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Spinosad-mediated effects in the post-embryonic development of *Partamona helleri* (Hymenoptera: Apidae: Meliponini)☆

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ABSTRACT

The use of insecticides based on metabolites found in live organisms, such as the insecticide spinosad, has been an option for the control of agricultural pests because of the allegedly low toxicological risk for nontarget arthropods, such as stingless bees. In the current study, we evaluate the effects of chronic oral exposure to spinosad during the larval phase on survival, developmental time, body mass, midgut epithelial remodeling, and the peritrophic matrix (PM) of *Partamona helleri* stingless bee workers. Worker larvae that were raised in the laboratory were orally exposed to different concentrations (0, 6.53, 13.06, 32.64, and 3,264 ng. a.i. bee^{-1}) of spinosad (formulation), and the resulting survival, developmental time, and body mass were studied. The concentration of spinosad recommended for use in the field (3,264 ng. a.i. bee^{-1}) reduced the survival of workers during development. Also, sublethal concentrations of spinosad delayed the development and caused morphological changes in the midgut epithelium. Finally, the chronic exposure of larvae to 32.64 ng. a.i. bee^{-1} spinosad also altered the remodeling of the midgut during metamorphosis and affected the organization of the PM of larvae, pupae, and adults. Our data suggest possible environmental risks for using spinosad in cultures that are naturally pollinated by stingless bees.

1. Introduction

Stingless bees are important pollinators of both native and cultivated plants in the Neotropics (Camargo and Pedro, 2013). These bees have been exposed to synthetic insecticides used to control agricultural pests on a large scale. Insecticide intoxication compromises the individual health and maintenance of the colonies of these nontarget bees (Lima et al., 2016; Tomé et al., 2012, 2017). Brazil has the most biodiverse assortment of these bees (Pedro, 2014; Morais et al., 2018), and it is one of the world's largest consumers of synthetic insecticides (Schreinemachers and Tipraqsa, 2012; FAO, 2019).

The use of insecticides from natural origin has been considered a viable alternative for the control of agricultural pests and has been considered a low risk for pollinators compared to using synthetic insecticides. Spinosad, for example, is a mixture of compounds derived from the fermentation of bacterial actinomycetes (*Saccharopolyspora spinosa*) (Sparks et al., 2001). This insecticide acts in the insects' nervous system, affecting the nicotinic acetylcholine receptors and the γ-aminobutyric acid (GABA) receptors (Salgado, 1998; Salgado and Sparks, 2005) primarily. Exposure to spinosad leads to hyperexcitation, paralysis associated with neuromuscular fatigue, and even death (Salgado, 1998; Williams et al., 2003; Monteiro et al., 2019).

Spinosad was formerly reported to be non-harmful to nontarget arthropods (Thompson et al., 2000); consequently, its use in plant protection and pest control quickly became commonplace (Sparks et al., 2001; Sarfraz et al., 2005). However, the selectivity of spinosad has become questionable, since lethal and/or sublethal effects on different insects have been reported (Bond et al., 2004; Stark et al., 2004; Morandin et al., 2005; Tomé et al., 2015a; Gómez-Escobar et al., 2018; Fernandes et al., 2019; Monteiro

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et al., 2019; Morales et al., 2019). In addition, some studies demonstrated the presence of spinosad residues in pollen, nectar, and/or water in agricultural fields (Vega et al., 2005; Bargańska et al., 2013; Kasiotis et al., 2014) with concentrations of this insecticide reaching up to 1.2 μg/L in river water (Vega et al., 2005). This contamination of food sources can potentially expose pollinating insects to spinosad (Abdel Razik, 2019) that could hypothetically be transported into the colonies; it would then be used to feed the inhabitants, including immature insects (Krupke et al., 2012; Kasiotis et al., 2014).

Most toxicological studies of pollinators, including those investigating the chronic exposure of larvae, use the honey bee (*Apis mellifera*) as a model (Lima et al., 2016; Catae et al., 2018; Lopes et al., 2018; Menail et al., 2018; Tomé et al., 2019). However, feeding during honey bee development differs from feeding during stingless bee development. For stingless bees, the larvae receive the food at once, which comprises a mixture of pollen, nectar, and glandular secretions that are provided by the adults (Velthuis et al., 2003). Honey bee larvae only ingest glandular secretions produced by the hypopharyngeal glands of workers (royal jelly) in the first days of their lives; subsequently, they receive modified jelly with pollen (Kunert and Crailsheim, 1988; Babendreier et al., 2004). Therefore, the risk of exposure of stingless bees to spinosad and other insecticides derived from living organisms during post-embryonic development can be higher than the risk to honey bees (Barbosa et al., 2015).

