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Toxicity and genetic analysis of bees *Scaptotrigona bipunctata* after contamination with insecticide acephate

T. O. Diniz[1]*, N. C. Pereira[1], W. C. S. Pizzaia[1], A. A. Sinópolis-Giglioli[1], B. G. Silva[2], Y. M. Borges[3], T. A. Guedes[4], M. C. C. Ruvolo-Takasusuki[1]

[1] Universidade Estadual de Maringá – Departamento de Biotecnologia, Genética e Biologia Celular
[2] Escola Superior de Agricultura "Luiz de Queiroz" / USP – Departamento de Ciências Exatas
[3] Instituto Federal do Amazonas
[4] Universidade Estadual de Maringá – Departamento de Estatística

*** Author for correspondence: tamirisd.biologa@gmail.com**

Abstract. The meliponines can be found in tropical and subtropical regions and is observed in the majority of Latin America. Scaptotrigona bipunctata are stingless bees that build colonies that possess between 2,000 and 50,000 individuals. This study aimed to estimate the toxicity of the insecticide acephate after oral and contact contamination in S. bipunctata. Results revealed differences between the two types of contamination and indicated that S. bipunctata is tolerant to acephate since the mortality rate was low. The findings indicated that there were expression changes of isoenzyme esterases after contamination with the insecticide, which was able to partially inhibit almost all of the enzymes identified in this study. In addition, morphological changes were identified in the midgut of the bees in the first 24 hours of contamination. However, after 48 hours it was observed the regeneration of the peritrophic membrane and other structures, contributing to the survival of the insects. It is essential to propose measures to minimize the impact of agrochemicals on pollinators and this study provides support for investigations on pesticide toxicity in stingless bees.
Keywords: organophosphate, esterases, midgut

Introduction

The meliponines, also known as stingless bees, are bees that have atrophied sting, have variable size and belongs to the Apidae family (Silva et al., 2012). *Scaptotrigona bipunctata* belongs to this class of bees and are found in Bolivia, Paraguay and Peru (Witter & Nunes-Silva, 2014). In Brazil, this species is generally found in the Pampas, Atlantic Forest and Pantanal biomes (Camargo & Pedro, 2013). These species possess interesting biological characteristics and it can be used in managed pollination (Venturieri et al., 2011) in tropical and subtropical regions (Slaa et al., 2006). However, there are few studies showing the susceptibility of *S. bipunctata* to insecticides (Santos et al., 2016).

Although the main function of pesticides is to protect agricultural crops against diseases and pests, the application of these substances can cause contamination of soils, water and food, and have negative effects on non-target organisms, such as bees (Desneux et al., 2007). Organophosphates are one of the major classes of insecticides used in pest control, which, together with carbamates and pyrethroids, corresponds to approximately 40% of the pesticide market. This class of insecticides has a broad spectrum of action, causing severe damages to the nervous system of contaminated individuals (Coutinho et al., 2005). It acts by phosphorylating the enzyme acetylcholinesterase, responsible for inactivating the neurotransmitter acetylcholine, blocking its catalytic activity (Bastos et al., 2015).

Acephate belongs to the organophosphates class and is used against pests in several crops, such as cotton, peanuts, potatoes, broccoli, citrus, kale, cauliflower, cloves, chrysanthemum, beans, tobacco, melons, peppers, cabbage, tomatoes and soy (Mapa, 2017). In Brazil, this insecticide is

considered moderately toxic, but its use is prohibited in the United States and Europe.

The efficiency of these insecticides can be measured by their action under the target and non-target insects, in particular, bees. The isoenzyme esterases are used as indicators or biomarkers for pesticides (Dmitryjuk et al., 2014). These isoenzymes are part of a diverse group of hydrolases capable of catalyzing the cleavage and formation of ester bonds and are widely distributed in animals, plants and microorganisms.

Esterases are divided into four classes: acetylesterase, (E.C. 3.1.1.6), arylesterases (E.C. 3.1.1.2), carboxylesterases (E.C. 3.1.1.1), and cholinesterases, that includes acetylcholinesterases (E.C.3.1.1.7) and pseudocholinesterases (E.C. 3.1.1.8 (Healy et al., 1991). Many of them have a wide range of possible substrates and various functions in the metabolism of exogenous and endogenous compounds acting on behaviour and development of insects (Montella et al., 2012). In addition, esterases play an important role in insecticide resistance (Shin et al., 2016) and may have the ability to metabolize or degenerate substances before toxic effects (Claudianos et al, 2015).

