Acta Scientiarum
http://periodicos.uem.br/ojs/acta
ISSN on-line: 1807-8672
Doi: 10.4025/actascianimsci.v45i1.58412

Toxicological evaluation of different pesticides in *Tetragonisca angustula* Latreille (Hymenoptera, Apidae)

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ABSTRACT. The stingless bee *Tetragonisca angustula* is an important pollinator of different agricultural and native crops. This study evaluated changes in the relative activity of esterases and critical electrolyte concentration in brain cells after exposure to pesticides malathion and thiamethoxam. Lethal concentration 50% showed greater toxicity of thiamethoxam in relation to malathion. Esterases EST-3 and EST-4 (carboxylesterase) were partially inhibited after contamination by contact and ingestion of malathion and contamination by contact with thiamethoxam, suggesting participation of these esterases in the metabolization of these compounds. The lowest critical electrolyte concentration (CEC) was found after contamination by malathion ingestion (0.15 M), indicating changes in gene expression. The alterations observed in the intensity of EST-3 and EST-4 and the chromatin structure indicate that pesticides can act in gene expression and be used as biomarkers of contaminant residues. Furthermore, knowing the susceptibility of *T. angustula* bees to pesticides, it would be possible to use this species for biomonitoring environmental quality in preserved areas and agroecosystems.

Keywords: chromatin; esterases; isoenzymes; stingless bee.

Received on March 29, 2021.
Accepted on January 11 2022.

Introduction

Pollination is a key factor in the production management of many crops worldwide (Ollerton, Winfree, & Tarrant, 2011) and the decreasing availability of natural pollinators may cause limitations to both higher biodiversity and non-crop pollination services (Potts et al., 2016; Powney et al., 2019). Supposedly, this decline is mainly due to the unsustainable use of agricultural practices (Ekroos et al., 2020; Redhead, Powney, Woodcock, & Pywell, 2020), and among the various aspects related to this, the excessive use of insecticides surely pose risks to populations of pollinating bee species (Woodcock et al., 2016), as well as losses in floral and nesting resources, increased pressure from diseases and parasites (Goulson, Nicholls, Botías, & Rotheray, 2015), including infestations by *Acarapis woodi* (Rennie) (Acari: Tarsonemidae) and *Varroa jacobsoni* Oudemans (Acari: Varroidae) (Westcott & Winston 1999).

Most studies on the toxicity of pesticides to bees are carried out in the northern hemisphere with *Apis mellifera*, since this region does not house stingless bees that are typical of a tropical climate (Wu, Anelli, & Sheppard, 2011; Fisher, Colman, Hoffmann, Fritz, & Rangel, 2018; Milone, Chakrabarti, Sagili, & Tarpy, 2021). Recent studies have shown the effects of pesticides on native bees, including *Scaptotrigona bipunctata* (Moreira et al., 2018) *Melipona scutellaris* (Domingues, Inoue, Silva-Zacarin, & Malaspina, 2020), *Scaptotrigona postica* and *Tetragonisca angustula* (Rosa-Fontana, Dorigo, Galaschi-Teixeira, Nocelli, & Malaspina, 2020).

T. angustula has adapted to rational hives and its honey is popular, allowing commercialization. These stingless bees are also important pollinators of native species of plants (Giannini et al., 2020), mainly strawberries (Abrol, Gorka, Ansari, Al-Chamdi, & Al-Kahtani, 2019). During foraging in agricultural regions, these pollinators come into contact with pesticide residues, which can compromise pollination, development of colonies and the bee products (Ruvolo-Takasusuki et al., 2015; Silva, Melo, & Blanco, 2016), but as the effects of pesticides in this species are limited, toxicological evaluations are still needed.

Stingless bees have half of the xenobiotic detoxifying enzymes when compared to *Drosophila melanogaster* and *Anopheles gambiae* (Claudianos et al., 2006). Isoenzymes like esterases participate in organism detoxification in many animal species (Shin & Smartt, 2016), including metabolizing or degrading these substances before their toxic activity on the target organism (Aïzoun et al., 2013). Esterases act in the metabolism of different classes of exogenous and endogenous substances, in the development and behavior of insects, and in functions related to reproduction and digestion (Montella, Schama, & Valle, 2012). In *A. mellifera,* Hashimoto, Ruvolo-Takasusuki and Toledo (2003), observed partial inhibition of some esterases after contamination with the thiamethoxam insecticide. Inhibition patterns detected with thiamethoxam have the potential to be used for detecting insecticide residues. Additionally, in the native species *S. bipunctata*, thiamethoxam promoted significant changes in isoenzyme EST-4 (Type II cholinesterase), which is associated with neonicotinoid detoxification (Moreira et al., 2018).

