Spinosad-mediated effects on the walking abilities, midgut, and Malpighian tubules of Africanized honey bee workers

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Abstract

BACKGROUND: The global decline in *Apis mellifera* colonies is attributed to multiple factors, including pesticides. The bioinsecticide spinosad was initially recognized as safe for non-target organisms; however, its toxicity has been changing this concept. Here, we investigated the survival, behavioral changes, and structural changes in the midgut and Malpighian tubules of *A. mellifera* treated orally with a spinosad formulation. RESULTS: The field-recommended concentration of spinosad killed 100% of the bees. The concentrations (LC_5 and LC_{50}) of spinosad altered the behavioral activity, reducing the walking distance and velocity, and increasing the resting time in comparison to control. The LC_{50} caused disorganization of the epithelia of tested organs and induced a perceived oxidative stress and cell death. CONCLUSION: The present work provides new insights into the debate about the role of bioinsecticides in the mortality of Africanized honey bees. Even at very low concentrations, the

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spinosad formulation is toxic for the vital organs midgut and Malpighian tubules and had adversely affected walking behavior. This detailed evaluation of the impact of the bioinsecticide on *A. mellifera* will contribute to the clarification of probable mediated disturbances caused by spinosad formulations, which can be used to develop more sustainable protocols in agriculture.

Keywords: *Apis mellifera*; bioinsecticide; behavioral; cell death; peritrophic matrix

1. Introduction

The honey bee *Apis mellifera* (Linnaeus 1758) (Hymenoptera: Apidae) has high economic importance. This species has been used as a successful pollinator in several agricultural crops worldwide, and as a source of valued products such as wax, honey, propolis, and royal jelly.[1] In the last few decades, the honey bee population has been declining worldwide.[2-4] This decline has been associated with multiple factors, including climate change, habitat fragmentation, introduction of exotic species, parasites, pathogens, malnutrition, and use of agrochemicals.[2,5,6]

The bioinsecticide spinosad consists of a mixture of spinosyns obtained from the actinomycete *Saccharopolyspora spinosa*. This bioinsecticide acts on the nicotinic acetylcholine receptors in the nervous systems of insects[7] and is used in the control of pests in different crops, including cotton, bean, corn, soybean, and tomato, which can be also be visited by *A. mellifera* in search of food resources.[1] Spinosad was recently introduced in Brazil and was initially recognized as safe for non-target organisms, thereby increasing its use.[8] However, spinosad's cumulative effects and toxicity to non-target organisms, including bees, have been reported;[9-11] thus, it is important to understand the sublethal effects of biopesticides on non-target organisms.[12-14]

Non-target organisms, including natural enemies of agricultural pests, pollinators, and detritivores can be affected by exposure to sublethal concentrations of insecticides for long periods before the pesticides fully degrade in the environment.[10,15] The sublethal pesticide toxicity can manifest in a variety of ways, interfering with animals' behavior, development, and reproduction. In bees, the sublethal effects include malnutrition of colonies, reproduction impairment, and malformation in the offspring.[13,15,16]

The sublethal effects of pesticides may include changes in behavior, neural and locomotor disorders[17], and memory and learning impairments on bees.[18] In addition, the sublethal effect of pesticides may include damage to the different organs of bees, such as the midgut and Malpighian tubules. For example, the phenylpyrazole insecticide fipronil and the neonicotinoid imidacloprid can damage the midgut larval cells of *A. mellifera*.[19,20] As with the midgut, alterations caused by the ingestion of imidacloprid in the Malpighian tubule of *A. mellifera* adults have also been reported.[21]

In the present study, we assessed the effects of the spinosad formulation on the survival and locomotory behavior of workers of Africanized *A. mellifera,* exposed orally under laboratory conditions. We also investigated the negative effects of spinosad formulation in the midgut and Malpighian tubules of workers. These organs are responsible for food digestion and excretion, respectively, and can be used as models for understanding the mode of action of pesticides.

