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RESEARCH ARTICLE

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Toxicity and effects of the neonicotinoid thiamethoxam on *Scaptotrigona bipunctata* lepeletier, 1836 (Hymenoptera: Apidae)

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Abstract

The neonicotinoid thiamethoxam is widely used in different agricultural crops, and it has a spectrum of action against insects, affecting both pests and pollinators, such as bees. In this study, the effects of exposure to sublethal concentrations of thiamethoxam on stingless bees *Scaptotrigona bipunctata* were evaluated. Foragers bees were exposed to the insecticide and subjected to genetic biochemical, histochemical, and morphological analyses after 24, 48, and 72 h of ingestion. Analysis of isoenzyme esterases revealed significant alterations in the relative activity of EST-4, a type II cholinesterase. Evaluation of the *S. bipunctata* brain revealed changes in the state of chromatin condensation according to the exposure time and concentration of neonicotinoid compared with the control. Morphological changes were observed in the midgut of this species at all concentrations and exposure times, which may interfere with various physiological processes of these insects. We can conclude that, although thiamethoxam at the concentrations evaluated did not cause high mortality, it induced concentration-dependent changes in bees by activating enzymes related with the protection for xenobiotic, internal morphology and probably these changes may lead to alterations in the activity of bees.

KEYWORDS
chromatin, esterases, midgut, stingless bee

1 | INTRODUCTION

The meliponines, also known as stingless bees, exhibit biological characteristics ideal for pollination,[1] making them efficient pollinators in some cultures.[2]

Scaptotrigona bipunctata (Lepeletier) (Hymenoptera: Apidae) is distributed in South America (Bolivia, Brazil, Paraguay, and Peru). In Brazil, it can be found in the states of Acre, Ceará, Maranhão, Minas Gerais, Paraná, Pará, Rio Grande do Sul, Rio de Janeiro, and Santa Catarina.[3] Its colonies are populous, and have a specific scent, similar to the smell of coconut, and although they show great potential as pollinators, research on the susceptibility of this bee to insecticides is limited.

Among the threats to bees, disintegration and loss of habitat,[4,5] migratory apiculture, high levels of parasites,[5] and intensive use of pesticides are highlighted.[5–8]

Neonicotinoids are pesticides used extensively to control agricultural crop pests[9] and they are based on nicotine,[10] acting as agonists of the acetylcholine in the nicotinic receptor[11] not hydrolyzed by acetylcholinesterase, which causes hyperexcitation of the insect's nervous system.[12] Due to their wide application and persistence in both soil and water, neonicotinoids become bioavailable to pollinators in sublethal concentrations for much of the year.[13]

Thiamethoxam belongs to neonicotinoid class and is classified as a systemic insecticide, because it has the ability to translocate through the sap throughout the plant to all tissues[14,15] including the pollen[16,17] and nectar.[9,17] Lundin et al.[18] after analyzing 268 publications involving neonicotinoid researches, found that thiamethoxam was the second most studied component with 34%, in 73 studies. Recently, articles evaluating the exhibition of the thiamethoxam on pollinators have been pointed out as sublethal effects on behavior, locomotion, and

memory,[17,19–27] metabolism and immunity,[28–31] reproduction,[32–38] and synergistic effects of additional pesticides with neonicotinoids.[39,40]

The efficiency of these insecticides can be measured by their action under target insects and also nontargets such as bees.[41] Enzymes as glutathione-*S*-transferase (GST), polyphenol oxidase (PPO),[42] and esterases[42–45] have been used as indicators or biomarkers for pesticides.[42,46] Specifically, esterases are a highly diversified subclass of hydrolytic enzymes,[46] classified into four distinct classes: acetilesterases (E.C. 3.1.1.6), arilesterases (E.C. 3.1.1.2), carboxilesterases (E. C. 3.1.1.1), and colinesterases, that includes acetilcolinesterases (E.C. 3.1.1.7) and pseudocolinesterases (E.C. 3.1.1.8).[47] Esterases present a wide range of functions in the metabolism of several classes of exogenous and endogenous compounds, acting on the development and behavior of insects, such as on odorant degradation and functions related to reproduction and digestion.[48] Also, they are important in resisting insecticides,[49–52] having the ability to metabolize or degrade these substances before exerting a toxic effect on the organism.[53,54]

In addition to the isoenzymes analyzes, changes in chromatin integrity are an important tool to identify the presence of external agents.[55–59] Therefore, nuclear basophilia has been studied in order to analyze the levels of DNA-protein complexation in the chromatin, based on the use of the cationic dye Toluidine Blue (TB).[60] If TB staining occurs in the presence of Mg^{2+} íons, the competition for phosphate groups available from DNA and RNA also begins. CEC was defined as the concentration of Mg^{2+} íon that abolishes the metachromasy (exhibits greenish color). This concentration varies according to the degree of chromatin compaction and higher values are obtained in the condensed chromatin.[61,62]

Morphophysiological analyzes can also be used as a criteria for analyzing exposure to agrochemicals.[57,63–67] Thus, in the middle intestine of the bees occurs most of the metabolism of the ingested insecticides, occurring interactions between chemicals that at least in part, can be influenced by detoxification enzymes such as glutathione-*S*-transferases and esterases.

Since stingless bees are more sensitive to insecticides, that the use of these compounds in agriculture are threatening survival of the colonies, that in most cases, are located near the crops, therefore, it is important to evaluate the toxicity of these components on stingless bees in order to ensure their protection.[68,69] Thus the objective of this study was to evaluate the toxicity of the insecticide thiamethoxam in *S. bipunctata*, by assessing changes in the relative activity of isoenzymes esterases, chromatin in brain, and morphology of the midgut.

2 | MATERIALS AND METHODS

2.1 | Biological material

Adult *S. bipunctata* foragers were collected at the entrance of the colony when they returned from foraging, at the Fazenda Experimental de Iguatemi (FEI) (23°25'S and 51°57'O) of the Universidade Estadual de Maringá and taken to the Laboratory of Genética Animal do Departamento de Biotecnologia, Genética e Biologia Celular da Universidade Estadual de Maringá.

2.2 | Bioassays

The insecticide used was Actara 250 WG containing 250 g of thiamethoxam active ingredients (a.i.) per kilogram (kg) (Syngenta International, Basel, GA, Switzerland). The main solution was prepared diluted in water, based on the recommended dose for the Citrus pest, *Orthezia praelonga* (5×10^5 μg a.i./L), due to the presence of citricultural crops in the region. Subsequently, preliminary tests were carried out to determine sublethal concentrations (0.50×10^2 μg a.i./L, 0.75×10^2 μg a.i./L, 1×10^2 μg a.i./L, 1.25×10^2 μg a.i./L, 2.5×10^2 μg a.i./L) from stock solution.

