



## Toxicity and motor changes in Africanized honey bees (*Apis mellifera* L.) exposed to fipronil and imidacloprid

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*Manuscript received on March 16, 2015; accepted for publication on November 27, 2015*

### ABSTRACT

This study evaluated the *in vitro* toxicity and motor activity changes in African-derived adult honey bees (*Apis mellifera* L.) exposed to lethal or sublethal doses of the insecticides fipronil and imidacloprid. Mortality of bees was assessed to determine the ingestion and contact lethal dose for 24 h using probit analysis. Motor activities in bees exposed to lethal ( $LD_{50}$ ) and sublethal doses ( $1/500^{\text{th}}$  of the lethal dose) of both insecticides were evaluated in a behavioral observation box at 1 and 4 h. Ingestion and contact lethal doses of fipronil were  $0.2316 \pm 0.0626$  and  $0.0080 \pm 0.0021$   $\mu\text{g}/\text{bee}$ , respectively. Ingestion and contact lethal doses of imidacloprid were  $0.1079 \pm 0.0375$  and  $0.0308 \pm 0.0218$   $\mu\text{g}/\text{bee}$ , respectively. Motor function of bees exposed to lethal doses of fipronil and imidacloprid was impaired; exposure to sublethal doses of fipronil but not imidacloprid impaired motor function. The insecticides evaluated in this study were highly toxic to African-derived *A. mellifera* and caused impaired motor function in these pollinators.

**Key words:** environment, pesticides, pollinators, contamination, phenylpyrazoles, neonicotinoids.

### INTRODUCTION

Honey bees are very important pollinators of crops and native vegetation, as well as producers of honey and other goods. Bees promote the cross-pollination of vegetables, increasing genetic diversity in species and improving the production of fruit and seeds (Klein et al. 2007). The value of pollination to agriculture, provided primarily by bees, is >\$200 billion per annum worldwide, and in natural ecosystems it is thought to be even greater (Lebuhn et al. 2013).

Managed bee colonies have suffered considerable losses in recent years, and reduced diversity of native bees has been observed worldwide (Oldroyd 2007, Stokstad 2007, Van Engelsdorp and Meixner 2010). A number of potential causes of reduced bee populations globally have been investigated, including habitat destruction and the scarcity of floral sources, the presence of pathogens and parasites (Oldroyd 2007, Williams et al. 2010), climate changes (Van Engelsdorp and Meixner 2010), improper management, queen-related issues (Williams et al. 2010), and high use of pesticides and adjuvants (Van Engelsdorp et al. 2009, Ciarlo

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et al. 2012). An additional cause of bee colony loss is the syndrome Colony Collapse Disorder (CCD). Colony Collapse Disorder is associated with multiple factors and characterized by rapid losses of worker bees, with few or no dead bees in or near the hives, abandoned brood and stored food, and delayed invasion of hive pests, has caused high colony losses (Oldroyd 2007, Van Engelsdorp et al. 2009).

Some colony losses are normal in beekeeping; however, it is clear that about once per decade, apiarists suffer unusually heavy colony losses, and there is a lack of understanding of the factors contributing to these declines (Oldroyd 2007). A recent major shift in agriculture has been the development and extensive deployment of phenylpyrazole and neonicotinoid pesticides that replace many of the older pesticides. These recently developed pesticides are used extensively on field, vegetable, turf, and ornamental crops, some of which are pollinated by bees (Ellis 2010). Due the high toxicity of these insecticides, some countries have banned the use of phenylpyrazoles (Ghisi et al. 2011), and the European Union restricts the use of three neonicotinoids (clothianidin, imidacloprid, and thiametoxam) for a period of two years, to review the risk of these pesticides to bees (Environmental Protection Agency 2015).

