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RESEARCH ARTICLE

The effects of field-realistic doses of imidacloprid on *Melipona quadrifasciata* (*Apidae*: *Meliponini*) workers

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Abstract

The presence of Brazilian native bees can improve tomato production by increasing pollination effectiveness. However, the extensive use of pesticides in tomato cultures may be harmful to bees. Imidacloprid-based insecticides are used in tomato plantations because of its high efficiency against tomato pests. This study investigated the effects of oral intake of field-realistic concentrations of imidacloprid by *M. quadrifasciata* workers, a stingless native bee from Brazil and effective pollinators of tomato crops. The oral intake of sucrose syrup added with 10, 35, or 70 ppb of imidacloprid did not increase the mortality rate when compared with the control group. However, we observed a reduction in the workers' motility and food consumption. We also treated *M. quadrifasciata* workers with sucrose syrup mixed with an imidacloprid-based insecticide (Evidence 700 WG®, Bayer), with the final concentration of 250 ppb of imidacloprid. This treatment did not cause visible alterations of the intestine absorptive cells of the bees' midgut and did not increase DNA damage. Therefore, the observed reduction of food consumption and locomotion behavior of *M. quadrifasciata* workers may contribute to the global effort to understand the contribution of neonicotinoids on bees' population decline process.

Keywords Stingless bees · Neonicotinoids · Insecticide · Electron microscopy · Comet assay · Pollinators

Introduction

Brazil is among the most significant agricultural producing country, and much of its production depends on bee pollination (Giannini et al. 2015a, b). A loss of about $12–14 billion for Brazilian agriculture was estimated if bees are extinguished (Novais et al. 2016; Giannini et al. 2015a, b; Santos et al. 2018). Wild bees, such as of *Melipona*, *Xylocopa*, *Centris*, and *Bombus* genus (Giannini et al. 2015b), perform an essential part of this pollination service in Brazil. The introduced bees *Apis mellifera* does another part. Recently, Brazilian beekeepers are resistance to rent their hives for pollination services because of the heavy pesticide use in Brazilian agriculture (Santos et al. 2018).

Native bees' pollination improves tomato production (*Solanum lycopersicum* L.), because they can vibrate the poricidal anther of the tomato flower, which increases the stigma pollen load, fruit size, and production (Bispo dos Santos et al. 2009; Silva-Neto et al. 2017). The stingless bee *Melipona quadrifasciata* has been studied as an effective tomato pollinator in Brazil because of its vibrating behavior (Bispo dos Santos et al. 2009). However, there is a

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prophylactic and excessive use of pesticides in tomato crops because of phytosanitary problems and the farmers' fear of losing their crops (Reis-Filho et al. 2009) that may be affecting the pollinating bees. Imidacloprid-based insecticides are used in tomato plantations due mainly to its high efficiency against tomato pests (MAPA 2019).

Imidacloprid is an insecticide from the neonicotinoid family regularly used in many crops. Only in Brazil, more than 39,000 tons of this active ingredient were commercialized in 2018 (MAPA 2019). Imidacloprid contamination has been found in some beehives of different countries in pollen with an average concentration of 19.7 and maximum 912 μg/Kg (ppb), honey/nectar with an average concentration of 6 and maximum 72.8 ppb, and wax with an average concentration of 26.5 and maximum 45 ppb (Sanchez-Bayo and Goka 2014). In Colorado, USA, 13% of wild bees are contaminated with imidacloprid (Hladik et al. 2016). Even with the use of imidacloprid and other neonicotinoids forbidden in European Union (EU) since 2013 because of its effects on bees (EFSA 2013; Auteri et al. 2017), it is used globally, including across South America, North America, Asia, and Australasia.

In the last decade, there have been an increasing number of studies about the toxic effects of imidacloprid and other neonicotinoids on bees. However, the knowledge about bees' detoxification mechanisms on neonicotinoids is limited (Gong and Diao 2017). It is known that the exposure to neonicotinoids can suppress immune response genes, rendering bees more susceptible to parasites (Gong and Diao 2017; Aufauvre et al. 2014). Studies with residual levels of imidacloprid did not demonstrate increase in bees' mortality (Cheng Zhu et al. 2017). However, laboratory tests have demonstrated that imidacloprid affects bees' brain memory centers development (Tomé et al. 2012; Decourtye et al. 2004a, b), affecting bees foraging activity and olfactory learn (Decourtye et al. 2004a, b). Furthermore, honeybees collected in areas with regular pesticide application have more DNA damages than those collected in free pesticide areas (Hayat et al. 2018).