All concentrations obtained were analyzed using an ingestion test and were added to previously prepared food candies (70 g of sugar cake and 40 g of honey). It was mixed with the insecticide in the calculated proportions (g/mL), ensuring a final concentration in the solid food. In addition, candy is used by beekeepers as complementary alternative for food.

Four bioassays were performed in triplicate, with 15 adult *S. bipunctata* workers by concentration, totaling 270 individuals by test. Each experiment was established to evaluate the methods analyzed. To replicates and control groups, it was employed glass bottles containing filter paper, a water-soaked cotton swab, and another candy container containing a sublethal concentration of thiamethoxam. The control conditions were the same as those used for the treatments; however, only candy was supplied as food. The flasks were maintained in 28 ± 2°C, UR 70% ± 10%, for 24, 48, and 72 h, and the different evaluations were performed in these intervals. After 24 h, the concentration lethal to 50% of the exposed insects (LC_{50}) was determined based on mortality.

2.3 | Esterases analyzes

The esterases were evaluated after 24, 48, and 72 h, by polyacrylamide gels electrophoresis at 12% concentration for separation gels and stacking gels at 4%.[70,71] Head/thorax samples from each surviving insect were macerated in 0.1% β-mercaptoethanol and 10% glycerol, and centrifuged at 16 128*g* for 10 min in a refrigerated centrifuge at 4°C (Sigma 3K30). From the supernatant, an aliquot of 20 μL was applied to the gel. The vats of the electrophoresis system were filled with 0.1 M tris-glycine buffer (pH 8.3) and electrophoresis was performed at 200 V for 6 h at 4°C. For identification of the esterases, the gels were incubated for 30 min in 50 mL of 0.1 M sodium phosphate buffer, pH 6.2. After, the gel was incubated in 50 mL of 0.1 M sodium phosphate buffer (pH 6.2), *n*-propanol (5 mL), Fast Blue RR salt (0.06 g), and the substrates α-naphthyl acetate (0.04 g) and β-naphthyl acetate (0.04 g), previously diluted in 1 mL of acetone, in the dark for 40 min. The esterases were visualized on the gels as brown (α-esterases) or red (β-esterases) bands.

2.4 | Esterases characterization

2.4.1 | Substrate inhibition test

For the inhibition tests, the organophosphate malathion (Dipyl Chemistry Industry), *para*-chloromercuriobenzoate (*p*CMB), eserine sulfate,

and phenylmethylsulfonyl (PMSF) (Sigma Chemical Co.) were used. The gels were incubated for 30 min in 0.1 M sodium phosphate buffer (pH 6.2) containing each inhibitor solution, and then the staining solution containing the inhibitors was also added. Control gels were prepared simultaneously using the same samples under identical conditions, but were devoid of inhibitors. According to the inhibition tests, the esterases were classified as cholinesterases (acetylcholinesterases or pseudocolinesterases), arylesterases, acetylesterases, and carboxylesterases following established criteria at Healy et al.[47]

2.5 | Critical electrolyte concentration (CEC)

Following the protocol described by Vidal and Mello[62] brains of surviving bees were dissected after 24, 48, and 72 h of ingestion, placed in physiological solution (0.1 M NaCl, 0.1 M Na_2HPO_4, and 0.1 M KH_2PO_4), extended on microscopy slides, with acetic acid (45%) and crushed underneath a coverslip. Microscopy slides were frozen in liquid nitrogen and the coverslip 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 and for the three exposure periods, nine slides containing the dissected material were used, totaling 144 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 the slides were washed in distilled water and air dried, being bleached in Xylol for 15 min, the light microscopy were assembled in Entellan and analyzed and photographed under a Zeiss standard optical microscopy. The cells nuclei stained violet were the controls and the green color correspond to the CEC point.

2.6 | Light microscopy

Following exposure to the insecticide at 0.50×10^2, 1.25×10^2, and 2.5×10^2 μg a.i./L for 24, 48, and 72 h, surviving *S. bipunctata* individuals and insects not exposed to the pesticide were anesthetized under cold temperatures, and the midgut was dissected in solution (0.1 M NaCl, 0.1 M Na_2HPO_4, and 0.1 M KH_2PO_4).The samples were fixed in aqueous Bouin's solution (picric acid, formaldehyde, and acetic acid) for 12 h, dehydrated in an alcohol series of increasing concentrations (70%, 80%, 90%, and 100%), 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).[72] The analyses were performed under Olympus light microscope and sections were photographed using a digital camera.

2.7 | Scanning electron microscopy (SEM)

For SEM, midguts were dissected from bees after exposure to the thiamethoxam for different periods and concentrations (0.50×10^2 μg a.i./L, 1.25×10^2 μg a.i./L, and 2.5×10^2 μg a.i./L), and then fixed in aqueous Bouin's solution for 12 h, being dehydrated in an alcohol series of increasing concentrations. The 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–FEI of the Microscopy Center of the Complex of Research Support Centre (COMCAP) of the Universidade Estadual de Maringá/Paraná/Brazil.

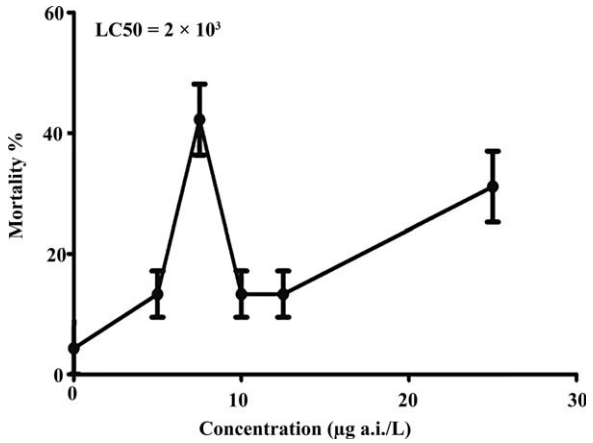

FIGURE 1 Average mortality of *S. bipunctata* exposed to five concentrations of the neonicotinoid thiamethoxam by ingestion for 24 h. The graph was created with GraphPad Prism 5.00. LC_{50} was estimated by means of Probit regression with SPSS 22.0

2.8 | Data analysis

The normality, homogeneity, and additivity of the mortality data were verified using the following tests: Shapiro-Wilk and Bartlett, respectively, in software R 3.2.2 (R Core Team, 2013). Box-plots were produced by using software R with interface R Studio 0.98.1056, with package ggplot2. Logistic regression was conducted with IBM SPSS 22.0, comparing the survival under each exposure with that of the control. IBM SPSS 22.0 was used for the Probit regression analyses, and the results from the bioassays were used to estimate the lethal concentrations.

3 | RESULTS

3.1 | Toxicity

Following oral exposure to the neonicotinoid thiamethoxam, statistical analyses indicated normality of the errors obtained through the Shapiro-Wilk test, at $W = 0.9404$; $P = .2939$. The Bartlett test revealed a Bartlett's K^2 value of 0.7543; $P = .9799$, confirming the homogeneity of the variance.