Multiple deleterious effects of exposure to spinosad have been recently reported for stingless bees. For instance, this insecticide reduced pupal body mass and caused deformities in individuals of the stingless bee *Melipona quadrifasciata* (Barbosa et al., 2015). Spinosad also compromised general group activity, enhanced the autophagy, apoptosis and oxidative stress in the midgut (Araujo et al., 2019a) and changed the abundance of gut bacteria of *Partamona helleri* (Botina et al., 2019). However, studies investigating the toxic effects of spinosad during the development of stingless bees, particularly in the workers, are nonexistent.

The species *P. helleri* has been used as an important model to understand the toxicological impact of pesticides on stingless bees (Tomé et al., 2015a, 2015b; Bernardes et al., 2018; Araujo et al., 2019a; Botina et al., 2019). In the current study, we investigated the effects of chronic oral exposure to spinosad on survival, developmental time, and body mass of *P. helleri* workers. In addition, the effects of the exposure to spinosad during larval development were studied for the midgut epithelial remodeling during metamorphosis and the organization of the peritrophic matrix (PM) of larvae, pupae, and adult bees. Midgut and PM are responsible for the digestion and absorption of nutrients and protection of the midgut epithelium, respectively (Hegedus et al., 2009), and sublethal effects during post-embryonic development on them may reduce the lifespan of bees.

2. Material and methods

2.1. Rearing of stingless bee workers

Rearing combs containing eggs and food were collected in four colonies of *Partamona helleri* kept in the Central Apiary at the Universidade Federal de Viçosa, Minas Gerais/Brazil (20° 75′ S and 42° 86′ W) and were transferred to the laboratory where the experiments occurred.

The rearing protocol for the workers was adapted from the methods described by Campos and Coelho (1993) and Bernardes et al. (2018). New polyethylene 96-well microplates that were coated and covered with Africanized bee wax were used as artificial breeding cells. The larval diet was collected with a surgical aspirator (MA520 Aspiramax, Grupo NS, São Paulo, Brazil) from cells of colonies. The eggs were transferred to the artificial cells containing 40 μL of larval treatment diet (37 μL of pure larval feed + 3 μL of spinosad solution; described in section 2.2) or the larval control diet (37 μL of pure larval feed + 3 μL of distilled and ionized water). Each artificial breeding cell received one egg.

The microplates were maintained in glass chambers under 97 ± 3% relative humidity (RH) during larval feeding for approximately five days, then they were maintained at 80 ± 3% RH throughout the rest of development (≈41 days). A saturated solution of sodium chloride (NaCl) was used within the chambers to control RH after the larval feed (Winston and Bates, 1960) because high RH causes an accumulation of water inside the artificial cells, which results in submerged larvae and death (Menezes et al., 2013). The samples were kept in the dark in an incubator at 28 ± 1 °C. All the instruments used to collect and create the bees and to store or collect the larval diet were autoclaved or sterilized in UV light in a biosafety chamber.

2.2. Insecticide use, larval exposure, and survival

Partamona helleri larvae were treated with the commercial formulation of spinosad (Tracer, 480 g of active ingredient (a.i.) L^{-1}, concentrated suspension; Dow AgroScience, Santo Amaro, SP, Brazil) available and registered for agricultural use in Brazil. A stock solution of spinosad at 81.6 g a.i. ha^{-1} was prepared by diluting the maximum rate recorded for the field (170 μL of Tracer per liter of water) according to the regulations of the Brazilian Ministry of Agriculture, Livestock, and Supply for the control of the whitefly, *Bemisia tabaci* (Hemiptera: Sternorrhyncha: Aleyrodidae), and tomato leafminer, *Tuta absoluta* (Lepidoptera: Gelechiidae) (MAPA, 2019), both of which are frequently present in agricultural areas visited by *Partamona* sp. (Santos and Nascimento, 2011).

Larvae (20 larvae/treatment/colony) were orally exposed to spinosad at the immediate onset of the experiment via a contaminated diet during post-embryonic development. Different doses of spinosad (6.53, 13.06, 32.64 and 3,264 ng. a.i. bee^{-1}) were used for each treatment, following dilutions of the recommended field concentration (81.6 ng a.i. μL^{-1}): 1/500, 1/250, 1/100, and 1/1. The control consisted of exposure to distilled and ionized water to measure the natural mortality of immature workers.

