Journal of Economic Entomology, XX(X), 2018, 1–9
doi: 10.1093/jee/toy140
Research Article

Responses of Honey Bees to Lethal and Sublethal Doses of Formulated Clothianidin Alone and Mixtures

Jianxiu Yao,[1] Yu Cheng Zhu,[1,3] and John Adamczyk[2]

[1]USDA-ARS, Southern Insect Management Unit, Stoneville, MS 38776, [2]USDA-ARS, Southern Horticultural Research Unit, Poplarville, MS 38470, and [3]Corresponding author, e-mail: yc.zhu@ars.usda.gov

Subject Editor: Raul Narciso Guedes

Received 26 January 2018; Editorial decision 2 May 2018

Abstract

The widespread use of neonicotinoid insecticides has sparked concern over the toxicity risk to honey bees (*Apis mellifera* L. (Hymenoptera: Apidae)). In this study, feeding treatments with the clothianidin formulation at 2.6 ppb (residue concentration) or its binary mixtures with five representative pesticides (classes) did not influence on adult survivorship, but all treatments caused significantly lower body weight than controls. Most binary mixtures at residue levels showed minor or no interaction on body weight loss, and synergistic interaction was detected only from the mixture of clothianidin + λ-cyhalothrin. Chlorpyrifos alone and the mixture of clothianidin + chlorpyrifos significantly suppressed esterase (EST) activity, while most treatments of individual pesticides and mixtures had no effect on EST and glutathione S-transferase (GST) activities. However, ingestion of clothianidin at 2.6 ppb significantly enhanced P450 oxidase activity by 19%. The LC_{50} of formulated clothianidin was estimated at 0.53 ppm active ingredient, which is equivalent to 25.4 ng clothianidin per bee (LD_{50}) based on the average sugar consumption of 24 µl per bee per day. In addition to mortality, ingestion of clothianidin at LC_{50} significantly reduced bee body weight by 12%. P450 activities were also significantly induced at 24 and 48 h in clothianidin-treated bees, while no significant difference was found in GST and EST activities. Further examinations revealed that the expression of an important *CYP9q1* detoxification gene was significantly induced by clothianidin. Thus, data consistently indicated that P450s were involved in clothianidin detoxification in honey bees. Although the honey bee population in Stoneville (MS, United States) had sixfold lower susceptibility than other reported populations, clothianidin had very high oral toxicity to bees.

Key words: clothianidin, honey bee, binary mixture, esterase, cytochrome P450

The impact of insecticides to honey bees, particularly neonicotinoid insecticides, has received substantial attention (Godfray et al. 2014, Sánchez-Bayo 2014). Seed treatment and root drenching are methods commonly used to apply neonicotinoids in the field. These insecticides systemically travel through plant tissues and protect all parts of the crops with high efficacy against some arthropod pests (Pisa et al. 2015). Neonicotinoids are also applied as foliar insecticides in the United States to control sucking insect pests, such as tarnished plant bugs (*Lygus lineolaris*), thrips, and the stink bug complex, and the foliar sprays pose the potential direct contact risks to foraging bees (Zhu et al. 2015).

Except thiamethoxam does not accumulate in soil (Hilton et al. 2015), most neonicotinoids are water soluble, and accumulate in soils, water puddles, and floral resources (Samson-Robert et al. 2015, Schaafsma et al. 2015). However, thiamethoxam converts to the more toxic compound clothianidin in plant and insect (Nauen et al. 2003). The concentration of neonicotinoids in agricultural water source, nectar, and pollen of crops might be sufficient to substantially impact honey bees (Botías et al. 2015, 2016; Samon-Robert 2015; Hladik et al. 2016). It was found that colonies still exhibited normal health when hive pollens were contaminated with 2.9 ppb clothianidin, but colony health deteriorated when clothianidin contamination reached 11 ppb (Krupke et al. 2012). The impaired learning memory and homing failure of honey bees were documented after exposure to sublethal doses (or environmental dosage) of neonicotinoids (Aliouane et al. 2009, Henry et al. 2012, Yang et al. 2012, Williamson and Wright 2013). However, other studies stated that risk of exposure to realistic field concentrations of neonicotinoids had very low or even no risk, because the laboratory and semi-field studies might not fully represent real field situations (Cutler and Scott-Dupree 2007, Cresswell et al. 2012). In realistic situation, actual exposure to pesticides for bees was worse than that of laboratory investigation because bees prefer to feed on sugar solutions contaminated with neonicotinoid (Kessler et al. 2015) and bees are also typically exposed to a cocktail of variety of pesticide residues. In addition, the pesticide exposure may cause bee colonies

more prone to pathogen infestations, or vice versa due to potential synergistic interaction between them (Vidau et al. 2011, Sánchez-Bayo et al. 2016).

To simulated field and in-hive situations, we used a formulated clothianidin (23.6% active ingredient) in this study to investigate the chronic toxicities at environmental dosage exposure (Johnson et al. 2010). To understand the potential additive or synergistic interactions, honey bee workers were treated with clothianidin alone and binary mixtures of clothianidin with five commonly used pesticides, pyrethroid (λ-cyhalothrin), organophosphate (chlorpyrifos), carbamate (oxamyl), fungicide (tetraconazole), and herbicide (glyphosate). Comparative activity assays of three detoxification enzymes were also conducted to reveal any physiological alternation in pesticide-treated honey bee workers. In addition, we increased clothianidin to LC_{50} level to verify the effects on body weight suppression and on induction of P450 oxidase activity and their gene regulations in clothianidin-treated bees.

Materials and Methods

Honey Bee Colony Maintenance

Bee colonies originally came from Mid-south local beekeepers in Arkansas and Mississippi. In total, 17 honey bee colonies were established and maintained using standard apiculture practices in an isolated space of Stoneville Wildlife Management Area (Stoneville, MS). Apivar mite control strips (Véto-pharma Inc., New York, NY) were applied twice in 2016 to suppress *Varroa destructor* (varroa) mite populations. In addition, an oil trap (35 × 45 cm tray filled with vegetable oil) was installed at the bottom of each colony for *Varroa* mite and small hive beetle (*Aethina tumida*) monitoring and control. From April to May of 2016, frames with more than 50% coverage of healthy broods were transferred to laboratory incubators (33 ± 0.5°C; 65% ± 3 RH; no light) (Winston 1987, Ellis 2008). Newly emerged workers were transferred daily into cages and were provided one scintillation vial of 50% sucrose solution and one scintillation vial of d-H_2O at the top of the cage. Caged bees were maintained in incubators at the conditions described above.

Chemicals

The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO): protease inhibitor (cocktail tablets), α-naphthyl acetate, fast blue B salt, 1-chloro-2,4-dinitrobenzene (CDNB), L-glutathione reduced (GSH), acetylthiocholine iodide (ATC), 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB), umbelliferone (7-hydroxycoumarin), 7-ethoxycoumarin (7-EC), oxidized glutathione (GSSG), glutathione reductase, β-nicotinamide adenine dinucleotide phosphate (reduced β-NADPH). Six formulated pesticides Belay (23.6% clothianidin, Valent), Karate (22.8% λ-cyhalothrin, Syngenta), Vydate 3.77CLV (42% oxamyl, DuPont), Lorsban 4E (44.6% chlorpyrifos, Dow), Domark 230ME (20.5% tetraconazole fungicide, Valent), and Roundup PowerMax (48.7% glyphosate herbicide, Monsanto) were purchased from local agricultural suppliers near Stoneville, MS (Table 1). The reason why we used commercial formulation (normally used by farmers), instead of technical grade of the pesticides, was better to simulate field situation and to include potential additive and/or synergistic toxicity to bees from formulating reagents (Zhu et al. 2014, Mullin et al. 2015).