Other groups have demonstrated pesticide toxicity in *A. mellifera* and have implicated the role of pesticides in the decline of bee populations. The exposure of bees to pesticides has been associated with changes in behavior (Schneider et al. 2012, Henry et al. 2012, Zaluski et al. 2015), reductions in colony queen production (Whitehorn et al. 2012), morphologic alterations (Silva Cruz et al. 2010), foraging and survival reduction (Henry et al. 2012), damage to mitochondrial bioenergetics (Nicodemo et al. 2014), and negative impacts on honey bee health (Boncristiani et al. 2012).

Determining the toxicity of systemic pesticides such as phenylpyrazoles and neonicotinoids on

honey bees is essential to establishing guidelines for controlled use in crops pollinated by bees. Our study was designed to establish the contact and ingestion lethal doses ( $LD_{50}$ ) of fipronil and imidacloprid for African-derived *Apis mellifera* and to assess the motor changes in bees having ingested or contacted a lethal dose ( $LD_{50}$ ) or a sublethal dose ( $1/500^{\text{th}}$  of the  $LD_{50}$ ) of these insecticides.

## MATERIALS AND METHODS

These experiments were conducted at the Beekeeping Production Area of Lageado Experimental Farm, Faculty of Veterinary Medicine and Animal Science, UNESP, Botucatu, São Paulo, Brazil,  $22^{\circ}50'30.16''\text{S}$ ;  $48^{\circ}25'41.90''\text{W}$ , with a humid subtropical (Cfa) climate and an average elevation of 623 m.

We utilized foragers of African-derived *A. mellifera* L. (Hymenoptera: Apidae), collected from five colonies free of diseases or parasites, each with a naturally mated queen. These bees initiate foraging when they are approximately 23 days old (Winston 1991). A bee trap was installed in the entrance of the beehive and was closed during the collection of bees; thus, only bees that returned from the field (foragers) were collected. A total of 720 bees was collected between 7:00 a.m. and 8:00 a.m. and anesthetized in a freezer at  $-10^{\circ}\text{C}$  for 1–2 min (Zaluski et al. 2015). Two forms of exposure were used to assess fipronil and imidacloprid toxicity: ingestion of contaminated food and contact with the diluted insecticide.

We utilized the active ingredient (a.i.) fipronil from the commercial formulation used in the field: Regent<sup>®</sup> 800WG–800 g a.i. fipronil/kg–(80% m/m); Inert Ingredients – 200 g/kg (20% m/m) (BASF Agri-Production SAS) as well as imidacloprid from the commercial formulation Appalus<sup>®</sup> 200 SC–200 g a.i. imidacloprid/L– (20% m/v); Inert Ingredients–892 g/L (89.20% m/v) (Consagro Agroquímica Ltda). Solutions of  $1\text{ g L}^{-1}$  Regent<sup>®</sup>

800WG and Appalus® 200 SC were prepared separately in distilled water and the doses used in all tests were diluted from this solution. For all dilutions used in the present study, we considered only the amount of a.i. fipronil or imidacloprid, not the amount of inert ingredients. No solubility problems were observed. The solutions were stirred vigorously during preparation and prior to use to ensure that they were always at the same concentration.

The oral LD<sub>50</sub> was determined according to the methodology described by Miranda et al. (2003) with modifications proposed by Zaluski et al. (2015). Bees were placed in disposable wooden boxes (25.0 × 15.0 × 10.0 cm), with screens on the sides. They remained unfed for 3 h and then received 1 mL of food (50% honey syrup) in a plastic tube (50 × 10 × 10 mm). The consumption of 50 µL, the average uptake by one bee (Crane 1990), was associated with the consumption of 0.00; 0.05; 0.10; 0.20; 0.40, or 0.80 µg of fipronil; and 0.000; 0.025; 0.050; 0.100; 0.200, or 0.400 µg of imidacloprid. The contaminated food was provided for 3 h and was then replaced with uncontaminated sugar syrup. The amount of uneaten contaminated food was recorded to check the approximate dose ingested by bees in each box.