The survival of *P. helleri* larvae was evaluated by monitoring each individual daily during their development time. The observations were made by briefly removing the wax caps from the rearing cells during the evaluation and placing them again at the top of the cells. Individuals without spiracle movement or with dark integument were considered dead and were removed to avoid fungal contamination. The number of larvae represented in each treatment was 78, 77, 79, 79, and 65 for doses 0 (control), 6.53, 13.06, 32.64, and 3,264 ng a.i. bee^{-1} of spinosad, respectively, for a total of 378 larvae. This number varied because of differences in the number of eggs that did not hatch and had to be removed from the analysis.

2.3. Development time, body mass, and sex determination

The developmental time, determined in days from egg hatching to adult emergence, was analyzed for each individual. The treated adults that survived the spinosad exposure and control had their body mass measured at different days: 15 days (larva), and 30 days (dark-eyed pupa) after egg hatching, as well as newly emerged adult (0 day of age). To measure body mass, 16 insects from each treatment at each stage were removed from the polyethylene microplates, transferred to a microcentrifuge tube (1.5 μm) and

weighed with an analytical scale (model XS3DU, Mettler Toledo, Columbus, OH). All individuals were weighted in the same tube individually. Posteriorly, part of the same weighted individuals was used in the histology and PM bioassays.

The sex of individuals was checked at the dark-eyed pupae stage (about 25 days after egg hatching) by visual inspection under the stereomicroscope (SZ2-ILST, Olympus Corporation, Tokyo, Japan). The males (less than 2% of individuals) were recognized by the presence of goniostil (gonopods) and by distinct external morphology of the abdomen compared to females (Barbosa et al., 2015; Bernardes et al., 2018) and were discharged.

2.4. Histology of the midgut

Workers treated (n = 4) with the concentration of 32.64 ng a.i. bee^{-1} of spinosad and control subjects (n = 4) were collected at three stages of development: larvae, 15 days after hatching; dark-eyed pupae, 30 days after hatching; and newly emerged adults. This concentration was selected as it has been previously shown to induce alterations in the midgut and the behavior of *P. helleri* foragers (Araujo et al., 2019a).

The specimens were dissected in a saline solution for insects (0.1M NaCl, 0.1M KH_2PO_4, 0.1M Na_2HPO_4), and the midgut of each sample was transferred to a Zamboni fixative solution (4% paraformaldehyde, Sorensen's phosphate buffer, and saturated picric acid solution) for two hours at room temperature (25 ± 2 °C). The organs were washed three times in phosphate-buffered saline (0.1M PBS, pH = 7.6), dehydrated in an ascending series of ethanol (70%, 80%, 90%, and 99%) for five minutes each and embedded in historesin (Leica, Biosystem Nussloch, Wetzlar, Germany). Sections of five-μm thickness were obtained using an automatic microtome, stained with hematoxylin and eosin (HE), and analyzed under an Olympus BX53 light microscope with an Olympus DP73 digital camera (Olympus Optical Corp., Tokyo, Japan).

2.5. WGA-FITC labeling

WGA-FITC labeling was performed to evaluate the presence of glycoconjugates and polysaccharides containing β-1-4 *N*-acetyl-glucosamine residues in the PM in the midgut lumen of larvae, pupae, and adults. Twenty new sections of each sample (see section 2.4) of treated and control bees were washed in PBS 3 times, incubated with FITC-conjugated lectin (WGA-FITC, Sigma-Aldrich, # L4895, Israel), and diluted (1:400) in 0.1M PBS for 1 hour. After a triple wash in PBS, the sections were stained with diamidino-2-phenylindole (DAPI; Biotium, Inc., Hayward, CA, USA, 1:500) for 30 min to label the cell nuclei. Sections were washed three times again and mounted with 50% sucrose solution. Finally, the sections were analyzed and photographed using the fluorescence microscope.

Quantification of the fluorescence intensity of WGA-FITC was performed on the images with Image-ProPlus 4.5 software (Media Cybernetics, Silver Spring, USA). For this quantification, five images obtained with a final magnification of 1600X of each midgut of both treated and control individuals at each stage of development were randomly selected.

2.6. Statistics

Survival data were analyzed using Kaplan–Meier curves to estimate survival. The general similarity between the curves was tested by log-rank and the paired comparisons using the Holm–Sidak method ($p < 0.05$). Individuals withdrawn during the experiment for midgut extraction and those that did not emerge for up to 50 days, i.e., those that were deformed, were treated as censored.