The toxicity studies on bees are concentrated on *Apis* and *Bombus* genera, ignoring important local species in different biome (Barbosa et al. 2015), with different nesting strategies (Sgolastra et al. 2019). Until the year of 2015, there were 268 studies about the effects of neonicotinoids on bees, 75% of which were about *A. mellifera* (Lundin et al. 2015). It is already known that the effect of pesticides in domestic and wild bees is variable and depends on their foraging behavior intrinsic sensitivity, life cycle and nesting activity (Arena and Sgolastra 2014; Del Sarto et al. 2014; Sgolastra et al. 2019).

Therefore, in this study we investigated the effects of the oral intake of imidacloprid by workers of *M. quadrifasciata* in environmentally relevant concentrations (Sanchez-Bayo and Goka 2014). We evaluated the mortality rate of *M. quadrifasciata* at such concentrations and if they are enough to cause locomotion impairment. We also analyzed the effects of a commercial formulation of imidacloprid (Evidence®) on gut epithelial cells and on hemocytes DNA integrity.

Material and methods

Chemicals

We conducted the mortality and locomotion impairment experiments with 98–100% purity crystals of imidacloprid from Sigma-Aldrich (USA). The intestine morphology evaluation and the Comet assay test were conducted with the imidacloprid based insecticide Evidence 700WG®, 700 g/kg (Bayer).

Bee colonies characterization

According to Kerr and Santos-Neto (1956), workers of *M. quadrifasciata* take on foraging tasks from the 30th day after emergence until the end of their lives (around 60 days). We performed our experiments with adult forager workers (supposedly from 30 to 60 days after emergence), from six colonies of stingless bees *M. quadrifasciata*, captured when leaving the hives for foraging in the morning. We obtained the colonies from a commercial supplier of domesticated colonies (Meliponary Bee Jataí, Cambará—PR, Brazil). They were maintained under field conditions in the Institute of Biological Sciences at the Federal University of Goiás (Goiânia—GO, Brazil, 16°36' S, 49°15' W).

Mortality and locomotion experiments

We evaluated in this experiment if bees had their mortality rate and locomotion ability altered when fed with food contaminated with imidacloprid in environmentally relevant concentrations for honey/nectar (Sanchez-Bayo and Goka 2014). We collected the forager workers when they were leaving the colonies in the morning, transferred to four disposable wooden boxes of 15 × 15 × 15 cm. Each experimental group was composed of 15 bees/box. We kept the boxes in an incubator at 28 ± 1 °C and relative humidity of 70% ± 5%, during the experimental period. The first 24 h were the adaptation period to minimize the stress effect. We fed all bees with a 50% sucrose solution during this period. After the adaptation period, we fed the bees of each box through an adapted bird feeder for 7 days with 40 ml of 50% sucrose solution added with different concentrations of imidacloprid (10, 35, and 70 μg/L—ppb).

We solubilized the imidacloprid in acetone at 4 μg/ml. After that, 100, 350, and 700 μl of this solution were added in 40 ml of the sucrose syrup, with final acetone concentrations of 0.25, 0.875, and 1.75%, respectively. To ensure no

interference of acetone in syrup consumption, in the control group, 300 μl of acetone was added in the sucrose syrup, corresponding to a concentration of 0.75% of acetone. Studies with *Melipona scutellaris* with 2% acetone in the control group showed no visible toxic effects (Lourenço et al. 2012); honeybees apparently are not affected by concentrations lower than 2.6% of acetone in food (Clinch et al. 1972; Gregorc et al. 2018) and the OECD protocols recommend not to exceeding 5% of acetone (OECD 2017). We checked the syrup consumption, and the number of bees alive was checked every 24 h for 7 days (168 h). The syrup consumption was checked through the feeders' weight, discounting the daily evaporation rate. We performed this experiment in triplicate using bees of different hives.

At the end of 168 h, we captured the surviving bees, one at a time, and allowed them to move in a wooden/glass lane of 5 × 5 × 60 cm for 30 s. The floor of this lane had a centimeter scale and their locomotion was recorded.