To determine the amount of contact constituting the LD<sub>50</sub> dose, adult bees were collected, anesthetized, and transferred to a cage (a disposable PET plastic tray, 25 × 15 × 10 cm). They received 2 µL of solution containing different amounts of fipronil (0.000; 0.002; 0.004; 0.008; 0.016, or 0.032 µg); or imidacloprid (0.000; 0.005; 0.010; 0.020; 0.040, or 0.080 µg) on the thorax, applied with an automatic micropipette (Mettler Toledo Company). Bees received sugar syrup ad libitum during all contact tests (Zaluski et al. 2015).

The doses used to calculate the LD<sub>50</sub> were based on preliminary tests. We tested the responses of 10 bees to each dose to determine the ingestion and contact LD<sub>50</sub>, and all tests were performed in

triplicate. Bees that showed behavioral alterations or lethargy before the tests were rejected and replaced by healthy bees. Bees were kept in the dark after consumption of the contaminated food, at room temperature (25 ± 1 °C), with humidity between 60 and 65%. The number of dead bees in each treatment was recorded 24 h after the beginning of the tests, and the results were used to calculate the LD<sub>50</sub> (Zaluski et al. 2015). In all tests, the mortality in the control groups was less than 5%.

To study motor function in bees exposed to insecticides, we collected 480 adult bees and exposed them by ingestion and contact to an LD<sub>50</sub> or sublethal dose of fipronil or imidacloprid. The sublethal dose supplied to bees corresponded to 1/500<sup>th</sup> of the LD<sub>50</sub> of ingestion (assuming that a bee ingested 50 µL of syrup); and contact determined in the present study. Fipronil LD<sub>50</sub> of ingestion and contact were 0.2316 and 0.0080 µg/bee, respectively; and sublethal doses of ingestion and contact were 0.0004 and 0.000016 µg/bee, respectively. Imidacloprid LD<sub>50</sub> doses of ingestion and contact were 0.1079 and 0.0308 µg/bee, respectively; and sublethal doses of ingestion and contact were 0.0002 and 0.00006 µg/bee, respectively. The collection and exposure of bees were performed as described for the LD<sub>50</sub> measurements.

The motor activity of bees was assessed at 1 and 4 h after exposure, according to the methodology described in Zaluski et al. (2015). The tests were performed in the laboratory using a wooden box (60 × 35 × 04 cm) divided into 5 lanes (50 × 05 × 04 cm), containing a fluorescent lamp in the top and covered with glass through which the bees could be observed. The tests were performed in the dark, with the box tilted at 45° and the lamp turned on, stimulating locomotion of the bees by positive phototaxis (Lambin et al. 2001). The bees were released into the box, one per lane, and the time that it took each bee to travel 50 cm was recorded.

For each tested dose and each time assessed, 10 bees were exposed to fipronil and imidacloprid, and 10 served as controls. All tests were performed in triplicate.

The ingestion and contact LD<sub>50</sub> were determined on the basis of mortality of bees per dose, using probit analysis with maximum likelihood. The results of the motor activity analyses were first tested for normality (Anderson–Darling test) and homogeneous variance (Levene’s test); if significant deviations were detected ( $p < 0.05$ ), the data were compared by non-parametric Mann–Whitney  $U$  test and presented as the median and interquartile intervals (Q1–Q3). A  $p$  value of less than 0.05 was considered significant. Data analyses were performed using Minitab statistical software (v. 17, State College, PA).

## RESULTS

The mean ingestion and contact LD<sub>50</sub> (24 h) of fipronil and imidacloprid for African-derived *A. mellifera* are  $0.2316 \pm 0.0626$  and  $0.0080 \pm 0.0021$  µg/bee; and  $0.1079 \pm 0.0375$  and  $0.0308 \pm 0.0218$  µg/bee, respectively (Table I). Both insecticides were more toxic when administered by contact than by ingestion.