Assay of Dose Responses of Bees to Clothianidin Oral Treatment

The acute toxicity of a formulated clothianidin (Belay 23.6% clothianidin) to 8-d-old honey bees was determined in a 48-h feeding bioassay. The assay consisted of triplicate cages (25 bees per cage) for each of six tested concentrations plus control (total 21 cages). Before feeding assay, tested bees were starved for 4 h, and then fed with a 50% sucrose solution containing clothianidin formulation at concentrations of 1.5, 2.4, 3.84, 6.14, 9.83, 15.73 mg/liter, as well as sugar solution-only as control. Mortality was recorded after honey bees were exposed via feeding to clothianidin-containing sugar solution for 48 h. The median lethal concentration (LC_{50}) of clothianidin formulation was determined using probit analysis (see details in Data Analysis section).

Testing Sublethal Oral Toxicities of Clothianidin Alone and Its Binary Mixtures With Five Pesticides

The residue concentrations of six commonly used pesticides were determined mostly according to the maximal detection levels by Johnson et al. (2010) and Mullin et al. (2010). Bøhn et al. (2014) and other internet source (http://www.fao.org/docrep/009/a0209e/a0209e0d.htm) were also referred for the concentrations of glyphosate in soybean used in this study. These concentrations included: clothianidin (Belay) at 0.011 mg/liter, λ-cyhalothrin (Karate) at 7.3 mg/liter, oxamyl (Vydate) at 0.179 mg/liter, tetraconazole (Domark) at 0.084 mg/liter, glyphosate (Roundup) at 35 mg/liter, and chlorpyrifos (Lorsban) at 1.86 mg/liter (Table 1). A total of 60 cages (25 bees per cage) were used in this feeding treatment experiment with five cages per individual insecticide or its binary combination with clothianidin. Fresh sugar solutions containing clothianidin and its binary mixtures were prepared weekly to replace old solutions.

Experimental Design for Testing Influences of Clothianidin on Enzymatic Activities and Body Weight Changes

As for median lethal exposure, the experiment was conducted with a clothianidin-treated sugar solution containing 2.25 mg/liter of Belay

Table 1. Pesticide name, manufacturer, percentage of active ingredient, and feeding treatment concentration

Common name	Commercial name	Manufacturer	Concentration of active ingredient (%)	In-hive residue levels[a] (ppb)	Feeding concentration (formulation mg/liter)
Clothianidin	Belay	Valent	23.6	2.6	0.011
λ-Cyhalothrin	Karate	Syngenta	22.8	1672	7.3
Oxamyl	Vydate	DuPont	42	75	0.179
Tetraconazole	Domark	Valent	20.5	17	0.084
Glyphosate	Roundup	Monsanto	48.7	17000	35
Chlorpyrifos	Lorsban	Dow AgroScience	44.6	830	1.86

[a]See Testing Sublethal Oral Toxicities of Clothianidin Alone and Its Binary Mixtures With Five Pesticides section for citations in Materials and Methods.

(approximately 0.53 ppm of clothianidin which was determined in this study with methods described in Assay of Dose Responses of Bees to Clothianidin Oral Treatment section and results in Acute Oral Toxicity of Clothianidin to Honey Bee Workers section) as the treatment, along with a sugar solution-only as control. Each treatment consisted of three replicates (cages) and each cage contained 30 honey bee workers at age of 3 d old.

To monitor how honey bees respond to sublethal and median lethal concentration of clothianidin (LC_{50}), three major midgut detoxification enzyme (esterase [EST], glutathione S-transferase [GST], and P450) activities were examined after 1-wk sublethal exposure, and a time course (6, 12, 24, 48, 72 h) of median lethal exposure, respectively (Yao et al. 2018). At each time-course point (feeding for 6, 12, 24, 48, and 72 h) of median lethal exposure, midguts of nine surviving bees were collected via dissections on ice. Three midguts were pooled as one sample and homogenized for EST, GST, and P450 activity assays and qRT-PCR analysis.

To measure the influence on bee body weight after clothianidin sublethal exposure 3-wk and median lethal (at LC_{50}) for 72 h, respectively, a group of six bees were weighed as one measurement and total seven measurements were conducted for each treatment using analytical balance.

Enzyme activity assays

Enzyme activities of EST, GST, and cytochrome monooxygenase (P450) were assayed in surviving bees after feeding treatments with clothianidin (at LC_{50}). Enzyme preparations and enzyme activity quantifications were processed according to the procedures of Zhu et al. (2017a). Relative enzyme activities were calculated as the ratio of the activity of clothianidin treatment to the activity of untreated control after 6-h feeding.

Analyzing clothianidin mediated midgut CYP gene expression

Previous phylogenetic studies have shown that 46 cytochrome P450 genes (*CYP*) from honey bees were clustered into four groups (*CYP3*, *CYP4*, *CYP2*, and mitochondrial *CYP*) (Claudianos et al. 2006). Here, four cytochrome P450 genes from *CYP3* clan (*CYP6A3*, *CYP6Q1*, *CYP9Q1*, and *CYP9Q2*) were selected based on their representatives of CYP detoxification families (Mao et al. 2011). Total RNA of each dissected midgut sample (three guts per sample) was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA), following the procedures from the manufacturer. Total RNA (1.0 µg) was first treated with DNase I (Invitrogen) to remove potential genomic DNA contamination. The treated total RNA (10 ng) was used as a template in Bio-Rad iScript one-step RT-qPCR reaction (Bio-Rad, Hercules, CA). RT-qPCR was performed with a reverse transcription (50°C for 5 m and 95°C for 1 m) followed by PCR amplifications for 40 cycles of 95°C for 5 s, 56°C for 30 s on Bio-Rad CFX Connect real-time system (Bio-Rad). The normalized abundance of *CYP* genes to internal reference gene (*actin*) was calculated (ΔCt) for clothianidin feeding and control samples. The relative transcript level of each *CYP* gene in clothianidin-treated bees (relative to that of untreated control) was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Data Analysis

SAS probit analysis (SAS/STAT 9.2 User's Guide, Cary, NC) was used to calculate LC_{50} values of clothianidin dose response (feeding) assay, and chi-square test was applied to ensure the goodness-of-fit of the model. The data of enzyme activities, mortality, and body weight were first tested to determine whether they were normally distributed using Minitab software, and then ANOVA was performed for mean separation (at $P < 0.05$) to determine 1) the statistical differences in mortality and body weight of honey bees after insecticide feeding; and 2) the difference of enzyme activities in bees after feeding treatments with different insecticides using post hoc Tukey test (McHugh 2011).