Intestine morphology

To test a scenario of acute high intoxication, for morphological investigations, we used imidacloprid in a concentration higher than 40 times higher the average concentration found in contaminate honey/nectar. We collected forager workers when they were leaving the colonies in the morning, transferred to two disposable wooden boxes of 15 × 15 × 15 cm. Each experimental group was composed of 18 bees/box. We kept the boxes in an incubator at 28 ± 1 °C and relative humidity of 70% ± 5%, during the experimental period. The first 24 h were the adaptation period to minimize the stress effect. We fed all bees with a 50% sucrose solution during this period. After the adaptation period, the groups were fed with sucrose syrup (control group) and sucrose syrup added with Evidence® with the final concentration of imidacloprid of 250 μg/L (ppb) for 3 days (72 h).

We analyzed the cells from the ventriculus (midgut) which are responsible for digestion and food absorption. For ultrastructure analysis, three bees of each experimental group had their ventriculus dissected in cacodylate buffer (pH 7.2), fixed for 12 h in 2.5% glutaraldehyde and post-fixed in 1% osmium tetroxide for 2 h. Dehydration was carried out in a progressively increasing acetone series, and embedding was in Epon. Ultrathin sections were stained with 2% uranyl acetate, 0.2% lead citrate and analyzed in the transmission electron microscope JEOL, JEM 2100 of the Laboratory of High-Resolution Microscopy (LabMic) of the Federal University of Goiás, under 80 kV.

Comet assay

The comet assay is a technique that comparatively quantifies the DNA breakages between experimental groups, indicating unrepaired DNA damage (Olive and Banath 2006). To perform the test the same group of bees, which had the ventriculus epithelium analyzed, were used. We anesthetized the bees using an ether chamber. We transferred the bees' hemolymph to a plate with an EDTA solution. Each specimen was injured twice, in the thorax and the abdomen, using a watchmaker forceps and a 5-mL syringe. We deposited hemolymph on the bottom of the hollowed plate and then transferred to 1.5 ml tubes. We mixed the hemolymph of each replica (15 bees) into a single tube, forming a cell pool of all subjects for each treated group. The final volume was equivalent to 0.5 mL per tube. The tubes were centrifuged two times at 3000 rpm for 3 min. Then, 100 μL of supernatant was discarded, followed by the addition of 100 μL more EDTA solution, completing the pipe volume again to 0.5 ml for the third centrifugation. This procedure was required to eliminate all impurities and increase the sample quality.

We prepared slides with an agarose precoating "Normal Melting" 1.5%. Then, 10 μL of cell suspension were soaked in 120 μL of agarose "low melting point" 0.5%, in a water bath at 37 °C. This mixture was placed on slides prepared with the agarose precoating normal melting1.5% and covered with a coverslip. We placed the blades in the refrigerator for 2 min to solidify the material. After this time, we removed the coverslips and immersed the slides in lysis buffer for 24 h.

After another 24 h, we placed the slides into horizontal electrophoresis, incubating them in an alkaline buffer for 30 min. The electrophoretic run was performed for 25 min at 25 V and 300 amps. The neutralization was carried out with at 0.4 M Tris buffer (pH 7.5) solution for 5 min. After neutralization, we washed the slides twice with distilled water and put to dry in an inclined position. We fixed the samples with absolute ethanol for 5 min. The DNA was stained with 100 μL of a solution containing Sybr Green® I (10,000 x in DMSO), in the dark, for 30 min. The stained material was fixed with 20 μL of Vectashield® reagent (1.5 μg/mL). After fixation, we placed the coverslip back on the blade. All sections were examined under epifluorescence microscopy Axio Imager 2 (Zeiss®), using a range of 515–560 nm excitation filter for green fluorescence. The nucleoids cells were visualized using × 20 magnification, and fluorescent images were captured using the Comet Imager version 2.2 software (MetaSystems, Altlussheim, Germany).

