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Spinosad-mediated effects on survival, overall group activity and the midgut of workers of *Partamona helleri* (Hymenoptera: Apidae)

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ABSTRACT

Populations of stingless bees have declined around the world and pesticides have been indicated as one of the possible causes of this decrease. Spinosad, which is synthesized from the fermentation process produced by the soil actinomycete *Saccharopolyspora spinosa*, is one of the most used bioinsecticides today. This study aimed to evaluate the possible effects of spinosad (formulation) on survival, general group activity and the processes of autophagy, apoptosis and oxidative stress in two organs (midgut and brain) of workers of *Partamona helleri*, after 24 h of oral exposure. Workers were orally exposed to different concentrations of spinosad. The concentration (8.16×10^{-3} mg a.i./mL) that led to the mortality of approximately half the number of treated bees was considered LC_{50} and was used in behavior, histology and immunofluorescence bioassays. The results revealed that bee survival was substantially reduced with increasing spinosad concentrations. The LC_{50} of the bioinsecticide compromised general group activity, caused morphological alterations in the midgut and intensified the processes of autophagy, apoptosis and oxidative stress in this organ. The brain, on the other hand, did not present significant alterations under the tested conditions. The data obtained demonstrate, therefore, that spinosad negatively affects individual survival, general group activity and the midgut epithelium of *P. helleri*.

1. Introduction

Discussions involving the possible causes of bee colony declines include diseases, parasites, inappropriate management by beekeepers (i.e., through of the excessive use of coumaphos), malnutrition, habitat fragmentation and/or pesticides (Ratnieks and Carreck, 2010; Tomé et al., 2012; Schwarz et al., 2014; Feng et al., 2017). The impact of pesticides on bee decline has received special attention in recent years, since several studies have shown negative effects, both lethal and sublethal, on a number of bee species (Wu et al., 2015; Revision in Barbosa et al., 2015a; Bernardes et al., 2018).

Among the most commonly used pesticides are neonicotinoids such as acetamiprid, imidacloprid, and thiamethoxam (Shi et al., 2017; Codling et al., 2018) and bioinsecticides, mainly azadiractin and spinosad (Bernardes et al., 2017; Gómez-Escobar et al., 2018). Rossi et al. (2013) and Catae et al. (2018) reported neural damage and learning impairment of *Apis mellifera* after the use of imidacloprid, while Lopes et al. (2018) found that the bioinsecticide spinosad can cause significant alterations in the midgut of these bees, affecting their ability to digest and absorb food.

The bioinsecticide spinosad is known to be a nicotinic acetylcholine receptor agonist that interferes with γ-aminobutyric acid receptors in the nervous system (Sparks et al., 2001). This bioinsecticide is a member of the spinosyn family and is composed of a mixture of tetracyclic-macrolide compounds that are produced by the fermentation of the soil actinomycete *Saccharopolyspora spinosa* (Bacteria: Actinobacteridae) (Sparks et al., 2012; Cabrera-Marín et al., 2016; Arena et al., 2018). Spinosad is classified as a low-risk bioinsecticide and is therefore approved for use in organic farming by several regulatory agencies in more than 80 countries (Biodini et al., 2012; Huang et al., 2016). Although the limits of potential exposure for most chemicals are not directly lethal to pollinating insects, sublethal doses of some of

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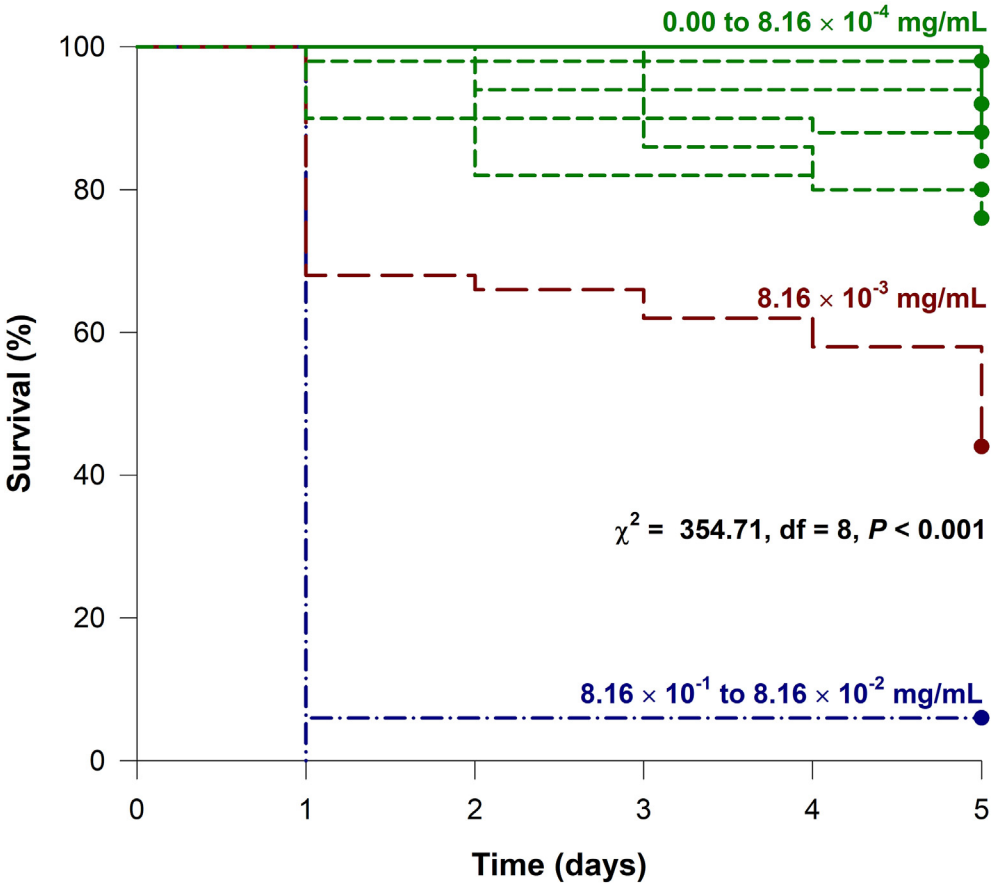

