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Toxicity and effects of combined agrochemical in *Scaptotrigona bipunctata* bees

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Abstract. The commercial insecticide Fastac Duo is a combined insecticide, widely used in different crops, acting on insects, affecting both pests and pollinators, such as bees. In this study, the effects of sublethal concentrations of Fastac Duo in stingless bees *Scaptotrigona bipunctata* were evaluated. Worker forager bees were exposed to the insecticide and histochemical and morphological analyses were conducted after 24, 48 and 72 h of ingestion. Brain analysis of *S. bipunctata* revealed changes in the chromatin condensing state according to exposure time and insecticide concentration when compared to the control group. Morphological changes were observed in the midgut in all concentrations and exposure times, which may interfere in several physiological processes. In conclusion, although the concentrations used in the study did not cause high mortality, it induced changes in the internal morphology that can lead to changes in bee activity.
Keywords: Histology, SEM, CEC, stingless bees

Introduction

Bees are the main pollinating agents, providing an extremely important ecosystem service (Barbosa et al., 2017) maintaining the fundamental ecological processes involved in plant reproduction, being vital for crops production (Ramírez et al., 2018).

Scaptotrigona bipunctata (Lepeletier) (Hymenoptera: Apidae) is a social bee with atrophied stinger, native from South America (Bolivia, Brazil, Paraguay and Peru). In Brazil, this species is found on the following states: Acre, Ceará, Maranhão, Minas Gerais, Paraná, Pará, Rio Grande do Sul, Rio de Janeiro and Santa Catarina (Camargo e Pedro, 1836). Their colonies are highly populated and although they are extremely good pollinators, research on their susceptibility is limited.

With advances in agriculture, deforestation, urban development and other anthropic activities, bee biodiversity is threatened (Barbosa et al., 2017). However, the unsustainable use of agricultural ecosystems and the excessive use of agrochemicals are considered the main causes of losses on bee diversity (Sanchez-Bayo e Goka, 2014).

With this in mind, studies focused on pollinators health should consider the contamination through flowers from crops treated with agrochemicals. Fastac Duo is an insecticide with both systemic and contact action, composed of two different chemical groups: neonicotinoid (acetamiprid – 100 g/L) and pyrethroid (alpha-cypermethrin – 200 g/L).

Neonicotinoids have become the most widely used class of insecticides in the world, with large-scale applications ranging from plant protection, veterinary products and fish farming. This agrochemical act as agonists of post-synaptic nicotinic acetylcholine receptor on the central nervous system of the insect, causing a blockage of signal transmission (Bridi et al., 2018). On the other hand, pyrethroids act on the sodium channels of the nerve cell membrane, altering depolarization and nerve impulse conduction insecticides and are efficient when used in low dose (Santos et al., 2007).

The effectiveness of agrochemicals may be evaluated through their action on target and non-target insects. Changes in chromatin integrity are an important tool to identify external stressors (Santos et al., 2014). Similarly, morphophysiological analyses are important to analyze exposure to agrochemicals (Tavares et al., 2015), since the midgut is responsible for most of the metabolism of ingested insecticides.

As stingless bees are more sensitive to insecticides than other groups of bees it is important to evaluate the toxicity of these compounds to ensure their protection (Arena e Sgolastra, 2014). Thus, this study aimed to evaluate the toxicity and establish the lethal concentration (LC_{50}) of the commercial insecticide Fastac Duo in contaminated *S. bipunctata* bees, investigating changes in the chromatin structure of brain cells and changes in the midgut morphology of bees orally exposed to this insecticide.

Methods

Biological material

Adult *S. bipunctata* forager workers were collected at the entrance of the colony, when they returned from foraging, in the meliponary of the Fazenda Experimental de Iguatemi (FEI) (23°25'S and 51°57'O), from Universidade Estadual de Maringá and taken to the Animal Genetics Laboratory of the Department of Biotechnology, Genetics and Cell Biology of the Universidade Estadual de Maringá.

Bioassays

Initially, preliminary tests were performed to determine the Fastac Duo concentrations to be used (0.0265 g a.i./L; 0.053 g a.i./L; 0.0795 g a.i./L and 0.106 g a.i./L) and all concentrations tested were added to the syrup, a mixture of water and sugar.

Bioassays were performed in quadruplicate, with 15 bees per concentration, totaling 60 individuals for each group. 300 individuals were evaluated in every bioassay period. Glass bottles containing filter paper, a cotton soaked in water and a container containing syrup with one of the insecticide concentrations were used in the bioassays. For the control group conditions were the same, but the syrup provided contained only sugar and water. The bioassays were kept at 28 ± 2°C, RH 70% ± 10%, for 24, 48 and 72 h, and the different evaluations were performed. After 24, 48 and 72 h, the lethal concentration for 50% of exposed insects (LC_{50}) was determined based on mortality.

Chromatin changes

To investigate possible changes in the brain chromatin structure, the protocol described by Vidal and Mello was used (1989). The brains of surviving bees were dissected after 24 h, 48 h and 72 h of insecticide intake, placed in saline solution for insects (NaCl 0.1 M, Na_2HPO_4 0.1 M and KH_2PO_4 0.1 M), extended in microscopy slides, with acetic acid (45%) and crushed under a glass slide. Microscopy slides were frozen in liquid nitrogen and the glass slide removed when it reached room temperature. The material was fixed in ethanol:acetic (3:1 v/v) acid for 1 min and the slide was washed in ethanol for 5 min.

For each treatment, nine slides were used, totaling 135 slides analyzed. These were stained for 20 min with TB 0.025% in a McIlvaine buffer (pH 4.0), contained different $MgCl_2$ concentrations (0.0; 0.02; 0.05; 0.08; 0.10; 0.12; 0.15; 0.20; and 0.30 mol/L). Then, slides were washed in distilled water and air-dried, bleached in Xylol for 15 min, assembled in Entellan, analyzed and photographed under a Zeiss standard optical microscopy. The cell nuclei stained violet were the controls and the green color corresponding to the CEC point.