Morphophysiological analyzes can also be used to study the effects of exposure to agrochemicals (Oliveira et al., 2014). The majority of ingested insecticides are metabolized in the midgut of the bees, thus detoxifying enzymes, especially esterases (Moreira et al., 2018), can control the interaction of insecticides.

Considering the widespread use of organophosphates insecticides in crops that attract bees and the consequences for these non-target organisms this study aimed to find the lethal concentration (LC_{50}) to oral and contact exposure to acephate in S. bipunctata, as well as identify possible morphological variations and expression changes of esterases.

Methods

Biological material

Adult bees of *S. bipunctata* were obtained from six colonies located at the Experimental Farm of Iguatemi, from Universidade Estadual de Maringá (UEM) (23º 25 'S, 51º 57' O, UEM, Maringá, PR, Brazil) and taken to the Laboratory of Genetics Animal from the Department of Biotechnology, Genetics and Cell Biology of UEM.

Bioassays

The commercial insecticide Orthene® 750BR, Arysta Lifescience, possess 75% of active ingredient (a.i.). Preliminary tests were made to determine the sublethal concentrations (contact: 0.1, 0.2, 0.3, 0.4 and 0.5 mg a.i./ mL, ingestion: 5, 6, 7, 8 and 9 µg a.i./ mL).

For each bioassay 270 *S. bipunctata* bees were collected. Five concentrations of the insecticide were used, in triplicate, containing 15 bees each, plus the control group. The number of bees in each glass was determined considering the size and behaviour of the individuals.

In order to ensure genetic variability and obtain genuine toxicological deductions, each experiment was composed of bees from different colonies. Bees were collected at the entrance of the colonies and transferred to glass bottles (18 cm x 13 cm) and stored in a BOD incubator, maintained in 28 ºC ± 2 °C and UR 70 ± 10%. Bees were kept under these conditions for 72 hours and dead individuals were counted after 24, 48 and 72 hours of exposure.

For the contact bioassay, 1 mL of the insecticide were added into the filter paper and left to dry. After dried, glass bottles were assembled as described above. For ingestion bioassay, the insecticide was placed into the food, which consisted of a liquid mixture of crystal sugar and water (1:1). To guarantee that the bees would feed efficiently small pieces of wood were placed into the food recipient to serve as support.

Esterases analyzes

Head and thorax of worker bees were homogenized in propylene tubes of 1.5 mL containing 60 µL of β- mercaptoethanol solution and glycerol 10 %. After that, the samples were centrifuged at 2,000 rpm for 10 minutes at 4° C.

PAGE electrophoresis at 12% was conducted with Tris-Glicina buffer 0,1 M pH 8,3 at 200 V, for 4 hours at 4° C. After the electrophoresis, the gels were incubated for 30 minutes in 50 mL of sodium phosphate buffer (0.1 M pH 6.2). Soon after, the buffer was discarded and the staining solution (50 mL of 0.1 M sodium phosphate buffer pH 6.2; 0.03 g of α-naphthyl acetate; 0.03 g of β-naphthyl acetate; 0.06 g of Fast Blue RR Salt) were added.

The gel was incubated until the bands appeared. Finally, the gels were kept in preservative solution, composed of 75% acetic acid and 10% glycerol dissolved in distilled water for at least 24 hours and digitized.

Light microscopy

After contamination with insecticide in the concentrations of 5, 6, 7, 8 e 9 µg a.i./ mL for 24, 48 and 72 hours surviving *S. bipunctata* bees were anesthetized under low temperature, and the midgut was dissected in solution (NaCl 0.1 M, Na2HPO4 0.1 M e KH2PO4 0.1 M). 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 lm 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 Olympus light microscope and sections were photographed using a digital camera.

Data analysis

The normality of the response variable (mortality) was verified using the following tests: Shapiro-Wilk, Cramér-von Mises and Lilliefors, in software R 3.5.0 (R Core Team, 2018). In order to investigate the behavior of the response variable in relation to probability distribution, histograms and density plot were produced. The Kruskal-Wallis test was used to verify if there was a difference in the number of mortality according to concentration levels. Logistic regression was performed to identify if there was a difference in the mortality rate associated with each concentration of the insecticide.