Fig. 1. Survival of *Partamona helleri* workers exposed orally to different concentrations of spinosad (8.16×10^{-1}, 8.16×10^{-2}, 8.16×10^{-3}, 8.16×10^{-4}, 8.16×10^{-5}, 8.16×10^{-6}, 8.16×10^{-7} and 8.16×10^{-8} mg a.i/mL) diluted in 50% sucrose. Survival curves coded with the same color and shape were not significantly different from one another based on pairwise multiple comparisons using Bonferroni Method ($p > 0.05$). The survival curve of the control group (diet with only 50% sucrose) is encoded with a solid line. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

them may adversely affect these individuals, influencing the development of colonies (Williamson et al., 2014; Revision in Lima et al., 2016).

The sublethal effects may be characterized by physiological or behavioral changes in surviving bees and these responses may indicate that the functional integrity of the organism has been affected, which contributes to their incapacity to perform habitual tasks (Tavares et al., 2015). Such incapacity can provide irreparable damage to bee populations because in agroecosystems these insects survive by feeding on flowers that grow on the shores of fields and in some semi-natural habitats (Whitehorn et al., 2012).

Among Brazilian bees, the best known are those belonging to the Meliponini tribe, which are popularly called “stingless indigenous bees” (Camargo and Pedro, 1992; Vollet-Neto et al., 2018). Some studies that have evaluated the sublethal effects of bioinsecticides with these bees have indicated the occurrence of malformations during development (Barbosa et al., 2015b) and impairment of flying activity (Tomé et al., 2015a). Spinosad induces antifeeding effects (Bernardes et al., 2017) and influences individual flight takeoff (Tomé et al., 2015b), development of the reproductive system and queen morphology (Bernardes et al., 2018) in the genus *Partamona*.

In the present work, we evaluated the effects of the formulation of the bioinsecticide spinosad on survival, general group activity and the processes of autophagy, apoptosis and oxidative stress in two organs (midgut, and brain) of workers of *Partamona helleri*, after 24 h of oral exposure.

2. Materials and methods

2.1. Test organisms

Adult *Partamona helleri* workers were collected from four colonies in the rural area of Viçosa and were maintained in the Central Apiary at the Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil (20° 75′S 42° 86′W). The midgut and brains of forager workers (dark black color) were used in the bioassays of survival, morphology, and immunofluorescence, while newly emerged workers (greyish) were used in general group bioassays. Midgut and brain were chosen because they are the organs responsible for food digestion and absorption and, for important cognitive processes, respectively (Catae et al., 2018). The newly emerged bees were used because they were not yet capable of flying and flight would compromise our ability to record their activities (Tomé et al., 2012).

The adult workers were collected at the entrance of each hive when leaving the nest using a glass Erlenmeyer flask. The newly emerged workers were gently removed from the honeycombs of the colonies with tweezers after we opened them and transferred to Petri dishes. Then, the Erlenmeyer flasks and Petri dishes with the bees were taken to the laboratory where they were kept in an incubator at 28 °C and 80% humidity in the dark for a period of 1 h for acclimatization.