Midgut alterations

Scanning electron microscopy (SEM) and light microscopy were used to verify possible variations in the midgut of the bees. For SEM, surviving bees were anesthetized under cold temperatures and the midgut was dissected in a physiological solution for insects, then fixed in aqueous Bouin's solution (picric acid, formaldehyde and acetic acid) for 12 h, being dehydrated in an alcohol series of increasing concentrations (70%, 80%, 90% and 100%). Samples were submitted to a critical-point dry (Leica CPD 030) and covered by gold dust on the metallizer Shimadzu IC-50. Analyses were performed using the MEV QUANTA 250 of the Microscopy Center of the Complex of Research Support Centre (COMCAP) of the Universidade Estadual de Maringá/Paraná/Brazil.

For light microscopy, after exposure to the insecticide Fastac Duo surviving bees were anesthetized and the midgut was dissected in saline solution for insects. Samples were fixed in aqueous Bouin's solution for 12 h, dehydrated in an alcohol series of increasing concentrations, diaphanized in xylene (100%), paraffin-embedded and sectioned into 6 µm slices using a microtome Leica RM 2250. Then, sections were spread on glass slides, rehydrated, and stained with Hematoxylin and eosin (H/E). The analyses were performed under an Olympus light microscope and sections were photographed using a digital camera.

Data analysis

The Shapiro-Wilk test was used to investigate the assumption of normality of the mortality variable. The Kruskal-Wallis test was used to verify whether there were median differences in working-class mortality according to the concentration level. To verify the ideal concentration of the insecticide the binary regression model with probit binding function was used. For the diagnostic analysis of the model, the normal probability graph with simulated envelope was used and for the adjustment, quality deviance was used. The significance level was 5% and the statistical analyses were performed at the R software version 3.6.2 (R Core Team, 2019).

Results and discussion

All the assumptions for the suitability of the model were met. Regarding the diagnostic analysis of

the model, it was found that there was no violation in the assumptions of the residues in all periods tested. The Shapiro-Wilk test showed that the hypothesis of normality of bee mortality was not attended in all experimental periods (p-value < 0.05). Once the hypothesis of normality was not satisfied, the Kruskal-Wallis nonparametric test was applied, which showed that there were significant differences in mortality regarding the concentration levels and the test showed that there were significant differences in all experimental periods (p-value < 0.05).

After 24, 48 and 72 h of oral contamination it was observed that the mortality mean and median was higher at the highest concentration (0.106 g a.i./L) (Figure 1). While the variability of workers mortality, the same behavior was observed after 24 h and 48 h (Figure 1 a and b), however, after 72 h the concentration 0.0795 g a.i./L allows greater variability of the lethality of workers (Figure 1 c).

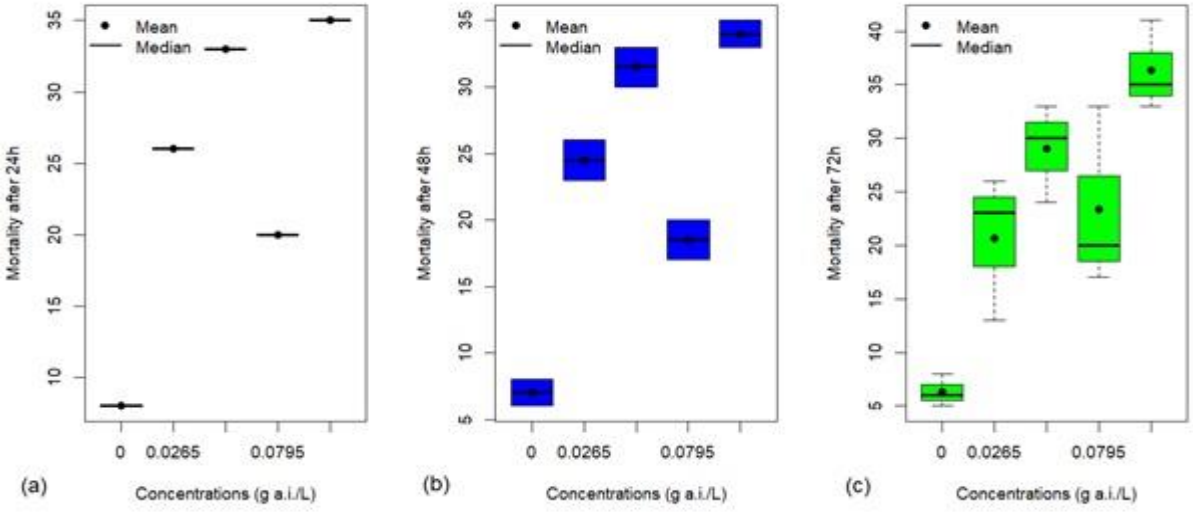

Figure 1. Mortality boxplot of *S. bipunctata* after (a) 24, (b) 48 and (c) 72 h of oral contamination with the insecticide Fastac Duo.

Considering the logarithmic scale at concentrations, to interpret the value of the lethal concentration (LC_{50}) that causes the death of 50% of the individuals exposed to Fastac Duo, it was necessary to apply the exponential to the obtained values and as a result the estimated LC_{50} values were 0.07 g a.i./L, for 24 h, 0.10 g a.i./L, for 48 h and 0.0665 g a.i./L after 72 h of contamination (Figure 2).

The agrochemical used in this study is a combination of products of different chemical classes, considered highly toxic (Adapar, 2018). To date there have been no studies on lethal and sublethal effects of combined products for bees, however, there are several studies on the effects of insecticides from both chemical classes.

The LC_{50} observed in this study were higher when compared to those reported in the literature for *Melipona quadrifasciata* bees 2.35×10^{-8} g/bee (Tomé et al., 2015) and *S. postica* 4.25×10^{-8} g/L (Soares et al., 2015) treated with the neonicotinoid imidacloprid and, *S. bipuncata* $2\text{x}10^{-6}$ g a.i./L (Moreira et al., 2018) orally contaminated with the neonicotinoid thiamethoxam; *S. tubiba* 0.70 ppm (Moraes et al., 2000) contaminated with the pyrethroid deltamethrin and *S. bipunctata* 2.72×10^{-8} g a.i./L (Pereira, 2017) orally contaminated with the pyrethroid cypermethrin. Thus, this species has a certain degree of tolerance to the insecticide Fastac Duo because although it is an agrochemical of synergistic effect, the LC_{50} was higher when compared to common insecticides.

