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Evaluation of Motor Changes and Toxicity of Insecticides Fipronil and Imidacloprid in Africanized Honey Bees (Hymenoptera: Apidae)

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Abstract

Honey bees are important pollinators and are essential in beekeeping. Honey bees get exposed to systemic pesticides while foraging in contaminated fields, and it is important to know the toxicity (LD_{50}) and evaluate the impacts of bees' exposure to these molecules. Fipronil and imidacloprid are systemic pesticides widely used in Brazil and other countries. The objective of this study was to determine the LD_{50} (24 hours) and evaluate motor changes in Africanized honey bee foragers exposed to lethal and sublethal doses of fipronil and imidacloprid. To determine the LD_{50}, foraging honey bees were exposed by ingestion and contact to five doses of fipronil (Regent 800WG®) and imidacloprid (Appalus 200SC®) insecticides. After 24 hours of exposure, the number of dead bees was counted, and the results were subjected to probit analysis. The motor activity of bees exposed by ingestion or contact to LD_{50} and sublethal doses ($1/500^{th}$ of the LD_{50}) of both pesticides was assessed 4 hours after exposure using a behavioral observation box. The ingestion and contact with LD_{50} of fipronil were 0.0528±0.0090 and 0.0054±0.0041 µg/bee, respectively; the ingestion and contact with LD_{50} of imidacloprid were 0.0809±0.0135 and 0.0626±0.0080 µg/bee, respectively. Bees exposed to lethal and sublethal doses of both insecticides experienced significant motor alterations compared to the control, except for exposure to sublethal doses of fipronil by contact. Fipronil and imidacloprid are highly toxic and promote motor changes in bees. Thus, it is important to establish management methods to reduce pollinators' exposure to these pesticides.

Introduction

Pollination by wild animals is a key ecosystem service that is linked to human well-being through the maintenance of ecosystem health and function, wild plant reproduction, crop production and food security (Potts et al., 2016). Bees, including western honey bees (*Apis mellifera*), bumble bees and solitary bees, are the prominent and economically most important group of pollinators worldwide; 35% of the global food crop production depends on pollinators (Klein et al., 2007). The worldwide economic value of the pollination service provided by insect pollinators was estimated in €153 billion annually for the main crops used directly for human food (Gallai et al., 2009).

Among pollinators, bees are the most important group, visiting more than 90% of the leading 107 global crop types; and *A. mellifera* is the most commonly managed bee in the world for crop pollination (Klein et al., 2007). Furthermore, the beekeeping that is based in the nurturing of honey bees to explore honey, wax, pollen, propolis, royal jelly, and apitoxin (bee venom) has high importance in employment and income generation.

Despite their importance, intense reductions of managed and wild bees have been observed worldwide (Oldroyd, 2007; Stokstad, 2007; van Engelsdorp & Meixner, 2010; van der Sluijis et al., 2013; Goulson et al., 2015). Many factors has been investigated as responsible for the overall reduction of pollinators, including habitat destruction,

reduction of floral resources, presence of pathogens and parasites, climate change, and indiscriminate use of pesticides for crop protection (for review, see Potts et al., 2010, 2016; González-Varo et al., 2013; Goulson et al., 2015).

The use of pesticides in crops to control insect pests, nematodes, weeds, and diseases has harmed the survival of native pollinators and beekeeping practices (Sanchez-Bayo & Goka, 2016). Among pesticides widely used, fipronil (phenylpyrazole) and imidacloprid (neonicotinoid) are systemic insecticides used for seed treatment, foliar application, and soil treatment in many crops. Because they are systemic, they contaminate all parts of the plants, including the nectar and pollen (Bonmatin et al., 2015), which are resources collected by worker bees. Besides the cultivated plants, water sources and shrubs and plants growing in nearby areas may be contaminated (Krupke et al., 2012) due to the dispersion of residues by drift and lateral water flow (Greatti et al., 2006; Sanchez-Bayo et al., 2007), increasing pollinators' exposure to these molecules.