Changes in chromatin may also indicate the presence of external elements in the insect organism (Falco, Hashimoto, Fermino, & Toledo, 2010; Santos et al., 2014). Critical Electrolyte Concentration (CEC) is a methodology that allows identification of alterations in chromatin structure (Vidal & Mello, 1989), and as a consequence the gene expression. Studies on nuclear basophilia evaluate the levels of DNA-protein complexation in chromatin, using the Toluidine Blue (TB) cationic dye (Vidal, 1987). Therefore, when somatic cells are treated with TB without the presence or at lower concentrations of Mg^{2+} for the CEC point, chromatin exhibits metachromasia (violet color). If the Mg^{2+} concentration corresponds to the CEC value, there will be no DNA metachromasia and the color displayed will be green (Vidal & Mello, 1989). This concentration varies according to the level of chromatin packing, the highest values being obtained when the chromatin is condensed (Mello, 1997).

Stingless bees can be exposed to pesticides in different ways, through ingestion, contact, inhalation (aerosol), varying according to the species due to the characteristics of foraging, phenology, and flight hours (Sluijs et al., 2013). Contact with these compounds can promote long-term effects since pyrethroid, neonicotinoids, and organophosphate insecticides represent the greatest risk exposure for *A. mellifera*, *Bombus* spp. (Sanchez-Bayo & Goka, 2014), *S. bipunctata*, *Tetragonisca fiebrigi* (Dorneles, Souza, & Blochtein, 2017), and *A. mellifera* L. larvae (Dai, Jack, Mortensen, Bustamante, & Ellis, 2018).

However, bioinsecticides derived from naturally occurring substances (Barbosa, Meyer, Guedes, & Smagghe, 2015), have also been indicated for causing changes in bees *Melipona quadrifasciata* (Tomé, Martins, Lima, Campos, & Guedes, 2012), and *Partamona helleri* (Bernardes, Barbosa, Martins, & Lima, 2017; Bernardes, Tomé, Barbosa, Guedes, & Lima, 2018). Another major concern is the synergism that can occur between different pesticides (Rinkevich et al., 2015), which can lead to a deregulation of bee-pathogen interaction, which can negatively affect the survival, as observed in *A. mellifera* exposed to fipronil (Aufauvre et al., 2012) and *Melipona colimana* exposed to thiamethoxam when infected with *Nosema ceranae* (Macías-Macías et al., 2020).

Thus, this study was developed to evaluate the sensitivity of native stingless bee *T. angustula* to contamination with insecticides, the mortality rate due to contamination, changes in the relative activity of esterase, and to check for chromatin alterations, at the brain level.

Material and methods

Insects and insecticides

Adult *T. angustula* bees were collected from different hives located in the campus of the State University of Maringá, Paraná (23°24'40''S; 51°56'23''W). After collection, bees were subjected to dose-mortality tests. Bioassays were performed with commercial insecticides Malathion 500 EC Cheminova registered at the Ministério da Agricultura Pecuária e Abastecimento - MAPA 0159870 (active ingredient malathion) and ACTARA 250 WG Syngenta registered at the Ministério da Agricultura, Pecuária e Abastecimento - MAPA 10098 (active ingredient thiamethoxam).

Dilutions of Malathion EC 500 were made according to the manufacturer's recommendation for the control of green aphid (*Myzus persicae*) and small fruit borer (*Neoleucinodes elegantalis*), pests of tomato plants. Dilutions of Actara 250 WG were made in accordance with the manufacturer's recommendation for the control of leafhopper *Mahanarva fimbriolata*, a sugarcane pest. From these dilutions, the sublethal concentrations of each insecticide used in the bioassays were defined, as listed in Table 1.

Table 1. Information on the commercial insecticides, manufacturers, active ingredients, and concentrations used in the bioassays.