2.2. Insecticide and survival bioassays

We used the insecticide spinosad in its commercial formulation (Tracer, 480 g active ingredient (a.i.)/L, concentrated suspension; Dow AgroSciences, Santo Amaro, SP, Brazil). A stock solution of spinosad at 0.816 mg a.i./mL was prepared by diluting the commercial formulation at the maximum rate recorded for field (i.e., 17 mL of Tracer per 100 L of water) in a 50% aqueous sucrose solution according to the regulations of the Brazilian Ministry of Agriculture for the control of the white fly (*Bemisia tabaci*) and the tomato leafminer (*Tuta absoluta*) (MAPA, 2018). To minimize the amount of stock solution produced, we diluted only 170 µL of Tracer in 100 mL of 50% aqueous solution. This insecticidal solution was used as the basis to obtain seven more diluted

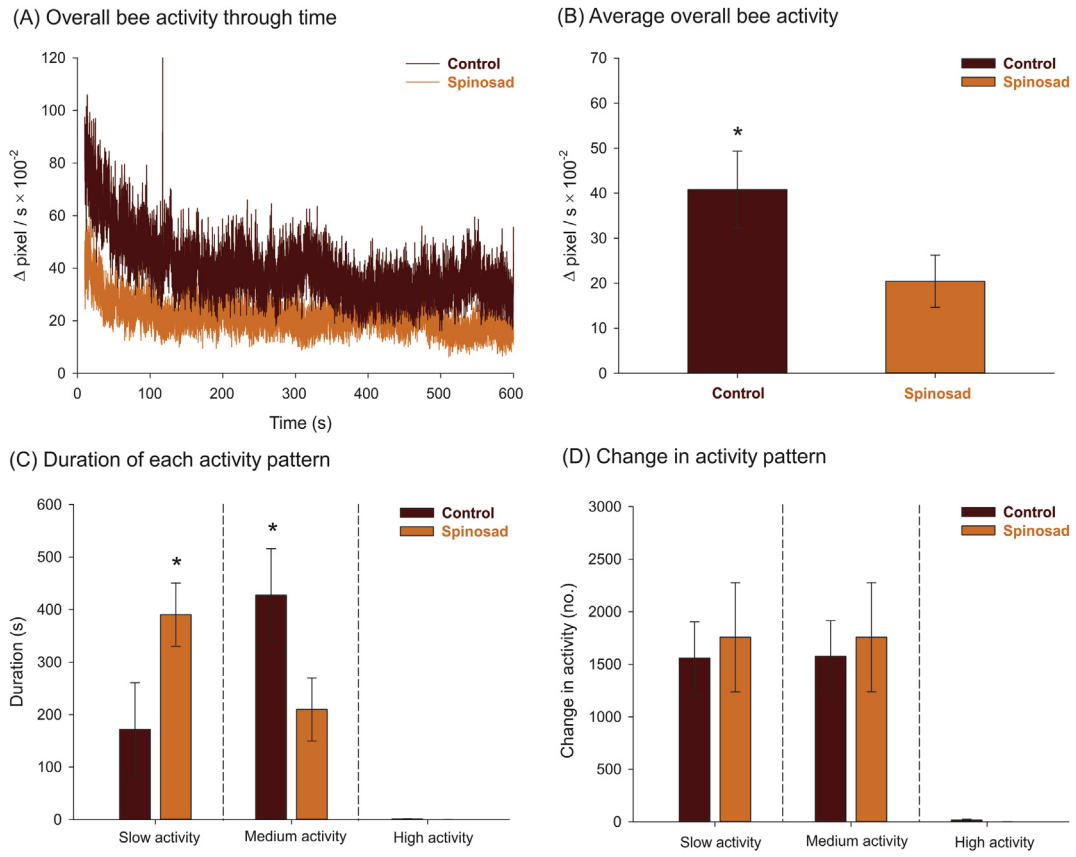

Fig. 2. General activity of adult workers of *Partamona helleri* after 24 h of oral exposure to LC_{50} of spinosad, evidencing: profile of general activity over time (A), average global activity (B), duration of each activity pattern (C) and change of -activity pattern (D). Asterisks denote significant differences by *t*-tests ($p < 0.05$) and whiskers represent standard errors.