Results and Discussion

Acute Oral Toxicity of Clothianidin to Honey Bee Workers

The dose response assay revealed that the 48-h oral toxicity (LC_{50}) of Belay (23.6% clothianidin) was 2.25 ± 0.36 mg/liter with 95% CI [1.99, 2.51] in young adult bees ($Pr > \chi^2 = 0.084$) or approximately 0.53 ppm active ingredient of clothianidin. In neonicotinoids, chemical structure played a significant role in their toxicity against honey bees. The nitro-substituted neonicotinoids (clothianidin, dinotefuran, imidacloprid and its metabolites, thiamethoxam, nitenpyram) appear the most toxic to bees through both oral ingestion and contact, while the cyano-substituted compounds (acetamiprid and thiacloprid) exhibit a much lower toxicity (Decourtye and Devillets 2010). By using topical application, Iwasa et al. (2004) ranked the neonicotinoid insecticides based on their 24-h LD_{50} as follows: for the nitro group: imidacloprid (18 ng per bee), clothianidin (22 ng per bee), thiamethoxam (30 ng per bee); and for the cyano group: acetamiprid (7 µg per bee) and thiacloprid (15 µg per bee). Although Iwasa et al. (2004) provided some useful information for assessing neonicotinoid toxicity, certain aspects should be considered to improve the assessment, such as longer treatment times (>24 h) and treatment methods which may represent real situations of field pesticide exposures. Mostly, honey bees are exposed to insecticides through in-hive feeding on contaminated pollens and field sprays. In this study, we simulated in-hive feeding exposure by incorporating clothianidin into sugar solutions and the oral toxicity of clothianidin was 25.4 ± 4.1 ng per bee with 95% CI [22.5 ng per bee, 28.4 ng per bee] based on the average contaminated sugar solution consumption per bee per day is approximately 24 µl (Free and Spencer-Booth 1958, Alkassab and Kirchner 2016, Zhu et al. 2017b). Our data were closed to the bee population (LD_{50} = 26.9 ± 4.9) tested by Alkassab and Krichner (2016) in Germany. All data indicated clothianidin is a relatively high toxic neonicotinoid insecticide to honey bees.

By comparing our data with published data from other researchers, our honey bee population showed the higher tolerance than others. Laurino et al. (2011) reported that the 48-h oral toxicity (LC_{50}) of clothianidin to an Italian strain (*A. m. ligustica* strain) was 0.077 ppm (2.69 ng per bee), and the contact (spraying) toxicity was 2.9 ppm. The oral toxicities to our local bee population were 0.53 ppm (25.4 ng per bee). The susceptibility of our population to clothianidin was surprisingly (five- to ninefolds) lower than that of Italian strain (LD_{50} = 2.69 ng per bee) (Laurino et al. 2011) and the population (LD_{50} = 3.8 ng per bee) reported by EFSA (2013). Many factors might contribute to the variable clothianidin susceptibility in different honey bee populations, including different insecticide formulations, bee ages, seasons, and colony health levels (Blacquière et al. 2012, Steinmann et al. 2015). However, the genetic background of a population is an inevitable factor, which may result in different metabolic abilities to detoxify pesticides. The Mississippi Delta is a heavy crop production area that receives multiple pesticides applications annually, and the frequency of pesticide spraying is much higher (once per month or more on cotton) during crop growing seasons (Catchot et al. 2014). It is possible the environmental

residue levels of clothianidin in the Mid-south agricultural area of the United States are higher than in other cropping areas because of constant usage. As the same as other insect species, honey bees in this area have gone through constant insecticide selections, becoming more tolerant to insecticides. The reduced clothianidin susceptibility in our local honey bee population needs to be verified with multiple populations within the same area and technical grade chemical, although some formulating reagents may aggregate the toxicity of active ingredient (Zhu et al. 2014). In spite of the potential development of tolerance, our local bee population is still very sensitive to clothianidin, and it is still necessary to minimize clothianidin exposure during the crop blooming period. The situation, meanwhile, necessitates the monitoring of different pesticide contaminants inside bee hives in the area.

Sublethal Impacts of Clothianidin and Its Binary Mixtures (at Residue Concentration via Feeding) on Honey Bee Workers

Clothianidin was widely used for seed treatment or foliar spraying to control a variety of insect pests, particularly the sucking insects in Mid-south United States. The compound makes its way to pollens through both seed treatment and foliar sprays. Foraging bees may bring back insecticide-contaminated pollens and nectars back their hives as a major food source. Clothianidin residues in pollen samples (collected from diverse locations in Greece) ranged from 6.1 to 1,273 ng/g in pollen (Kasiotis et al. 2014). In this study, clothianidin at 2.6 ppb (approximately 11 ng/g) was used to assess its sublethal effect on honey bees, because the concentration represented a documented residue levels in North American apiaries by Johnson et al. (2010).

Effect on bee mortality

Synergistic/additive toxicity is a big concern for beekeepers, because using formulated premixtures and tank mixing are common practices in field crop pest control. Overall, no significant difference (F[11, 48] = 0.64, P = 0.786) was found in honey bee mortality between control and treatments of each individual pesticides or binary mixtures of clothianidin with other pesticide classes. Failure to detect acute toxicity and synergistic/additive effect of the clothianidin mixtures did not warrant the safety of clothianidin residue to honey bees, because increasing applications of clothianidin and sporadic detections of high concentrations of contaminants (Girolami et al. 2012, Kasiotis et al. 2014, Rundlöf et al. 2015) could certainly increase clothianidin concentration in bee bread, which might be high enough to kill exposed honey bees. Thereby, the clothianidin residue in bee pollens in the Mid-south agricultural areas might be higher than 2.6 ng/g (Johnson et al. 2010), or even higher than those reported by Dively (2012) and Sánchez-Bayo and Goka (2014), and synergistic toxicity would be detectable as previously observed in imidacloprid mixtures at LC_{20} (Zhu et al. 2017a).

Effect on bee body weight

Although the sublethal treatments of individual pesticides and mixtures did not incur higher mortality than control, three individual pesticides and all mixtures induced adverse sublethal effects on honey bee workers by reducing body weight in treated bees. With the exception of clothianidin, λ-cyhalothrin, and oxamyl, the treatments of other individual pesticides and five binary mixtures caused significant body weight loss (F[11, 51] = 2.43, P = 0.016) (Fig. 1). Interestingly, clothianidin only or λ-cyhalothrin only had no significant effect on body weight loss, but their binary mixture led to