Results and discussion

Regarding the normality assumption of the continuous variable mortality, the tests Shapiro-Wilk, Cramér-von Mises and Lilliefors indicated absence of normality (p-value = 6.324e-12); (p-value = 8.215e-09); (p-value = 2.2e-16). Kruskal-Wallis test showed that there was no difference in the number of deaths according to the concentration levels (p-value = 0.245).

On the other hand, the binomial regression model showed that in the initial doses, approximately decreasing responses are observed, followed by an increase in response with increasing doses. These results obtained by the logistic adjustment reflect the high toxicity of the insecticide in the lower doses, as well as highly toxic effects in the higher doses, finally showing that there is no difference in the dosage used with effect on mortality.

The LC_{50} values estimated for *S. bipunctata* showed that the highest toxicity was observed when the bees were contaminated by ingestion with the insecticide (Table 1).

The estimated values for LC*50* were lower than the concentrations used in this study. The lowest concentrations (contact: 0.1 mg a.i./ mL; ingestion: 5 µg a.i./ mL) used in this study were the ones that most affected the survival of individuals (Table 2).

The mortality rate of *S. bipunctata* workers contaminated with acephate by contact was higher in 24 hours. After this, mortality rates were comparable to the ones observed in the control group. The concentration of 0.1 mg a.i./ mL showed the highest mortality rates. The same occurred in the contact bioassay, where the heights mortality rates were observed at the concentration of 5 µg a.i./ mL.

These results indicate that *S. bipunctata* shows tolerance to acephate, which leads to the bees death in the first 24 hours, both with contamination by contact and by ingestion. In a study carried out by Baptista et al. (2009), *A. mellifera* workers were contaminated with 0.075 g p. c./ 100 mL^{-1} of acephate by contact, spraying and ingestion. In all treatments, it was found that, after 24 hours of contamination, more than 90% of the infected bees died. It is worth noting that the dose used in this study was higher than that used in the present study, however *S. bipunctata* were more tolerant to acephate than *A. mellifera.*

Falco et al. (2010) observed similar effects, where *A. mellifera* were contaminated via oral with lowers concentrations of the neonicotinoid thiamethoxam showed higher mortality. Gajger et al. (2017) contaminated *A. mellifera* queens with thiamethoxam and inferred that lower doses of the insecticide impair development and reproductive traits.

In general, organophosphates insecticides have relatively low toxicity through oral contamination, because they are rapidly metabolized, however, some compounds may remain in the insect body and cause toxicity when ingested. Based on this assumption, Sanchez-Bayo e Goka (2014) reported that ingestion of the organophosphate chlorpyrifos can be dangerous to *A. mellifera* due to its high toxicity and a large amount of residue found in pollen and honey.

The lower mortality rates showed in the contact bioassay are probably related to the differences in thickness and chemical composition of the cuticle (Bacci et al., 2006). The cuticle in stingless bees is formed of an outer lipid layer composed of several classes of chemical compounds that diverge between species and are genetically established (Abdalla et al., 2003; Leonhardt et al., 2015). These particularities in the cuticle may favour or prevent insecticides penetration, presenting higher or lower toxicity, depending on the bee species.

Dorneles et al. (2017) estimated the toxicity of chlorpyrifos and phosmet insecticides for *S. bipunctata* and the results indicated that these stingless bees are tolerant to chlorpyrifos, however, both insecticides are potentially dangerous to this species. When the contamination was through ingestion of contaminated pollen or nectar, the toxicity was reduced during the detoxification process that occurs mainly in the midgut and fat body.

The lower mortality rates may be related to different factors. In the case of contact contamination, bees may have moved away from the contaminated filter paper, remaining in the upper parts of the glasses. When the contamination occurred by ingestion, the bees probably ate less food after noticing the insecticide.

Organophosphates are known for their irreversible inhibition of the enzyme acetylcholinesterase, inactivating it and causing the death of the insect (Elhanany et al., 2001). Despite the low mortality in contact and ingestion bioassays, the amounts of acephate used for oral contamination show its toxicity.