The estimated LC_{50} by ingestion after 24 h was 2 μg a.i./L, as can be observed in Figure 1. The estimated value was higher than the sublethal concentrations tested in *S. bipunctata*.

The 0.75×10^2 μg a.i./L sublethal thiamethoxam concentration affected the survival of *S. bipunctata* (logistic regression; χ^2 test = 12.349; $P \leq .001$), resulting in an average of 57.77% survivors, being estimated that after the exposition, the probability for mortality increases 16%. After exposure to 2.5×10^2 μg a.i./L, which was the highest concentration of thiamethoxam evaluated, average survival

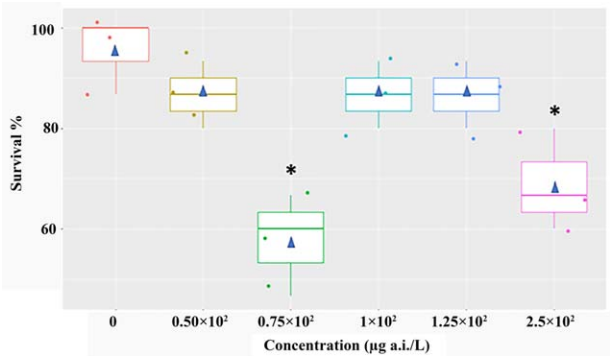

FIGURE 2 Boxplot showing the survival of bees following oral exposure to the neonicotinoid thiamethoxam for 72 h. The minimum and maximum values are presented, and the first and third quartiles are described. The central line represents the median, and the triangle represents the mean. $^*P < .005$ compared with the control on binary regression [Color figure can be viewed at wileyonlinelibrary.com]

was also significantly affected (logistic regression; χ^2 test = 8.242; $P = .004$), yielding in the mean of 68.88%, with the estimate of mortality 11% higher after exposition. Concentrations of 0.50×10^2, 1×10^2, and 1.25×10^2 µg a.i./L resulted in an average survival of 86.66%, and did not significantly interfere with the survival of the bees (logistic regression; $P \geq .05$) (Figure 2).

3.2 | Esterases isoenzymes

At 0.50×10^2 µg a.i./L, no esterase inhibition was observed at any period of exposure. At 0.75×10^2 µg a.i./L, partial inhibition of EST-4 occurred after 72 h. This esterase was partially inhibited in response to the 1×10^2 µg a.i./L treatment after 24 and 48 h, and, partial inhibition was observed with 1.25×10^2 µg a.i./L after 24 h, and total inhibition in 2.5×10^2 µg a.i./L after 24 and 72 h of exposure (Figure 3).

3.3 | Esterases characterization

In *S. bipunctata*, five esterases were identified, named EST-1 to EST-5, based on their electrophoretic mobility (Figure 3). EST-1, EST-2, and EST-3 were classified as α-esterases due to their preference for hydrolyzing α-naphthylacetate, and EST-4 and EST-5 were classified as β-esterases based on their preference for hydrolyzing of β-naphthylacetate. According the established criteria to Healy et al.[47] EST-1 and EST-2 were classified as cholinesterases I, and EST-4 and EST-5 are classified as cholinesterases II. The inhibition pattern of EST-3 did not permit its classification (Table 1).

3.4 | Critical electrolyte concentration (CEC)

In the cytochemical analyses, differences between treated and control insects were observed for all exposure periods and different concentrations tested. After 24 h exposure, the chromatin in treatments 0.75×10^2 µg a.i./L presented the CEC value of 0.20 M, which differed from the response observed with the other doses at this time point (Table 2; 24 h after exposure). As the period of exposure to chromatin became more compacted, as can be observed in all treatments after 72 h of exposure, the chromatin presented a CEC value of 0.30 M or higher (Table 2; 48 h after exposure and 72 h of exposure).

3.5 | Light microscopy and scanning electron microscopy (SEM)

The midgut was characterized morphologically in insects which were exposed (Figures 4B–D, 5E–J, 6B,D,F, and 7B–J) and not exposed with thiamethoxam (Figures 4A, 6A,C,E, and 7A). F4-F7

The external surface of the midgut was lined with internally disposed circular muscle fibers, and the outer surfaces of the midgut were marked by the insertion of the esophageal or cardiac valve and musculature lacking anelations. This was followed by the medial and posterior portions, characterized by the presence of regular transverse constrictions, corresponding to folds formed by invaginations of the circular fibers (Figures 4A and 7A).

The midgut is formed by a simple epithelium composed of digestive and regenerative cells resting on the basal lamina (Figures 4A and 6A). The digestive cells are cylindrical, presenting the basophilic cytoplasm with the nucleus located in the median region (Figures 4A and 6E). Microvilli project from the apical region of these cells towards the lumen, and are associated with nutrient absorption (Figure 6A,C,E). Regenerating cells were observed in groups, called nidi or nests of regeneration, located near the basal lamina, presenting a spherical shape and a bulky nucleus that occupied much of the cytoplasmic volume (Figure 4A).

In the lumen, the peritrophic membrane is composed of innumerable overlapping lamellar layers, delimiting the endo and ectoperitrophic spaces, separating the epithelium from the luminal content (Figures 4A and 6A,C).

The morphological characterization of *S. bipunctata* was similar to that reported by Landim[73] for *Apis mellifera* and *Scaptotrigona postica*.

After oral exposure to thiamethoxam at concentrations of 0.50×10^2, 1.25×10^2, and 2.5×10^2 µg a.i./L for 24, 48, and 72 h, the bee intestine presented numerous morphological changes. No treatment of 0.50×10^2 µg a.i./L in 24 h showed a more acidophilic cytoplasm when compared to the control. The epithelium was less developed, with remnants of peritrophic membrane, in which, as microvilli, were more prominent (Figure 4B). A longitudinal musculature demonstrated accentuated anelations, and musculature which was already circular exhibited folds less evident (Figure 7B).

In 48 h of exposure, a longitudinal musculature was observed, with less undulations, where the circular musculature was fewer dilated, but with more undulations (Figure 7C). In addition, the detachment of the epithelium was evidenced, due to the separation with the basal lamina, being this dispersed in the lumen. Peritrophic membrane evidences were found, without nests of regeneration, and in the digestive cells the microvilli were more elongated (Figure 4C).

In 72 h, the circular musculature was dilated (Figure 7D), with absence of organized cells in the epithelium, due to its release into the lumen, identifying vestiges of peritrophic membrane (Figure 4D).