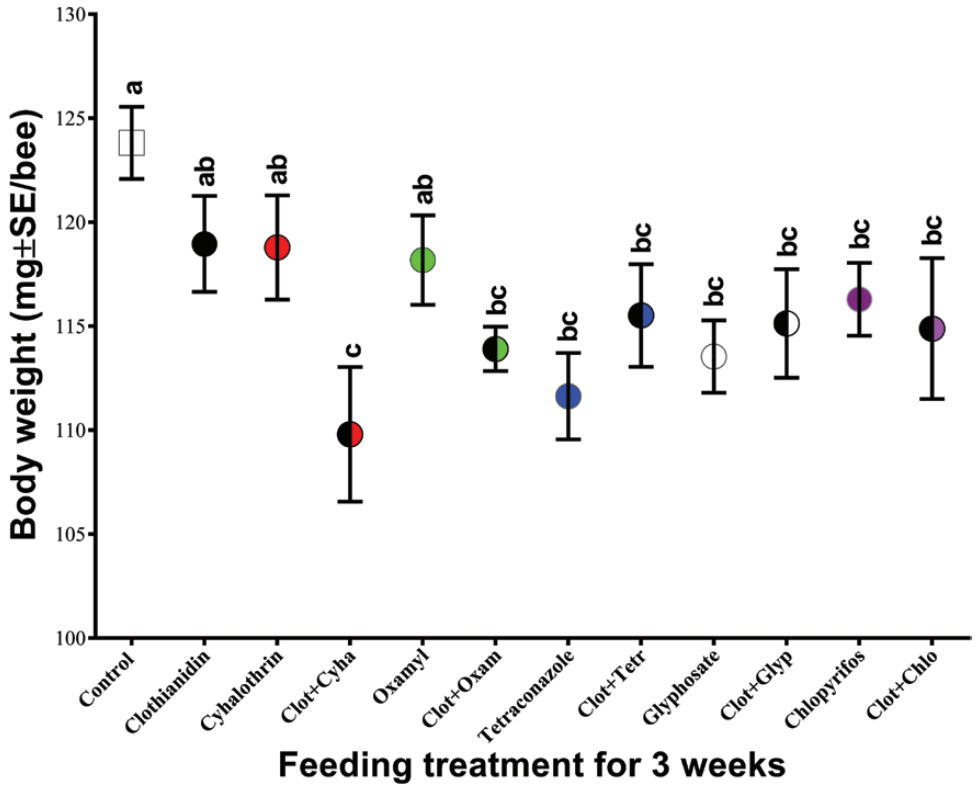

Fig. 1. Sublethal effects on honey bee workers body weight changes after 3 wk of feeding treatments with formulated clothianidin (Belay) alone and binary mixtures with five representative pesticides (classes) at residue concentrations. Means sharing no letter on the top of bars are significantly different ($P < 0.05$). Pesticide name abbreviation: Clot: clothianidin; Cyha: λ-cyhalothrin; Oxam: oxamyl; Tetr: tetraconazole; Glyp: glyphosate; and Chlo: chlorpyrifos.

significantly less body weight (Fig. 1). The interaction of two different insecticide classes (clothianidin + λ-cyhalothrin) synergistically and significantly ($P < 0.05$) reduced body weight in treated bees (Table 2). Intoxicated bees showed hyper activity, which may impair coordination and movement for finding food, and subsequently caused body weight loss in clothianidin-treated bees.

Effect on detoxification enzymes

Besides assessing sublethal impacts on body weight, we further examined effects of clothianidin and binary mixtures on honey bee physiology, specifically on the activity of three detoxification enzymes. Results showed that overall GST activity was not significantly influenced by any of individual insecticide treatments (Fig. 2A). But, the

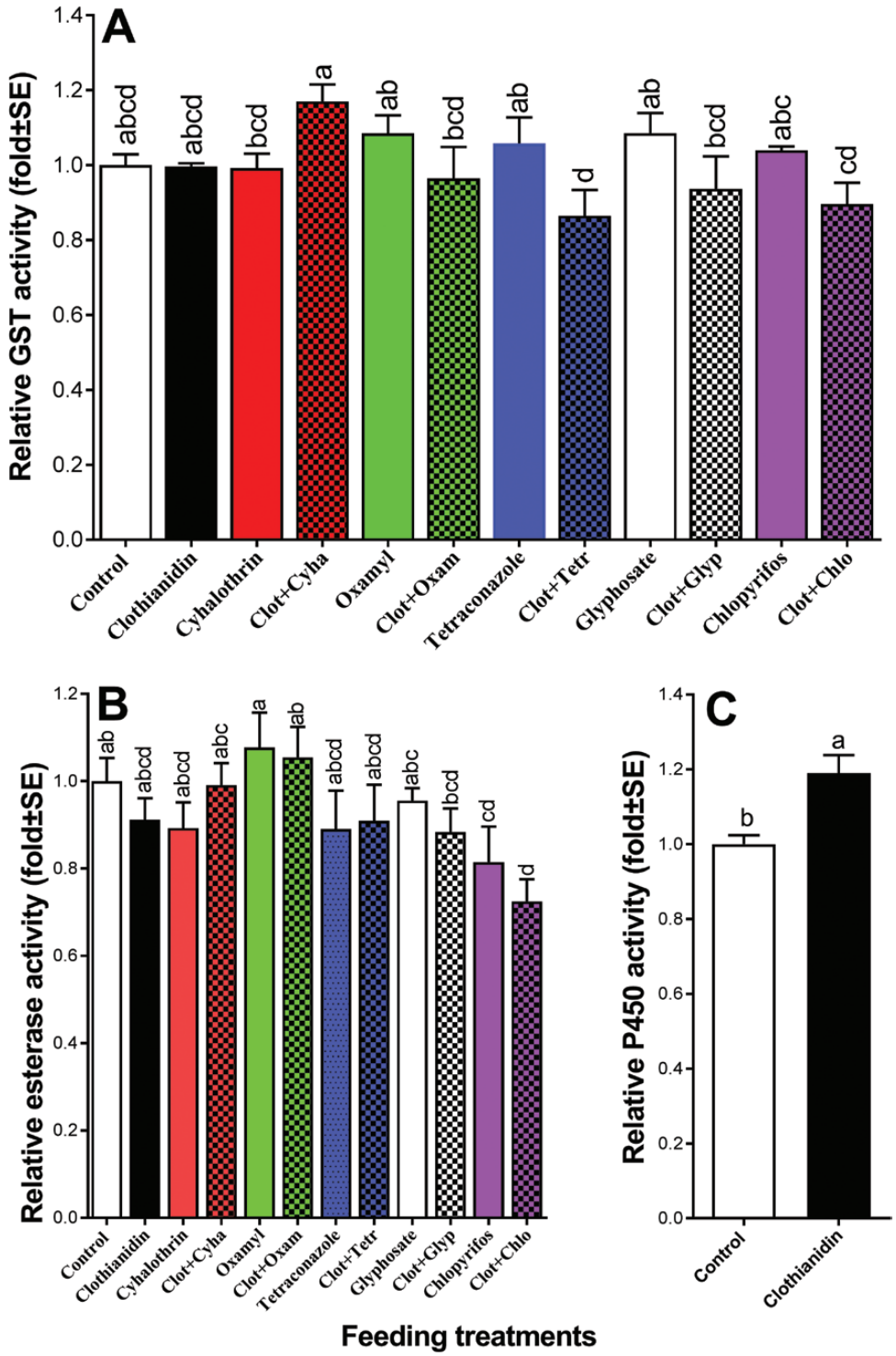

Fig. 2. Relative detoxification enzyme activities (to untreated control) in survivors of honeybee adults after feeding treatments with binary mixtures of clothianidin with five representative pesticides at residue concentrations for a week. (A) GST activities in honey bee adults; (B) EST activities in honey bees; (C) P450 oxidase activities in honey bee midguts. Means sharing no letter on the top of bars are significantly different ($P < 0.05$). Pesticide name abbreviation: Clot: clothianidin; Cyha: λ-cyhalothrin; Oxam: oxamyl; Tetr: tetraconazole; Glyp: glyphosate; and Chlo: chlorpyrifos.

bees treated with binary mixtures of clothianidin and λ-cyhalothrin exhibited the highest GST activity, while the bees treated with binary mixture of clothianidin and tetraconazole showed the lowest GST activity (F[11, 24] = 2.409, P = 0.035). EST activities (Fig. 2B) were only suppressed (F[11, 24] = 2.34, P = 0.04) in bees treated with chlorpyrifos and the binary mixture of chlorpyrifos and clothianidin, as expected given the mode of action of organophosphates (Fukuto 1990). P450 activities in honey bee midguts were significantly induced (t[4] = 3.548, P = 0.024) by 19% after a week feeding on clothianidin-containing sugar solution (Fig. 2C), suggests the potential involvement of P450s in clothianidin metabolic detoxification.