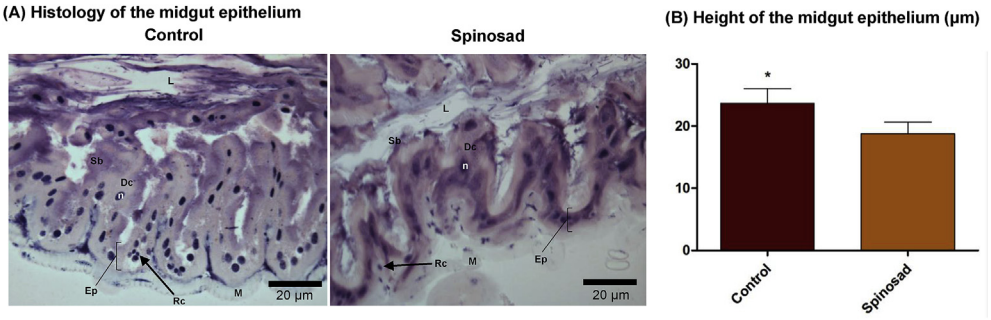

Fig. 3. Histological sessions showing the morphological changes observed in the midgut of *Partamona helleri* workers treated with LC_{50} of spinosad in relation to the control group (A) and midgut thickness of treated or untreated (control) bees with LC_{50} of spinosad (B). In the midgut epithelium (Ep) the nuclei (n) of digestive cells (Dc) with their striated borders (Sb) and regenerative cells (Rc) are shown. Note the midgut lumen (L) and a layer of muscle cells (M). Asterisks denote significant differences by *t*-tests ($p < 0.05$) and whiskers represent standard errors.

concentrations that were used in the experiment (see below).

Ten forager bees from each colony were separately transferred to new plastic pots with volumes of 250 mL, with each of the four pots corresponding to an experimental unit. The bees were orally exposed to the bioinsecticidal solution by a feeder made of perforated microcentrifuge tube (1.5 mL), which was inserted into a hole in the wall of each plastic pot. The bioinsecticide was used in the following concentrations: 8.16×10^{-1}, 8.16×10^{-2}, 8.16×10^{-3}, 8.16×10^{-4}, 8.16×10^{-5}, 8.16×10^{-6}, 8.16×10^{-7} and 8.16×10^{-8} mg a.i./mL. The oral exposure to spinosad lasted for 3 h. Subsequently, the bees were fed with only 50% uncontaminated sucrose solution. As a control, ten foragers bees from each colony were used. They were fed with 50% sucrose solution during the experimental period.

The survival of bees exposed to spinosad was recorded every 24 h, until the first bees of the control group die, which happened on the 5th day, after exposure of the experimental group to the bioinsecticide. The concentration of spinosad that led to the mortality of approximately half the number of treated bees (8.16×10^{-3} mg a.i./mL) was considered in this work, the lethal concentration of 50% (LC_{50}).

2.3. General group activity (video tracking)

General activity bioassays were performed using four newly emerged workers from each of the four colonies, this is, the experimental unit encompassed a Petri dish with four bees from a single colony and, therefore, colonies were used as replicates.

Bees exposed to LC_{50} of spinosad or control diet were treated as mentioned in section 2.2. These bees were transferred to a Petri dish, which was 9 cm in diameter and 2 cm in height, was lined with filter paper (Whatman no. 1) and had its inner walls covered with Teflon® polytetrafluoroethylene (PTFE) (Dupont, Wilmington, DE, USA) to prevent the insects from escaping because this makes the walls of the Petri dish smooth (Tomé et al., 2012).