2. Material and methods

2.1. Insects and insecticides

Forager workers (21–60 days old) and nurse workers (14–21 days old) of *A. mellifera* were obtained from five colonies at the Apiary of the Federal University of Viçosa (UFV), Minas Gerais, Brazil. Foragers were collected in a glass Erlenmeyer flask, which was positioned at the exit of the colony to enable capture of the specimens as they exited the hive after being stimulated by hand beats. In order to carry out video tracking, flightless young bees or nurse workers (14–21 days old) were removed carefully from the colony combs with forceps and transferred to Petri dishes. The insecticide used to treat the bees was spinosad in suspension, concentrated at 480 g a.i/L (Tracer 480 SC, Dow AgroSciences, Santo Amaro, SP, Brazil). Foragers were used in the toxicity assay and in the morphology assay because they go out to collect food resources in the field, offering a more realistic scenario than nurses do. Then, we assumed that the foragers are more susceptible to the exposure to agrochemicals used in the crops than nurses are. For the behavior assays, we used nurses because, differently of foragers, nurses do not fly and the flight interferes with the recording of the behavior.[17]

2.2. Toxicity bioassays

The forager bees from five different colonies were brought to the insectary (Department of General Biology of UFV) and transferred to plastic pots separated by colony (10 bees per colony/pot). The bees were exposed orally, following protocols for *A. mellifera*.[22,23] The control group received 1.5 mL of 50% sucrose solution in a perforated microcentrifuge tube inserted into a hole in the wall of each plastic pot. Treated bees were orally treated with 1.5 mL of 50% sucrose solution with the following spinosad (active ingredient) concentrations: 0.816, 0.816×10^{-1}, 0.816×10^{-2}, 0.816×10^{-3}, 0.816×10^{-4}, 0.816×10^{-5}, 0.816×10^{-6}, and 0.816×10^{-7} mg/mL.

We used the concentration of 0.816 mg/mL because it is the label rate used in the field.[14] This concentration corresponds to the field-recommended concentration of spinosad (100 L ha^{-1}) for the control of the white fly *Bemisia tabaci* (Hemiptera: Aleyrodidae) on tomato crops in accordance with the recommendations of the Brazilian Ministry of Agriculture.[24] After 1 h of the fasting period, the diets were offered for 3 h; thereafter, they were replaced by 50% sucrose solution without the bioinsecticide for 21 h.[14] During this period of 24 h, the bees were kept in the incubator at 34°C, 80% humidity in the dark. The concentration-response curve was estimated from data considering the eight concentrations used, besides the control. Three h of exposure was chosen because if we increase the time of exposure to a lower concentration of spinosad, bees could ingest more insecticide and dye. In this case, the rate of survival would be very low to plot the concentration-response curve.[14]

To determine the average amount of diet each bee ingested (dose-response), the bees were orally exposed to 1.5 mL of sucrose solution with spinosad concentrations of 7.7×10^{-3} mg/mL and 6.1×10^{-4} mg/mL. The control group received 1.5 mL of sucrose solution. After an adaptation period of 1 h in the incubator, the diets were offered to the forager bees for 3 h (the microtubes containing the diets were weight before and after the exposure). The microtubes containing contaminated diet were replaced by 50% sucrose solution without the bioinsecticide for 21 h.[14] Bees were kept in controlled conditions in the incubator as described above. Immobile bees with no reaction were considered dead.

2.3. Video tracking (walking behavior)

The recording of the behavior of nurse bees was carried out 24 h after oral exposure to LC_5 and LC_{50} of spinosad. Exposure was made as described above in item 2.2. Exposed and unexposed bees were transferred individually to Petri dishes placed flat over a table (9.0 cm diameter and 2 cm height), with 18 replicates being used for each treatment (n = 54). The bottom of each Petri dish was covered with filter paper (Whatman No. 1), and the opening was covered with transparent PVC film, to prevent the escape of bees. After an adaptation period of 1 h in the Petri dish, the activity of the bees, including the walked distance, mean velocity, and rest time were recorded during 10 min with a digital CCD camera coupled to a computer equipped with video-tracking software (ViewPoint LifeSciences, Montreal, Canada).[10,17] The bioassays were performed at 25 ± 2 °C and under artificial fluorescent light in the morning.