Worker bees are exposed to pesticides through direct contact during or immediately after pulverization or by ingestion of contaminated resources (nectar, pollen, water); and the exposure to high doses of insecticides in field may be sufficient to kill a bee immediately (Sanchez-Bayo & Goka, 2014). Residual levels of systemic insecticides can contaminate the resources collected by bees for long periods and can be stored in nests (Chauzat et al., 2006; Mullin et al., 2010; Orantes-Bermejo et al., 2010). In the nest, the contaminated resources are used to feed larvae and adults, generating physiological and behavioral changes in bees and compromising the productivity and colony maintenance (Orantes-Bermejo et al., 2010; Zaluski et al., 2015).

In Brazil, fipronil and imidacloprid insecticides have authorization for commercialization in various formulations and to control pests in sugarcane, cotton, rice, bean, corn, barley, soybean, wheat, and other crops (Ministério da Agricultura, Pecuária e Abastecimento, 2016; Agência Nacional de Vigilância Sanitária, 2016). Currently, the authorization of the use of imidacloprid is under review process and the aerial application of fipronil and imidacloprid are forbidden in flowering crops (Ministério da Agricultura, Pecuária e Abastecimento, 2013).

The release of pesticides on crops requires regulatory measures that include LD_{50} tests for pollinators, and are based on European protocols (Sanchez-Bayo & Goka, 2016). The LD_{50} value is determined based on laboratory tests corresponding to the dose capable of causing the death of 50% of the population over a period of 24 or 48 hours. Currently, the tests to regularize the use of pesticides do not regard the damages that bee exposure to doses below the LD_{50} (sublethal doses) can cause to honey bees individually and to the entire colony (Zaluski et al., 2015; Sanchez-Bayo & Goka, 2016).

In Brazil are found Africanized honey bees originated from crossing of imported African *Apis mellifera scutellata* with local populations of European *A. mellifera* previously introduced. The poly-hybrid bees resulting from these crossings expressed high defensive behavior, have good resistance to diseases, and are excellent pollinators (Pereira & Chaud-Netto, 2005). Some studies performed with Africanized bees demonstrate that fipronil is highly toxic to newly emerged bees (Roat et al., 2013) and to foragers (Zaluski et al., 2015); whereas studies performed by de Almeida Rossi et al. (2013) demonstrate that imidacloprid is highly toxic to newly emerged bees.

Considering the wide use of fipronil and imidacloprid in agricultural crops and the presence of Africanized bees in Americas that are widely used in beekeeping and crop pollination, is important to determine the LD_{50} and potential behavioral changes that worker bees can have if they are exposed to lethal or sublethal doses of these molecules. In this study, the acute LD_{50} of fipronil and imidacloprid insecticides for Africanized *A. mellifera* foragers was evaluated by ingestion and contact testing. Additionally, based in neurotoxic effects of fipronil and imidacloprid (Brown et al., 2006; Narahashi et al., 2010) that can trigger motor changes in bees, were conducted tests to evaluate the motor activity of bees exposed to LD_{50} and sublethal doses ($1/500^{th}$ of the LD_{50}) of these insecticides.

Material and Methods

The experiment was developed in the Beekeeping Production Area of Lageado Experimental Farm, Faculty of Veterinary Medicine and Animal Science, UNESP, Botucatu, São Paulo State, Brazil (22°50'30"S; 48°25'41"W), in a humid subtropical climate, and an average elevation of 623 m. The area where the experimental apiary is located and bees were collected to perform the experiments is free of application of pesticides, what reduces the risk of bees' contamination during foraging. Taking into account that foraging honey bees are most likely to be exposed to pesticides, all tests were performed with forager honey bees to better understand effects of exposition of these bees to fipronil and imidacloprid.