Cytochemical analyses showed differences between treated and untreated bees in the experimental periods. After 24 h of oral contamination the brain chromatin of *S. bipunctata* bees from contaminated with Fastac Duo at the concentration 0.053 g a.i./L and 0.106 g a.i./L showed the CEC value of 0.30 M, indicating its condensation, which differed from the observed in the control group, which showed a CEC point of 0.20 M (Figure 3 a).

After 48 h the concentration 0.053 g a.i./L and 0.106 g a.i./L showed CEC value of 0.30 M, a similar response to the 24 h test (Figure 3 b). Finally, after 72 h it was observed that the chromatin condensation on the brain of *S. bipunctata* bees in all treatments since CEC values were between 0.20 M and 0.30 M (Figure 3 c).

The CEC analysis showed that changes occured in the chromatin structure of the brain cells of *S. bipunctata* after contamination with Fastac Duo. Neonicotinoid insecticides act as nicotinic acetylcholine receptor (nAChR) agonists in a similar way as nicotine, but it is more potent and more selective for the receptors of the post-synaptic membrane of insects than in mammals (Pacífico-da-Silva et al., 2016). This binding is persistent since neonicotinoids are insensitive to the action of the acetylcholinesterase enzyme and the activation of

acetylcholine receptors is abnormally prolonged, causing hyperexcitability of the central nervous system due to continuous and uncontrolled transmission of nerve impulses. Symptoms resulting from intoxication include tremors, seizures (possibly central nervous system collapse) and death (Faria, 2006).

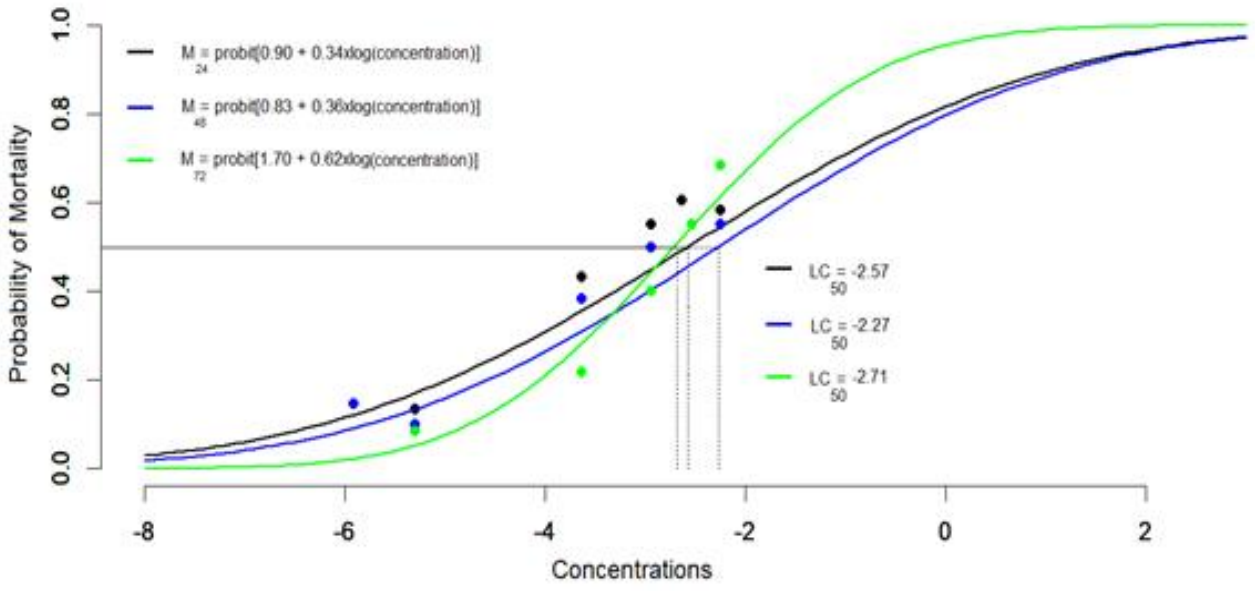

Figure 2. Lethal concentration curve of *S. bipunctata* mortality after oral contamination with the insecticide Fastac Duo.

Pyrethroid insecticides act on sodium channels, interfering in their opening and closing and prolonging the entry time of Na^+ ions into the cell. At relatively high concentrations, pyrethroids bind to the inotropic receptor complex of γ-aminobutyric acid (GABA) blocking chlorine channels and their activation, which leads to hyperexcitability of the insects' central nervous system (Santos et al., 2007). In addition to the lethal effects, sublethal effects can also induce behavioral changes (Rossi et al., 2013). Tison et al. (2016) investigated chronic exposure to sublethal doses of thiacloprid in *Apis mellifera* and observed that this neonicotinoid caused foraging impairment, difficulty to return to the colony, losses in navigation performance and communication. Besides, thiacloprid residue levels increased in both foragers and nestmates over time. Thany et al. (2015) studied the retraction of the *A. mellifera* proboscis and concluded that acetamiprid (neonicotinoid) impaired this movement in the tested doses. According to Freitas and Pinheiro (2010), pyrethroid insecticides at recommended levels of application in the field seems to affect the ability of the honeybees to return to the hive.

The results showed that after 24, 48 and 72 h of agrochemical consumption the CEC values were higher when compared to the control group. Similar results were found by Falco et al. (2010) using the neonicotinoid thiamethoxam in *A. mellifera*. The authors showed that the CEC value can change after contamination with the agrochemical. Studies using thiamethoxam observed chromatin condensation in *S. bipunctata* brains (Moreira at al., 2018).

Santos et al. (2014) investigated the oral contamination of *Diatraea saccharalis* with the insecticides fipronil, malathion, cypermethrin and neem oil, and the results suggested that the activation and inactivation of genes may be acting on defense mechanisms. Although this study did not use neonicotinoid, cypermethrin was used. The results obtained with *S. bipunctata* corroborate this report, because chromatin condensation observed in our study may be related to gene inactivation, allowing an alternative to bee survival after oral contamination.

Furthermore, Catae et al. (2014), verified that after ingestion of thiamethoxam there was significant damage to the bee organism, affecting the distribution of specific proteins in the brain, indicating impairment in oxygen supply, synapses and neuronal degeneration, which may explain deficits in learning and memory processes, that can compromise the individual's behavior and colony health.