2.4. Histology and morphometry

Forager bees [control (n = 5) and LC_{50}-treated (n = 5)] were dissected in insect physiological solution (0.1 M NaCl, 20 mM KH_2PO_4 or 20 mM Na_2HP_4). The midgut and Malpighian tubules were transferred to Zamboni's fixative solution (4% paraformaldehyde, Sorensen's phosphate buffer, and saturated picric acid solution) for 2 h at room temperature. The samples were then washed in PBS (phosphate buffered saline, 0.1 M), dehydrated in increasing series of ethanol (70–100%) and embedded in historesin (Leica Microsystems). Serial sections of 5 μm were obtained using a Leica 2255 automatic microtome with a glass knife. The sections were stained with toluidine blue, analyzed, and photographed under an Olympus BX53 microscope coupled with an Olympus DP 73 digital camera. The images were used for the morphometric analyses. The thickness of the midgut epithelium, the brush border, and the nuclear area of the Malpighian tubules was determined with Image Pro Plus 4.0 software (Media Cybernetics, Silver Spring, MD, USA).

2.5. Peritrophic matrix

Ten fixed midguts (five controls and five treated LC_{50}) were washed three times for 30 min each, in PBS/1% Triton X-100 (PBST) and incubated for 24 h at 4 °C in primary anti-peritrophin 55 kDa antibody (Cell Signaling) in PBS (1:500). Subsequently, the samples were washed three times and incubated with a FITC-conjugated rabbit anti-IgG secondary antibody (Sigma) in PBS (1:500) for 24 h at 4 °C, followed by three washes in PBS for 10 min each. After washing, samples were incubated for 30 min in 10 g/mL FITC-conjugated lectin (WGA-FITC, Sigma-Aldrich) diluted in 0.1 M PBS. The midguts were then washed in PBS, dehydrated in ascending series of ethanol (70-100%) and embedded in historesin (Leica Microsystems). Serial sections (5 μm) were obtained using a Leica 2255 automatic microtome with a glass knife, processed in the dark. As a negative control, five midguts were treated as described above, excluding incubation in the primary antibody. Sections were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Biotium Inc., Hayward, CA, USA) for 30 min. The sections were prepared with 50% sucrose solution and analyzed and photographed using an Olympus BX60 epifluorescence microscope with a WB filter.

2.6. Cell death and oxidative stress

Fixed midguts and Malpighian tubules from control and LC_{50}-treated individuals were washed and incubated for 24 h at 4 °C in the primary antibody solution (1) anti-cleaved-caspase-3 (cleaved) (1:500) or (2) anti-peroxidase (1:800) (Sigma-Aldrich). Five midguts and five Malpighian tubules were used for anti-cleaved-caspase-3 incubation, and five of each organ were also used for anti-peroxidase incubation. The midguts were washed in PBS followed by incubation in an IgG-FITC secondary antibody, as described above. After washing, the cell nuclei were labeled with TO-PRO-3 (Life Technologies) for 30 min and mounted in Mowiol solution (Fluka). As a negative control, five midguts and five Malpighian tubules were treated as described above, excluding the incubation in the primary antibody (Supp Fig 1). The samples were analyzed and photographed under a Zeiss confocal microscope LSM 510 (Carl Zeiss) in fluorescence mode, at the Nucleus of Microscopy and Microanalysis at the Federal University of Viçosa (NMM/UFV). Fluorescence intensity was quantified using Image-Pro Plus 4.5 software (Media Cybernetics, Silver Spring, USA). For the quantification of the label intensity for the cell death and oxidative stress, six images with a 20X objective (total area = 0.828 mm^2) per midgut and Malpighian tubules were arbitrarily selected.