The study used Africanized *A. mellifera* (Hymenoptera: Apidae) workers aged 20 days collected in a single hive, in order to reduce genetic variations that can interfere in LD_{50} tests (Suchail et al., 2001; Zaluski et al., 2015). A bee trap was installed in the entrance of the beehive and was closed during the collection of bees; thus, only aged bees that returned from the field (foragers) were collected. Seven hundred and twenty bees were collected between 7:00 a.m. and 8:00 a.m. and anesthetized in a freezer at -10 ºC for 1–2 minutes to determine ingestion and contact LD_{50} (Zaluski et al., 2015).

In all tests, the active ingredients used were from commercial formulations that are used in the field: fipronil (Regent 800WG®) and imidacloprid (Appalus 200 SC®). A stock solution containing 1g L^{-1} of these insecticides was prepared separately in distilled water, considering only the amount of fipronil and imidacloprid active ingredient present in commercial formulations. There were no problems with solubility. The doses used in the tests were prepared from

these solutions that were stirred during preparation and before use to ensure that they were always at a proper concentration.

The ingestion LD_{50} was determined followed OECD guidelines (1998a) according to the method described by Miranda et al. (2003) with modifications proposed by Zaluski et al. (2015). After collection, groups of 10 bees were placed in disposable wooden boxes (25 × 15 × 10 cm) with side screens, and they remained unfed for 3 hours. Then, the bees received 1 mL of food (honey syrup - 50%) in a plastic tube (50 × 10 × 10 mm). The syrup consumption of 50 µL per bee, the volume corresponding to the average consumption per bee (Crane, 1990), has been associated with consumption of 0.00, 0.010, 0.020, 0.050, 0.100, 0.200, and 0.400 µg of fipronil and 0.000, 0.012, 0.025, 0.050, 0.100, 0.200, and 0.400 µg of imidacloprid. The contaminated food was provided to bees for 3 hours and then exchanged for uncontaminated food. The volume of contaminated syrup unconsumed by each group of bees was measured to confirm the approximate dose ingested by bees in each box.

To determine the contact LD_{50}, were followed OECD guidelines (1998b).Collected honey bees were anesthetized and directly transferred to disposable Petri dishes (25 × 15 × 10 cm) with a perforated lid to allow adequate ventilation. Then, with an automatic micropipet, the bees received 2 µL of solutions containing different amounts of fipronil (0.0000, 0.0017, 0.0035, 0.0070, 0.0160, 0.0320, 0.0640 µg) or imidacloprid (0.0000, 0.0050, 0.0100, 0.0200, 0.0400, 0.0800, 0.1600 µg) in the thorax. During all contact tests, the bees received sugar syrup *ad libitum*.

Doses used to determine the LD_{50} were based on preliminary tests conducted using the same method described above. All tests were performed in triplicate, using 10 bees for each dose tested. Bees that showed behavioral changes or lethargy before the tests were rejected and replaced with healthy bees. The bees were kept in a B.O.D. incubator at a temperature of 33 ± 1 ºC and humidity between 60 and 70%. The occurrence of behavioral alterations in bees 3 hours after exposure to insecticides was observed. Twenty-four hours after starting the tests, the number of dead bees per treatment was recorded, and the results were used to determine LD_{50}.

To study motor function in bees exposed to fipronil and imidacloprid, 360 adult bees were collected and exposed by ingestion and contact to LD_{50} or sublethal doses of these insecticides. The sublethal dose supplied to bees corresponded to 1/500th of the LD_{50} of ingestion and contact determined in the present study. The collection and exposure of bees were performed as described for the LD_{50} measurements.

The motor activity of bees was assessed 4 hours after exposure, according to the method described by Zaluski et al. (2015). The tests were performed in the laboratory using a wooden behavioral box (60 × 35 × 04 cm) divided into five 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, 10 bees were exposed to the pesticides and 10 served as controls. All tests were performed in triplicate.

The ingestion and contact LD_{50} were determined on the basis of the mortality of bees per dose using probit analysis with maximum likelihood. The results of the motor activity analyses 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. 16, State College, PA, USA).