Commercial insecticide	Company	Chemical group	Active ingredient (a.i.)	Crop	Concentrations	
					Contact (g a.i. L^{-1})	Ingestion (g a.i. L^{-1})
Malathion EC 500	Cheminova	Organophosphate	Malathion	Tomato (borer)	3.1×10^{-5}	5×10^{-3}
					3.5×10^{-5}	7.5×10^{-3}
					3.7×10^{-5}	0.125
					5×10^{-5}	2.5×10^{-2}
					1.25×10^{-3}	3.7×10^{-2}
Actara 250 WG	Syngenta	Neonicotinoid	Thiamethoxam	Sugarcane	3×10^{-4}	3×10^{-5}
					4.2×10^{-4}	4.8×10^{-5}
					6×10^{-4}	6×10^{-5}
					1.2×10^{-3}	1.2×10^{-4}
					1.8×10^{-3}	1.8×10^{-4}

a.i.: active ingredient

Dose-mortality bioassays

Bees were collected in plastic bottles and cold immobilized for 1 min in a freezer. Then, they were placed in previously mounted Petri dishes.

Bioassay by contact: Bees collected were placed in Petri dishes (150 x 20 mm) containing food candies (80 g sugar cake and 40 g honey) and filter paper (150 mm diameter) soaked in 1 mL solution containing the insecticide. Twenty bees were placed in each Petri dish and four dishes were used per insecticide treatment. Bioassays were performed in three replicates with insecticide and a control. The control board contained food and filter paper soaked in water. The exposure period was 24 h. Then, dead bees were counted and the survived bees were removed for electrophoretic analysis. Bees completely motionless were considered to be dead.

Bioassay by ingestion: Bees collected were placed in Petri dishes (150 x 20 mm) containing filter paper soaked in water and a container of insecticide mixed with food candies (80 g sugar cake and 40 g honey). All remaining procedures were the same as in the first bioassay. The control board contained food only. Concentrations of insecticides used are presented in Table 1. Data were analyzed in the statistical software SPSS 22.0 and the lethal concentrations (LC_{50}) were obtained by Probit.

Electrophoretic analysis of esterases

Esterases were analyzed by polyacrylamide gel electrophoresis (PAGE) using the standard methods described by Davis (1964) and Laemmli (1970). Surviving *T. angustula* bees of the bioassays by contact or ingestion were stored at -20°C. Electrophoretic analyses were performed using insecticide-exposed surviving and control bees. The head of each stingless bee was separated from the rest of the body and individually homogenized in polypropylene tubes containing 0.1% β-mercaptoethanol solution and 10% glycerol. Homogenate was centrifuged at 10,000 *g* for 10 min at 4°C in a Jouan centrifuge MR23. Vertical electrophoresis was performed using PAGE gel at 8% concentration and stacking gel at 5% concentration. In the vats, it was used 0.1 M tris-glycine buffer (pH 8.3) and electrophoresis was performed at 200 V for 5 h at 4°C. To identify the esterases, the gel was incubated for 30 min. in 0.1 M sodium phosphate buffer (pH 6.2). After incubation, the solution was discarded, added with the staining solution prepared with 0.1 M sodium phosphate buffer (pH 6.2), Fast Blue RR Salt, α-naphthyl acetate, and β-naphthyl acetate substrates, previously solubilized in acetone. Gels were incubated at room temperature for 1 h, and esterase bands were identified by their color: the black band (α-esterase), the red band (β-esterase) and qualitatively determined according to the intensity of staining (Zhu & Gao, 1999).

Cytochemical analysis

T. angustula were exposed by ingestion or contact as above mentioned to malathion and thiamethoxam, and after 24 h they were used for cytochemical analysis. The malathion concentrations tested for the CEC analysis were 3.1×10^{-5} g a.i. L^{-1} (contact), 7.5×10^{-3} g a.i. L^{-1} (ingestion) and 0.125 g a.i. L^{-1} (ingestion). Thiamethoxam concentrations tested were 4.2×10^{-4} g a.i. L^{-1} (contact), 6.0×10^{-4} g a.i. L^{-1} (contact), 4.8×10^{-5} g a.i. L^{-1} (ingestion) and 6.0×10^{-5} g a.i. L^{-1} (ingestion). Dishes for contact bioassays contained food and filter paper soaked in water only. In ingestion bioassays, control dishes contained only food.