After 24 h of contamination, it was observed loosening of the longitudinal musculature (Figure 4 b – d), which was also thinner (Figure 4 c – e), muscle relaxation (Figure 5 b – e), epithelium disorganization (Figure 5 b – e), remnants of the peritrophic membrane (Figure 5 b – e) and detachment of the epithelium from the basal lamina (Figure 5 d – e).

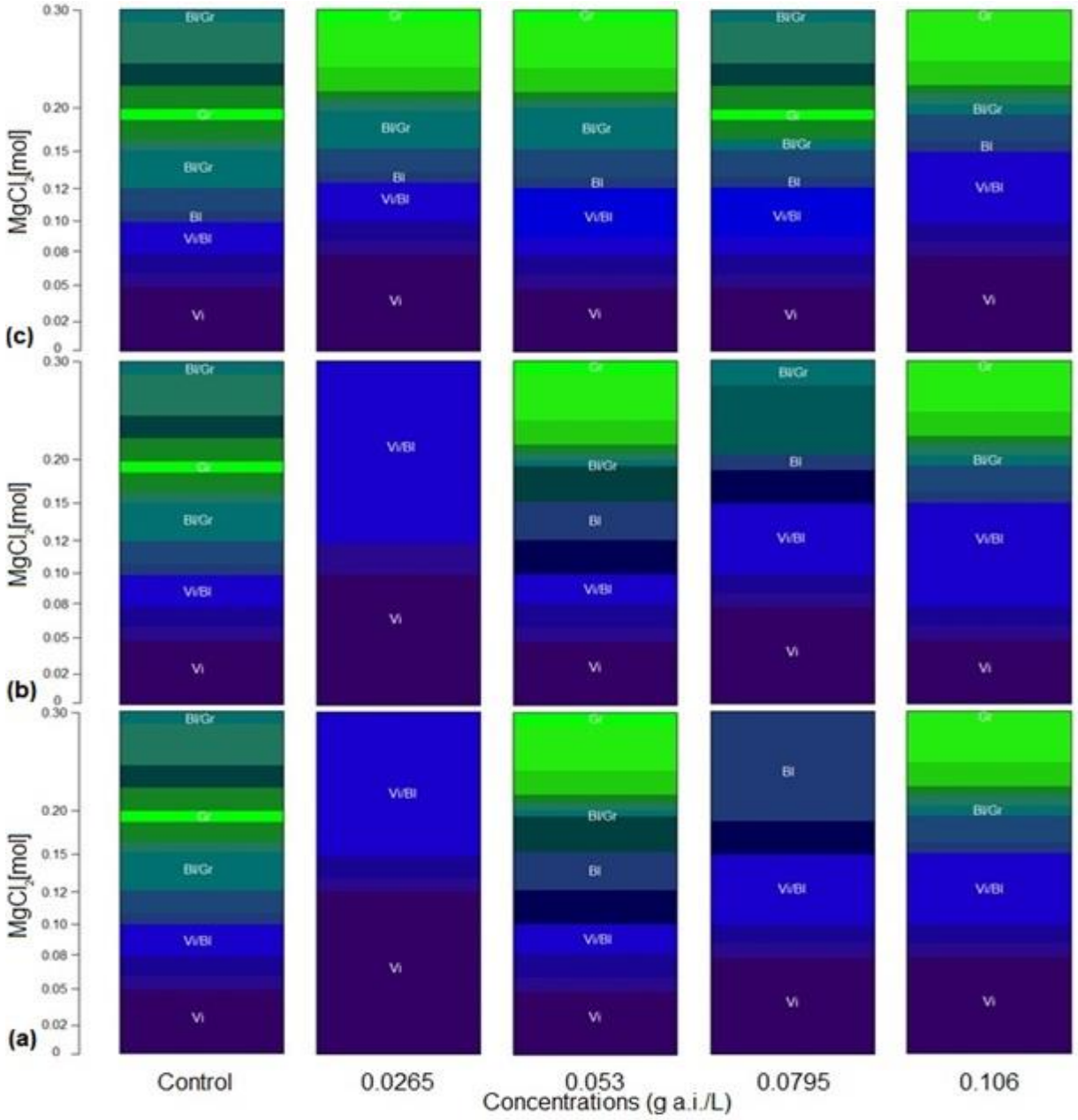

Figure 3. Nuclear basophilia of *S. bipunctata* workers brain after (a) 24, (b) 48 and (c) 72 h of oral contamination with the insecticide Fastac Duo [stained with 0.025% toluidine blue (TB) added of $MgCl_2$ in various concentrations (mol/L)]. Bl, blue; Gr, green; Vi: violet.

After 48 h there was a loosening (Fig 6 c – e) and thinning (Fig 6 b – e) of the longitudinal musculature, intestine distension (Fig 6 c and e) and relaxation of the musculature in all treatments (Fig 6 c – e). Also, it was observed loss of digestive cells from the lumen and epithelial disorganization in all treatments and degradation of the peritrophic membrane (Fig 7 b – e).

After 72 h longitudinal muscle fibers were totally loose (Figure 8 b – e), there was degradation of digestive cells and loss to the lumen, disorganization of the epithelium and degradation of the peritrophic membrane (Figure 9 b – e).

The midgut is the main means of contact of the insects with the environment and it is where nutrient absorption and metabolization of chemicals occurs, which makes it an important organ for toxicity studies (Landim, 2009). The midgut of *S. bipunctata* bees orally contaminated with the insecticide Fastac Duo showed morphological changes after exposure on all the periods tested.

The peritrophic membrane is an envelope that forms around the food in the ventricular lumen, separating it from the epithelial cells, protecting the ventricle cells from damages caused by food that was not digested, therefore this system occurs on insects that feed on solids (Landim, 2009).

In addition to the excretory function, the digestive cells produce digestive enzymes and absorb

digestion products in the ventricular lumen (Landim, 2009). The degradation of digestive cells and their elimination in the lumen, in addition to the loosening of the longitudinal musculature, offered

evidence of the degenerative effects of the agrochemical used in this study. Also, continuous exposure to sublethal doses of agrochemicals, such as thiamethoxam, it is harmful to the organs responsible for digestion and detoxication (Catae et al., 2014, Oliveira et al., 2014, Diniz et al., 2020).