Results

The ingestion and contact fipronil LD_{50} (24h) were 0.0528 ± 0.0090 µg/bee and 0.0054 ± 0.0041 µg/bee (Figures 1A and 1B), respectively. The ingestion and contact imidacloprid LD_{50} (24h) were 0.08092 ± 0.0135 and 0.0626 ± 0.0080 µg/bee (Figures 2A and 2B), respectively. From these values, were calculated the sublethal doses equivalent to 1/500th of the LD_{50} to be used in motor activity tests: fipronil sublethal doses of ingestion and contact were 0.0001056 and 0.0000108 µg/bee, respectively; and imidacloprid sublethal doses of ingestion and contact were 0.0001618 and 0.0001252 µg/bee, respectively.

Behavioral changes verified during the LD_{50} tests included agitation, seizures, tremors, and paralysis in bees. These behaviors occurred in higher frequency in bees exposed to doses between 0.100 and 0.400 µg in the ingestion tests for both insecticides, between 0.0160 and 0.0640 µg for contact tests with fipronil, and between 0.0400 and 0.1600 µg in contact tests with imidacloprid.

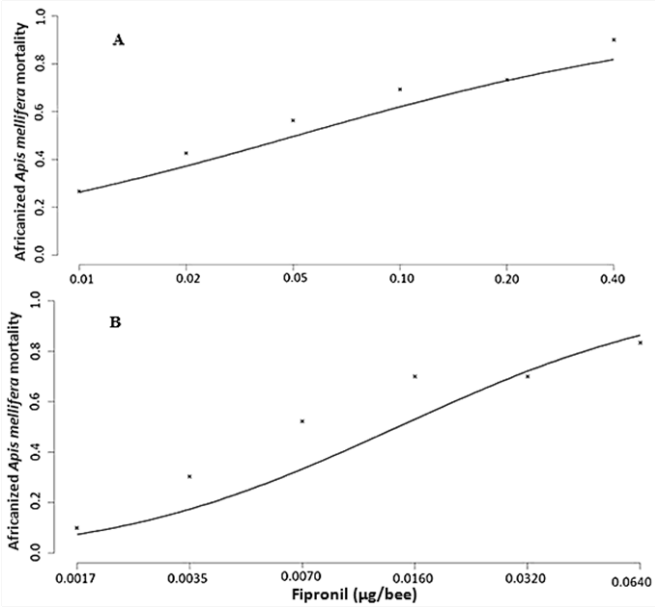

Fig 1. Mortality of Africanized *Apis mellifera* (24 hours) after the intoxication with fipronil by ingestion (A), and by contact (B).

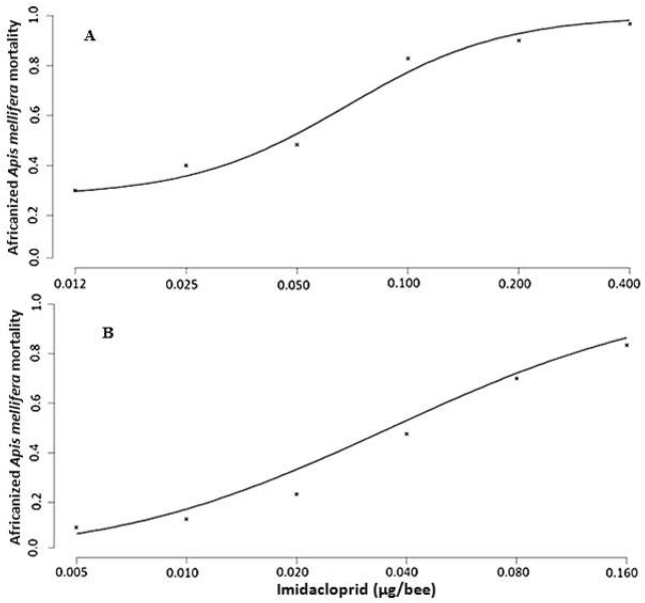

Fig 2. Mortality of Africanized *Apis mellifera* (24 hours) after the intoxication with imidacloprid by ingestion (A), and by contact (B).