For the analyses of the development time and body mass, the treatment group of 3,264 ng a.i. bee^{-1} was excluded because its high mortality made it impossible to obtain a satisfactory sample size. Because of the non-independence between individuals from the same colony, a selection of models was chosen between mixed models that included the colony as a random effect and models with no random effect. This selection was based on the lower value of the Akaike information criterion with correction for small sample sizes (AICc). Thus, the development time data were submitted for analysis of variance and the least significant difference (LSD) test for multiple comparisons with Holm–Sidak *p*-values adjusted method ($p < 0.05$). For the body mass data, mixed linear models (LMMs) were set up.

WGA-FITC signal emission data were subjected to multi-factor analysis of variance (ANOVA two-way; treatment x age) and the LSD test for multiple comparisons with Holm–Sidak *p*-values adjusted method ($p < 0.05$). The images were randomly sampled from individuals from different colonies.

When necessary, development time, body mass, and WGA-FITC data were transformed by Box-Cox ($\frac{y^{\lambda}-1}{\lambda}$, $\lambda = 0.34$) to fit the Gaussian distribution. The residuals were checked by visually inspection (residuals vs. fitted values and normal Q-Q plot), Shapiro-Wilk test and Bartlett test to verify distribution, suitability, and homoscedasticity in all models. All analyses were performed using the software R (R Core Team, version 3.4.4, 2018).

3. Results

3.1. Survival during post-embryonic development

The chronic oral exposure to concentrations of 6.53, 13.06, and 32.64 ng a.i. bee^{-1} of spinosad for *P. helleri* workers during larval development did not significantly alter survival in comparison to the control (Fig. 1). However, the highest concentration (3,264 ng a.i. bee^{-1}) led to a significant reduction in survival ($\chi^2 = 91$, df = 4, $p < 0.001$), and only about 10% of bees survived until the end of the experiment. More than 50% of individuals treated with 3,264 ng a.i.

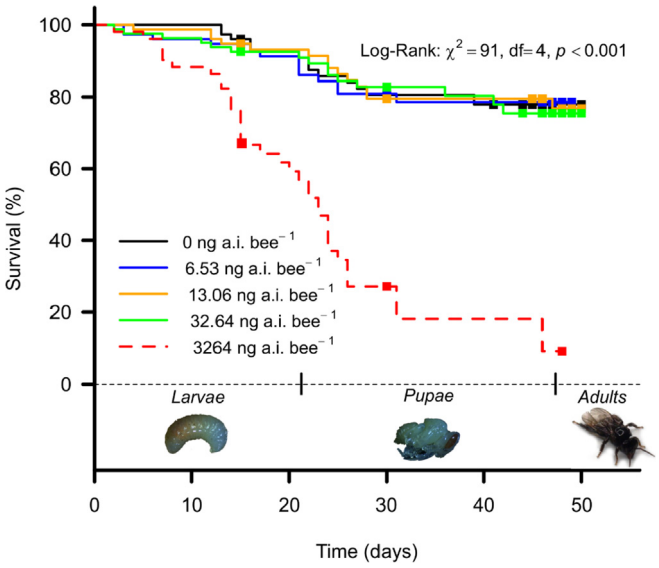

Fig. 1. Survival curves of the stingless bee *Partamona helleri* that were orally exposed to different concentrations of spinosad during their larval stage. The filled squares in the curves indicate censored data. The dotted-line curve differs significantly from continuous lines by the Holm–Sidak method ($p < 0.05$).

bee^{-1} died before they reached the pupal stage (Fig. 1).

3.2. Development time and body mass

The ingestion of 32.64 and 13.06 ng a.i. bee^{-1} spinosad during the development of *P. helleri* significantly affected the pupation time ($F_{3, 200} = 7.73$, $p < 0.01$; Fig. 2A), as well as the time of adult emergence ($F_{3, 120} = 3.3$, $p = 0.023$; Fig. 2B). For the control bees, the mean time ±standard error of beginning pupation was 20.94 ± 0.36 days; the time to emergence was 46.89 ± 0.36 days. In treated bees that ingested 32.64 ng a.i. bee^{-1} spinosad, the times for pupation and emergence onset were 21.40 ± 0.19 days and 47.71 ± 0.19 days, respectively. In the cases of bees that ingested 13.06 ng a.i. bee^{-1} spinosad, the times for pupation and emergence onset were 21.77 ± 0.20 days and 47.73 ± 0.20 days, respectively.

