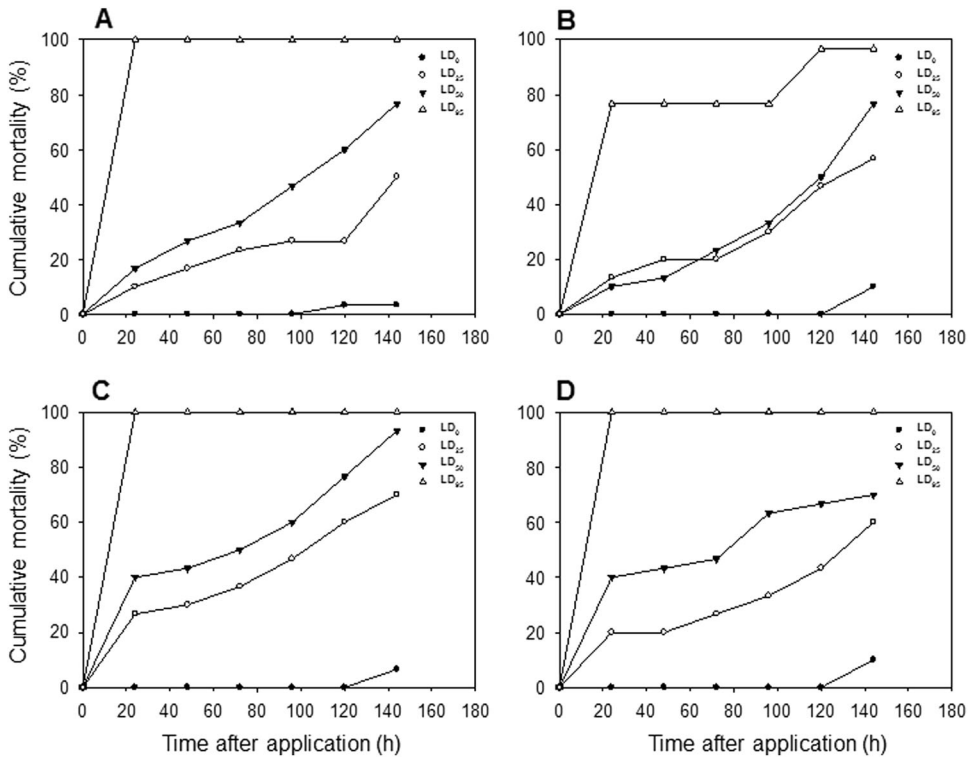

Fig. 1 Cumulative mortality of foraging honey bees, *Apis mellifera,* after application of lethal and sublethal doses of dimethioate (**a**), fenpropathrin (**b**), imidacloprid (**c**), and spinetoram (**d**)

observed for fenpropathrin (Fig. 1b), imidacloprid (Fig. 1c) and spinetoram (Fig. 1d).

Hazard assessment

The hazard assessment for each chemical is listed in Table 2. A hazard quotient of <50 is used to define an insecticide that is harmless to *A. mellifera*. A moderate toxicity quotient was defined as ranging between 50–2500, while a dangerous hazard quotient was defined as >2500 (EPPO 2003). Based on the hazard quotient method, fenpropathrin, dimethoate, spinetoram and imidacloprid all posed a hazard to *A. mellifera* in this investigation. The results showed that fenpropathrin, spinetoram and imidacloprid posed a moderate hazard to *A. mellifera.* Dimethoate was defined as dangerous, while diflubenzuron was deemed harmless to *A. mellifera.*

Survival of *A. mellifera* after exposure to insecticides residues

Mortality of *A. mellifera* was high on citrus branches 3 days after treatment (Table 3). Fenpropathrin and dimethoate caused 100% mortality, while imidacloprid and spinetoram

Table 3 Mortality of honey bees, *Apis mellifera,* after exposure to field–aged residues of various insecticides on citrus used for management of Asian citrus psyllid, *Diaphorina citri*

Insecticides	Mortality after application insecticides (%)				
	Before application	3 days	7 days	14 days	21 days
Untreated control	0.0 ± 0.0a	2.5 ± 2.5c	7.5 ± 2.5dc	0.0 ± 0.0c	0.0 ± 0.0a
Imidacloprid	0.0 ± 0.0a	40.0 ± 0.0b	22.5 ± 9.6cb	15.0 ± 5.8b	0.0 ± 0.0a
Fenpropathrin	0.0 ± 0.0a	100.0 ± 0.0a	40.0 ± 8.2ba	15.0 ± 5.8b	0.0 ± 0.0a
Spinetoram	0.0 ± 0.0a	34.8 ± 1.6b	7.5 ± 9.6dc	10.0 ± 0.0cb	0.0 ± 0.0a
Diflubenzuron	–	5.0 ± 2.9c	2.5 ± 2.5d	2.5 ± 5.0c	0.0 ± 0.0a
Dimethoate	–	100.0 ± 0.0a	60.0 ± 16.3b	30.0 ± 8.2a	0.0 ± 0.0a

Mean percentage mortality followed by different letters within each day is significantly different ($p < 0.05$)

caused 40 and 34.8%, respectively, which were statistically greater than that observed in controls ($df = 5$; $F = 493.12$; $p < 0.001$). There was no significant difference in mortality caused by fenpropathrin and dimethoate ($p > 0.05$) (Table 3). Only 5% mortality was observed on diflubenzuron residues 3 days after treatment, which was not statically different from the control ($p > 0.05$).