Impacts of Clothianidin (Belay at LC_{50} Dose via Feeding) on Honey Bee Workers

Effect on bee body weight

The exposure to the median lethal dose of Belay (2.25 mg/liter) caused significant (12%) body weight deduction (105.63 ± 2.76 mg per bee) than that of the control (118 ± 1.47 mg per bee) (t[12] = 3.615, P = 0.004) (Fig. 3). The data indicated that the impact of clothianidin on body weight was greater at LC_{50} than that at the lower residue concentration. We observed that neonicotinoids spraying at higher concentration coursed bees to fall down to the bottom of cages quicker than that the lower concentration. The hyper activity appeared earlier and last longer in treated bees with higher concentration compared to the lower concentration. Both falling to the bottom of cages and hyperactivity prevented bees from reaching food sources, and the treatment with higher concentration caused more weight loss than lower concentrations. In vertebrates, clothianidin intoxication caused the reductions of growth, development, and reproduction; and the impaired weight gain was associated with the reduction or cessation of feeding (Gibbons et al. 2015). Thereby, body weight loss might be a common phenomenon of orally treated bees with neonicotinoids. Similar situations were also observed in imidacloprid-treated bees that consumed significantly less sugar solution in a 2-wk exposure (Zhu et al. 2017b). In addition to the impact of clothianidin on body weight and mortality, we are optimizing a method for assessing whether those survivors are able to fly normally.

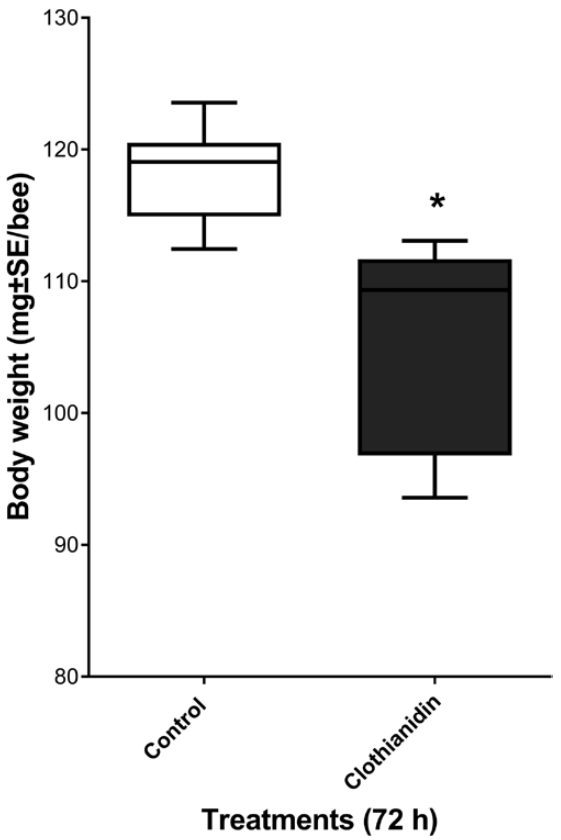

Fig. 3. Body weight (mg ± SE/bee) of honeybee survivors after 72 h of feeding on median lethal dose of clothianidin. The asterisk '*' on the top of mean bar indicates a significant difference from untreated control (P < 0.05).

Effect on midgut detoxification enzymes activities

Three major detoxification enzymes (EST, GST, and P450) were examined in honey bee midguts after ingestion of clothianidin-containing sugar solution at a LC_{50} dose. Data showed that, in general, EST (F[4, 16] = 0.994, P = 0.439) and GST (F[4, 16] = 3.299, P = 0.058) activities were not influenced by clothianidin feeding treatments (Fig. 4A and B). Conversely, the midgut P450 activity was significantly induced (F[1, 8] = 40, P = 0.002) by 34.5% at 24 h and 20.5% at 48 h, respectively (Fig. 4C). It is known that neonicotinoids undergo oxidative degradation and lead to nontoxic metabolites, and this was proposed as major resistance mechanism in many insects (Nauen and Denholm 2005). Clothianidin's metabolic process in mice includes N-methyl and O-methylene hydroxylation and nitro reduction, in which P450s and GST both act in nitro reduction and dechlorination (Ford and Casida 2006). Iwasa et al. (2004) demonstrated neonicotinoid detoxification was mainly through P450s oxidation in honey bees. The suppression of P450s by applying piperonyl butoxide could significantly synergize imidacloprid toxicity by 5.2-fold, while EST and GST inhibitors did not significantly influence the imidacloprid toxicity (Zhu et al. 2017a), which indicating the predominant role of P450s in neonicotinoid detoxification in honey bees. Similarly, the elevated P450s activity was also documented as resistance mechanism in many insect species, including *Lepitinotarsa decemlineata*, *Franklienella occidentalis*, *Bemisia tabaci*, *Musca domestica*, and *Blattella germinica* etc. (Casida and Durkin 2013). Therefore, the induction of P450 activity is an indication of increase of metabolic detoxification in honey bee guts after ingestion of clothianidin.

Effect on midgut CYP genes expression profiles

The importance of P450s prompted further examination to reveal whether the gene overexpression was involved in honey bee detoxification. We confirmed that the clothianidin feeding treatment led to the significant upregulations of *CYP9q1* and *CYP6a3* expressions (Fig. 5A) and the transcript level of *CYP9q1* was constitutively upregulated from 1- to 1.2-folds at 6 and 12 h of clothianidin feeding treatment, respectively (Fig. 5B). Mao et al. (2011) reported *CYP9q* was associated with acaricide detoxification and proposed that the upregulation of *CYPs* expressions might link to quick neonicotinoids detoxification in bees. The constitutive upregulation of *CYPs* expressions was linked with neonicotinoid resistance in *Myzus pericae* and *Bemisa tabaci* (Rauch and Nauen 2003, Puinean et al. 2010), e.g., the overexpression of *CYP6CY3* might be associated with neonicotinoid resistance in *M. pericae* too (Puinean et al. 2010). Thereby, the induction of P450 activity and upregulation of *CYPs* consistently supported the major role of P450s in neonicotinoid detoxification in honey bees.