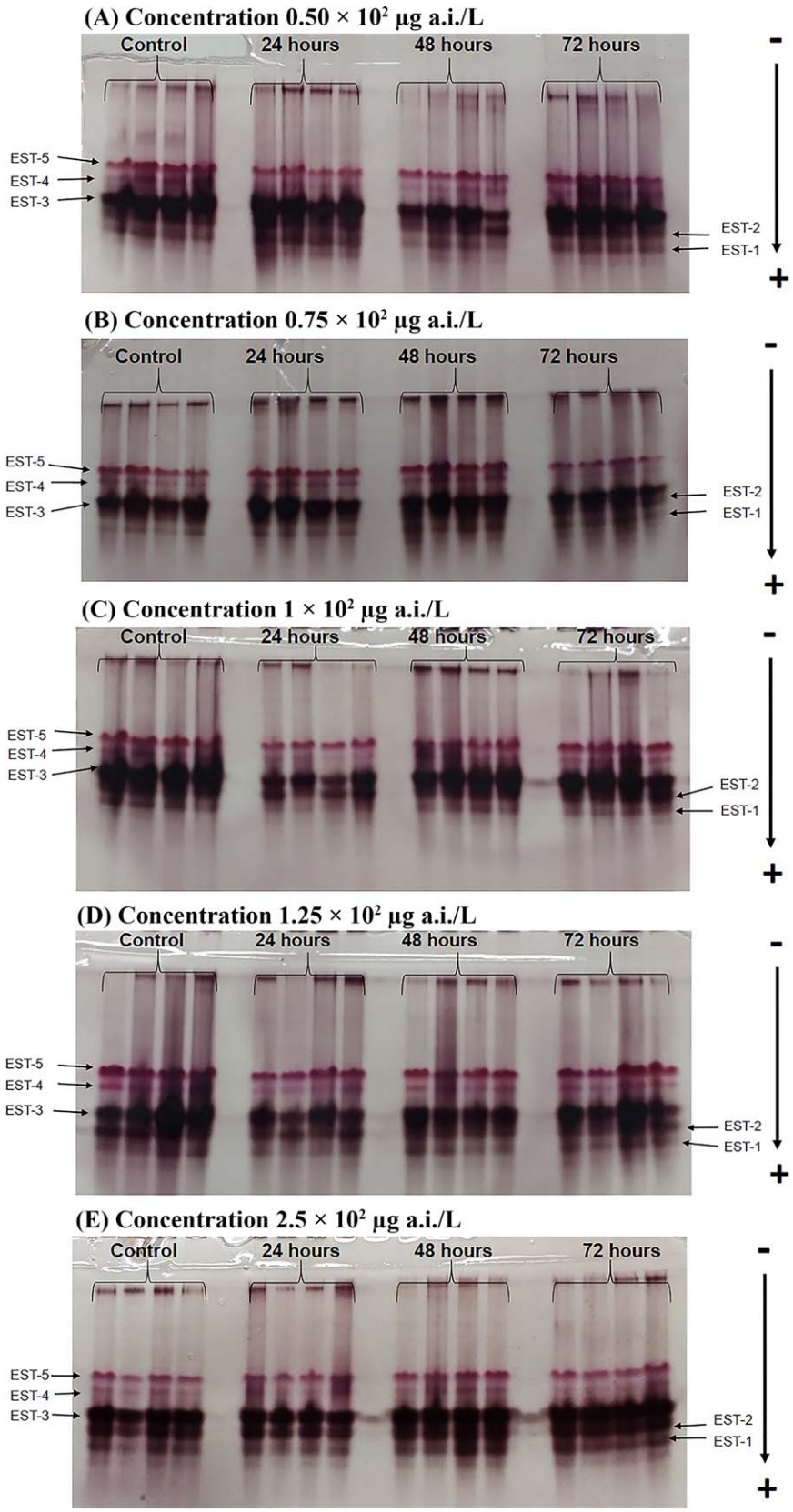

FIGURE 3 Electrophoretic profile of polyacrylamide gel esterases following ingestion of different concentrations of the neonicotinoid thiamethoxam [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Inhibition and classification of esterases with the use of inhibitors in *S. bipunctata* according Healy et al.[47]

Esterases	Organophosphate (Malathion)	Eserine sulfate	p-CMB	PMSF	Classification
EST-1	+	+	+	+	Cholinesterase I
EST-2	+	+	+	+	Cholinesterase I
EST-3	–	+	+	+	Without classification
EST-4	+	+	–	+	Cholinesterase II
EST-5	+	+	–	+	Cholinesterase II

Abbreviation: pCMB, *p*-chloromercuribenzoate acid; PMSF, phenylmethylsulfonyl fluoride.
Degrees of inhibition: (+) inhibition (–) absence of inhibition.

When exposed to 1.25×10^2 μg a.i./L insecticide in 24 h, a musculature exhibited to be looser, with less prominent folds, but circular musculature was very dilated (Figures 5E and 7E). There was absence of organized epithelium, with digestive cells being released into the lumen. In addition, the disruption and disorganization of the peritrophic membrane was detected (Figure 5E).

According to the exposure period, it was verified that after 48 and 72 h, the same changes were certified as in 24 h, but more accentuated (Figures 5F,G and 7F,G).

In the treatment of 2.5×10^2 μg a.i./L in 24 h, it was characterized by epithelial absence, more dilated musculature presenting deformations (Figure 7H). The absence or reduction of the circular folds,

TABLE 2 Nuclear basophilia of the *S. bipunctata* worker brain after 24, 48, and 72 h exposed with oral thiamethoxam [stained with 0.025% toluidine blue (TB) added of $MgCl_2$ in various concentrations (mol/L)]