2.7. Statistics

Concentration-mortality bioassay data were submitted to probit analysis to estimate toxicological parameters LC_5 and LC_{50} (PROBIT PROC; SAS Institute, 2008). [25] Behavioral data were submitted to analysis of variance (ANOVA) and to Tukey's HSD test when necessary (Sigmaplot v 12.5). Morphometric data were submitted to the Kruskal-Wallis non-parametric test (Sigmaplot v 12.5). The fluorescence data were submitted to Tukey's test (Significance, $P < 0.05$) performed with the program GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, California, USA).

3. Results and discussion

3.1 Sublethal concentrations of spinosad negatively affected behavior of foragers of *A. mellifera*

The probit model was adequate for the data obtained from the concentration-mortality bioassay for spinosad formulation based on the low value of χ^2 and high *p*-value (Fig. 1). The

estimated LC_{50} and LC_5 for spinosad obtained with the probit model were 7.7×10^{-3} mg/mL and 6.1×10^{-4} mg/mL, respectively. The mean consumption of contaminated diet during 3 h of exposure was 6.6 µL/bee. Thus, the estimated oral doses for bees exposed to LC5 and LC50 were 4.03×10^{-3} µg and 5.08×10^{-2} µg a.i/bee, respectively. The field-recommended concentration of spinosad (0.816 mg/mL) was 100% lethal to *A. mellifera* workers (Fig. 1) in laboratory conditions, which was also seen in *Melipona quadrifasciata* bees.[14] These results reveal that the concentration of spinosad recommended for use in the field can expose the pollinators (i.e., non-target species) to sublethal concentrations of spinosad in lab conditions.[12-14] The field concentration (0.816 mg/mL) shows the worst possible scenario considering the laboratory conditions.

Although the probability of this concentration reaching the bees is low in field conditions due to the degradation by light and by microorganisms,[26,27] the LC_{50}, which was approximately 220-fold lower than the field concentration, still has toxic effects in bees. The honey bees exposed to spinosad at LC_5 and LC_{50} had adversely affected walking behavior. The control group walked longer distances and was faster (1,600 ± 55 cm and 2.8 ± 0.09 cm/s on average, respectively) than LC_5-treated individuals (750 ± 122 cm and 1.4 ± 0.17 cm/s on average) and LC_{50}-treated individuals (1,100 ± 150 cm and 2 ± 0.23 cm/s on average, respectively) ($P < 0.001$) (Figs 2A and 2B). The control bees did not stop walking, whereas for LC_5- and LC_{50}-treated individuals, the resting time was 100 ± 40 and 50 ± 28 seconds, respectively ($P = 0.035$) (Fig. 2C). These negative effects on behavior caused by spinosad may compromise other behaviors, such as the foraging activity of *A. mellifera* workers in the field,[28] colony maintenance, and longevity because of the reduction in survival.[18] Sublethal doses of spinosad also negatively affected the behavior of workers of the stingless bee *M. quadrifasciata*, impairing their flight performance and reducing their respiration rate,[10] locomotion, and orientation.[28] The toxic effects of spinosad may be due its interaction with acetylcholinesterase receptors in the central nervous system, disrupting the excitatory stimulus transmission,[7,29] which includes initial flaccid paralysis, tremors, and death.[26]

3.2. Spinosad damaged the midgut and Malpighian tubules

The ingestion of spinosad formulation caused disorganization of the midgut and Malpighian tubule epithelia of LC_{50}-treated forage workers. The midgut of the control group

consisted of a columnar epithelium (57.3 ± 15.5 μm height) with an evident brush border (5.8 ± 2.0 μm height), whereas in the spinosad treated group, there was a marked decrease in the height of the digestive epithelium (22.8 ± 5.4 μm) ($P < 0.001$), digestive cells without brush border, and few clusters of regenerative cells were seen. In the Malpighian tubule cells, there was an intense vacuolization and cytoplasmic degradation in the treated individuals. In addition, Malpighian tubule cells in the control had a nuclear area of 41.5 ± 6.9 μm^2 with evident nucleoli, whereas, in the treated workers, the nuclear area was smaller (17.1 ± 5.4 μm^2) ($P < 0.001$) and with condensed chromatin (Fig. 3).