Several authors have evaluated the toxicity of organophosphates insecticides in bees. Moraes et al. (2000) determined the lethal dose (LD_{50}) of malathion for the *Scaptotrigona tubiba* (LD_{50} >0.04 mg/ bee), Macieira et al. (1989) found an LD_{50} for *Trigona spinipes* (LD_{50} 0,26 mg/ bee) and Batista et al. (1975) established an LD_{50} for *A. mellifera* (LD_{50}

0,18 mg/ bee). Baptista et al. (2009) found the toxicity of acephate to *A. mellifera*, which when supplied orally, was able to cause the mortality of almost all bees 20 hours after its application.

In this study, it was possible to verify that *S. bipunctata* is tolerant to acephate because even though that was high mortality rates after 24 hours of contamination, in 72 hours the organism of the bees was stabilized. This can be explained with the esterases expression being similar to the control group, morphological changes in the midgut and reduction of mortality rates.

In addition, acephate was highly toxic to adult forage bees, presenting a LC_{50} of 3.470 µg a.i./ mL after 24 hours of contamination. Dorneles et al. (2017) found a LC_{50} of 0.0112 µg a.i./ µL when *S. bipunctata* was contaminated with chlorpyrifos and a LC*50* of 0.0245 µg a.i./ µL when contaminated with phosmet. These data reinforce that stingless bees are more tolerant to the insecticide used in our study.

Five esterases were identified in *S. bipunctata*, nominated according to their electrophoretic mobility (EST-1 to EST-5). EST-1, EST-2 and EST-3 are classified as α-esterases due to its propensity to hydrolyze α-naphthyl acetate, while EST-4 and EST-5 are classified as β-esterases because of its predilection for hydrolyzing α-naphthyl acetate. According to the criteria determined by Healy et al. (1991) EST-1 and EST-2 were classified as cholinesterases I and EST-4 and EST-5 are classified as cholinesterases II. However, due to the inhibition pattern presented by EST-3, it was not possible to indicate its classification (Moreira, 2018).

The relative activity of isoenzymes esterases in *S. bipunctata* contaminated with acephate showed different patterns of inhibition when compared to the control group. On the first 24 hours of contamination by contact, it was observed a total inhibition of EST-4 in almost every concentration of the insecticide. After 48 hours EST-4 was expressed again in all the concentrations used, and at the end of the experiment, after 72 hours, there was no inhibition of any esterase (Figure 1).

Table 1. Toxicity parameters obtained after 24 hours of contamination by contact and ingestion with acephate insecticide in *S. bipunctata*

	Contact bioassay	Ingestion bioassay
LC_{10}	1.640 mg a. i./ mL	11.842 µg a. i./ mL
LC_{50}	0.052 mg a. i./ mL	3.470 µg a. i./ mL
LC_{99}	0.002 mg a. i./ mL	1.017 µg a. i./ mL

Table 2. Mortality rate of *S. bipunctata* contaminated with acephate insecticide for 72 hours (A): Contact; (B): Ingestion

(A)	Concentration	24 h	48 h	72 h	Total	Mortality(%)
	Control	1	1	0	2	4.44
	0.1 mg a. i./ mL	15	1	2	18	40.0
	0.2 mg a. i./ mL	8	1	3	12	26.7
	0.3 mg a. i./ mL	6	1	1	8	17.8
	0.4 mg a. i./ mL	2	0	1	3	6.7
	0.5 mg a. i./ mL	4	2	2	8	17.8

(B)	Concentration	24 h	48 h	72 h	Total	Mortality(%)
	Control	1	0	0	1	2.22
	5 µg a. i./ mL	12	2	4	18	40.0
	6 µg a. i./ mL	6	3	1	10	22.2
	7 µg a. i./ mL	4	1	3	8	17.8
	8 µg a. i./ mL	1	0	1	2	4.44
	9 µg a. i./ mL	3	2	0	5	11.1

For the ingestion test, after 24 hours of contamination, esterases were not inhibited, however, after 48 hours only EST-3 was not totally inhibited. Furthermore, none of the esterases at the concentration of 9 µg a.i./ mL was inhibited or partially inhibited. After 72 hours, only EST-2 and EST-4 continued to be inhibited at the concentrations of 5 to 8 µg a.i./ mL (Figure 2).