		Thiamethoxam (ingestion)				
Stain	Control	0.50×10^2 μg a.i./L	0.75×10^2 μg a.i./L	1×10^2 μg a.i./L	1.25×10^2 μg a.i./L	2.5×10^2 μg a.i./L
24 h after exposure						
TB	Vi	Vi	Vi	Vi	Vi	Vi
TB + $MgCl_2$ 0.02 mol/L	Vi	Vi	Vi	Vi	Vi	Vi
TB + $MgCl_2$ 0.05 mol/L	Vio/Bl	Vi	Vi	Vi	Vi	Vi
TB + $MgCl_2$ 0.08 mol/L	Vio/Bl	Vi	Vi	Vi	Vi	Vi
TB + $MgCl_2$ 0.10 mol/L	Bl	Vi	Vi	Vi	Vi	Vi/Bl
TB + $MgCl_2$ 0.12 mol/L	Bl	Vi	Vi/Bl	Vi/Bl	Vi/Bl	Vi/Bl
TB + $MgCl_2$ 0.15 mol/L	*Gr**	Vi/Bl	Vi/Bl	Vi/Bl	Vi/Bl	Vi/Bl
TB + $MgCl_2$ 0.20 mol/L	Bl/Vi	Vi/Bl	*Gr**	Vi/Bl	Vi/Bl	Vi/Bl
TB + $MgCl_2$ 0.30 mol/L	Bl/Vi	*Gr**	Bl/Vi	Vi/Bl	Vi/Bl	*Gr**
CEC value (mol/L)	0.15	0.30	0.20<CEC>0.30	>0.30	>0.30	0.30
48 h after exposure						
TB	Vi	Vi	Vi	Vi	Vi	Vi
TB + $MgCl_2$ 0.02 mol/L	Vi	Vi	Vi	Vi	Vi	Vi
TB + $MgCl_2$ 0.05 mol/L	Vio/Bl	Vi	Vi	Vi	Vi	Vi
TB + $MgCl_2$ 0.08 mol/L	Vio/Bl	Vi	Vi	Vi	Vi	Vi
TB + $MgCl_2$ 0.10 mol/L	Bl	Vi	Vi	Vi	Vi	Vi
TB + $MgCl_2$ 0.12 mol/L	Bl	Vi/Bl	Vi/Bl	Vi/Bl	Vi	Vi/Bl
TB + $MgCl_2$ 0.15 mol/L	*Gr**	Vi/Bl	Vi/Bl	Vi/Bl	Vi/Bl	Vi/Bl
TB + $MgCl_2$ 0.20 mol/L	Bl/Vi	Vi/Bl	Vi/Bl	Vi/Bl	Vi/Bl	Vi/Bl
TB + $MgCl_2$ 0.30 mol/L	Bl/Vi	*Gr**	Vi/Bl	Vi/Bl	*Gr**	*Gr**
CEC value (mol/L)	0.15	0.30	>0.30	>0.30	0.30	0.30
72 h after exposure						
TB	Vi	Vi	Vi	Vi	Vi	Vi
TB + $MgCl_2$ 0.02 mol/L	Vi	Vi	Vi	Vi	Vi	Vi
TB + $MgCl_2$ 0.05 mol/L	Vio/Bl	Vi	Vi	Vi	Vi	Vi
TB + $MgCl_2$ 0.08 mol/L	Vio/Bl	Vi	Vi	Vi	Vi	Vi
TB + $MgCl_2$ 0.10 mol/L	Bl	Vi	Vi	Vi/Bl	Vi	Vi/Bl
TB + $MgCl_2$ 0.12 mol/L	Bl	Vi/Bl	Vi/Bl	Vi/Bl	Vi/Bl	Vi/Bl
TB + $MgCl_2$ 0.15 mol/L	*Gr**	Vi/Bl	Vi/Bl	Vi/Bl	Vi/Bl	Vi/Bl
TB + $MgCl_2$ 0.20 mol/L	Bl/Vi	Vi/Bl	Vi/Bl	Vi/Bl	Vi/Bl	Vi/Bl
TB + $MgCl_2$ 0.30 mol/L	Bl/Vi	*Gr**	*Gr**	*Gr**	*Gr**	*Gr**
CEC value (mol/L)	0.15	0.30	0.30	0.30	0.30	0.30

Abbreviations: Vi, violet; Bl, blue; Gr*, green.

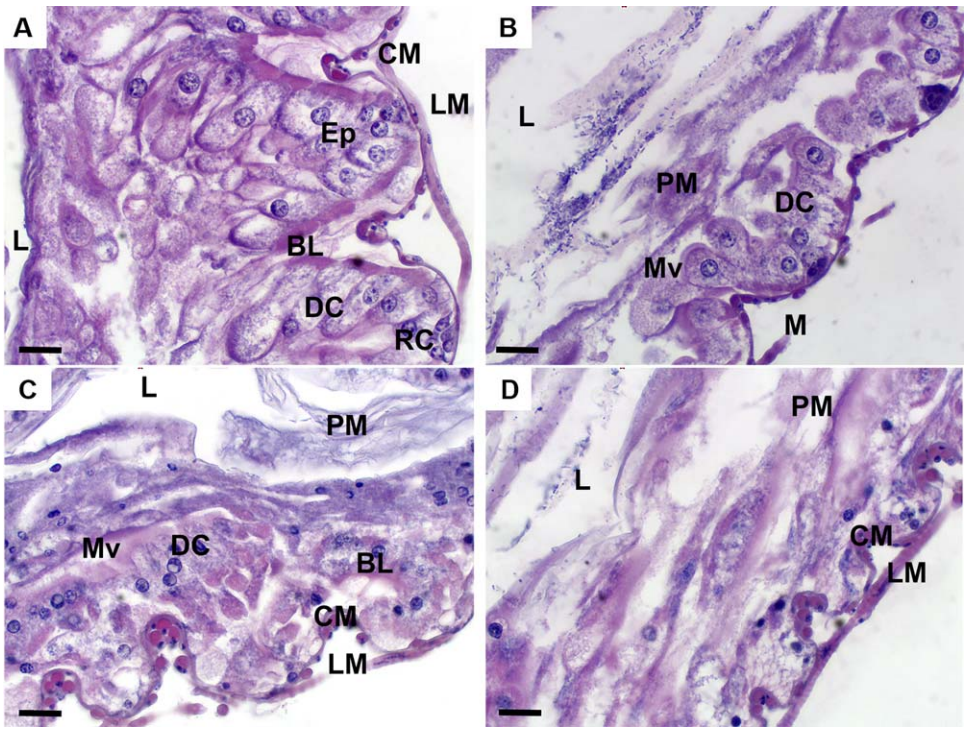

FIGURE 4 Photomicrography showing the midgut of the *S. bipunctata*. A, control; B, 0.50 × 10^2 μg a.i./L in 24 h; C, 0.50 × 10^2 μg a.i./L in 48 h; D, 0.50 × 10^2 μg a.i./L in 72 h; Ep, epithelium of the midgut; CM, circular musculature; LM, longitudinal musculature; M, musculature; BL, basal lamina; DC, digestive cells; RC, regenerative cells; PM, peritrophic membrane; L, lumen; Mv, microvilli. Hematoxylin-Eosin staining. Scale bar: 20 μm [Color figure can be viewed at wileyonlinelibrary.com]

remnants of the peritrophic membrane, and detachment of the basal lamina were also confirmed (Figure 5H).

The modifications reported after 48 h of exposure were similar to those described in 24 h (Figures 5I and 7I). However, within 72 h, fragments of epithelial cells were observed in the lumen. Microvilli were also identified in this treatment (Figures 5J and 6B,D,F).

4 | DISCUSSION

Insect survival after exposure to a lower concentration (0.75 × 10^2 μg a.i./L) was affected when compared to the highest concentration (2.5 × 10^2 μg a.i./L). During the experiments, it was observed that, in smaller concentrations, there was a considerable consumption of food with insecticide, which may have provided high mortality. Kessler et al.[22] carried out feeding trials with *A. mellifera* and *Bombus terrestris*, with three agricultural neonicotinoids (thiamethoxam, imidacloprid, and clothianidin) and verified those bees preferred to feed on the sucrose solutions containing the neonicotinoids. These results indicated that bees cannot detect the neonicotinoids and are not repelled by their presence, being considerable risk to these species.