Dimethoate and fenpropathrin residues caused 40 and 60% mortality, respectively, 7 days after treatment, which were significantly higher than in controls ($df = 5$; $F = 23.64$; $p < 0.001$) (Table 3). There was no statistical difference in mortality following exposure to residues of fenpropathrin, spinetoram, and imidacloprid at 14 days post treatment ($p > 0.05$) (Table 3). At 21 days after treatment, residues of all insecticide tested on citrus were no longer toxic to *A mellifera* (Table 3).

Phenoloxidase (PO) activity

PO activity was highest in the control treatment as compared with those exposed to insecticide residues at 14 days after treatment (Fig. 2a). Exposure to fenpropathrin residue caused the lowest PO activity (Fig. 2a). Total PO coincided with measured PO activity after exposure to 14 days residues (Fig. 2c). PO was greater following exposure to insecticide residues than the untreated control at 21 days after exposure (Fig. 2b). The total PO response was similar to measured response with PO activity at 21 days after exposure; exposed *A. mellifera* showed higher total PO than the untreated control (Fig. 2d). Fenpropathrin caused the highest increase in PO activity and total PO as compared with the untreated control at 21 days following treatment (Fig. 2b, d).

Discussion

Beneficial insects and insecticide treatments can contribute to management of *D. citri* and HLB in citrus (Qureshi and Stansly 2010). However, incompatibility of these two tactics for management of *D. citri* has been documented given non-target effects on natural enemies (Monzo et al. 2014). The beekeeper industry in citrus is also susceptible to non-target impact of *D. citri* management given potential poisoning of *A. mellifera.* Citrus crops with long bloom periods are frequently sprayed throughout the entire season (8–15 times per years) to manage *D. citri* (Tiwari et al. 2011; Kanga et al. 2016). To better protect *A. mellifera*, it is important to understand how field residues of sprays on the target crop may inevitably affect *A. mellifera*.

Imidacloprid is considered moderately toxic to *A. mellifera* by direct contact as reported from previous laboratory experiments (Stark et al. 1995; Suchail et al. 2001; Schmuck et al. 2001; Bailey et al. 2005). Synthetic pyrethroids are also highly lethal to *A. mellifera* (Hassan et al. 1986). These modes of action were included among the common types of insecticides used in citrus to manage *D. citri* and other pests of cultivated citrus. Our investigation confirmed that residues of organophosphate, pyrethroid, and neonicotinoid insecticides on citrus are incompatible with foraging *A. mellifera* due to both acute and chronic toxicity. Of those insecticides used against *D. citri* tested here, only diflubenzuron was non-toxic even when topically applied at 1000 ng/µl. Exposure of bees to field aged residues also indicated lack of toxicity with this active ingredient. Diflubenzuron does not influence learning of harnessed honey bee foragers; however, there may be subtle impacts on learning behavior (Abramson et al. 2004; Rabea et al. 2010). Overall, our current results suggest that diflubenzuron can be used to target *D. citri* during bloom period in citrus without devastating damage to foraging *A. mellifera.*

In addition to mortality, exposure to minute doses of insecticides may cause sublethal effects on *A. mellifera*, including impaired foraging ability, altered behavior, decreased life span, and changes in reproduction and development (Johansen et al. 1983; Johansen and Mayer 1990; Tasei et al. 2000; Morandin et al. 2005; Desneaux et al. 2007; Gradish et al. 2010; Karahan et al. 2015). Our investigation suggests that exposures of *A. mellifera* to