Esterases play numerous roles in insects, including detoxification of xenobiotics, presenting alterations in expression or in relative activity after the contact of the insects with different compounds. Organophosphates act by binding to these enzymes, in particular, the enzyme acetylcholinesterase, inhibiting its action resulting in accumulation of acetylcholine in the synapse. This leads to hyperexcitability due to the continuous and uncontrolled transmission of nerve impulses, including tremors, seizures, and eventually central nervous system collapse and death (Barboza et. al, 2018).

It was observed total inhibition of EST-4 in almost all of the insecticides concentrations on the first 24 hours. After 48 hours, EST-4 was expressed again in all of the concentrations and at the end, after 72 hours, there was no esterase inhibition.

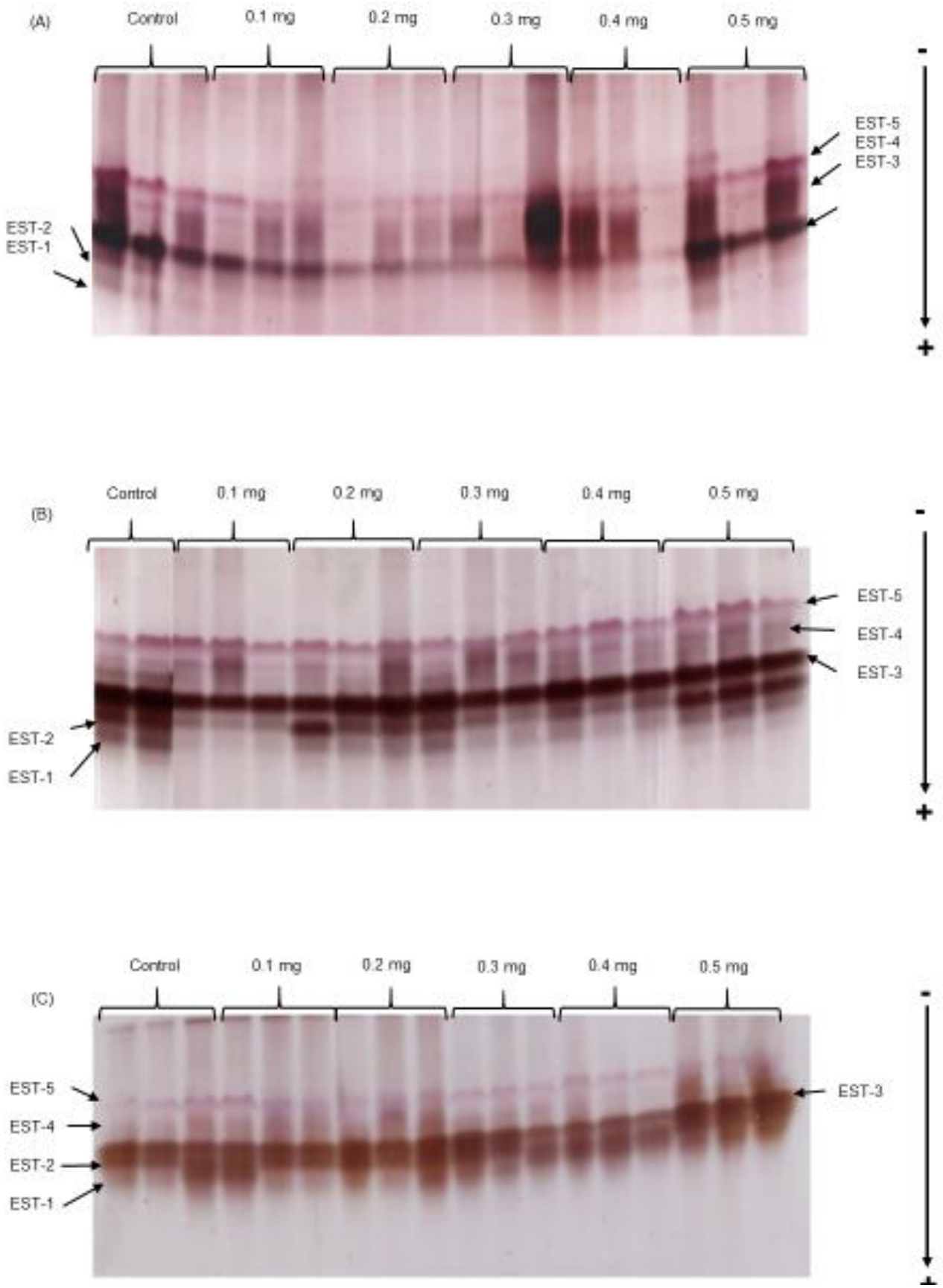

Figure 1. Electrophoretic profile of esterases in *S. bipunctata* after contact with acephate in different exposure periods. (A), 24; (B), 48; (C), 72 hours.

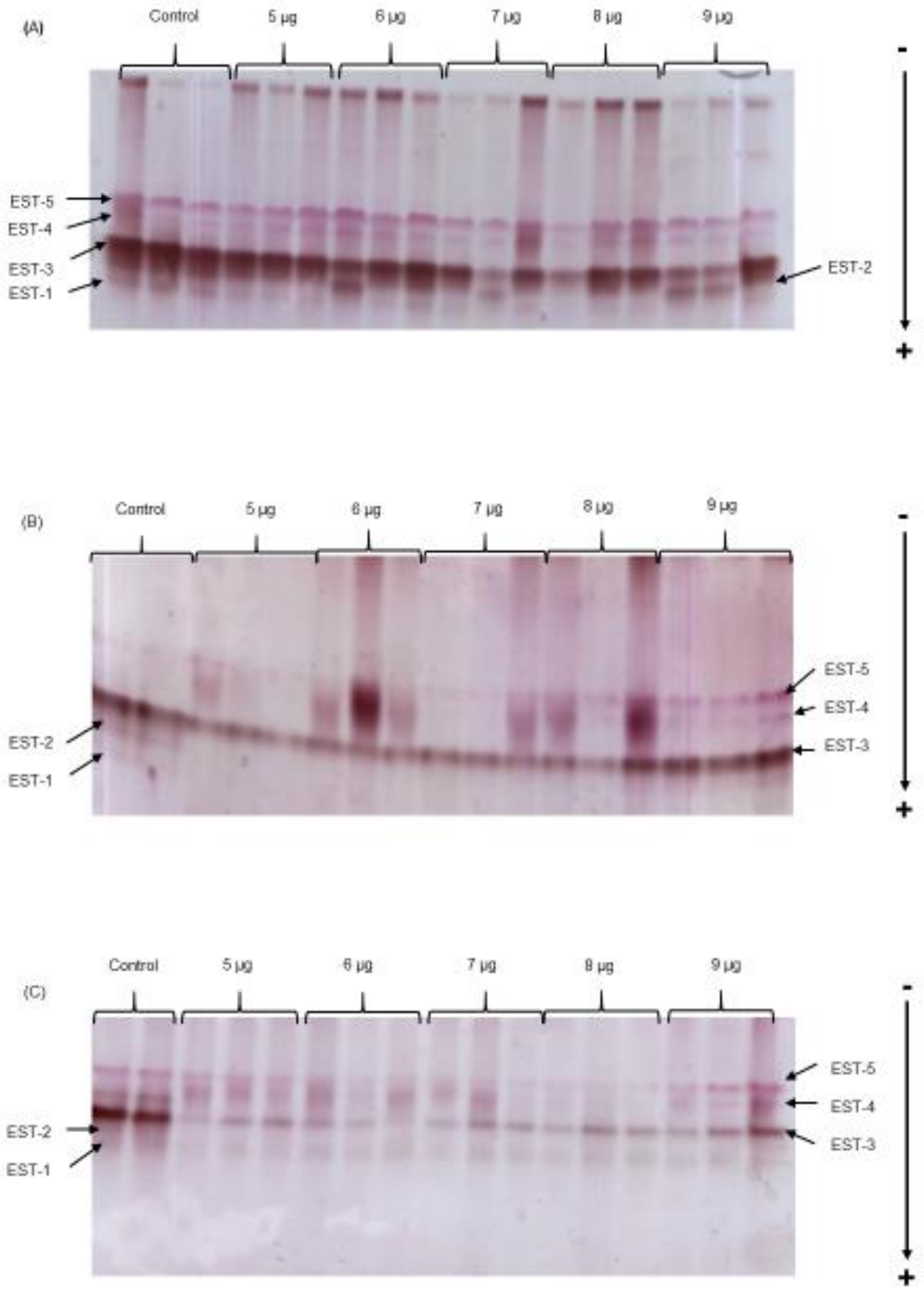

Figure 2. Electrophoretic profile of esterases in *S. bipunctata* after acephate ingestion in different exposure periods. (A), 24; (B), 48; (C), 72 hours.