Following the protocol described by Vidal and Mello (1989), the heads of each stingless bee were dissected, placed in physiological solution (0.1 M NaCl, 0.1 M Na_2HPO_4, and 0.1 MKH_2PO_4), and the brain was removed and placed on slides containing acetic acid (45%). Then, brain samples were crushed under coverslips; microscope slides were frozen in liquid nitrogen, the coverslip was then removed and the slide was fixed in ethanol: acetic acid solution (3:1 vv^{-1}) for 2 min. After this period, samples were washed in 70% ethanol for 5 min, stained with 0.025% toluidine blue (Merck, Germany) for 20 min., in a McIlvaine buffer (pH 4.0), containing different $MgCl_2$ (Merck) concentrations (0.0; 0.02; 0.05; 0.08; 0.10; 0.12; 0.15; 0.20; and 0.30 mol L^{-1}).

Slides were left in the dye for 20 min, washed in distilled water and placed to dry at 25 ± 2°C. Dried slides were dipped into xylene for 15 min and covered with a coverslip and Entellan (Merck). Slides were observed under a Zeiss optical microscope to detect changes in chromatin at the brain level (Mello, 1997).

Results

Lethal Concentration 50

Analysis of LC_{50} values demonstrated that, for both malathion and thiamethoxam, *T. angustula* was sensitive to contact with thiamethoxam, more toxic to this species (Table 2).

Table 2. Lethal concentrations 50% (LC50) of *T. angustula* bees exposed to malathion and thiamethoxam, both by contact and ingestion.

Pesticide	Exposure route	LC_{50} (g a.i. L^{-1})	95% Confidence interval
Malathion	contact	6.67×10^{-6}	-----
Malathion	ingestion	1.66×10^{-2}	0.54467 – 2.18486
Thiamethoxam	contact	9.73×10^{-4}	0.06343 – 0.34581
Thiamethoxam	ingestion	1.27×10^{-3}	-0.00039 – 0.5357

a.i: active ingredient.

Esterases

Electrophoretic analysis of *T. angustula* bees revealed changes in esterase bands. The contact with malathion reduced the intensity of the EST-3 at concentrations of 3.1×10^{-5} and 3.7×10^{-5} g a.i. L^{-1} and the EST-4 at 3.1×10^{-5} a.i. L^{-1} (Table 3). Electrophoresis of individuals exposed to thiamethoxam by contact showed partial inhibition of EST-3 and EST-4 at the concentration of 6×10^{-4} g a.i. L^{-1} (Table 3).

The ingestion of malathion partially inhibited the EST-3 at the concentration 0.125 g a.i. L^{-1} and EST-4 at concentrations of 0.125 and 2.5×10^{-2} g a.i. L^{-1} (Table 3). For thiamethoxam, no esterase changes were observed.

Table 3. Inhibition of esterase activity in *T. angustula* after contact or ingestion of malathion or thiamethoxam. The minus symbol (-) indicates a lack of inhibition and the symbol (+) indicates partial inhibition.

Contact exposure			Ingestion exposure		
Concentration	Esterase		Concentration	Esterase	
Malathion (g a.i. L^{-1})	EST-3	EST-4	Malathion (g a.i. L^{-1})	EST-3	EST-4
3.1×10^{-5}	-	+	5×10^{-3}	-	-
3.5×10^{-5}	-	-	7.5×10^{-3}	-	-
3.7×10^{-5}	+	+	0.125	+	+
5×10^{-5}	-	-	2.5×10^{-2}	-	+
1.25×10^{-3}	-	-	3.7×10^{-2}	-	-
Thiamethoxam (g a.i. L^{-1})			Thiamethoxam (g a.i. L^{-1})		
3×10^{-4}	-	-	3×10^{-5}	-	-
4.2×10^{-4}	-	-	4.8×10^{-5}	-	-
6×10^{-4}	+	+	6×10^{-5}	-	-
1.2×10^{-3}	-	-	1.2×10^{-4}	-	-
1.8×10^{-3}	-	-	1.8×10^{-4}	-	-

a.i.: active ingredient.