The general activity of the bees was recorded 24 h after the

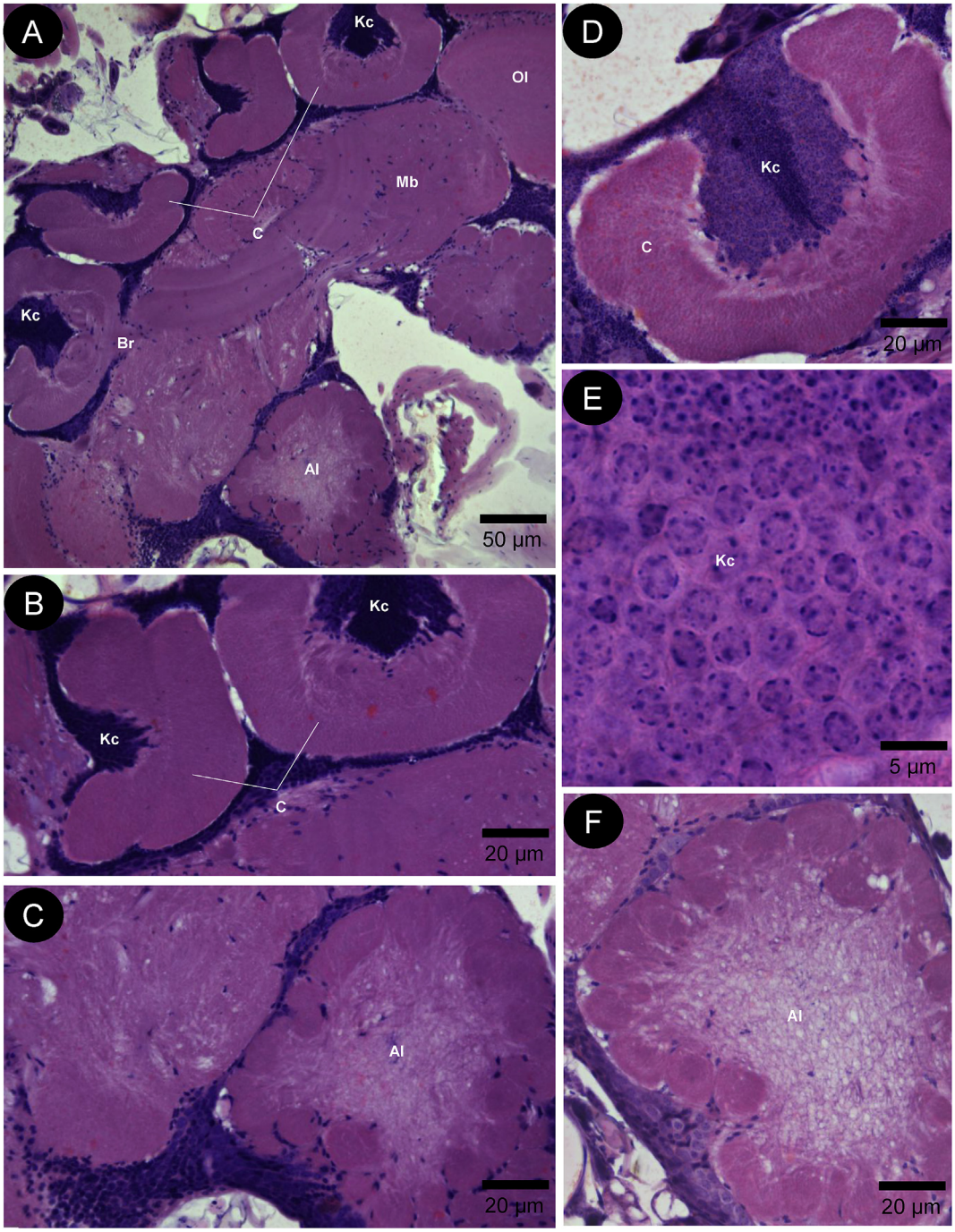

Fig. 4. Histological section of the brains of *Partamona helleri* workers comparing individuals treated with spinosad LC50 (A, B and C) and control (D, E, and F). Kenyon cells (Kc), calix (C), basal ring (Br), mushroom bodies (Mb), optic lobe (Ol) and antennal lobe (Al).

beginning of oral exposure to the treatment at room temperature (25 °C ± 2) and under artificial light. The activities were digitally captured for 10 min, using a digital charge-coupled device (CCD) camera connected to a computer equipped with a video-tracking system (ViewPoint Life Sciences, Montreal, Canadá). This system determines the overall activity by changing captured pixels by a fraction of time (Δ pixels/s x 10^{-2}), which corresponds to the sum of any change in the position and posture of individuals within the arena (Tomé et al., 2015a). Therefore, movements including walking, wings' opening, head movement, and grooming were considered as "general activity" and counted as pixel change over time. The overall activity of the groups of bees was standardized from the protocol proposed by Lima et al. (2015), which classifies the activity of individuals into three categories: low activity (variation less than 4 pixels/s x 10^{-2}), medium activity (variation between 4 and 8 pixels/s x 10^{-2}) and high activity (variation above 8 pixels/s x 10^{-2}).

2.4. Histology

Uncontaminated solutions (control, n = 4 individuals per colony) and LC_{50} (n = 4) of spinosad estimated for 24 h of exposure were given to new groups of foragers bees. After the exposure period, the bees were dissected in saline solution (0.1 M NaCl, 20 mM KH_2PO_4 and 20 mM Na_2HPO_4). The midgut and brain of each specimen were fixed in a Zamboni solution (2% paraformaldehyde, containing 15% picric acid in 0.1 M sodium phosphate buffer) for 2 h at room temperature.