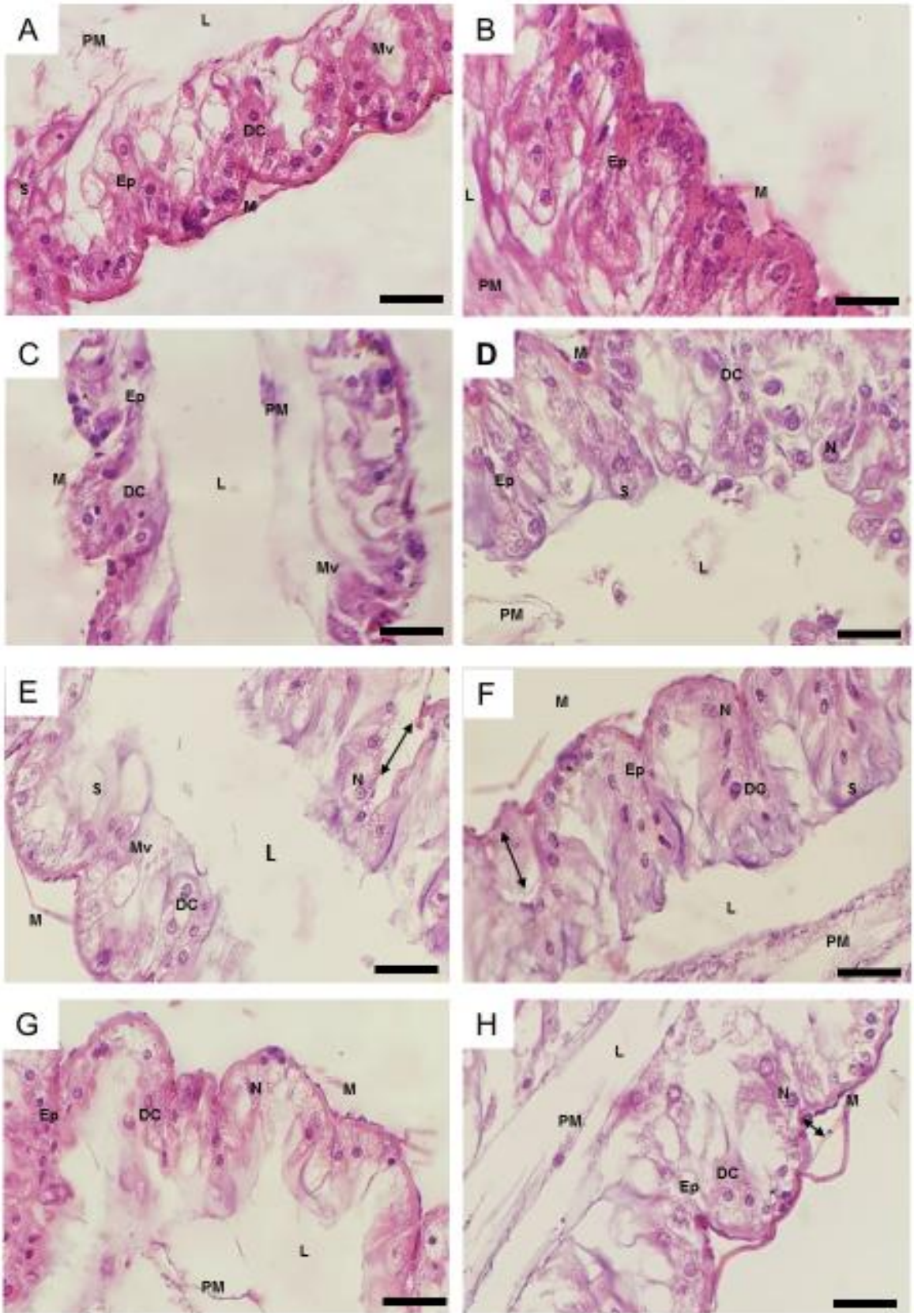

Figure 3. Photomicrography showing the midgut of *S. bipunctata*. a, control group; b, control group; c, 5 µg a.i./ mL in 24 h; d, 7 µg a.i./ mL in 24 h; e, 9 µg a.i./ mL in 24 h; f, 5 µg a.i./ mL in 48 h; g, 7 µg a.i./ mL in 48 h; h, 9 µg a.i./ mL in 48 h. Ep, midgut epithelium; M, musculature; BL, basal lamina; DC, digestive cells; *, regenerative cells; PM, peritrophic membrane; L, lumen; Mv, microvilli. Hematoxylin-Eosin staining. Scale bar: 20 µm.

With 24 hours of contamination by ingestion, none of the esterases was total inhibited, however, after 48 hours only EST-3 was not inhibited. Besides that, none of the esterases was inhibited at the concentration of 9 µg a.i./ mL. After 72 hours, only EST-2 and EST-4 were inhibited at the concentrations of 5 and 8 µg a.i./ mL.

After ingestion of this organophosphorus, there was a decrease in EST-4 activity at the concentrations 5 and 8 µg a.i./ mL, when compared to the control group. The results for EST-4, which was classified as cholinesterase type II, indicate their role in detoxification. Although higher mortality occurred at the lowest concentration and this esterases re-expressed after 72 hours, we suggest that its acting in the metabolization of this insecticide to a less toxic form.

Morphological characterization of the *S. bipunctata* midgut was similar to that reported by Moreira et al. (2018). After contamination by ingestion at the concentrations of 5, 7 and 9 µg a.i./ mL for 24 and 48 hours, the midgut exhibited several morphological changes.

After 24 hours of contamination, it was observed disorganization of the epithelium in all treatments. There was an increase of microvilli (Figure 3C), except for the treatment of 9 µg a.i./ mL (Figure 3E) which was similar to the control group (Figure 3a and 3B). In addition, there was cellular degeneration, the establishment of intercellular spaces and rupture of the musculature (Figure 3C), detachment of the epithelium from the basal lamina and absence of the peritrophic membrane.

After 48 hours there was a reduction in regeneration nests and the restructuring of the peritrophic membrane at the concentration of 5 µg a.i./ mL.