Bees that received the LD<sub>50</sub> of fipronil or imidacloprid by either route took longer to walk through the 50-cm track in motor activity tests than did bees in the control groups at all observation times (Table II). Bees that received the sublethal dose of fipronil by either route took longer to walk through the 50-cm track in motor activity tests than did bees in the control groups at all observation times. No differences were observed after ingestion or contact exposure to the sublethal dose of imidacloprid (Table II).

## DISCUSSION

In this study, we showed that fipronil and imidacloprid insecticides are highly toxic and

**TABLE I**  
Mean ingestion and contact lethal doses (LD<sub>50</sub> at 24 h) (µg/bee) and slopes in the probit analysis of the insecticides fipronil and imidacloprid for African-derived *Apis mellifera*.

Insecticide	Ingestion LD <sub>50</sub>	Slope	Contact LD <sub>50</sub>	Slope
Fipronil	$0.2316 \pm 0.0626$	1.93	$0.0080 \pm 0.0021$	1.87
Imidacloprid	$0.1079 \pm 0.0375$	1.39	$0.0308 \pm 0.0218$	1.27

Data are presented as the mean and standard deviations.

**TABLE II**  
Time (seconds) spent by African-derived *Apis mellifera* walking along a 50-cm track after exposure to the lethal dose (LD<sub>50</sub>) or sublethal dose (1/500<sup>th</sup> of the LD<sub>50</sub>) of fipronil or imidacloprid, by ingestion or contact, at 1 and 4 h after exposure.

Treatment	Lethal Dose (LD <sub>50</sub> )		Sublethal Dose	
	1 h	4 h	1 h	4 h
<b>Control</b>	10 (5–12)	8 (4–12)	4 (4–5)	7 (5–7)
<b>Ingestion fipronil</b>	20 (14–26)*	13 (10–17)*	8 (7–10)*	12 (8–14)*
<b>Control</b>	7 (5–15)	7 (5–15)	5 (4–6)	6 (5–7)
<b>Contact fipronil</b>	22 (15–38)*	21 (10–39)*	12 (9–13)*	9 (8–12)*
<b>Control</b>	4 (4–5)	7 (5–7)	8 (6–11)	15 (6–21)
<b>Ingestion imidacloprid</b>	17 (14–21)*	10 (9–12)*	13 (7–20)	18 (12–27)
<b>Control</b>	5 (4–6)	6 (5–7)	11 (8–13)	10 (9–15)
<b>Contact imidacloprid</b>	8 (7–10)*	10 (9–13)*	11 (7–14)	11 (7–15)

Data are presented as the median and interquartile intervals (Q1–Q3). \* $p < 0.01$  compared to control group by Mann–Whitney  $U$  test, within a single time bracket ( $n = 30$ ).

induce motor activity changes in African-derived *A. mellifera*. Insecticides are considered toxic to bees when the LD<sub>50</sub> is less than 2 µg per bee (Johansen and Mayer 1990). The values of ingested and contacted fipronil LD<sub>50</sub> presented in this study are within the ranges reported by Carrillo et al. (2013) and Zaluski et al. (2015). Toxicity data from Agence Nationale de Sécurité Sanitaire de l’Alimentation (France) reported LD<sub>50</sub> doses of ingestion and

through contact of 0.00417 and 0.00593  $\mu\text{g}$  fipronil/bee, respectively (AgriTox Database, 2015). The ingested  $\text{LD}_{50}$  values for imidacloprid are similar to those reported by Carrillo et al. (2013), and the contact tests are within the ranges reported by Cresswell (2011). Suchail et al. (2000) reported ingested  $\text{LD}_{50}$  values of imidacloprid about 0.005  $\mu\text{g}/\text{bee}$ ; and after contact application, the  $\text{LD}_{50}$  values were approximately 0.024  $\mu\text{g}/\text{bee}$  for *A. m. mellifera* and 0.014  $\mu\text{g}/\text{bee}$  for *A. m. caucasica*.