Similar results were found by Falco et al.[55] that showed that after oral test with thiamethoxam in *A. mellifera*, higher mortality, and food consumption were observed in treatments that contained lower concentrations of the insecticide. In this study was verified that contaminated food with low concentrations of the insecticide can be collected and transported to the beehive contaminating a larger number of individuals. Sublethal effects are not related to the immediate death of insects, but they can induce behavioral modifications.[58] Gajger et al.[33] when exposing *A. mellifera* queens to thiamethoxam concluded that low doses of the insecticide affect the development and reproductive traits. Grillone et al.[34] when using thiamethoxam in larvae of *Apis mellifera ligustica* throughout the development verified that remarkable effects were more present in smaller concentrations, such as death of larvae, late pupation, and deformed adult emerged.

Furthermore, the studied thiamethoxam was highly toxic to adult *S. bipunctata* foragers, presenting after 24 h a LC_{50} of 2 μg a.i./L. According to Valdovinos-Núñez et al.[74] after thiamethoxam test in stingless bee *Nannotrigona perilampoides*, they identified a LD_{50} (lethal dose to 50% of the exposed insects) of 0.004 μg/bee. Tests performed in the laboratory with *A. mellifera* found that the acute oral LD_{50} for thiamethoxam was 5 ng/bee.[10,75] Laurino et al.[76] performed oral test with thiamethoxam and found it to be lethal to adult *A. mellifera* bees following exposure up to 72 h from the start of the test. These data reinforce that stingless bees are more sensitive than honey bees when exposed to pesticides.[8,74,77]

Due to the exposure to neonicotinoid it is probable that cellular compromise has occurred altering the secretion and absorption in the cells resulting in a nutritional depletion and changes in different physiological processes. These modifications could cause changes in development, increase mortality, resulting in losses in colonies.

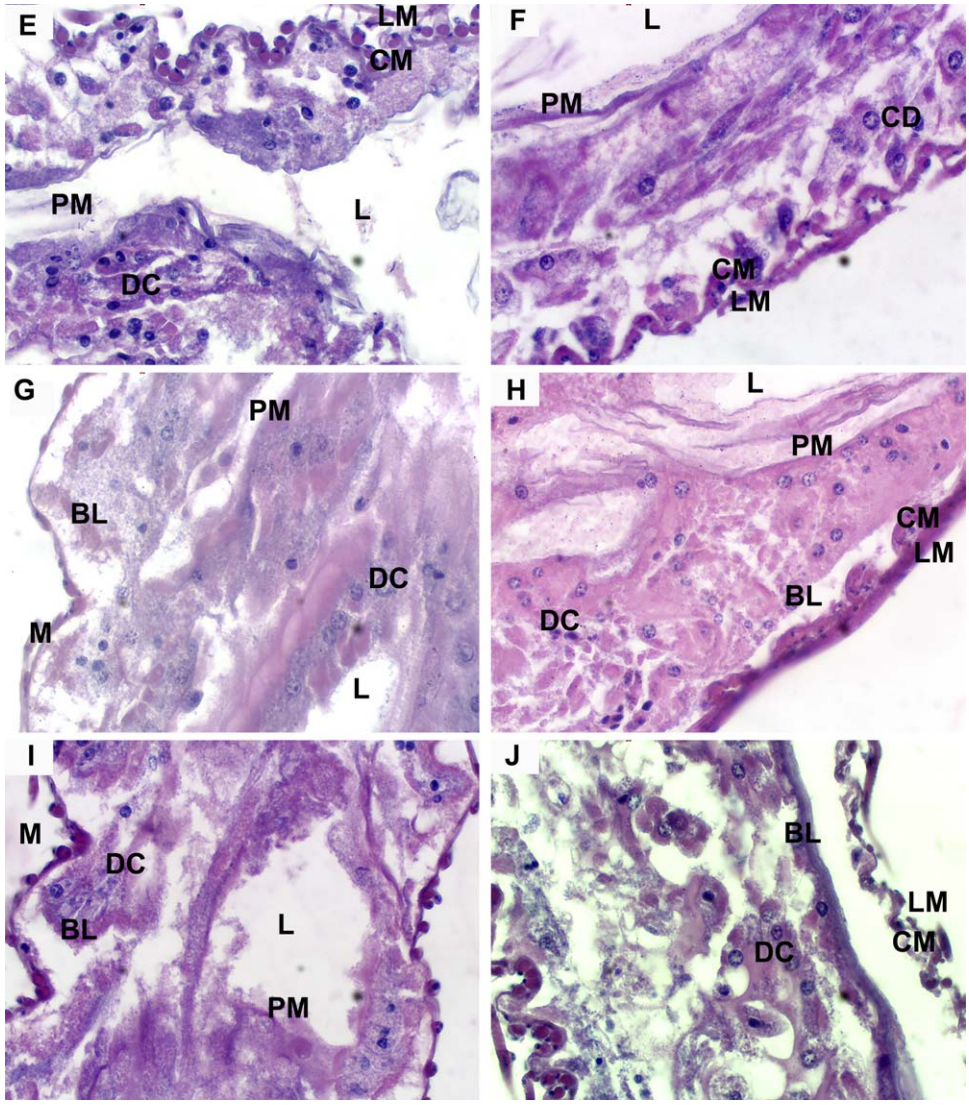

FIGURE 5 Photomicrography showing the midgut of the *S. bipunctata*. E, 1.25×10^2 μg a.i./L in 24 h; F, 1.25×10^2 μg a.i./L in 48 h; G, 1.25×10^2 μg a.i./L in 72 h; H, 2.5×10^2 μg a.i./L in 24 h; I, 2.5×10^2 μg a.i./L in 48 h; J, 2.5×10^2 μg a.i./L in 72 h. CM = circular musculature; LM = longitudinal musculature; M = musculature; BL = basal lamina; DC = digestive cells; PM = peritrophic membrane; L = lumen. Hematoxylin-Eosin staining. Scale bar: 20 μm [Color figure can be viewed at wileyonlinelibrary.com]

Among these enzymes, esterases that exert a number of functions in insects, including detoxification of xenobiotics, may present changes in the expression or relative activity after contact of the insects with the different compounds. However, these enzymes have been widely used as tools for the study of the effects of insecticides on insects.

Acetylcholinesterase is the target for insecticides such as neonicotinoids. Thiamethoxam mimics the action of acetylcholine and is not degraded by acetylcholinesterase. This compound binds to acetylcholine receptors on the membrane of postsynaptic cells, opening Na+ channels, promoting nervous hyperactivity, and the subsequent collapse of the nervous system. Nevertheless, as acetylcholinesterase is not the only target for the toxicity of cholinergic compounds[78] secondary targets such as butyrylcholinesterase (BChE) and other pseudocholinesterases may also be affected.