In tests that analyzed motor activity, it was found that bees exposed by ingestion or contact to the fipronil and imidacloprid LD_{50} took longer to pass through the 50-cm track compared to bees from the control group ($p < 0.05$; Mann–Whitney U Test) (Table 1).

In the exposure to sublethal doses, there was a reduction of motor activity in bees exposed by ingestion and contact to imidacloprid and in bees exposed by ingestion to fipronil ($p < 0.05$; Mann–Whitney U Test) (Table 2). For exposure by contact to a fipronil sublethal dose, there was no significant motor impairment ($p > 0.05$; Mann–Whitney U Test) (Table 2).

Table 1. Median and interquartile intervals (Q1−Q3) of time (seconds) spent by Africanized *Apis mellifera* to travel 50 cm 4 hours after exposure by ingestion or contact to LD_{50} of fipronil and imidacloprid insecticides.

Treatment	Exposure (µg/bee)	Time (s)
Control Ingestion	0.0000	6.00 (4.00 – 8.50)
LD_{50} Ingestion Fipronil	0.0528	35.50 (21.00 – 58.50)*
LD_{50} Ingestion Imidacloprid	0.0809	17.50 (13.00 – 29.25)*
Control Contact	0.0000	7.00 (5.00 – 10.00)
LD_{50} Contact Fipronil	0.0054	12.00 (8.75 – 14.50)*
LD_{50} Contact Imidacloprid	0.0626	19.00 (12.00 – 31.00)*

*$p < 0.05$ compared to the control group using the Mann–Whitney U Test.

Discussion

According to the toxicological classification of Johansen and Mayer (1990), pesticides whose LD_{50} is less than 2 µg/bee are highly toxic to *A. mellifera*. The results of this study demonstrate that fipronil and imidacloprid are highly toxic to Africanized honeybee foragers, causing motor and behavioral changes after exposure by ingestion or contact to lethal and sublethal doses of these systemic insecticides.

Table 2. Median and interquartile intervals (Q1−Q3) of time (seconds) spent by Africanized *Apis mellifera* to travel 50 cm 4 hours after exposure by ingestion or contact to sublethal doses (SD) of fipronil and imidacloprid insecticides.

Treatment	Exposure (µg/bee)	Time(s)
Control Ingestion	0.0000000	6.00 (4.00 – 8.50)
SD Ingestion Fipronil	0.0001056	9.00 (6.00 – 12.00)*
SD Ingestion Imidacloprid	0.0001618	10.00 (4.75 – 17.75)*
Control Contact	0.0000000	7,00 (5.00 – 10.00)
SD Contact Fipronil	0.0000108	7,00 (4.75 – 13.25)
SD Contact Imidacloprid	0.0001252	10,00 (5.75 – 14.75)*

*$p < 0.05$ compared to the control group using the Mann–Whitney U Test.

In order to evaluate the LD_{50} of the insecticide fipronil to European newly emerged bees, the ingestion and contact LD_{50} were 0.0041 and 0.0059 µg/bee, respectively (Agritox Database, 2016). As for the imidacloprid insecticide, the ingestion and contact LD_{50} values were 0.0810 and 0.0037 µg/bee, respectively (Agritox Database, 2016). In studies performed with Africanized newly emerged bees, the ingestion and contact LD_{50} of the insecticide fipronil were 0.00127 and 0.00106 µg/bee, respectively (Roat et al., 2013); for exposure with imidacloprid by contact LD_{50} was 0.0809 µg/bee (de Almeida Rossi et al., 2013). The values of ingestion LD_{50} for Africanized honey bee foragers in this study were higher for both insecticides, whereas the contact LD_{50} value was similar, comparing to European and Africanized newly emerged bees. Higher values of LD_{50} ingestion may be related to metabolization of toxic compounds due to the presence of enzymes in bees' digestive system, and Malpighian tubules (Miranda et al., 2003). LD_{50} values of fipronil in Africanized honey bee foragers exposed by ingestion and contact ranged from 0.19 to 0.28 µg/bee and 0.006 to 0.012 µg/bee, respectively (Carrillo et al., 2013; Zaluski et al., 2015). For imidacloprid, LD_{50} values ranged from 0.04 to 0.10 µg/bee (Suchail et al., 2001; Carrillo et al., 2013). Changes in LD_{50} values can occur due to the genetic variability of bees, origin of the population, methodology, and difference in detoxification ability of bees (Suchail et al., 2001).