Statistical analysis

We compared differences in consumption and locomotion between insecticides' concentrations and controls through Kruskal-Wallis test due to nonnormal distributed data ($w = 0.86132$, $p = 2.342 \times 10–7$ for consumption, $w = 0.68154$, $p = 3{,}318 \times 10–12$ for consumption of imidacloprid and $w = 0.69697$, $p = 1.865 \times 10–15$ for locomotion) and lack of homoscedasticity ($F = 3.3312$, $p = 90.0236$ for consumption,

F = 15.366, $p = 5.539 \times 10–8$ for consumption of imidacloprid and F = 7.6771, $p = 9.035 \times 10–5$ for locomotion) and Dunn test for post hoc comparisons. We estimated mortality through Kaplan-Meier (Efron 1988) survival analysis.

To test possible differences in Comet assay, we analyzed five slides for each replica. We randomly counted 20 nucleoids in each slide, amounting 100 cells per pool. We evaluated the Olive tail moment (OTM), which is the product of the percentage of DNA in the tail (%DNA) and the tail length (TL) (Olive et al. 1990). To satisfy the model assumptions, we performed a logarithmic transformation on the response variable values. We performed the statistical tests using Statistica software version 13 (Statsoft Inc., 2005, Tulsa, OK, USA) and R package. Results were considered significant when $p < 0.05$.

Results

The bees of the control group consumed approximately 0.12 ml of sucrose syrup/bee/day. The presence of imidacloprid in the sucrose syrup reduces the consumption by *M. quadrifasciata*. Even the lowest concentration of imidacloprid (10 ppb) was enough to reduce this consumption. We registered the lowest consumption when the syrup was contaminated with 35 ppb of imidacloprid, with each bee consuming approximately 0.025 ml of syrup/day. However, the consumption of the three treatment groups (10, 35, and 70 ppb) was statically the same (Fig. 1). Calculating the average daily ingestion of imidacloprid per bee, we observed the lowest value in the group of 10 ppb (less than 1 ng/ bee/day) and the highest in the group of 70 ppb (approximately 3.5 ng/ bee/day) (Fig. 2).

The proportion of living bees in all the experimental groups reduced over time (Fig. 3). However, the concentration of imidacloprid in the syrup did not influenced the mortality rate (Fig. 3). Although not influencing the survival rates in the experiment, the imidacloprid reduced the distance walked by the bees when fed by syrup contaminated with the concentrations tested (Fig. 4).

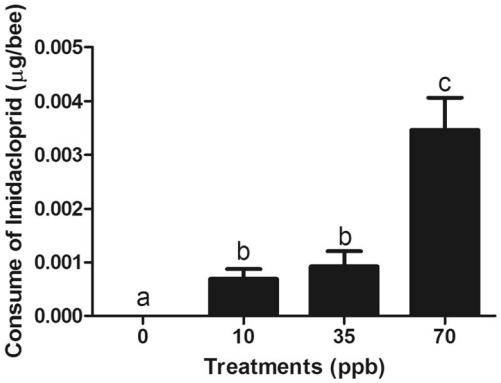

Fig. 2 Consume of imidacloprid by bees of control and treated groups. Different letters indicate a significant difference ($\chi^2 = 45.962$, $p = 0.0000000005777$)

Absorptive cells are the most common type of cell in the ventriculus epithelium; they are columnar with apical microvilli and basal membrane infoldings full of mitochondria (Fig. 5) The presence of digestive vacuoles and few agglomerates of degenerative figures (Fig. 5b) characterizes the apical portion of these cells. The absorptive cells have round nuclei located approximately at the middle of the cells. An evident nucleolus is usually observed in the nuclei (Fig. 5a). A muscular layer (Fig. 5c) was observed externally surrounding the ventriculus. We did not observe evident differences in the absorptive cells of the ventriculus epithelium of the bees treated with sucrose syrup or with syrup contaminated with commercial imidacloprid formulation (Evidence®).

We did not find significant difference in the OTM, between the average of the group treated with imidacloprid when compared with the negative control ($t = -0.828$, $p = 0.422$) (Table 1). Imidacloprid seems not to damage DNA extracted from hemocytes of worker bees of the species *Melipona quadrifasciata.*

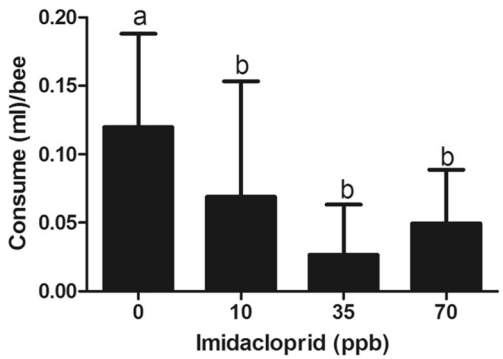

Fig. 1 Food consumption by bees of the control and treated groups. Different letters indicate a significant difference ($\chi^2 = 23.347$, $p = 0.00003419$)