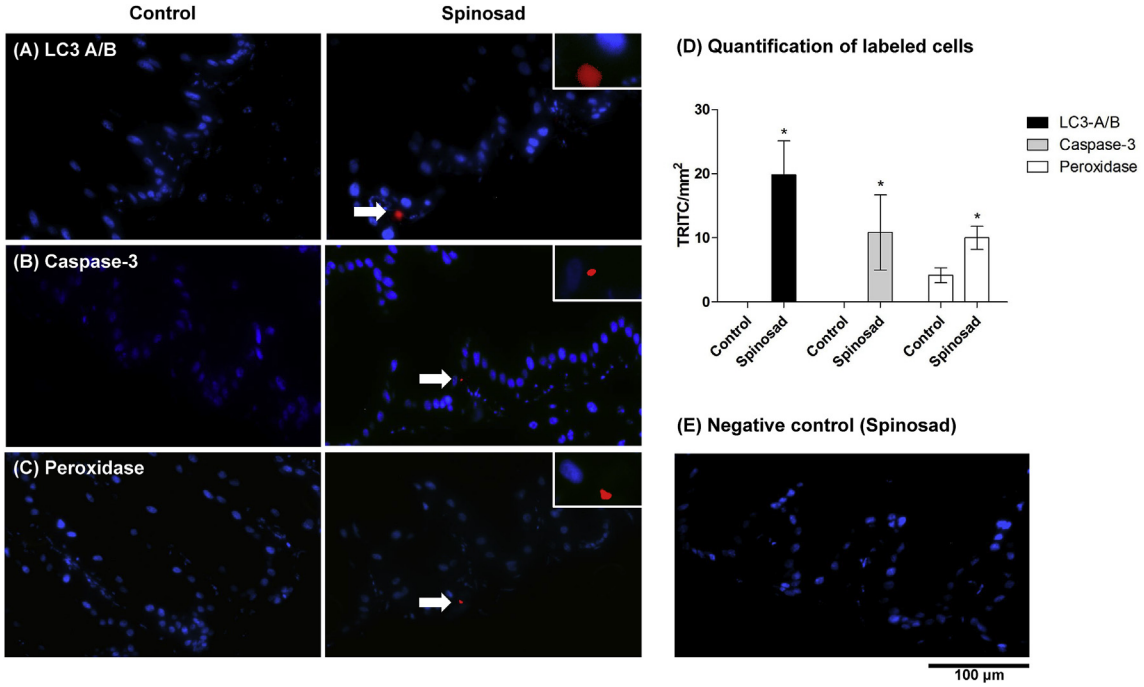

Fig. 5. Histological sections of the midgut of *Partamona helleri* workers showing markers for LC3 A/B (A), cleaved caspase-3 (B) and peroxidase (C), in untreated or treated bees with LC_{50} of spinosad. Number of cells labeled for the different antibodies (D) and negative control of the midgut of treated bees (E). The total area analyzed was 1.656 mm^2. Cell nuclei are blue (DAPI) and positive markings are red (arrows). Asterisks denote significant differences by *t*-tests ($p < 0.05$) and whiskers represent standard errors. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Subsequently, the samples were washed three times in 0.1 M phosphate buffered saline (PBS), dehydrated in a graded series of ethanol (70–99%), stained with historesin (Leica Biosystems, São Paulo, SP, Brazil) and sectioned with 7 µm thick glass knife in a Leica 2255 automatic microtome. Sections were stained with hematoxylin and eosin (HE), then were analyzed and photographed under an Olympus BX53 microscope coupled to an Olympus DP 73 digital camera (Olympus Optical Corp., Tokyo, Japan).

The images were used to measure the thickness of the midgut epithelium using Image-Pro Plus 4.5 software (Media Cybernetics, Silver Spring, USA). For this quantification, twelve images were obtained with a final magnification of 1600× both for bees of the control group and those treated with spinosad. These images were selected arbitrarily and the means were measured.

2.5. Immunofluorescence

The midguts and brains that were fixed as described above were washed three times with PBS and incubated at 0.1 M PBS / 1% Triton X-100 (PBST) for 2 h. The samples were then incubated overnight at 4 °C separately with the following primary antibodies diluted in PBS: anti-LC3 A/B (Cell Signalling Technology, Beverly, MA, EUA; 1: 100) related to autophagy; anti-cleaved caspase-3 (Sigma-Aldrich, St. Louis Mo., EUA; 1: 500) related to apoptosis; and anti-peroxidase (Sigma-Aldrich, St. Louis Mo., EUA; 1: 500) related to oxidative stress (Gonçalves et al., 2018; Lopes et al., 2018). The three immunolabels were performed in the midgut and brains from four bees exposed to the control or spinosad, totaling 24 individuals.

Subsequently, the organs were washed and incubated with TRITC conjugated secondary antibody (Thermo Fisher-Scientific, Waltham, Mass., EUA; 1: 500) in PBS overnight at 4 °C. After being subjected to the triple wash, the samples were dehydrated in ethanol, soaked in historesin and sectioned at a thickness of 7 µm. Subsequently, the sections were stained with diamidino-2-phenylindole (DAPI; Biotium, Inc., Hayward, CA, EUA; 1: 500) for 30 min, washed in PBS three times and mounted on histological slides using a 50% sucrose solution. Finally, the slides were analyzed under a fluorescent microscope Evos® FL (Advanced Microscopy Group, Bothell, WA, USA). The quantification of LC3 A/B, caspase-3 and peroxidase-labeled cells was performed in 20 sections using a final magnification of 1600× (total area = 1.656 mm^2). For the negative control, four midguts and brains from each treatment were treated as previously described, omitting the treatment with the primary antibodies.