Cytochemical analysis

Regarding cytochemical analysis, for the control stingless bees, the CEC values were within the range of 0.20 M < CEC < 0.30 M for all groups tested (Tables 4 and 5). *T. angustula* bees exposed to malathion both by contact at 3.1×10^{-5} g a.i. L^{-1} and ingestion at 0.125×10^{-3} g a.i. L^{-1} had the CEC value of 0.20 M (Table 4), which

was not different from the CEC value of the control stingless bees. However, when bees were exposed to malathion, by ingestion, at the concentration of 7.5×10^{-2} g a.i. L^{-1}, chromatin packing was indicated by the CEC value of 0.15 M (Table 4).

When thiamethoxam was used in both contaminations by contact and ingestion, no significant change was observed in CEC values when compared to the control, being the point of CEC 0.20 M (Table 5).

Table 4. Nuclear basophilia responses of the chromatin in brain of *T. angustula* workers after contamination by ingestion and contact with the insecticide malathion [stained with 0.025% toluidine blue (TB) added with MgCl2 in various concentrations (mol L^{-1})].

Stain	Control	Malathion (contact g a.i. L^{-1})	Malathion (ingestion g a.i. L^{-1})	
		3.1×10^{-5}	7.5×10^{-3}	0.125
TB	Vi	Vi	Vi	Vi
TB + $MgCl_2$ 0.02 mol L^{-1}	Vi/Bl	Vi/Bl	Vi/Bl	Vi/Bl
TB + $MgCl_2$ 0.05 mol L^{-1}	Bl	Vi/Bl	Vi/Bl	Vi/Bl
TB + $MgCl_2$ 0.08 mol L^{-1}	Bl	Vi/Bl	Bl	Vi/Bl
TB + $MgCl_2$ 0.10 mol L^{-1}	Bl	Bl	Bl	Bl
TB + $MgCl_2$ 0.12 mol L^{-1}	Bl/Gr*	Bl/Gr*	Bl/Gr*	Bl/Gr*
TB + $MgCl_2$ 0.15 mol L^{-1}	Bl/Gr*	Bl/Gr*	*Gr**	Bl/Gr*
TB + $MgCl_2$ 0.20 mol L^{-1}	*Bl/Gr**	*Gr**	Gr*	*Gr**
TB + $MgCl_2$ 0.30 mol L^{-1}	*Gr**	Gr*	Bl/Gr*	Gr*
CEC value (mol L^{-1})	0.20 < CEC < 0.30	0.20	0.15	0.20

Abbreviations: a.i., active ingredient; Vi, violet; Bl, Blue; Gr, Green; *, modification to green coloring; Shading in cells, point of CEC.

Table 5. Nuclear basophilia responses of the chromatin in brain of *T. angustula* workers after contamination by ingestion and contact with the insecticide thiamethoxam [stained with 0.025% toluidine blue (TB) added with MgCl2 in various concentrations (mol L^{-1})].

Stain	Control	Thiamethoxam (contact g a.i. L^{-1})		Thiamethoxam (ingestion g a.i. L^{-1})	
		4.2×10^{-4}	6×10^{-4}	4.8×10^{-5}	6×10^{-5}
TB	Vi	Vi	Vi	Vi	Vi
TB + $MgCl_2$ 0.02 mol L^{-1}	Vi/Bl	Vi	Vi	Vi	Vi
TB + $MgCl_2$ 0.05 mol L^{-1}	Bl	Vi/Bl	Vi/Bl	Vi/Bl	Vi/Bl
TB + $MgCl_2$ 0.08 mol L^{-1}	Bl	Vi/Bl	Vi/Bl	Vi/Bl	Vi/Bl
TB + $MgCl_2$ 0.10 mol L^{-1}	Bl	Bl	Bl	Bl	Bl
TB + $MgCl_2$ 0.12 mol L^{-1}	Bl/Gr*	Bl/Gr*	Bl	Bl	Bl
TB + $MgCl_2$ 0.15 mol L^{-1}	Bl/Gr*	Bl/Gr*	Bl/Gr*	Bl/Gr*	Bl
TB + $MgCl_2$ 0.20 mol L^{-1}	*Bl/Gr**	*Gr**	*Gr**	*Gr**	*Bl/Gr**
TB + $MgCl_2$ 0.30 mol L^{-1}	*Gr*	Gr*	Gr*	Gr*	*Gr**
CEC value (mol L^{-1})	0.20<CEC<0.30	0.20	0.20	0.20	0.20

Abbreviations: a.i., active ingredient; Vi, violet; Bl, Blue; *, modification to green coloring; Shading in cells, point of CEC.