The body mass of the workers was not impaired in any of the measured ages (15 days of age: $\chi^2 = 3.85$, df = 3, $p = 0.28$; 30 days: $\chi^2 = 4.77$, df = 3, $p = 0.19$; Adults, day 0: $\chi^2 = 1.76$, df = 3, $P = 0.62$). The body masses were 22.31 ± 1.80 mg for 15-day-old larvae, 24.02 ± 1.53 mg for 30-day-old pupae, and 19.20 ± 1.12 mg (mean ± standard error) for newly emerged adults.

3.3. Midgut epithelium

The ingestion of the sublethal concentration (32.64 ng a.i. bee^{-1}) of spinosad caused significant changes in the bees' midgut epithelium development (Fig. 3). The epithelium of treated or control larvae at 15 days old is pseudostratified, consisting of digestive cells with a thin, striated border and regenerative cells, both of which are laying upon a basal lamina. In the control larvae, digestive cells had a nucleus with condensed chromatin and cytoplasm without evident vacuolization (Fig. 3A and B). On the other hand, the epithelium of treated larvae had cells with nuclei with predominantly decondensed chromatin and cytoplasm with expressive vacuolization (Fig. 3C and D). Also, the midgut epithelium of the treated larvae had disintegrated and had cell cytoplasm projecting towards the lumen.

In the 30-day-old pupae, the midgut epithelium was formed from a layer of digestive cells —columnar type— and regenerative cells located in the basal portion (Fig. 3E–H). At this stage, cell disintegration was detected in the midgut epithelium, and cell debris was detected in the gut lumen, both in control and in the spinosad-treated bees.

In the midgut of newly emerged workers in the control group, there was a layer of juxtaposed digestive and regenerative cells, a prominent striated border, and scarce debris in the gut lumen (Fig. 3I and J). On the other hand, in the midgut of treated bees, the epithelium was disintegrated, and a tiny striated border and abundant cellular debris were seen in the lumen (Fig. 3K–L). In addition, a large number of cytoplasmic granules were detected in the digestive cells of treated adult bees (Fig. 3L).

3.4. Peritrophic matrix (PM)

The analysis of the images and the fluorescence signal intensity showed a significant reduction of PM staining in the midgut of *P. helleri* exposed to spinosad in the three analyzed stages ($F_{3, 26} = 12.93$, $p < 0.001$; Fig. 4). The values of the staining intensity of the PM differed significantly between control and treated (32.64 ng a.i. bee^{-1}) bees ($F_{1, 26} = 7.3$, $p = 0.012$), with the greatest difference observed between the adult groups. There was also a significant difference detected for the stages of development (larvae, pupae, and adults; $F_{2, 26} = 15.8$, $p < 0.001$), with the lowest staining intensity of the PM detected in the pupal stage. The interaction between treatments and stages of development was not significant ($F_{2, 24} = 1.6$, $p = 0.23$). The changes evidenced by the FITC-WGA staining are demonstrated in Fig. 5.

4. Discussion

The chronic oral exposure to spinosad (formulation) at a dose recommended for field use reduced the survival of *P. helleri* when the larvae ingested the contaminated diet under laboratory conditions. The decreased larval survival of *P. helleri* can reduce the number of workers in the colony and could compromise the dispersion, foraging, and maintenance of the population (Henry et al., 2012; Baron et al., 2017). Oral exposure to spinosad also impaired development and decreased survival of other non-target insects, such as the stingless bees *Melipona quadrifasciata* (Barbosa et al., 2015) and the lady beetles *Harmonia axyridis* (Galvan et al., 2005). These results demonstrated that under laboratory conditions, that represent a worst case scenario of larval