The overall intensity labeling was assessed for peroxidase and activated caspase-3 in the midgut and Malpighian tubules of LC_{50}-treated forage workers as a proxy to determine damage in the internal organs of the bees. There was an intense labeling for both peroxidase (~158.00 ± 105.70) and caspase-3 (~148.80 ± 97.13) in the midgut, whereas the fluorescent signal was very low (~13.75 ± 7.53 and ~1.25 ± 1.89 respectively) ($P < 0.001$) in the controls (non-exposed bees). Similarly to observed for the midgut, there was an intense labeling for both peroxidase (~67.25 ± 41.28) and activated caspase-3 (~150.00 ± 79.00) in the Malpighian tubules of LC_{50}-treated bees, whereas the fluorescence signal was barely or not detected (~1.00 ± 1.30 and ~0.00 ± 0.00 respectively) ($P < 0.001$) in the control (Figs 5, 6 and 7). These results confirmed that spinosad formulation is toxic to these two organs, as have been reported for other pesticides in bees,[21,30,31] and for the red palm weevil *Rhynchophorus ferrugineus* (Coleoptera: Dryophthoridae).[32]

Peroxidase labeling indicates a response to oxidative stress, followed by the synthesis of antioxidant enzymes for the maintenance of organ functions against spinosad treatment, as have been proposed for *A. mellifera* treated with imidacloprid.[21] The strong labeling of caspase-3 in the cells of these two organs shows that expression of peroxidase possibly might not recover the damage caused by spinosad, leading to the activation of the apoptotic pathway, as observed in the midgut of *Spodoptera littoralis* (Lepidoptera: Noctuidae) larvae after treatment with spinosad.[33] In *Spodoptera frugiperda* (Lepidoptera: Noctuidae), cells treated with spinosad have mitochondrial dysfunction with an increased production of reactive oxygen species, followed by cell death due to the activation of caspase-3.[34] The degeneration of the epithelium can be the main factor leading to individual mortality in bees, due to the deprivation of nutrient absorption in the midgut.[20,31,35]

Spinosad acts on the nicotinic acetylcholine receptors and the gamma-aminobutyric acid receptor in the nervous systems of target insects;[7] however, when it is used as a formulation, it can also affect other organs in bees, including the midgut and Malpighian tubules, as previously reported for *S. littoralis*[33] and *R. ferrugineus*.[32] Compounds of the insecticide formulation, including the surfactants, can, increase the persistence of pesticides in the environment,[36, 37] and increase insecticide toxicity too.[38] Damage observed in the digestive cells of honey bees treated with spinosad formulation also interfered with the synthesis of components of the peritrophic matrix. There was no chitin detection and very weak labeling for peritrophin, whereas for the control the peritrophic matrix was intact (Fig 4). The peritrophic matrix is responsible for the protection of the midgut epithelium against mechanical and chemical damages, as well as being a physical barrier against microorganisms.[39] The collapse of the peritrophic matrix affects nutrient uptake, signaling pathways, and transport processes[40] and leaves the midgut more vulnerable to pathogens[41,42] and can lead to death.[43]

Under the stressful conditions that occur after insecticide exposure, the organs may increase autophagic activity to degrade altered proteins and organelles, and finally, lead to the process of apoptosis.[44] In addition, it has been suggested that different pesticides (e.g. thiacloprid, fipronil, and imidacloprid) may increase the parasitic load of bees.[45-47] Taken together, our results confirmed that oral exposure to spinosad formulation in laboratory conditions is also capable of affecting non-primary target organs such as the midgut and Malpighian tubules. Thus, further studies are required on how spinosad formulation is capable of negatively interfering with these two organs.