Following the ingestion of neonicotinoid, there was a decrease in EST-4 activity compared with the control, except for the lowest concentration evaluated 0.50×10^2 μg a.i./L. Similarly, Hashimoto et al.[43] confirmed that the relative activities of EST-1, −2, −4, and −5 were partially inhibited after 24 h of oral exposure with thiamethoxam in *A. mellifera* workers. Ruvolo-Takasusuki et al.[44] showed that esterase inhibition also occurs in the egg and larval stages of *A. mellifera* upon exposure to thiamethoxam residues in food, identifying a 25% reduction in the relative activity of these enzymes.

The isoenzyme EST-4 was classified as a type II cholinesterase (pseudocholinesterase or butyrylcholinesterase) according to the classification criteria established to Healy et al.,[47] and, the results suggest its participation in the detoxification of this neonicotinoid in *S. bipunctata*. If we consider that, in the lowest concentration evaluated, there was lower mortality, and that this esterase showed no alteration, we suggest that it could be acting in the metabolization of this insecticide to a less toxic form. Besides that, the changes observed in the insect midgut would not motivate to immediate mortality, possibly because the action

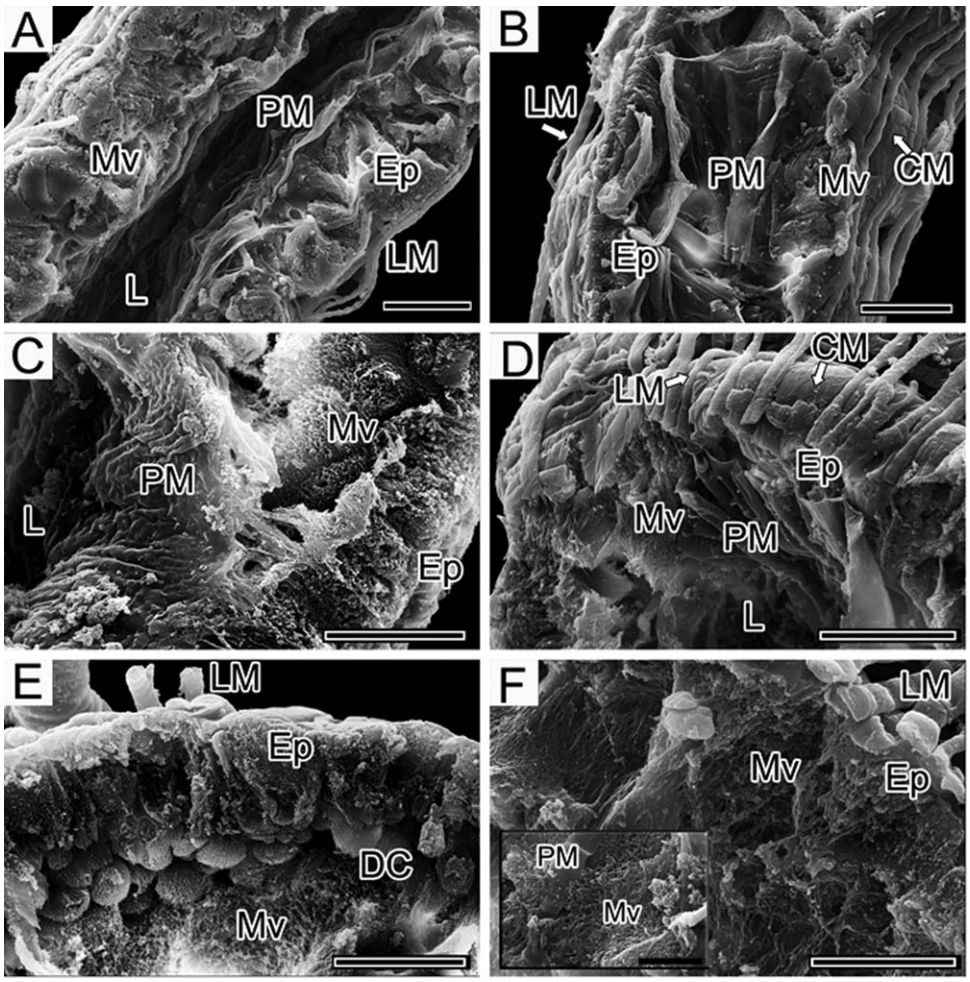

FIGURE 6 Scanning electron microscopy showing the midgut of *S. bipunctata* following ingestion of thiamethoxam. A, Control. Escale bar: 50 μm; B, 2.5 × 10^2 μg a.i./L in 24 h. Escale bar: 50 μm; C, control. Escale bar: 30 μm; D, 2.5 × 10^2 μg a.i./L in 24 h. Escale bar: 30 μm; E, control. Escale bar: 30 μm; F, 2.5 × 10^2 μg a.i./L in 24 h. Escale bar: 30 μm. Ep = epithelium of the midgut; PM = peritrophic membrane; Mv = microvilli; L = lumen; LM = longitudinal musculature; CM = circular musculature; DC = digestive cells

of the insecticide on its main target would have been reduced, especially acetylcholinesterase of the central nervous system, allowing a prolonged survival period. On the other hand, to the extent that neonicotinoid concentrations are increased and EST-4 is partially inhibited, higher concentrations of the insecticide could be compromising the morphophysiology of the intestine and nervous system, finally increasing mortality.

The analysis of critical electrolyte concentration showed that changes occur in the chromatin structure of *S. bipunctata* brain cells after thiamethoxam contamination. Taking that into account, it is suggested that the inhibition of EST-4 as an important insect detoxification enzyme could be associated with a decrease in gene expression, since condensed chromatin is normally inactive as the decondensed chromatin is active, according to Alberts et al.[79]

The results showed that after 24, 48, and 72 h exposure, the exposed bees showed higher CEC values than control bees. Falco et al.[55] explain modifications in the CEC values reflect structural changes in chromatin. The condensed chromatin has an CEC value higher than the decondensed chromatin, due to the interaction that occurs when as toluidine blue molecules are stacked, causing an increase in metachromasia and consequent enhance CEC value.[61,80,81]

Similar results were described by Rossi et al.[58] using the neonicotinoid imidacloprid. These authors observed chromatin condensation in *A. mellifera* brains. Falco et al.[55] in exposing bees to thiamethoxam, concluded that this affects the chromatin structure in the Malpighian tubules of *A. mellifera*, where the CEC value can be altered not only by the action of the insecticide, but also by the age or activity exercised by bee.

Santos et al.[59] conducted CEC tests with salivary glands of third and fifth instars larvae of *Diatraea saccharalis* submitted to oral contamination with the insecticides (fipronil, malathion, cypermethrin, and neem oil). In this study, several changes were detected in the chromatin structure. Authors concluded that gene activation and inactivation may be acting in the defense mechanisms, contributing to the selection and survival of resistant individuals. Although those authors did not use neonicotinoids, the results obtained with *S. bipunctata* may support this statement, since the chromatin condensation observed may be related to the genes inactivation, allowing an alternative response to neonicotinoid, and the bee survival after oral contamination.