In summary, this study and our previous study (Zhu et al. 2015) demonstrated a lower oral and contact susceptibility to clothianidin in honey bee population in the Delta region of Mississippi (United States) than those reported populations in other geographic locations

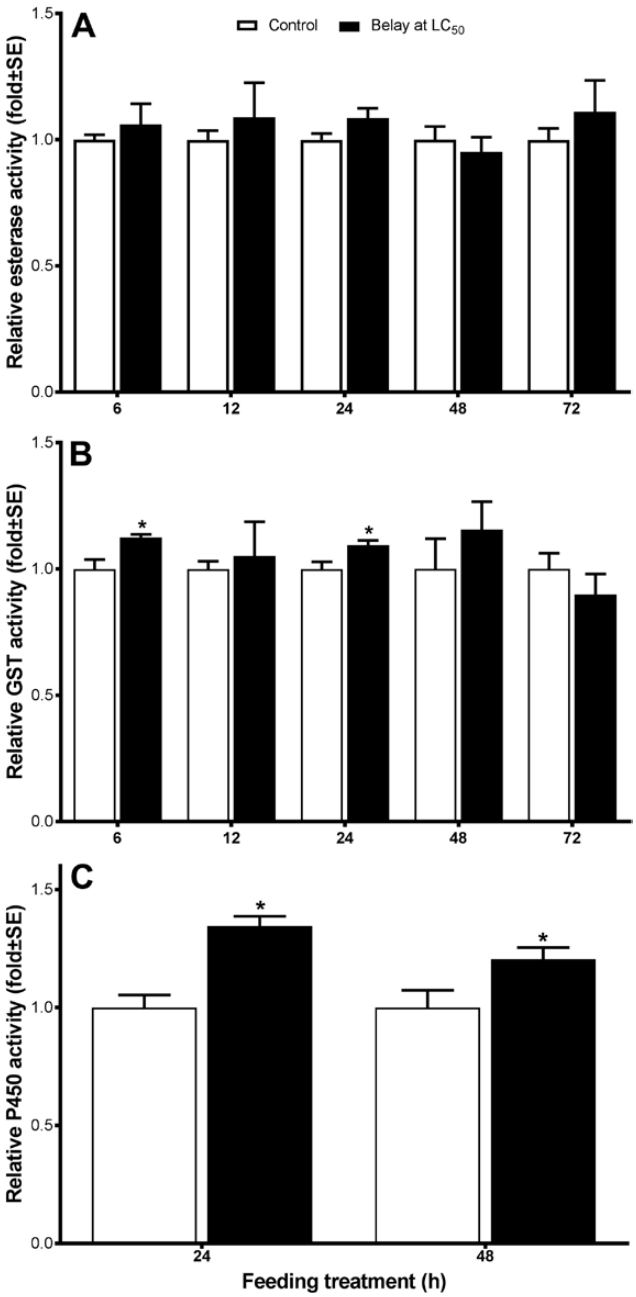

Fig. 4. Relative enzyme activities (to untreated control) in honey bee survivors after feeding treatment with clothianidin at LC_{50} for different time length. (A) EST activities in honeybee midgut; (B) GST activities in honeybee midgut; (C) P450 activities in honeybee midgut. The asterisks (*) indicate that treatment means differ significantly from control ($P < 0.05$) at different time points.

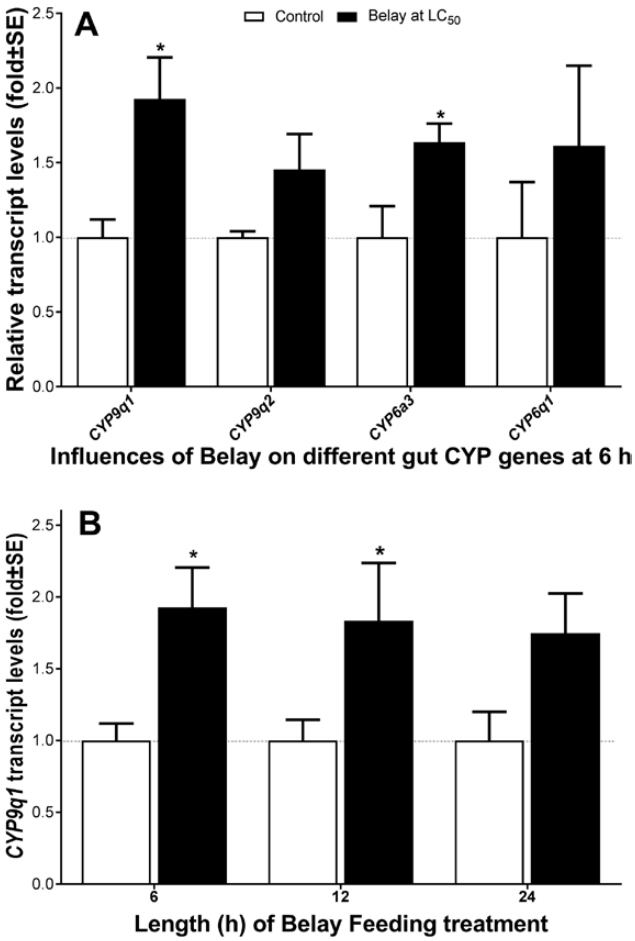

Fig. 5. Transcriptional changes of *CYP* genes in honey bee midguts after feeding treatment with clothianidin at LC_{50}. (A) Clothianidin mediated genes expressions of four midgut *CYPs*; (B) time-dependent regulation of midgut *CYP9q1* expression. The asterisks (*) indicate that treatment means differ significantly ($P < 0.05$) from untreated control.

Table 2. Pairwise comparison (Holm–Sidak multiple comparison test) of body weight loss in honey bees after 3-wk oral exposure to clothianidin

Treatments	Body weight/per bee (mg) ± SE	Statistics	*P*-value
Control vs clothianidin	123.8 ± 1.7 vs 119.0 ± 2.3	1.471	0.99
Control vs λ-cyhalothrin	123.8 ± 1.7 vs 118.8 ± 2.5	1.466	0.99
Control vs Clot + Cyha	123.8 ± 1.7 vs 109.8 ± 3.2	4.087	0.01*
Bela vs λ-cyhalothrin	119.0 ± 2.3 vs 118.8 ± 2.5	0.049	>0.99
Clothianidin vs Clot + Cyha	119.0 ± 2.3 vs 109.8 ± 3.2	2.67	0.46
λ-Cyhalothrin vs Clot + Cyha	118.8 ± 2.5 vs 109.8 ± 3.2	2.621	0.501

Clot = clothianidin; Cyha = λ-cyhalothrin. If *P*-value was ≤ 0.05, it indicated a significant difference between two treatments; otherwise, there was no significant difference.

*indicated the significant difference (Sgolastra et al., 2017) between control and the binary mixture (clothianidin and λ-cyhalothrin), suggesting the synergistic interaction existed between clothianidin and λ-cyhalothrin.

(Bailey et al. 2005, Laurino et al. 2011, EFSA 2013, Alkassab et al. 2016). The tolerance development in honey bees in the Mid-south region may fit the resistance evolution model of many insect pests due to frequent applications of a variety of pesticides. Besides testing sublethal effects of an important neonicotinoid insecticide, clothianidin, alone and its binary mixtures with five representative pesticides (classes), we also examined the adverse impact of clothianidin on honey bees at LC_{50} dose. Our data indicated that higher dose (LC_{50}) clothianidin significantly reduced honey bee body weight, but lower dose (2.6 ppb; Johnson et al. 2010) clothianidin alone did not have significant effect on body weight or mortality. However, the mixing of lower dose (reported residual levels) of clothianidin with λ-cyhalothrin showed synergism led to weight loss in bees, suggesting an interaction happened between two insecticides that have

different modes of actions. Finally, we detected a consistently higher P450 oxidase activity in clothianidin-treated bees at both residue and median lethal doses. Whether the induction of P450 was caused or/and correlated with neonicotinoid selection pressure is worth to conduct further examination to understand the role of specific P450s in clothianidin intoxication and detoxification.