The oral  $\text{LD}_{50}$  of fipronil and imidacloprid were higher when determined by contact exposure; this may be attributed to the action of detoxification enzymes that act when bees are exposed to pesticides orally. These detoxification enzymes are present in the digestive system, liver, or Malpighian tubules of honey bees (Miranda et al. 2003). However, regardless of the route of exposure, both pesticides presented high toxicity to adult bees and this fact emphasizes the importance of regulating the application of these insecticides during the flowering periods of crops attractive to bees.

Fipronil has an antagonistic action on gamma amino butyric acid (GABA) neurotransmitters and glutamate-activated chloride channels (Glu Cls) (Narahashi et al. 2010), and this insecticide can cause behavioral changes in bees that include agitation, spasms, tremors, and paralysis (Zaluski et al. 2015). Unlike fipronil, imidacloprid acts as an agonist to nicotinic acetylcholine receptors (nAChRs) present in high density in insect nervous tissue (Brown et al. 2006). Suchail et al. (2000) describe neurotoxic symptoms such as movement coordination problems, trembling, and tumbling in bees exposed to imidacloprid. The high toxicity and the behavioral changes that can occur in honey bees foraging in contaminated fields can reduce the performance and maintenance of whole colonies.

Phenylpyrazole and neonicotinoid insecticides differ from classic insecticides in that they become systemic in the plant, and can be detected in pollen

and nectar throughout the blooming period (Ellis 2010). This study demonstrates that bees exposed to  $\text{LD}_{50}$  of fipronil and imidacloprid experience motor changes. Evaluating motor changes in bees exposed to  $\text{LD}_{50}$  of fipronil and imidacloprid in the lab simulates acute toxicity of bees exposed to these pesticides in natural or agricultural ecosystems immediately after sprayed.

In sublethal doses, bees exposed to fipronil by ingestion and contact showed impaired motor activity; this did not occur with imidacloprid. Some pesticides can be metabolized to different compounds in insects (Singh 2012), and depending on the route of exposure and the dose, they can have reduced toxicity due to the action of detoxification enzymes (Miranda et al. 2003). Thus, the low amount of imidacloprid in the sublethal dose may be insufficient to cause motor changes by itself; however, the active substance or metabolites formed in the sublethal dose of fipronil may not be inactivated by detoxification enzymes, impairing motor function in honey bees. This study suggests that fipronil is more toxic than imidacloprid in sublethal doses, impairing the motor activity of bees. It is important to recognize that in this study, honey bees were exposed only once to the doses of fipronil and imidacloprid, and that in fields where these pesticides are sprayed, bees can suffer a repetitive exposure that can cause major damage.

Exposure to fipronil and imidacloprid while bees are collecting nectar, pollen, water, and resin, as well as the presence of these pesticides or metabolites in stored products in colonies of *A. mellifera* (Chauzat et al. 2006, Pareja et al. 2011) represent a risk for these pollinators, which may result in behavioral changes (Zaluski et al. 2015, Suchail et al. 2000) and impaired motor activity. Proper motor activity is essential to forager bees collecting resources for a colony; bees also communicate to unemployed nestmate foragers the location of food using a dance language (Von Frisch 1967). The exposure of bees to fipronil

and imidacloprid can lower the global fitness of the colonies and harm their maintenance. Studies conducted by Zaluski et al. (2015) demonstrate dramatic negative effects of a sublethal dose of fipronil to colonies and show colony maintenance completely hindered.

Further studies are necessary to evaluate the safety of the use of fipronil and imidacloprid in areas in which bees collect resources, including the duration that residual systemic pesticides may be present in the environment and contaminating plants that are attractive to bees. Determining the realistic field exposure of insect pollinators to fipronil and imidacloprid is essential, because, as this study demonstrates, even in sublethal doses, fipronil can cause impairment of motor functions in bees and hinder their performance. The use of these insecticides should be restricted in crops that could present a contamination risk to bees, and measures should be undertaken to replace fipronil, imidacloprid and associated insecticides with products that have lower toxicity to pollinators.

#### ACKNOWLEDGMENTS

To the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the scholarship granted to the first author.

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