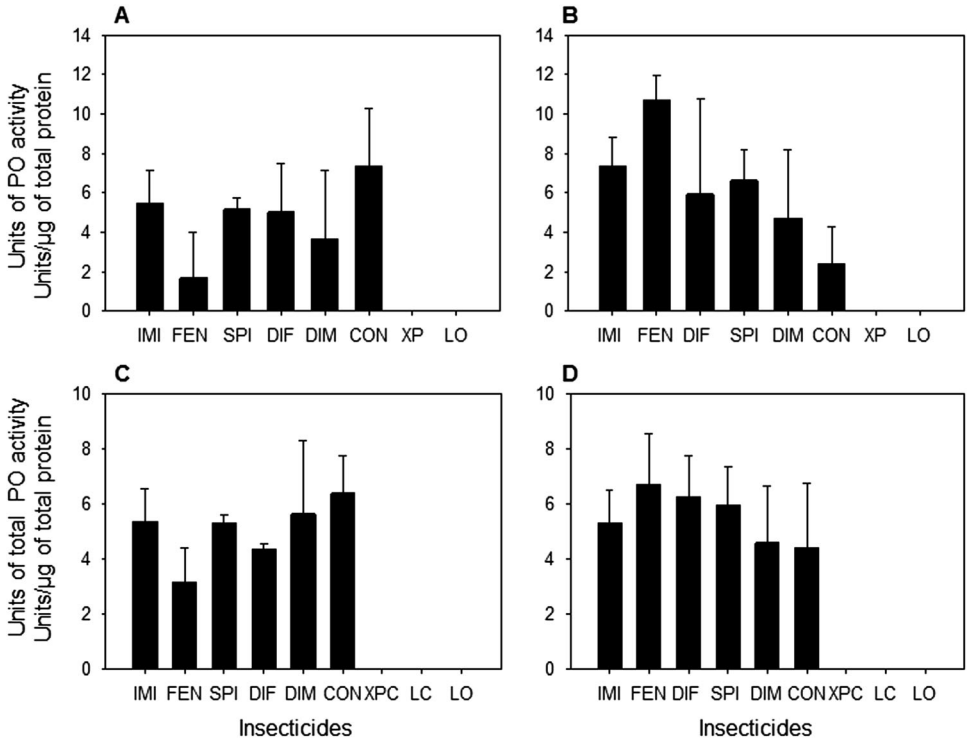

Fig. 2 Unit of PO activity per unit total protein measured from *Apis mellifera* after exposure to 14 (**a**) and 21 day old (**b**) residues of various insecticide applied to citrus and units of total PO per unit total protein after exposure to 14 (**c**) and 21 day old (**d**) residues: imidacloprid (IMI), fenpropathrin (FEN), spinetoram (SPI), diflubenzuron (DIF), 1XPBS (XP), Ldopa only (LO), Ldopa + Chymo (LC), 1XPBS + Chymo (XPC), control (CON)

insecticide residues in citrus could interfere with the melanization/immune process by interfering with the PO cascade. In vitro studies of *Micromelalopha troglodyta* cells treated with insecticides and other chemicals showed that dimethoate and fenpropathrin produced moderate inhibition of diphenolase activity of tyrosinase (Tang et al. 2009). However, organophosphate exposures of wax moth, *Galleria mellonella* (Linnaeus), and Colorado potato beetle, *Leptinotarsa decemlineata* (Say), submerged at the LD_{50} elevated total hemocyte count, PO activity, and encapsulation response (Dubovskiy et al. 2013). Therefore, we used PO activity and total PO activity as an indicator of humoral immunity after exposure to insecticide residues. *A. mellifera* exposed to 14-day-old insecticide residues experienced moderate mortality and deceased PO and total PO levels in general, as compared with the controls. Although 21-day-old residues did not yield mortality of *A. mellifera,* PO activity and total PO activity was elevated following exposures at this duration post treatment (Fig. 2). Therefore, our results suggest that exposures to 21-day-old residues may no longer inhibit humoral immune responses, but rather cause stimulation (Fig. 2b, d). This hypothesis warrants further direct testing.

PO activity is a useful measure with respect to insecticide exposure and its degree of inhibition is related to toxicity (James and Xu 2012). Although *D. citri* can be effectively managed with insecticides, undesirable non-target effects must be considered. Protection of *A. mellifera* in heavily managed citrus agroecosystems does not require further justification (Free 1993). Using PO as a biomarker in *A. mellifera* is interesting because this insect is an important pollinating agent, sensitive to environmental contaminants (Wallwork-Barber et al. 1982; Smith and Wilcox 1990; Rabea et al. 2010). Biochemical parameters used in determining the probable cause of lethal and sublethal effects under field conditions or monitoring for the combined effects of long-term exposures to insecticides or other toxicants are useful in diagnoses of detrimental non-target effects caused by human chemical input (Giesy and Graney 1989).

In summary, increased use of insecticides for management of *D. citri* in citrus has likely negative associated impacts on *A. mellifera*. This investigation was initiated to realistically simulate field sprays in citrus to assess toxicity of residues of five commonly used insecticides against *A. mellifera*. Our results provide a quantifiable scale to help extension specialists and citrus growers with insecticide selection to maintain effective management of *D. citri* and minimize the risk to foraging *A. mellifera* during bloom periods. Overall, we found that susceptibility of *A. mellifera* to insecticides differed among compounds and residue age exposures. Diflubenzuron caused no observable negative impact at high exposure concentrations, making it the least hazardous product for use against *D. citri* during bloom. Further investigations are needed to determine sublethal effects of the entire suite of available insecticides used in

citrus on *A. mellifera*, particularly with respect to the effects on behavior and other biochemical parameters in addition to PO activity. The current investigation provides a baseline for such follow up research that is needed given the ever-increasing use of insecticides in citrus production.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Informed consent Informed consent was obtained from all individual participants included in the study.

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