2.6. Statistical analyses

The concentration-mortality bioassay data were subjected to a survival analysis using the procedure Survival LogRank ($p < 0.05$) (SIGMAPLOT v 12.5, Systat Software, San Jose, CA, EUA). Survival curves were obtained by Kaplan–Meier estimators and the Bonferroni method was used as pairwise multiple comparisons ($p < 0.05$) (SIGMAPLOT v 12.5). The general group activity, thickness of the midgut epithelium and fluorescence data were submitted to *t*-tests at 5% of significance (SIGMAPLOT v 12.5).

3. Results

3.1. Time-mortality by oral exposure (survival)

The survival of workers of *P. helleri* was significantly impaired after the ingestion of increasing concentrations of spinosad when compared to the control (Log-Rank test: $\chi^2 = 354.71$, df = 8, $p < 0.05$). Twenty-four hours after the onset of exposure, all subjects treated with the field concentration (8.16×10^{-1} mg a.i./mL) were dead (Fig. 1). The survival of bees exposed to this concentration diluted by 10, 100 and 1000 times was 8%, 68%, and 100%, respectively.

The survival curve of the individuals exposed to 8.16×10^{-3} mg/mL (100× dilution) showed a significant difference ($p < 0.05$) in relation to the other treatments, with a total of 44% of individuals alive five days after ingestion of spinosad.

3.2. General activity in groups of stingless bees

The general activity profile of *P. helleri* over time showed that individuals who were exposed to the LC_{50} of spinosad and survived generally were less active than the control ones (Fig. 2A). Thus, the mean overall activity during the evaluation period was significantly reduced in spinosad-treated bees (10 min; $p < 0.05$; Fig. 2B). The duration of slow and medium activity levels also varied significantly, with a predominance of slow and medium activity in the treated and control bees, respectively ($p < 0.05$; Fig. 2C). However, there was no significant difference in levels of high activity (Fig. 2C) and the general activity pattern between the treated and control groups ($p > 0.05$; Fig. 2D).

3.3. Morphology of the midgut and brain

The midgut of foragers of *P. helleri* who ingested the uncontaminated diet presented an epithelium with a layer of columnar digestive cells and among them, regenerative cells, of smaller size, organized in nests located at the base of the epithelium (Fig. 3A). The mean height of this epithelium was 23.68 ± 2.36 μm (Fig. 3B) and in the apical regions of the digestive cells, a well-developed striated border (5.88 ± 0.79 μm) was observed. Individuals who received the spinosad formulation (LC_{50}) had a disordered and significantly thinner epithelium (18.76 ± 1.89 μm) when compared to the control. The regenerative cell nests were disorganized and the striated border was conspicuously lower (1.75 ± 0.33 μm).

In spite of the differences found in the gut epithelium of bees that survived to the spinosad exposure, there were no morphological differences in the brain of exposed bees compared to those not exposed to spinosad (Fig. 4).

3.4. Autophagy, apoptosis, and oxidative stress

In the histological sections of the midgut of bees subjected to the LC_{50}, positive fluorescence for LC3 A/B (mean 20 ± 5 labeled cells) and cleaved caspase-3 (11 ± 5 cells) was observed. The fluorescence results were negative for the control (Fig. 5A–B). In this organ, the mean number of peroxidase-positive cells was significantly higher in subjects treated with spinosad than the control diet (10 ± 1 and 4 ± 2 cells, respectively, $p < 0.05$; Fig. 5C–D). In the brain, however, no fluorescence was detected that was positive for any antibody, either in individuals treated with spinosad (LC_{50}) or exposed to the untreated diet (Supplementary Material 1).

4. Discussion

Toxicological data on adult workers of *P. helleri* fed diets of different concentrations of spinosad showed a great reduction in the survival of the bees with increased concentrations of the bioinsecticide. Similar data have been found in studies with *Apis mellifera* (using 20, 3, and 4.8 g a.i./100 L by 24 h - Rabea et al., 2010), *Bombus terrestres* (400 mg L^{-1} diluted in 1/10, 1/100, 1/1000 and 1/10000 by 72 h - Besard et al., 2011), *Melipona quadrifasciata* (5.0, 10.0, 17.5, 25.0, and 42.5 ng a.i./bee by 1 h - Tomé et al., 2015b) and *Scaptotrigona xanthotricha* (15.82 ng a.i./bee^{-1} by 1 h - Tomé et al., 2015a), evidencing that spinosad, especially when ingested, induces negative effects on the survival of several species of bees. This may occur because spinosad acts primarily on the nicotinic receptors and γ-aminobutyric acid receptor on the nervous system, which leads to death (Spark et al., 2001).