4. Conclusions

Our findings provide new insights into the debate about the role of bioinsecticides in the mortality of Africanized honey bees since even at very low concentrations (LC_5), *A. mellifera* workers underwent behavioral changes. These changes include the impairment of walking abilities, which could undermine the dynamics of colonies with reduced longevity, influence on foraging activity, or even loss of individuals who cannot return to the colony due to lack of orientation. In addition, our experiments indicate the need for additional tests to evaluate the toxicity of bioinsecticides, aiming to evaluate the behavioral changes and damages caused in non-target organs. Our study represents a significant contribution to support future assessments

of the risk of spinosad alone or in formulations for the development and validation of appropriate toxicological test methods for pollinators. In addition, a modification on the spinosad formulation can signify a great reduction in the toxicity to bees. Finally, although it originates from a natural source, the bioinsecticide spinosad does not guarantee safety for the honey bees because exposure of bees to this insecticide even at very low concentrations (dilutions of up to 220-fold; the concentration recommended for use in the field) is toxic for the vital organs midgut and Malpighian tubules.

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7. Figure Legends

Figure 1 - Concentration-mortality curve of *Apis mellifera* forage workers orally exposed to spinosad; the dotted lines represent the 95% fiducial limits of the curve.

Figure 2 - Behavior assessment (10 min of record) of *Apis mellifera* adult nurse workers. Individuals were orally exposed for 3 h to sucrose solution with spinosad (LC5 and LC50) and the behavior was recorded 21 h after exposure. Control receive only sucrose. (A) A representative graph exhibiting the walking distance. (B) Mean velocity. (C) Resting time. Bars represent the average (±SE of the average), and the different letters indicate significant differences among treatments based on Tukey's HSD test ($P < 0.01$).

Figure 3 - Histological section of the midgut [A and B] and the Malpighian tubules [C and D] of *Apis mellifera* forage workers stained with toluidine blue. Individuals were orally exposed for 24 h with 50% sucrose (control) and with sucrose solution with spinosad (LC_{50}). (A) Midgut of a control and (B) midgut of a treated individual (LC_{50}) with fragmented epithelium with cells without the brush border. (C) Malpighian tubules of a control and (D) Malpighian tubules of a treated individual (LC_{50}) with intense cell vacuolization. Ep-epithelium; N-nucleus; L-lumen; M-muscle; V-vacuoles.

Figure 4 - Histological sections of the midgut of *Apis mellifera* forage workers fed orally with 50% sucrose (control) and with sucrose solution with spinosad (LC_{50}). The cell nucleus was stained with DAPI (blue), while the peritrophic matrix was stained with the lectin WGA-FITC (for chitin, green) and anti-peritrophin (red).

Figure 5 - Whole mounts of midgut and Malpighian tubules of *Apis mellifera* forage workers fed orally for 50% sucrose (control) and sucrose solution with spinosad (LC_{50}). Labeling for peroxidase (green) was detected only in cells of treated bees, not occurring in the control. The nuclei of the epithelial cells were stained with TO-PRO-3 (red).

Figure 6 - Whole mounts of midgut and Malpighian tubules of *Apis mellifera* forage workers fed orally with 50% sucrose (control) and sucrose solution with spinosad (LC_{50}). Labeling for caspase-3 (green) was detected in cells in apoptosis. No staining was observed in the organs of control bees. The nuclei of the epithelial cells were stained with TO-PRO-3 (red).

Figure 7 - Fluorescence intensity for caspase-and peroxidase in the midgut and Malpighian tubules of *Apis mellifera* forage workers fed orally with 50% sucrose (control) and sucrose solution with spinosad (LC_{50}). Mean and standard deviation (±SD) are shown (bars). Different letters indicate significant differences based on Tukey's HSD test ($P < 0.001$).

Supplementary Figure 1 - Whole mounts of the midgut and Malpighian tubules of *Apis mellifera* foragers fed orally with sucrose solution (control) and sucrose solution with spinosad (LC50). Samples were incubated with secondary antibody (negative control for the immunohistochemistry assays) bees and no staining was observed in the organs. The nuclei of the epithelial cells were stained with TO-PRO-3 (red).