Catae et al. (2014) investigated the effects of thiamethoxam on the midgut and Malpighian tubules of *A. mellifera* and the results indicated that the insecticide is cytotoxic to bees. In the midgut, the damage was more evident in bees exposed to the insecticide on the first day. On the eighth day, the cells were ultrastructurally intact, suggesting a recovery. On the other hand, the Malpighian tubules showed noticeable alterations on the eighth day of exposure.

Epithelial degeneration detected in this study may be related to the degradation of the peritrophic membrane after oral contamination with Fastac Duo. In a similar study, using *A. mellifera* workers orally contaminated with the thiamethoxam, Oliveira et al. (2014) found that digestive and regenerative cells of the midgut showed morphological and histochemical alterations, such as cytoplasm vacuolization, increased apocrine secretion and increased cellular elimination in the lumen. Diniz et al. (2020) orally exposed *S. bipunctata* workers with the organophosphate insecticide acephate and observed epithelium disorganization, cellular degeneration and rupture of midgut musculature.

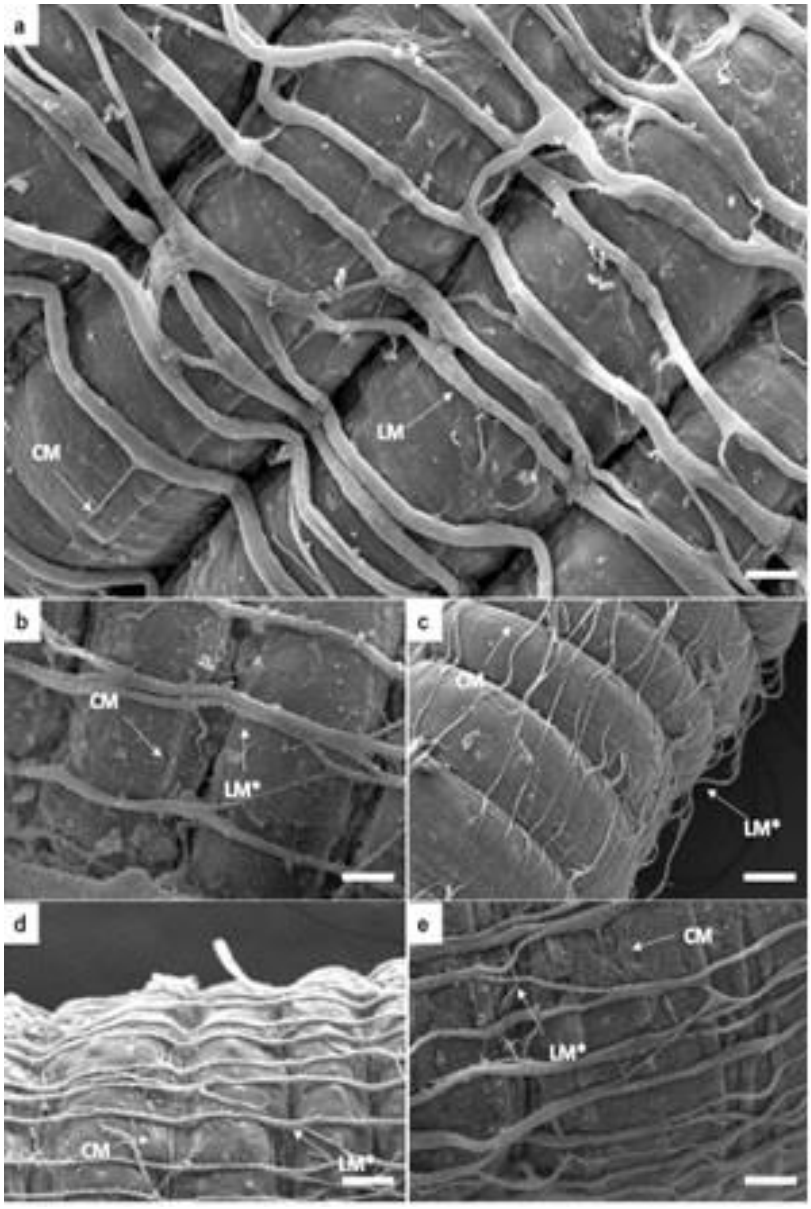

Figure 4. Scanning electron microscopy showing the midgut of *S. bipunctata* after 24 h of ingestion of the insecticide Fastac Duo. (a) control; (b), 0.0265 g a.i./L; (c), 0.053 g a.i./L; (d), 0.0795 g a.i./L; (e), 0.106 g a.i./L. CM, Circular musculature; LM, Longitudinal musculature; LM*, Longitudinal musculature showing alterations; T, Tracheoles. Scale bar: 50 µm.