Discussion

Stingless bees are susceptible to insecticides commonly used to control pests in different crops, and these compounds are becoming a threat to these non-target insects (Crenna, Jolliet, Collina, Sala, & Fantke, 2020). This is because, during foraging, contaminants can adhere to the body surface of these pollinators or be collected with nectar, pollen (Sgolastra et al., 2019; Crenna et al., 2020), or other products (Cham et al., 2019).

Despite being exposed to residual doses of insecticides, which are not always sufficient to cause the immediate death of bees, when the contaminants are taken to the hive, they end up putting the development of the colony at risk. This is due to the persistence of the products and their capacity for bioaccumulation (Shimshoni et al., 2019), which may cause morphophysiological, metabolic, genetic, and behavioral damage (Guedes, Smagghe, Stark, & Desneux, 2016), as well as changes in development and reduction in survival of bees, culminating in the extinction of populations (Lima, Martins, Oliveira, & Guedes, 2016).

These toxicological effects are variable both considering the period for which the different species of bees remain exposed to insecticides (Lima et al., 2016), as well as, according to age, stage of development, physiological conditions (Fairbrother, Purdy, Anderson, & Fell, 2014), and, species behavior (Guedes et al., 2016). Although variable, these effects can be used as indicative of the presence of contaminants in the environment, *A. mellifera* as well as *stingless bees* are important bioindicators of environmental quality (Quigley, Amdam, & Harwood, 2019; Sgolastra et al., 2019).

Among insecticides, compounds belonging to the class of organophosphates and neonicotinoids used for the control of agricultural pests stand out. Malathion is an organophosphate that acts directly on acetylcholinesterase, an important regulatory enzyme responsible for the control of neural transmission at synapses through the hydrolysis of acetylcholine (Soreq & Seidman, 2001). In our study, we found the lowest toxicity in the contamination by ingestion with malathion (LC_{50} 1.66×10^{-2} g a.i. L^{-1}), however, toxicity was higher in insects exposed by contact (LC_{50} 6.67×10^{-6} g a.i. L^{-1}). Costa, Grella, Barbosa, Malaspina and Nocelli (2015), evaluated the oral exposure to malathion in *Melipona scutellaris* and confirmed an LC_{50} of 2.01 ng a.i. μL^{-1} for 24 h and 0.81 ng a.i. μL^{-1} for 48 h. Diniz et al. (2020), identified in *S. bipunctata* an LC_{50} of 0.052 mg a.i. mL^{-1} for contact and 3.470 µg a.i. mL^{-1} for ingestion after exposure to the organophosphate acetate.

Among neonicotinoids, thiamethoxam is a second-generation insecticide (Buszewski, Bukowska, Ligor, & Staneczko-Baranowska, 2019), that acts by interfering with acetylcholine postsynaptic receptors (Pisa et al., 2017). In our study, we confirmed LC_{50} for thiamethoxam at 1.27×10^{-3} g a.i. L^{-1} for ingestion and 9.73×10^{-4} g a.i. L^{-1} for contact. Murcia, Zotti and Pech-Pech (2017), assessed the oral exposure to thiamethoxam and reported LC_{50} of 6.664 ng μL^{-1} for *T. angustula* and 0.1848 ng μL^{-1} for *S. xanthotricha*. Hashimoto et al. (2003), determined the LC_{50} for newly emerged *A. mellifera* at 21 days of age after exposure to thiamethoxam. The authors observed greater toxicity for younger bees with LC_{50} 4.7×10^{-5} mg mL^{-1} after ingestion and 3.21 mg mL^{-1} after contact. In *S. bipunctata*, exposure to thiamethoxam indicated an LC_{50} of 2×10^{3} µg a.i. L^{-1} 24 h after ingestion (Moreira et al., 2018).

In this study, *T. angustula* was more sensitive to exposure to both thiamethoxam and malathion by contact. As in this case, insecticides penetrate the insect cuticle and directly reach nerve endings, it is likely that even at very low concentrations, they will be enough to cause the death of non-target insects, such as bees (Moreira et al., 2018). However, if ingested, the insecticide needs to be absorbed in the intestine, and only then transported by hemolymph to the insect's nervous system, giving the body time to activate important metabolic detoxification systems, such as isoenzyme esterases.