There was also the loosening of the musculature and a slight detachment of the epithelium from the basal lamina (Figure 3H).

In addition, the changes observed in the midgut would not lead to immediate mortality. Because the action of the insecticide on its main target would have been limited, especially by the fact that after 48 hours, the cells of regeneration nests from the midgut can recompose it, as well as the peritrophic membrane, thus organizing a barrier against the action of the pesticide. Similarly, Hashimoto et al. (2003) observed a partial inhibition of EST-1 relative activity after 24 hours of oral contamination with neonicotinoid insecticide *A. mellifera*.

The digestive tract is the central means of contact of the insects with the environment, thus, the midgut exhibited morphological changes after exposure to acephate. After 24 hours of contamination with 5 µg a.i./ mL of elongated microvilli ware observed, possibly in an attempt by the exposed digestive cells to increase the uptake of nutrients to extend the survival of the insect.

Many regeneration nests were found in individuals contaminated with acephate, contrary to that observed Oliveira et al. (2014) in *A. mellifera* and Moreira et al. (2018) in *S.bipunctata* contaminated with thiamethoxam. These results indicate that oral exposure to the agrochemical could have interfered in the structure and function of regenerative cells, affecting its proliferation and differentiation in new digestive cells only shortly. After the critical period of mortality, the nidi were able to regenerate the cellular structure of the midgut, which may be responsible for the tolerance of *S. bipunctata* to the organophosphate acephate.

Conclusion

The insecticide organophosphate acephate is extremely toxic to *S. bipunctata*, especially at low concentrations, which was evidenced by higher mortality in the first 24 hours of contamination. After this critical period, the detoxification enzymes esterases are able to attenuate the toxicity of the insecticide. Also, due to the performance of regeneration nests of the midgut recomposition of the tissues can occur, contributing to the survival of bees.

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