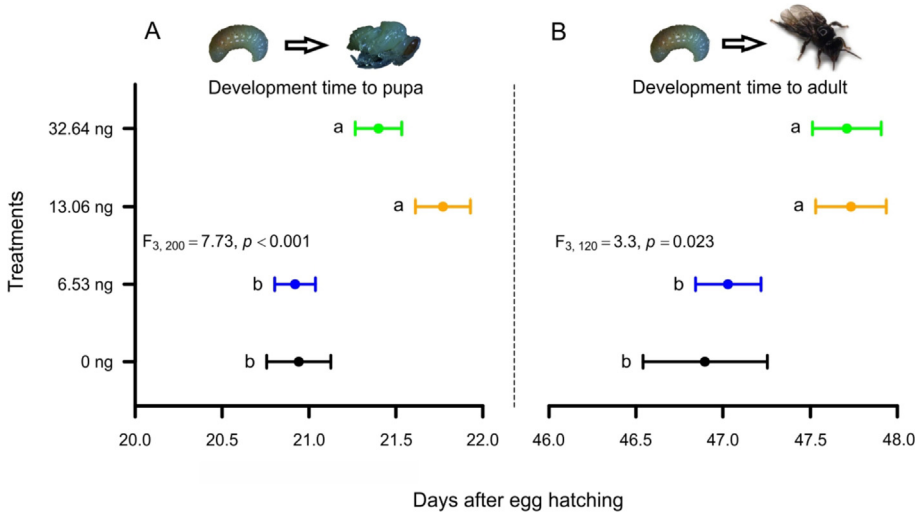

Fig. 2. Development of the workers of *Partamona helleri* that ingested a diet contaminated with spinosad during the larval stage. (A) Developmental time until larvae reach pupal stage. (B) Developmental time until the larvae reach adult stage. The dots represent averages and the longitudinal bars are the standard errors. The values on the y-axis represent the concentrations in ng a.i. bee^{-1}. Means followed by different letters differ significantly by the LSD test with Holm–Sidak *p*-values adjusted method ($p < 0.05$).

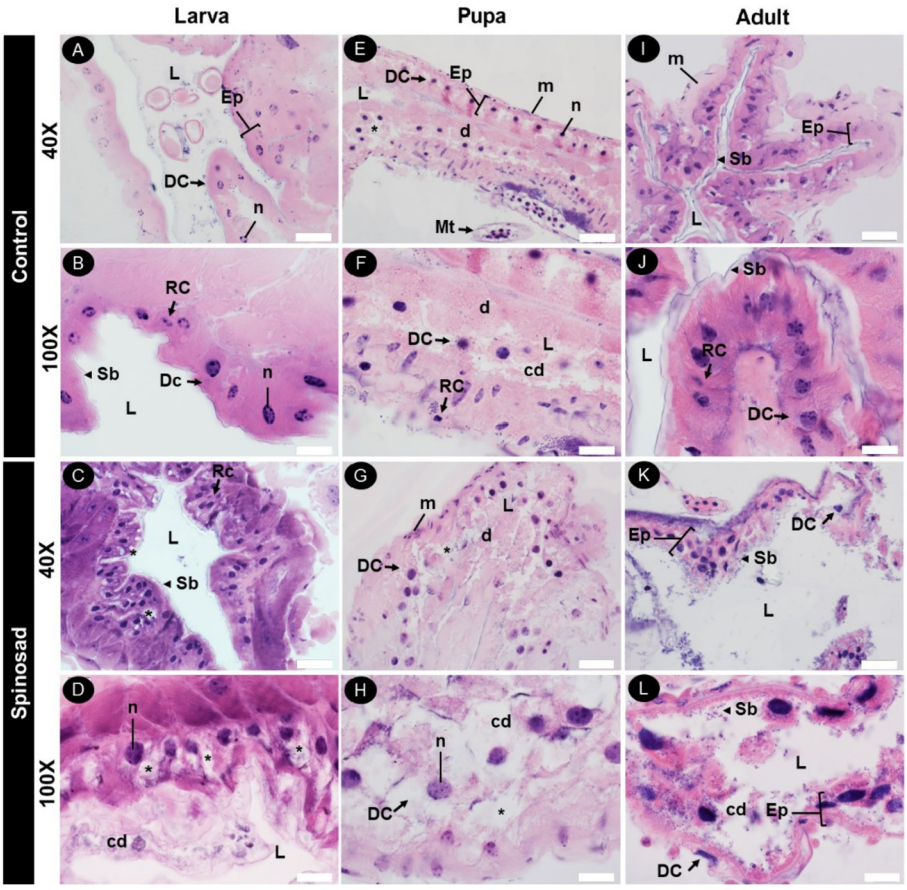

Fig. 3. Histological sections from the midgut of *Partamona helleri* workers in different developmental stages taken from bees treated with 32.64 ng a.i. bee^{-1} spinosad or the control bees. Abbreviations: digestive cell (DC), regenerative cell (RC), striated border (Sb), epithelium (Ep), lumen (L), nucleus (n), cell disintegration (cd), cell debris (d), muscular (m), cytoplasmic vacuolization (*). Bar = 20 μm.