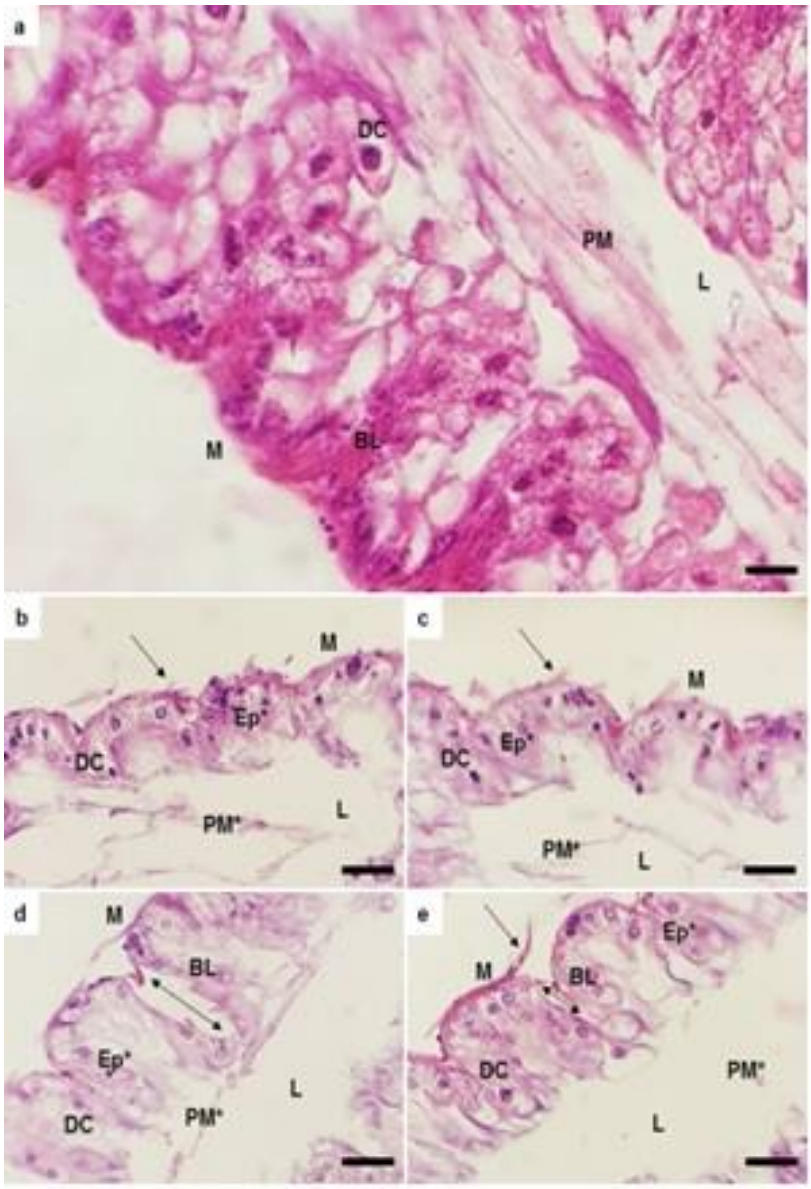

Figure 5. Photomicrography showing the midgut of *S. bipunctata* after 24 h of oral contamination with the insecticide Fastac Duo. (a), control; (b), 0.0265 g a.i./L; (c), 0.053 g a.i./L; (d), 0.0795 g a.i./L; (e), 0.106 g a.i./L. DC, Digestive cells; Ep, Epithelium; Ep*, Epithelium showing alterations; L, Lumen; BL, Basal lamina; M, Musculature; PM, Peritrophic membrane; MP*, Peritrophic membrane showing alterations, Musculature showing alterations; Basal lamina epithelium detachment. Hematoxylin-Eosin staining. Scale bar: 20 µm.

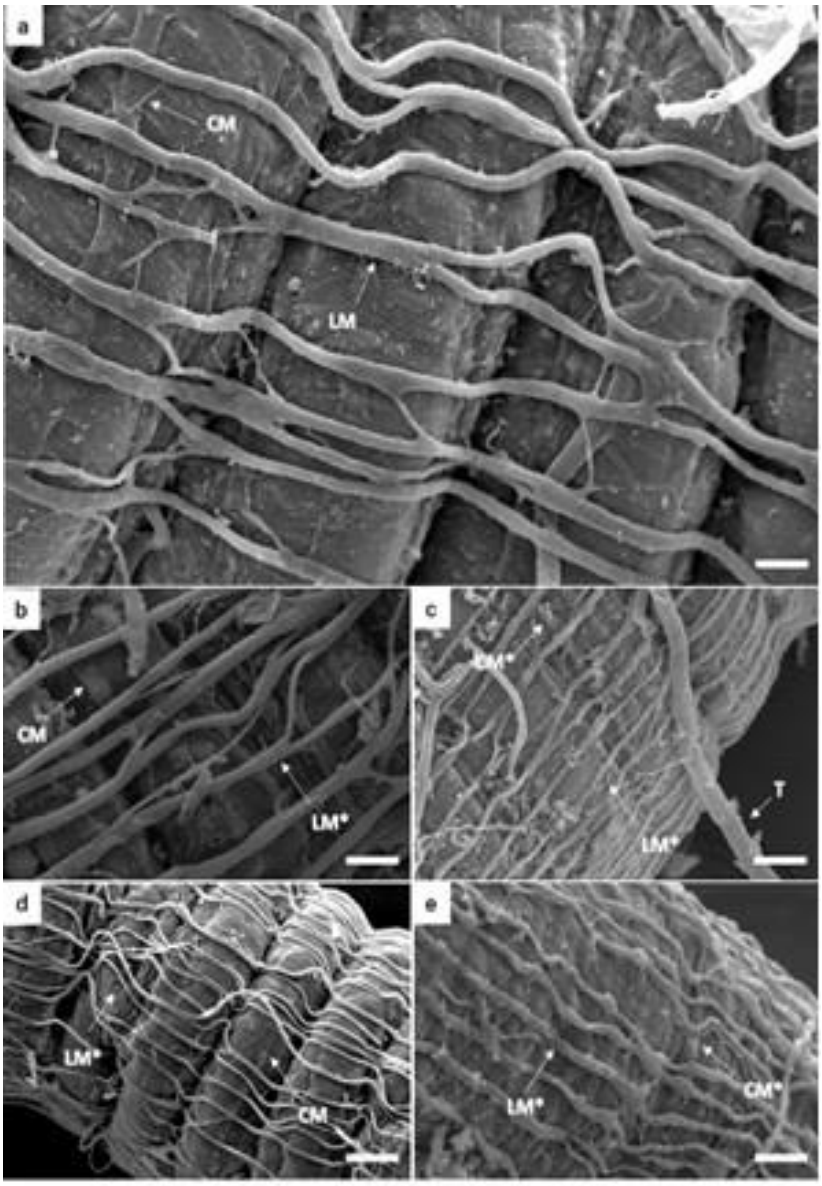

Figure 6. Scanning electron microscopy showing the midgut of *S. bipunctata* after 48 h of ingestion of the insecticide Fastac Duo. (a), control; (b), 0.0265 g a.i./L; (c), 0.053 g a.i./L; (d), 0.0795 g a.i./L; (e), 0.106 g a.i./L. CM, Circular musculature; MC*, Circular musculature showing alterations; LM, longitudinal musculature; LM*, longitudinal musculature showing alterations; T, tracheoles; Scale bar: 50 µm.