Acknowledgments

The authors are grateful to Dr. Clint Allen and Dr. Nathan Little of USDA-ARS in Stoneville, MS, United States for their valuable comments and suggestions to improve this manuscript. The authors also appreciate the laboratory assistance from Joel Caren, John Wiltcher, and Kenneth Adam Wells. Mention of a proprietary product does not constitute a recommendation or endorsement by the USDA. J.Y. and Y.C.Z. conceived and designed experiments; J.Y. and Y.C.Z. performed experiments; J.Y. and Y.C.Z. analyzed data; Y.C.Z. contributed reagents/material/analysis tools; J.Y., Y.C.Z., and J.A. wrote the paper.

References Cited

Aliouane, Y., A. K. El Hassani, V. Gary, C. Armengaud, M. Lambin, and M. Gauthier. 2009. Subchronic exposure of honeybees to sublethal doses of pesticides: effects on behavior. Environ. Toxicol. Chem. 28: 113–122.

Alkassab, A. T., and W. H. Kirchner. 2016. Impacts of chronic sublethal exposure to clothianidin on winter honeybees. Ecotoxicology. 25: 1000–1010.

Bailey J., C. Scott-Dupree, R. Harris, J. Tolman, and Harris B. 2005. Contact and oral toxicity to honey bees (*Apis mellifera*) of agents registered for use for sweet corn insect control in Ontario, Canada. Apidologie. 36: 623–633.

Blacquière, T., G. Smagghe, C. A. van Gestel, and V. Mommaerts. 2012. Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment. Ecotoxicology. 21: 973–992.

Bøhn, T., M. Cuhra, T. Traavik, M. Sanden, J. Fagan, and R. Primicerio. 2014. Compositional differences in soybeans on the market: glyphosate accumulates in Roundup Ready GM soybeans. Food Chem. 153: 207–215.

Botías, C., A. David, J. Horwood, A. Abdul-Sada, E. Nicholls, E. Hill, and D. Goulson. 2015. Neonicotinoid residues in wildflowers, a potential route of chronic exposure for bees. Environ. Sci. Technol. 49: 12731–12740.

Botías, C., A. David, E. M. Hill, and D. Goulson. 2016. Contamination of wild plants near neonicotinoid seed-treated crops, and implications for non-target insects. Sci. Total Environ. 566–567: 269–278.

Casida, J. E., and K. A. Durkin. 2013. Neuroactive insecticides: targets, selectivity, resistance, and secondary effects. Annu. Rev. Entomol. 58: 99–117.

Catchot, A., C. Allen, D. Cook, D. Dodds, J. Gore, T. Irby, E. Larson, B. Layton, S. Meyers, and F. Musser. 2014. Insect control guide for agronomic crops. Insect Control Guide Committee, Mississippi State University Extension Service Publication 2471, Starkville, MS.

Claudianos, C., H. Ranson, R. M. Johnson, S. Biswas, M. A. Schuler, M. R. Berenbaum, R. Feyereisen, and J. G. Oakeshott. 2006. A deficit of detoxification enzymes: pesticide sensitivity and environmental response in the honeybee. Insect Mol. Biol. 15: 615–636.

Cresswell, J. E., C. J. Page, M. B. Uygun, M. Holmbergh, Y. Li, J. G. Wheeler, I. Laycock, C. J. Pook, N. H. de Ibarra, N. Smirnoff, et al. 2012. Differential sensitivity of honey bees and bumble bees to a dietary insecticide (imidacloprid). Zoology. 115: 365–371.

Cutler, G. C., and C. D. Scott-Dupree. 2007. Exposure to clothianidin seed-treated canola has no long-term impact on honey bees. J. Econ. Entomol. 100: 765–772.

Decourtye A., Devillers J. 2010. Ecotoxicity of neonicotinoid insecticides to bees. *In* Thany S.H. (ed.), Insect nicotinic acetylcholine receptors. Advances in experimental medicine and biology, vol. 683. Springer, New York, NY.

Dively, G. P. 2012. Insecticide residues in pollen and nectar of a cucurbit crop and their potential exposure to pollinators. J. Agric. Food Chem. 60: 4449–4456.

EFSA. 2013. Conclusion on the peer review of the pesticide risk assessment for bees for the active substance clothianidin. EFSA J. 11: 3066.

Ellis, M. B. 2008. Homeostasis: humidity and water relation in honeybee colony. Master thesis, University of Pretoria, Pretoria, South Africa.

Ford, K. A., and J. E. Casida. 2006. Unique and common metabolites of thiamethoxam, clothianidin, and dinotefuran in mice. Chem. Res. Toxicol. 19: 1549–1556.

Free, J. B., and Y. Spencer-Booth. 1958. Observations on the temperature regulation and food consumption of honeybees (*Apis Mellifera*). J. Exp. Biol. 35: 930–937.

Fukuto, T. R. 1990. Mechanism of action of organophosphorus and carbamate insecticides. Environ. Health Perspect. 87: 245–254.

Gibbons, D., C. Morrissey, and P. Mineau. 2015. A review of the direct and indirect effects of neonicotinoids and fipronil on vertebrate wildlife. Environ. Sci. Pollut. Res. Int. 22: 103–118.

Girolami, V., M. Marzaro, L. Vivan, L. Mazzon, M. Greatti, C. Giorio, D. Marton, and A. Tapparo. 2012. Fatal powdering of bees in flight with particulates of neonicotinoids seed coating and humidity implication. J. Appl. Entomol. 136: 17–26.

Godfray, H. C. J., T. Blacquière, L. M. Field, R. S. Hails, G. Petrokofsky, S. G. Potts, N. E. Raine, A. J. Vanbergen, and A. R. McLean. 2014. A restatement of the natural science evidence base concerning neonicotinoid insecticides and insect pollinators. Proc. R. Soc. B Biol. Sci. 281: e0558.

Henry, M., M. Béguin, F. Requier, O. Rollin, J. F. Odoux, P. Aupinel, J. Aptel, S. Tchamitchian, and A. Decourtye. 2012. A common pesticide decreases foraging success and survival in honey bees. Science. 336: 348–350.

Hilton, M. J., T. D. Jarvis, and D. C. Ricketts. 2015. The degradation rate of thiamethoxam in European field studies. Pest Manag. Sci. 72: 388–397.

Hladik, M. L., M. Vandever, and K. L. Smalling. 2016. Exposure of native bees foraging in an agricultural landscape to current-use pesticides. Sci. Total Environ. 542: 469–477.

Iwasa, T., N. Motoyama, J. T. Ambrose, and R. M. Roe. 2004. Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera*. Crop Prot. 23: 371–378.

Johnson, R. M., M. D. Ellis, C.A. Mullin, M. Frazier. 2010. Pesticides and honey bee toxicity–USA. Apidologie. 41: 312–331.