The tests in this study showed that bees intoxicated by ingestion or contact with the pesticides LD_{50} had impaired motor activity. These changes can be explained by the neurotoxic action of fipronil and imidacloprid. Fipronil and the metabolites resulting from its degradation have an antagonistic action on gamma amino butyric acid (GABA) neurotransmitters and glutamate-activated chloride channels (GluCls) (Narahashi et al., 2010). Unlike fipronil, imidacloprid and its metabolites act as nicotinic acetylcholine receptor (nAChR) agonists, which provide the majority of the excitatory neurotransmission in the

insects' central nervous system (Deglise et al., 2002; Brown et al., 2006). Besides motor changes, the effects of these insecticides in the bees' nervous system trigger agitation, seizures, tremors, and paralysis, behaviors that were observed during LD_{50} tests.

The results of this study also show that motor abnormalities occur in bees exposed to sublethal doses of fipronil and imidacloprid. Studies by Colin et al. (2004) found that sublethal doses of fipronil and imidacloprid reduce the foraging activity of *A. mellifera.* These results indicate that low doses of these pesticides can compromise vital functions for the maintenance of colonies, inducing effects on the nervous system that drive alterations in the orientation and consequently in the behavior. These alterations can compromise the orientation of bees for the location and collection of resources for the colony.

Motor and behavioral changes, as observed during this study, may occur after the pulverization of blooming crops, in which the bees collect resources. In addition, fipronil and imidacloprid residues in nectar and pollen can be found when crop seeds are treated with these molecules during planting; this is due to the high persistence and systemic nature of these insecticides (Bonmatin et al., 2015). Repetitive spraying of these insecticides on crops results in increased environmental contamination, and surrounding areas also can be contaminated, representing a risk to wild pollinators and to commercial beekeeping (Greatti et al., 2006; Sanchez-Bayo et al., 2007). Bees' exposure to lethal and sublethal doses of systemic pesticides may increase motor and behavioral alterations due to continuous exposure of these pollinators to contaminated resources. The fipronil and imidacloprid detection in resources collected by bees and stored in hives (Chauzat et al., 2006; Pareja et al., 2011) confirmed the exposure of pollinators to sublethal doses of these insecticides, which can compromise the maintenance of whole colonies. Studies also have demonstrated the high toxicity of these molecules to native stingless bees (Tomé et al., 2012; Rondeau et al., 2014; Costa et al., 2015; Soares et al., 2015).

The use of the systemic insecticides fipronil and imidacloprid was banned in France, Italy, Germany, Slovenia (Ghisi et al., 2011), and Uruguay (Pareja et al., 2011) due to the high toxicity of these pesticides to pollinators. In Brazil, the authorization of the use of imidacloprid is under review process, and the aerial application of neonicotinoids (imidacloprid, tiamethoxam, clotianidine) and fipronil are forbidden in flowering crops (Ministério da Agricultura, Pecuária e Abastecimento, 2013).The review of the use authorization of fipronil and imidacloprid in countries where these products are authorized, such as Brazil, is important to reduce the risk that these substances represent to honey bees, whose loss affects beekeeping and compromises the survival and maintenance of native pollinators.

Measures to replace fipronil and imidacloprid and related insecticides with products with lower toxicity to pollinators must be taken in order to avoid honey bee losses and to preserve the native pollinators that are essential to the maintenance of ecosystems. The adoption of strategies to reduce the use of fipronil and imidaclopride on crops, including biological control and integrated pest management, can contribute to the conservation of bees and reduce the environmental contamination caused by the use of systemic pesticides.

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