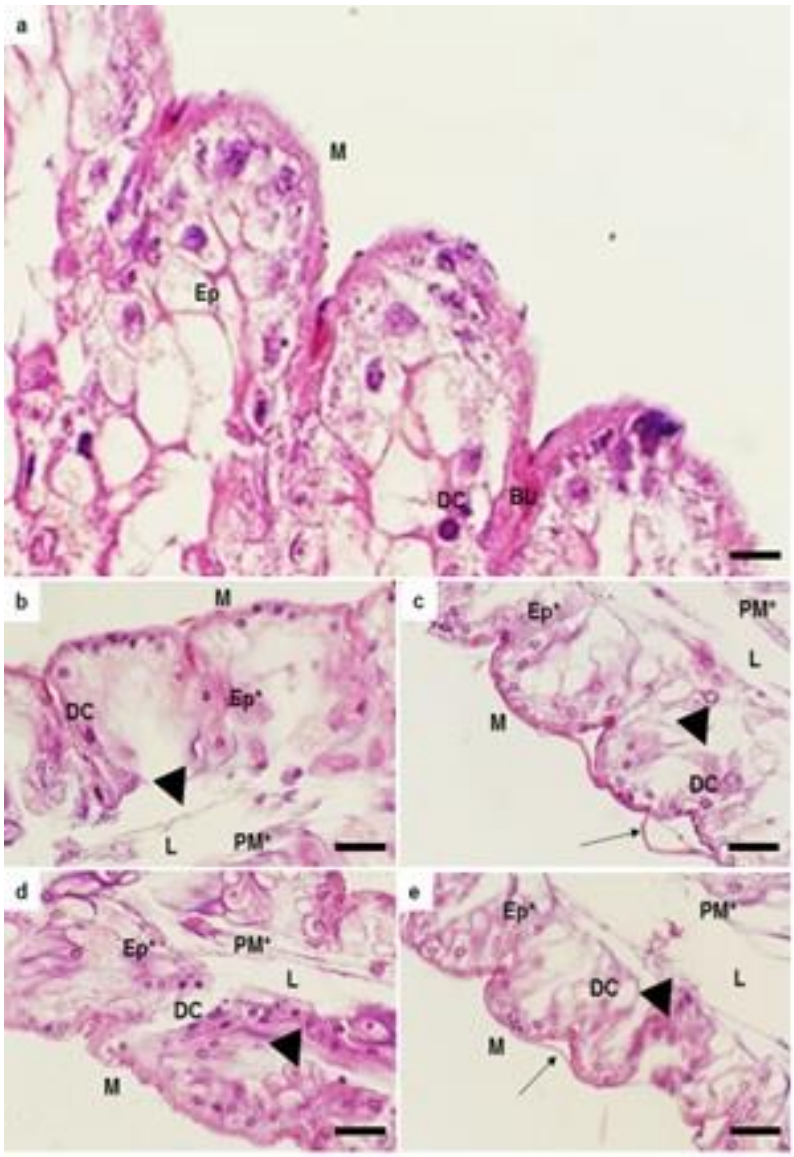

Figure 7. Photomicrography showing the midgut of *S. bipunctata* after 48 h of oral contamination with the insecticide Fastac Duo. (a), control; (b), 0.0265 g a.i./L; (c), 0.053 g a.i./L; (d), 0.0795 g a.i./L; (e), 0.106 g a.i./L. DC, Digestive cells; Ep, Epithelium; Ep*, Epithelium showing alterations; L, Lumen; BL, Basal lamina; M, Musculature; PM, Peritrophic membrane; MP*, Peritrophic membrane showing alterations, Musculature showing alterations; Basal lamina epithelium detachment; Loss of digestive cells to the lumen. Hematoxylin-Eosin staining. Scale bar: 20 µm.