Kasiotis, K. M., C. Anagnostopoulos, P. Anastasiadou, and K. Machera. 2014. Pesticide residues in honeybees, honey and bee pollen by LC-MS/MS screening: reported death incidents in honeybees. Sci. Total Environ. 485–486: 633–642.

Kessler, S., E. J. Tiedeken, K. L. Simcock, S. Derveau, J. Mitchell, S. Softley, J. C. Stout, and G. A. Wright. 2015. Bees prefer foods containing neonicotinoid pesticides. Nature. 521: 74–76.

Krupke, C. H., G. J. Hunt, B. D. Eitzer, G. Andino, and K. Given. 2012. Multiple routes of pesticide exposure for honey bees living near agricultural fields. PLoS One. 7: e29268.

Laurino, D., M. Porporato, A. Patetta, and A. Manino. 2011. Toxicity of neonicotinoid insecticides to honey bees: laboratory tests. Bull. Insectol. 64: 107–113.

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods. 25: 402–408.

Mao, W., M. A. Schuler, and M. R. Berenbaum. 2011. CYP9Q-mediated detoxification of acaricides in the honey bee (*Apis mellifera*). Proc. Natl. Acad. Sci. USA. 108: 12657–12662.

McHugh, M. L. 2011. Multiple comparison analysis testing in ANOVA. Biochem. Med. 21: 203–209.

Mullin, C. A., M. Frazier, J. L. Frazier, S. Ashcraft, R. Simonds, D. Vanengelsdorp, and J. S. Pettis. 2010. High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health. PLoS One. 5: e9754.

Mullin, C. A., J. Chen, J. D. Fine, M. T. Frazier, and J. L. Frazier. 2015. The formulation makes the honey bee poison. Pest. Biochem. Physiol. 120: 27–35.

Nauen, R., and I. Denholm. 2005. Resistance of insect pests to neonicotinoid insecticides: current status and future prospects. Arch. Insect Biochem. Physiol. 58: 200–215.

Nauen, R., U. Ebbinghaus-Kintscher, V. L. Salgado, and M. Kaussmann. 2003. Thiamethoxam is a neonicotinoid precursor converted to clothianidin in insects and plants. Pest. Biochem. Physiol. 76: 55–69.

Pisa, L. W., V. Amaral-Rogers, L. P. Belzunces, J. M. Bonmatin, C. A. Downs, D. Goulson, D. P. Kreutzweiser, C. Krupke, M. Liess, M. McField, et al. 2015. Effects of neonicotinoids and fipronil on non-target invertebrates. Environ. Sci. Pollut. Res. Int. 22: 68–102.

Puinean, A. M., S. P. Foster, L. Oliphant, I. Denholm, L. M. Field, N. S. Millar, M. S. Williamson, and C. Bass. 2010. Amplification of a cytochrome P450 gene is associated with resistance to neonicotinoid insecticides in the aphid *Myzus persicae*. PLoS Genet. 6: e1000999.

Rauch, N., and R. Nauen. 2003. Identification of biochemical markers linked to neonicotinoid cross resistance in *Bemisia tabaci* (Hemiptera: Aleyrodidae). Arch. Insect Biochem. Physiol. 54: 165–176.

Rundlöf, M., G. K. Andersson, R. Bommarco, I. Fries, V. Hederström, L. Herbertsson, O. Jonsson, B. K. Klatt, T. R. Pedersen, J. Yourstone, et al. 2015. Seed coating with a neonicotinoid insecticide negatively affects wild bees. Nature. 521: 77–80.

Samson-Robert, O., G. Labrie, M. Chagnon, and V. Fournier. 2015. Neonicotinoid-contaminated puddles of water represent a risk of intoxication for honey bees. PLoS One. 9: e108443.

Sánchez-Bayo, F. 2014. The trouble with neonicotinoids. Science. 346: 806–807.

Sánchez-Bayo, F., and K. Goka. 2014. Pesticide residues and bees–a risk assessment. PLoS One. 9: e94482.

Sánchez-Bayo, F., D. Goulson, F. Pennacchio, F. Nazzi, K. Goka, and N. Desneux. 2016. Are bee diseases linked to pesticides?—a brief review. Environ. Int. 89–90: 7–11.

Schaafsma, A., V. Limay-Rios, T. Baute, J. Smith, and Y. Xue. 2015. Neonicotinoid insecticide residues in surface water and soil associated with commercial maize (corn) fields in southwestern Ontario. PLoS One. 10: e0118139.

Sgolastra, F., P. Medrzycki, L. Bortolotti, M. T. Renzi, S. Tosi, G. Bogo, D. Teper, C. Porrini, R. Molowny-Horas, and J. Bosch. 2017. Synergistic mortality between a neonicotinoid insecticide and an ergosterol-biosynthesis-inhibiting fungicide in three bee species. Pest Manag Sci. 73: 1236-1243.

Steinmann, N., M. Corona, P. Neumann, and B. Dainat. 2015. Overwintering is associated with reduced expression of immune genes and higher susceptibility to virus infection in honey bees. PLoS One. 10: e0129956.

Vidau, C., M. Diogon, J. Aufauvre, R. Fontbonne, B. Viguès, J. L. Brunet, C. Texier, D. G. Biron, N. Blot, H. El Alaoui, et al. 2011. Exposure to sublethal doses of fipronil and thiacloprid highly increases mortality of honeybees previously infected by *Nosema ceranae*. PLoS One. 6: e21550.

Williamson, S. M., and G. A. Wright. 2013. Exposure to multiple cholinergic pesticides impairs olfactory learning and memory in honeybees. J. Exp. Biol. 216: 1799–1807.

Winston, M. L. 1987. The biology of the honey bee. Harvard University Press, Cambridge, MA.

Yang, E. C., H. C. Chang, W. Y. Wu, and Y. W. Chen. 2012. Impaired olfactory associative behavior of honeybee workers due to contamination of imidacloprid in the larval stage. PLoS One. 7: e49472.

Yao, J., Y. C. Zhu, J. Adamczyk, and R. Luttrell. 2018. Influences of acephate and mixtures with other commonly used pesticides on honey bee (*Apis mellifera*) survival and detoxification enzyme activities. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 209: 9–17.

Zhu, W., D. R. Schmehl, C. A. Mullin, and J. L. Frazier. 2014. Four common pesticides, their mixtures and a formulation solvent in the hive environment have high oral toxicity to honey bee larvae. PLoS One. 9: e77547.

Zhu, Y. C., J. Adamczyk, T. Rinderer, J. Yao, R. Danka, R. Luttrell, and J. Gore. 2015. Spray toxicity and risk potential of 42 commonly used formulations of row crop pesticides to adult honey bees (Hymenoptera: Apidae). J. Econ. Entomol. 108: 2640–2647.

Zhu, Y. C., J. Yao, J. Adamczyk, and R. Luttrell. 2017a. Synergistic toxicity and physiological impact of imidacloprid alone and binary mixtures with seven representative pesticides on honey bee (*Apis mellifera*). PLoS One. 12: e0176837.

Zhu, Y. C., J. Yao, J. Adamczyk, and R. Luttrell. 2017b. Feeding toxicity and impact of imidacloprid formulation and mixtures with six representative pesticides at residue concentrations on honey bee physiology (*Apis mellifera*). PLoS One. 12: e0178421.