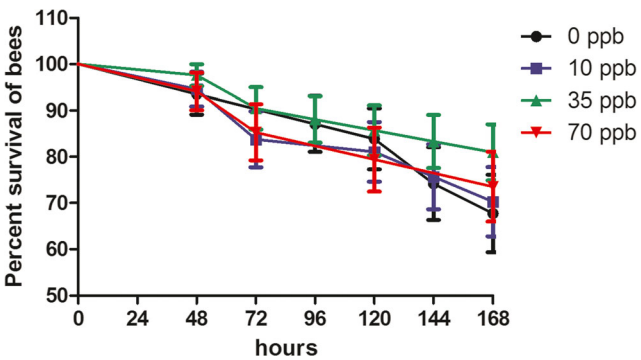

Fig. 3 Percent survival of bees comparing different treated groups. No significant difference was observed between the treated groups ($\chi^2 = 1.657$, $p = 0.6464$)

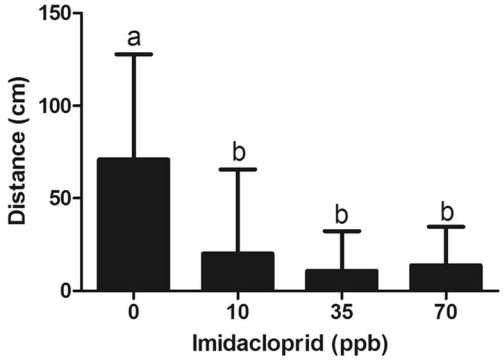

Fig. 4 Distance walked in 30 s by bees of the control and treated groups. Different letters indicate a significant difference. ($\chi^2 = 52.6463$, $p = 0$)

Discussion

The present study demonstrated the effects of environmentally relevant concentrations of imidacloprid on the Neotropical stingless bee *M. quadrifasciata*. In a compilation study, Sanchez-Bayo and Goka (2014) found an average concentration of 6 ppb of imidacloprid in the honey/nectar. The maximum concentration of imidacloprid in honey/nectar showed in literature was 72.8 ppb (Sanchez-Bayo and Goka 2014). Our results demonstrated that concentrations from 10 to 70 ppb in food are not enough to increase *M. quadrifasciata* mortality. However, we noticed other adverse effects. Food contamination by imidacloprid can reduce food intake by *M. quadrifasciata*. These data are similar to the ones observed