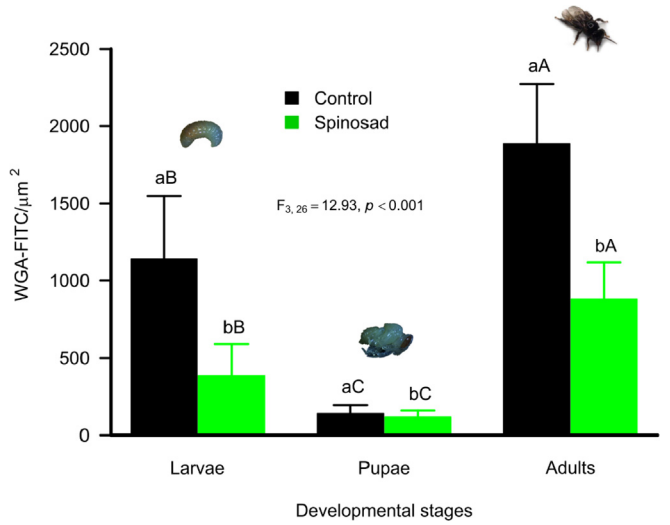

Fig. 4. Fluorescence intensity of WGA-FITC in the peritrophic matrix of the midgut of workers of *Partamona helleri* of control (untreated) bees or bees treated with 32.64 ng a.i. bee^{-1} spinosad in different developmental stages. Lower case letters indicate significant difference between treatments at each stage of development. Different capital letters indicate significant difference between the stages of development in each treatment by the LSD test with Holm–Sidak *p*-values adjusted method ($p < 0.05$).

exposure, spinosad may impose toxicological risks to populations of beneficial insects, such as pollinators. Residues of insecticides affect non-target organisms less than can be expected from laboratory tests because in natural conditions, the insecticides can be degraded by light and microorganisms. However, these degradation varies, depending on the insecticide and the environment conditions (Thompson et al., 2000; Caboni et al., 2006) and it can not be ignored that the half-life of insecticides may be sufficient for it to be found within the colony (Tomé et al., 2019), and impact the larval survival.

Spinosad treatment also increased the developmental time of *P. helleri* workers. Similar results were also described when immature queens of this species were chronically (oral route) treated with the insecticide azadirachtin (Bernardes et al., 2018). Alterations of the development time of queens and workers —as shown in this work— may have occurred because of disturbances in hormone titers associated with insect growth regulation (i.e., due to changes in juvenile hormones and/or ecdysteroids) (Dhadialla et al., 1998; Mordue and Nisbet, 2000; Pandey and Bloch, 2015) apparently caused by insecticides based on compounds found in living organisms. In eusocial bees, the division of labor is determined by age polyethism; delays in larval development may limit the behavioral repertoire of adult workers, and as a consequence for the delay, these changes may lead to alterations in the colony's activities such as foraging (Wu et al., 2011; Mateus et al., 2019). Spinosad treatment did not change body mass of the individuals. Both treated and control larvae acquired all food supplied, necessary to support the post-embryonic development of the bees (Campos and Coelho, 1993), suggesting that the contamination by spinosad, at least for the conditions used here, did not interfere