It is also possible that spinosad triggers behavioral changes, causing sublethal effects. In the specific case of *P. helleri*, LC_{50} exposure of spinosad (oral route) caused a significant loss in the general activity of groups of workers, indicating a change in the bees' behavior. In contrast, this bioinsecticide did not alter the general activity of *M. quadrifasciata*, but impaired flight activity in these bees, which may compromise foraging activity and, as a consequence, colony maintenance and survival (Tomé et al., 2015a). Considering that reduced general activity, as observed in *Neoseiulus baraki* (Acari: Phytoseiidae), can also cause losses in foraging, as well as decrease reproduction and dispersion (Lima et al., 2015), the lower activity in groups of *P. helleri* that were exposed to spinosad should be viewed with concern, since this parameter is related to the movement of the bees and their commitment can result in negative effects on reproduction, dispersion, foraging and, consequently, on the maintenance of the colony.

In this work, we also evaluated the effects of exposure to spinosad on *P. helleri* midgut epithelium. The histological results corroborate recent investigations in *A. mellifera* that similarly were orally exposed to spinosad and presented disorganization in the midgut epithelium, few nests of regenerative cells, and the absence of a striated border in digestive cells (Lopes et al., 2018). Such effects of spinosad may compromise gut functionality, since striated border size is related to the degree of absorption of food (Gonçalves et al., 2014), and nests of regenerative cells are important for increasing the population of these cells (Martins et al., 2006). These effects may contribute to the reduction of bees' useful life in general, as a result of impaired digestion and nutrient uptake (Oliveira et al., 2014).

By using LC3 A/B as a specific marker of autophagy it was possible to detect the occurrence of "autophagic cell death" (ACD) in the midgut of *P. helleri* workers exposed to spinosad. Previous studies have suggested that pesticides could cause ACD in the midgut of *A. mellifera* (Catae et al., 2014) and *Anticarsia gemmatalis* (Fiaz et al., 2018) and that ACD can be potentiated in certain organs after direct contact with these pesticides. Thus, it can be assumed that in the specific case of *P. helleri* the occurrence of cells labeled with LC3 A/B and, extrapolating from ACD, after exposure to spinosad, would respond to the presence of toxic molecules that were absorbed by the cells of the midgut in an attempt to eliminate these toxins and recycle the cellular constituents, as suggested by Fiaz et al. (2018).

The presence of caspase-3 and peroxidase-positive cells that are indicative of apoptosis and oxidative stress in the midgut, as observed in the present study, were also reported in studies with *A. mellifera* (Lopes et al., 2018), *Rhynchophorus ferrugineus* (Abdelsalam et al., 2016) and *Spodoptera frugiperda* (Yang et al., 2017). In these insects, spinosad induced structural and even ultrastructural cell alterations, such as mitochondrial dysfunction and inhibition of antioxidant enzyme activity, confirming that spinosad is capable of enhancing oxidative stress and, therefore, apoptotic cell death in the midgut of non-target insects. In addition, it has been found that sublethal doses of spinosad trigger oxidative stress in liver and brain cells of *Oreochromis niloticus* (Cichlidae: Pseudocrenilabrinae) as a result of the generation of ROS, and these cells can undergo apoptosis (Piner and Üner, 2012, 2013). Therefore, the detection of peroxidase and caspase-3 positive cells in the midgut of adult bees (forager) of *P. helleri* suggested that in this species, spinosad has effects similar to those described above, which may result in morphological alterations that impair organ function and, consequently, insect survival.

No morphological changes and/or immunofluorescence markers were found with any of the antibodies used in the *P. helleri* brain when analyses were performed 24 h after exposure to spinosad. This is an interesting fact because alterations in the optical lobes and mushroom bodies (Tomé et al., 2012), impairment of the development of the mushroom bodies (Rossi et al., 2013) and disorganization of Kenyon cells, with some evidence of cell death (Catae et al., 2018) have been reported in *M. quadrifasciata* and/or *A. mellifera*. These analyzes, however, were performed 72 h after exposure to imidacloprid (Tomé et al., 2012; Rossi et al., 2013; Catae et al., 2018). Thus, we speculated that there was a momentary increase in the metabolism of the detoxification system to pesticides (Feyereisen, 2006; Chaimanee et al., 2016) during the experimental period of the present study. This increase might have hindered the detection of cellular changes in the brain of treated *P. helleri*. Nevertheless, further investigation is needed.

Ultrastructure analyses and/or assays considering the effects of this bioinsecticide on the functions of metabolic enzymes, such as, glutathione S-transferase and carboxylesterase may also provide data to better understand the effect of this insecticide on the brain of *P. helleri*.

Collectively, the data from this study suggests that the bioinsecticide spinosad negatively affects individual survival and overall activity in groups of *P. helleri*. The results also suggest that this bioinsecticide can compromise the midgut in a short period of time, inducing oxidative stress and cell death by autophagy and apoptosis, resulting in epithelial degradation and, consequently, affecting the absorption and digestion of nutrients. These data should, therefore, be considered in future assessments related to the environmental impact of this bioinsecticide on non-target insects.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2019.03.050.

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