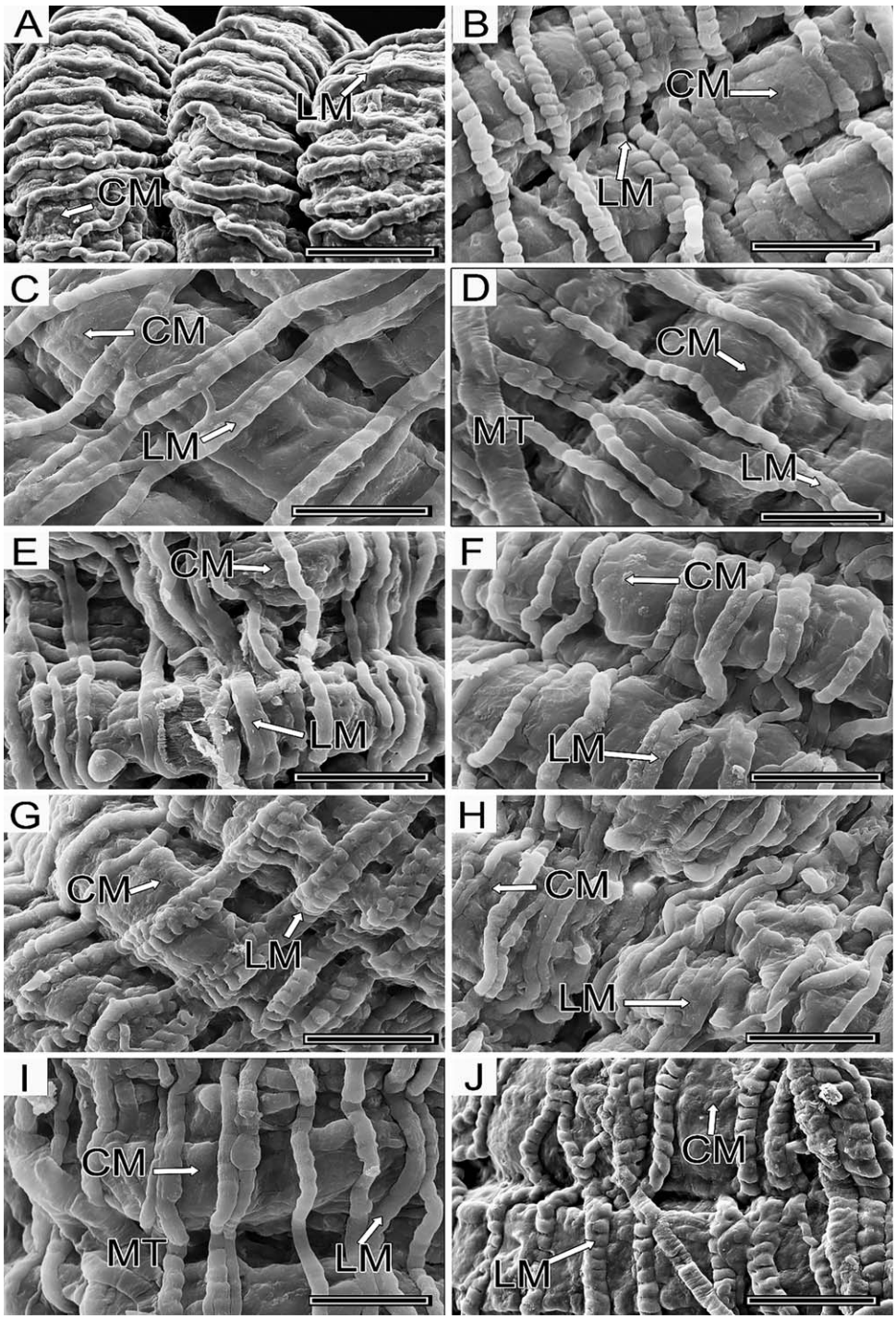

FIGURE 7 Scanning electron microscopy showing the midgut of *S. bipunctata* following ingestion of thiamethoxam. A, Control; B, 0.50×10^2 μg a.i./L in 24 h; C, 0.50×10^2 μg a.i./L in 48 h; D, 0.50×10^2 μg a.i./L in 72 h; E, 1.25×10^2 μg a.i./L in 24 h; F, 1.25×10^2 μg a.i./L in 48 h; G, 1.25×10^2 μg a.i./L in 72 h; H, 2.5×10^2 μg a.i./L in 24 h; I, 2.5×10^2 μg a.i./L in 48 h; J, 2.5×10^2 μg a.i./L in 72 h. LM = longitudinal musculature; CM = circular musculature; MT = malpighian tubules; Escale bar: 30 μm

As the digestive tract represents the main way of contact for the insects with the environment, the midgut presented morphological changes after exposure to the neonicotinoid. Morphological analysis of the treatments with 0.50×10^2 and 2.5×10^2 μg a.i./L with thiamethoxam for 24 hours showed the occurrence of elongated microvilli, probably an attempt of the digestive cells exposed to the neonicotinoid to increase the absorption of nutrients to prolong the insect survival. As the length of the exposure period increased, degradation of digestive cells and their elimination into the lumen provided evidence of the degenerative effects of this insecticide on these cells. The observed epithelial degeneration may be associated with degradation of the peritrophic membrane following oral contamination

with thiamethoxam, detected in the midgut of the contaminated bees.

In *A. mellifera*, the presence of cytoplasmic vacuolization, increased apocrine secretion, cell elimination,[57] ruptured cell membrane, abnormality, and decreased nuclei[65] emphasize the degenerative effects of the insecticide on the digestive cells, which are related to transport of ions and water, absorption of nutrients, as well as synthesis and secretion of digestive enzymes.[63,82–84]

Regarding the results, few regeneration nests were characterized in thiamethoxam-treated individuals, as observed by Oliveira et al.[57] in *A. mellifera* treated with the same insecticide. These results suggest that oral exposure to the agrochemical could have interfered with the structure and function of the regenerative cells, affecting their proliferation and differentiation into new digestive cells.

5 | CONCLUSION

Ingestion of sublethal concentrations of the neonicotinoid thiamethoxam in *S. bipunctata* may interfere with physiological processes of these insects. We can conclude that, although thiamethoxam at the concentrations evaluated did not cause high mortality, it induced concentration-dependent changes in bees. The changes resulting from this contamination may compromise physiological processes, which are fundamental to the survival of these pollinators. Thus, the changes detected reinforce the importance of studies investigating insecticides in stingless bees, seeking to ensure the preservation and protection of these species, and protecting their role as pollinators for the maintenance of plant diversity and for all who benefit from it.

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