Among these detoxification systems, mechanisms by enzymes such as esterases, stand out (Lushchak, Matyiishyn, Husak, Storey, & Storey, 2018). When evaluating these enzymes after exposure to insecticides, we found that bees exposed to both malathion and thiamethoxam, showed a change in the intensity of staining of EST-3 and EST-4, which indicates the inhibition of enzymes by the insecticides or their participation in hydrolysis. These results suggest that these regions may be used as biomarkers for the presence of residues of this insecticide in the environment.

Regarding thiamethoxam, EST-3 and EST-4 were partially inhibited after contact exposure. In the treatment by ingestion, in addition to these esterases not being altered, a higher concentration of the insecticide was necessary to cause the death of 50% bees. Probably, due to the intestinal absorption of this insecticide, metabolic detoxification systems were activated, possibly with the participation of these esterases, promoting the biotransformation of the compound, reducing the concentration of this insecticide when reaching the target site (Oost, Beyer, & Vermeulen, 2003; Panini, Manicardi, Moores, & Mazzoni, 2016).The same was observed by Hashimoto et al. (2003), who worked with exposure of *A. mellifera* to thiamethoxam by both contact and ingestion. The authors observed changes in esterases 1, 2, 4, and 5. Moreira et al. (2018), reported changes in the relative activity of EST-4 at different concentrations of thiamethoxam after oral ingestion.

With respect to malathion, EST-3 and EST-4 were altered both in bees exposed by contact and by ingestion. In this case, we also suggest the participation of esterases, especially EST-4, a carboxylesterase (Stuchi, Toledo, Lopes, Cantagalli, & Ruvolo-Takasusuki, 2014), that acts on the metabolic detoxification of this compound. As these isoenzymes can bind to malathion, which would explain the observed inhibition, hydrolyzing it to make it a less toxic compound for the insect, higher concentrations of the insecticide would be necessary to lead the insect to death (Oost et al., 2003; Panini et al., 2016). Thus, the direct action of EST-4 in metabolic detoxification of malathion, and possible secondary participation of EST-3 in this process, could explain the lower toxicity of this organophosphate to *T. angustula*.

Additionally, *A. mellifera* bees exposed to this organophosphate by ingestion (7.5×10^{-2} g a.i. L^{-1}) showed a reduction in the CEC value (0.15 M), indicating lower chromatin packing, and, consequently, an increase in gene expression (Falco et al., 2010). Moreira et al. (2018) found the CEC value of 0.30 M after 72 h of oral exposure to thiamethoxam, in *S. bipunctata*. In this same species, the fungicide Locker after 72 h of oral exposure indicated a CEC point of 0.30 M, showing high chromatin condensation (Diniz et al., 2020).

Probably, the activation of genes related to metabolic detoxification would be a very important factor to reduce the toxicity of insecticides, such as malathion, in contaminated organisms (Lushchak et al., 2018). Also, the results of changes in the relative activity of EST-3 and EST-4 associated with chromatin decondensation indicate the action of insecticides. Moreover, in the treatment with malathion, the return of metachromasia was found, which is related to the interaction between DNA and protein, which confirms the alteration in the gene expression of the exposed insects (Mello, Vidal, Dantas, & Monteiro, 1993). If an increase in gene expression occurs, other detoxifying enzymes could be synthesized to contribute to the metabolic detoxification of this contaminant.

Although studies evaluating chromatin condensation after exposure to insecticides are limited, they need to be developed since they demonstrate the action of insecticides also at the genetic level.

Conclusion

It is important to highlight that during foraging, stingless bees can collect pollen, nectar, and water contaminated with different pesticides, which further reinforces the need for studies to evaluate the action of these compounds mainly on native bees. The confirmed alteration in the brain chromatin and esterase enzymes after experiments with *T. angustula* evidence that these insecticides can initially promote damage to the insect and consequently the colony, affecting their development.

Acknowledgements

We thank the *Fundação CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior)*, for the doctoral scholarship, and the National Council for Scientific and Technological Development (CNPq) for funding our project (Process 311072/2019-4).

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