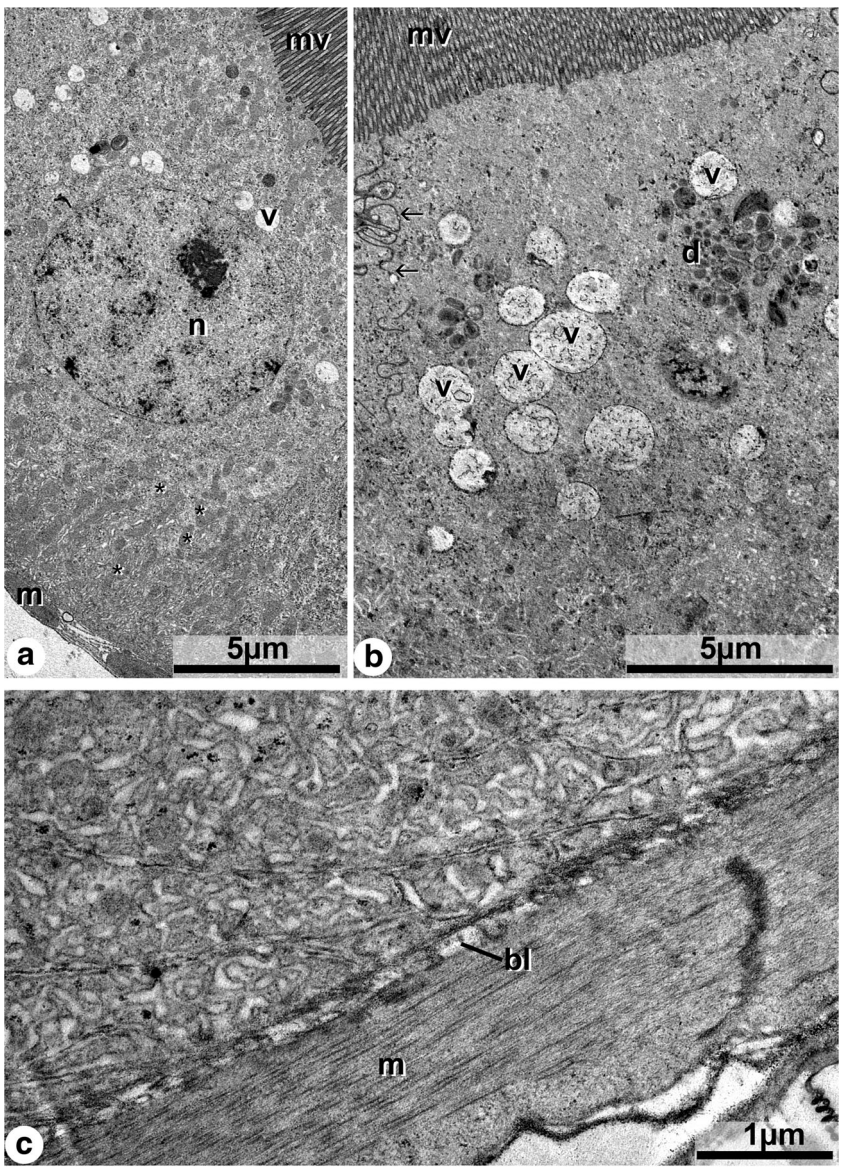

Fig. 5 Transmission electron micrographys of absorptive cells of the ventriculus. **a** Panoramic view of a cell with microvilli (mv), nucleus (n), apical vesicles (v), and basal mitochondria (*). The muscle layer (m) is surrounding the ventriculus epithelium. **b** Major magnification of the apical portion of an absorptive cell. Presence of cytoplasmic degenerative figures (d) and the septate junctions between neighbor cells (arrow). **c** Major magnification of the basal portion of an absorptive cell with the membrane debris. The basal laminae (bl) and the adjacent muscle layer (m)

Table 1 Mean and standard deviation of the OTM in hemocytes of worker bees of the species *Melipona quadrifasciata* exposed to Imidacloprid. A.U.: arbitrary units

Insecticides	Treatment	Media and standard deviation OTM (A.U.)
Imidacloprid	250 ppb	0.46 ± 0.11
Negative control	-	1.25 ± 1.37

in laboratory tests with *Bombus terrestris* (Thompson et al. 2015). A proportional reduction of food intake was noticed for honeybees when imidacloprid is added with acaricides (Gregorc et al. 2018). Kessler et al. (2015) demonstrated that bees prefer food containing low concentrations of imidacloprid and thiamethoxam, even though these pesticides caused less food consumption. It was also demonstrated that honeybees and bumblebees do not have gustatory neurons to these neonicotinoids (Kessler et al. 2015). The reduction of food intake must be associated with insecticide action on the nervous system.

It must be remarked that, in the present study, bees from the control group consumed approximately 0.12 ml of sucrose syrup/day (approximately 105 mg). It is surprising that a bee weighting approximately 80 mg (Ramalho et al. 1998) can consume more than its own body weight in a day. To compare, *M. scutellaris* worker weighs approximately 76 mg and consumes in laboratory conditions approximately 35 mg of sucrose syrup/day; *Scaptotrigona postica* worker weighs approximately 17 mg and consumes in laboratory conditions approximately 9.5 mg of sucrose syrup/day and *Apis mellifera* worker weighs approximately 79 mg and consumes in laboratory conditions approximately 50 mg of sucrose syrup/day (Dorigo et al. 2018). However, the small stingless bee *Tetragonisca angustula* which workers weigh approximately 4 mg consumes in laboratory conditions approximately 7.2 mg of sucrose syrup/day corresponding to almost twice its body weight (Dorigo et al. 2018). These data reinforce the need to investigate the biology of different bees' species, since these variations on the consume rates must be considered on the characterization of pesticide exposure risks.