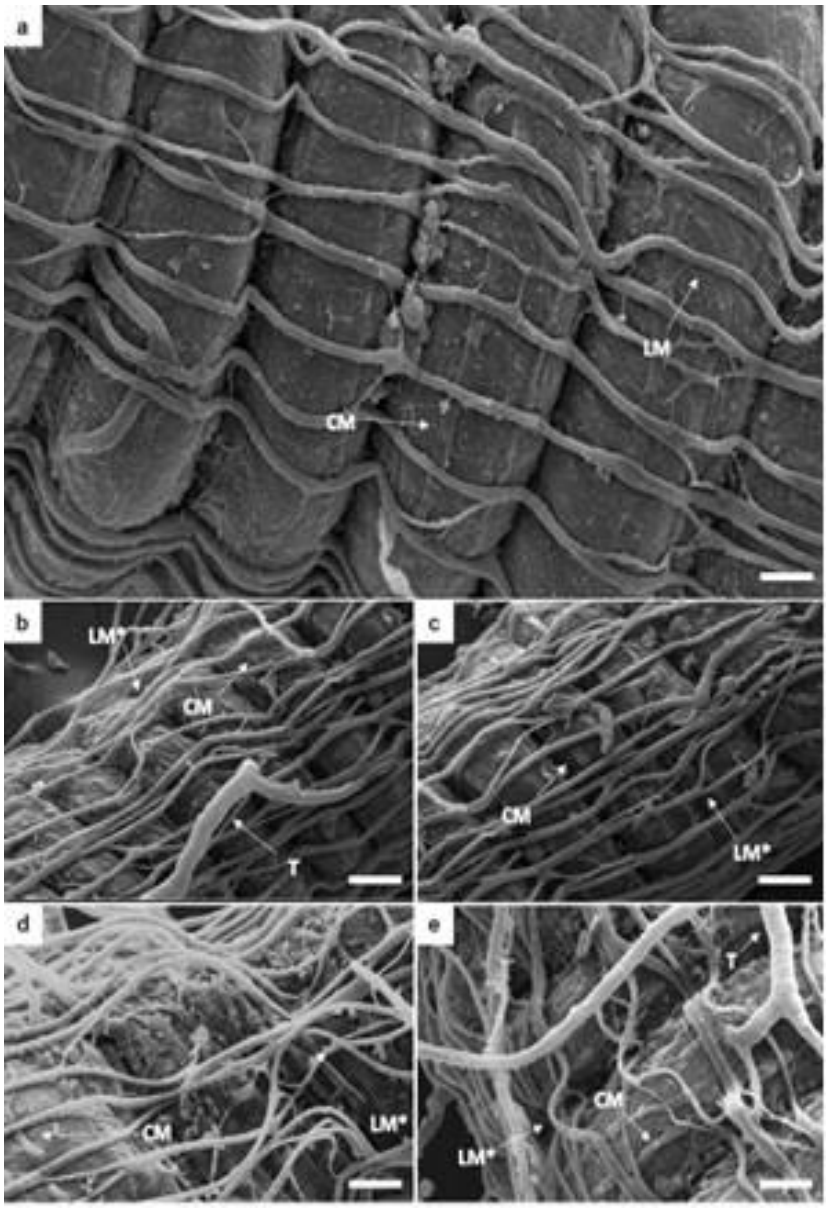

Figure 8. Scanning electron microscopy showing the midgut of *S. bipunctata* after 72 h of ingestion of the insecticide Fastac Duo. (a), control; (b), 0.0265 g a.i./L; (c), 0.053 g a.i./L; (d), 0.0795 g a.i./L; (e), 0.106 g a.i./L. CM, Circular musculature; LM, longitudinal musculature; LM*, longitudinal musculature showing alterations; T, tracheoles; Scale bar: 50 µm.

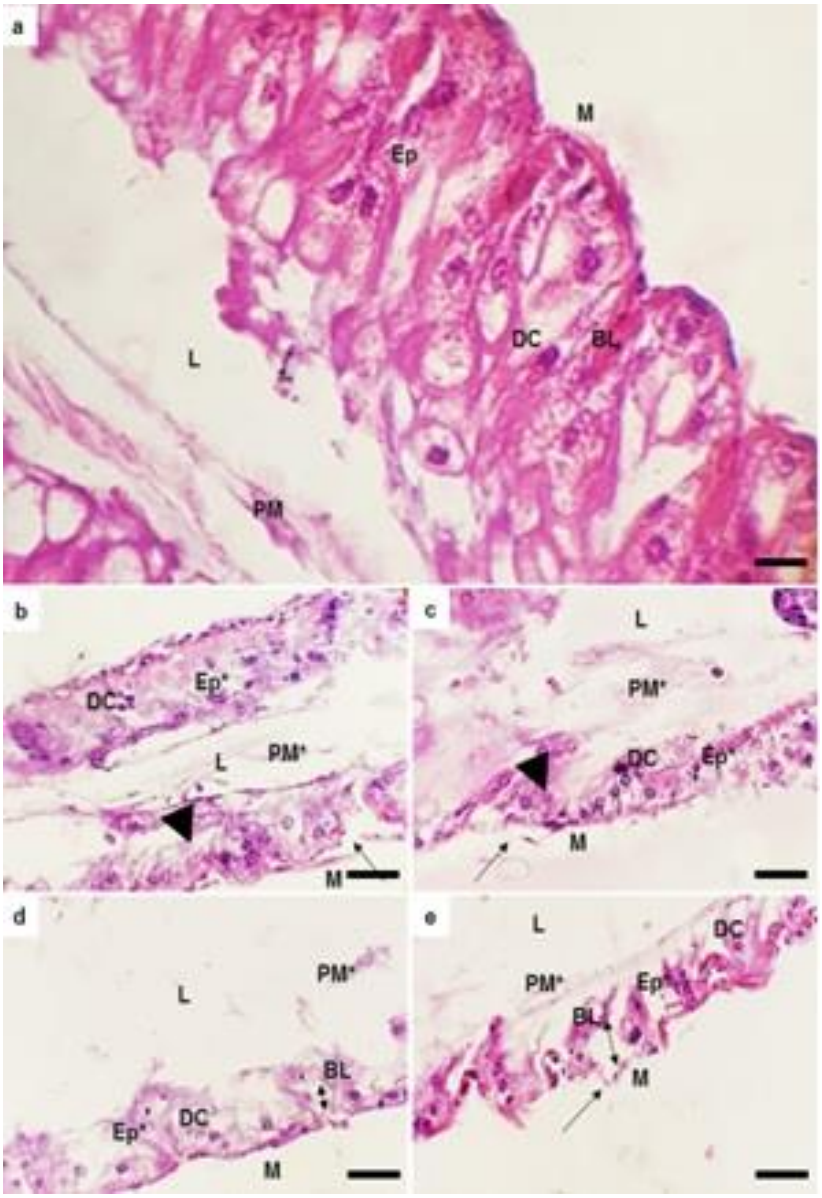

Figure 9. Photomicrography showing the midgut of *S. bipunctata* after 72 h of oral contamination with the insecticide Fastac Duo. (a), control; (b), 0.0265 g a.i./L; (c), 0.053 g a.i./L; (d), 0.0795 g a.i./L; (e), 0.106 g a.i./L. DC, Digestive cells; Ep, Epithelium; Ep*, Epithelium showing alterations; L, Lumen; BL, Basal lamina; M, Musculature; PM, Peritrophic membrane; MP*, Peritrophic membrane showing alterations, Musculature showing alterations; Basal lamina epithelium detachment; Loss of digestive cells to the lumen. Hematoxylin-Eosin staining. Scale bar: 20 µm.

Oliveira et al. (2014) contaminated *A. mellifera* bees with thiamethoxam and found nest of regeneration. The same was observed by Moreira et al. (2018) in *S. bipunctata* bees orally contaminated with thiamethoxam, and by Diniz et al. (2020) in studies using the acephate. These results suggest that oral exposure to agrochemicals can modify the structure and function of regenerative cells, affecting their proliferation and differentiation in new digestive cells. One of the functions of the regenerative cells is to restore the epithelium when digestive cells are eliminated (Landim, 2009). However, in our study, regenerative cells were not observed, which may indicate the degenerative effects of the agrochemical.

The action of insecticides on bees is considered negative and may influence various aspects of their life. Some responses occur through physiological and morphological changes, such as changes in muscles, epithelium and peritrophic membrane, which results in mortality and harmful

effects on behavior, impairing foraging and compromising colony survival (Roat et al., 2013).

Conclusion

In conclusion, oral contamination of *S. bipunctata* bees with Fastac Duo can modify morphophysiological essential processes for bee survival, although it does not cause high mortality. Therefore, this agrochemical can be considered dangerous to *S. bipunctata* health. Finally, it is essential to propose measures to minimize the impact of agrochemicals on pollinators, especially on native bees, to ensure its preservation.

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