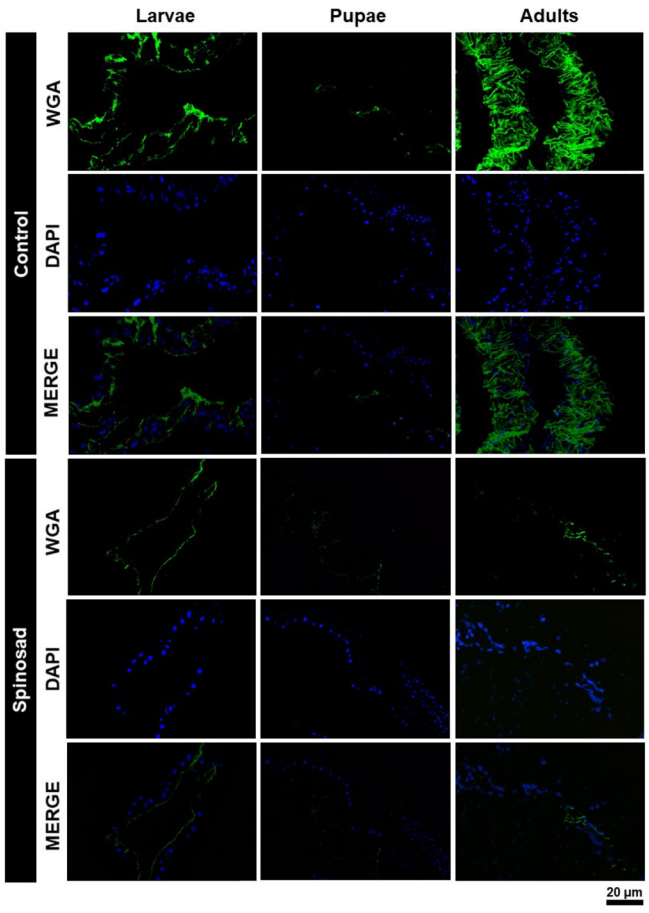

Fig. 5. Histological sections of the midgut of *Partamona helleri* workers stained with WGA-FITC (peritrophic matrix; green) and DAPI (nuclei; blue). The panels show the midguts of control bees and treated (32.64 ng ai. bee^{-1}) bees in larval (15 days after egg hatching), pupal (30 days old) and newly emerged adult stages.

with the food intake by *P. helleri* larvae.

The ingestion of spinosad at a field dose diluted by 100-fold (i.e., 32.64 ng a.i. bee^{-1}) altered the midgut epithelium of *P. helleri* larvae. Similar effects have recently been reported for the midgut of forager workers of these bees after acute oral exposure at a sublethal concentration (8.16×10^{-3} mg a.i./mL) of spinosad, which can result in damage to the digestive system (Araujo et al., 2019a). These effects require attention because the midgut is a non-target organ of spinosad (Salgado, 1998; Thompson et al., 2000; Sparks et al., 2001). Our data suggest that ingestion of spinosad rapidly compromises the intestinal epithelium of non-target insects, including at the larval stage. Supposedly, this can reduce the survival time of the bees and make them susceptible to other stressors, such as pathogens (Doublet et al., 2015). The occurrence of sublethal effects due to the ingestion of such a low dose of spinosad by the larvae is also of concern, as this can frequently occur in the field by consumption of contaminated pollen and nectar (Rortais et al., 2005; Krupke et al., 2012).

The epithelium of the midgut of bees undergoes extensive remodeling during pupation —which was observed in the present study— in both treated and untreated pupae. Specifically in dark-eyed pupae, this remodeling involves the death of larval digestive cells and epithelial restructuring through the proliferation and differentiation of regenerative cells (Serrão and Cruz-Landim, 2000; Neves et al., 2002; Martins et al., 2006; Cruz et al., 2007, 2011; 2013; Araujo et al., 2019b). Unlike in pupae, in newly emerged adult workers, the treatment with spinosad during larval development altered the midgut epithelium structure. Epithelial damage in the adult midgut of *Aedes aegypti* (Diptera: Culicidae) has also been recently reported in spinosad-treated larvae. In this insect, larval exposure interfered with the midgut cells at all stages of development, reducing the number of proliferating and enteroendocrine cells and leading to malformation of the midgut epithelium in adults (Fernandes et al., 2019). Therefore, the detection of many damaged cells in the midguts of adult *P. helleri* workers indicates that in this species, spinosad also affects the midgut epithelium similarly to treated mosquitoes.

Another sublethal effect observed after the ingestion of spinosad by *P. helleri* larvae was the reduction of the thickness of the PM. This sublethal effect was also reported for adult honey bee workers (Lopes et al., 2018) and in the PM of larvae of the leafworm, *Spodoptera littoralis* (Abouelghar et al., 2013) after oral exposure to spinosad. The PM protects the midgut epithelium against the mechanical and chemical action of food, and it also acts as a physical barrier to microorganisms (Lehane, 1997; Hegedus et al., 2009). In view of this, it is possible to infer that the oral exposure to spinosad during the larval stage not only impairs the development of the midgut of *P. helleri* workers, but also has a direct effect on the PM and may interfere with the health of bees, reduce the production of normal workers, and decrease the survival rate of these bees.

The ingestion of spinosad in the larval stage decreases survival and delays the development time of *P. helleri* workers, and the effect of chronic ingestion persists in the adults. Also, sublethal doses of spinosad cause damage to the midgut epithelium of larvae, compromise the epithelial remodeling of the midgut during metamorphosis and impair the organization of the PM, all of which could prejudice the function of the organ. To our knowledge, this is the first time that the sublethal effects of an insecticide derived from living organisms have been reported on the midgut of bees during the different stages. Finally, our data reinforce the possible environmental risks of using spinosad in cultures that are naturally pollinated by stingless bees, and also serves as a basis for further research on the toxicological effects of spinosyns on the post-embryonic development of native plant pollinators.

Declarations of interest

None.

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