We found that the ingestion of contaminated food reduced the walked distance by the bees. A similar result was also observed in honeybees (Medrzycki et al. 2003). Gill et al. (2012) observed that forager bees contaminated with imidacloprid were less efficient at collecting pollen and that the duration of the successful foraging bouts of bees contaminated with 10 ppb of imidacloprid and λ-cyhalothrin is longer than those of bees not contaminated. Bumblebees exposed to 10 ppb of thiamethoxam presented similar results, with lower visitation rates to apple flowers and less pollination efficiency (Stanley et al. 2015). Stanley et al. (2015) also observed increasing "flower switching" during each trip and suggested that an alteration of bees' behavior when visiting the flowers may explain the pollination failures. Other study demonstrated that nonlethal doses of neonicotinoids could alter the navigation memory of honeybees (Fischer et al. 2014). Imidacloprid can act in three distinct acetylcholine receptor subtypes in the insects' nervous system (Buckingham et al. 1997). Even though our study cannot exclude the hypothesis that the reduction of the walked distance is a conserving energy strategy due to lack of food, this effect and the reduction of food consumption may suggest a motor control difficulty caused by imidacloprid intake.

We did not find mortality increase in imidacloprid-treated groups compared with control. However, we detected a decrease in the proportion of living bees in all groups during the experiment, which must be related to aging and natural mortality. As the present study was performed with adult foragers leaving the hives, their presumably age is around 30–60 days, while the life expectancy for *M. quadrifasciata* is up to 60 days (Kerr and Santos-Neto 1956).

Our study demonstrated the presence of digestive vacuoles and degenerative figures in the absorptive cells. However, even using a higher concentration of imidacloprid (250 ppb), there was no evidence of cell death or increase of the digestive vacuoles. The vacuolization of absorptive ventriculus cells is probably associated with the aging process (Serrão and Cruz-Landim 1996) of stingless worker bees. This process is characterized by the presence of digestive and autophagic vacuoles (Serrão and Cruz-Landim 1996). *Melipona quadrifasciata* intestinal cells have large areas of cytoplasmic and nuclear disorganization, when exposed to deltamethrin (Brito personal observation). Honeybees have increasing vacuolization process of intestinal cells when treated with boric acid and fipronil and also the presence of pyknotic nuclei and alterations of mitochondrial membranes permeability, indicating cell death (Cruz et al. 2010).

We found that the concentration of 250 ppb of imidacloprid did not increase the DNA damage on *M. quadrifasciata* hemocytes using comet assay. Frantzios et al. (2008) found no genotoxic effects of imidacloprid in the species *Drosophila melanogaster* for larvae and adults through mutation tests and somatic recombination (SMART). However, other studies evaluating imidacloprid toxicity detected DNA damage in worms, amphibians, and humans, using the comet assay (Zang et al. 2000; Feng et al. 2004, 2005). According to Frantzios et al. (2008), the divergent results may be related to the technique used, to the studied genetic system, and to different levels of exposure. Comet assay studies with honeybees collected in different environments in Pakistan showed that those bees collected in agricultural zones exposed to pesticides have more DNA fragmentation than those collected in pesticide free zones (Hayat et al. 2018).

The apparent resistance of the *M. quadrifasciata* intestine absorptive cells and the hemocyte genetic material to injuries caused by imidacloprid must be better investigated. Neonicotinoid insecticides are specific agonists to acetylcholine receptors in insects' nervous system (Buckingham et al. 1997), and there is no previous evidence of its action on intestine cells or hemocytes DNA structure. It has been demonstrated that honeybees can detox their systems when contaminated with nicotine (Du Rand et al. 2015). However, it may have a high energetic cost to bees (Du Rand et al. 2015). How much this energy cost weakens bees and the entire hive must be assessed (Dively et al. 2015). Some studies also demonstrated that sublethal dosages of imidacloprid could change the expression pattern of immune and antioxidant genes in bee, making them more susceptible to opportunistic infections and diminishing the hive long-time survival (Dively et al. 2015; Li et al. 2017; Gregorc et al. 2018). Therefore, the observed reduction of food consumption and locomotion behavior of *M. quadrifasciata* workers observed in this study may contribute to the global effort to understand the contribution of neonicotinoids on bees' population decline process.

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