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Comparative toxicity of fipronil, malathion, and thiamethoxam on the stingless bee *Tetragonisca fiebrigi* (Schwarz, 1938)

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ABSTRACT. Stingless bees are important pollinators for various plant crops. We investigated the susceptibility of *Tetragonisca fiebrigi* to sublethal concentrations of insecticides fipronil, malathion, and thiamethoxam (administered through contact and ingestion) by determining the LC_{50} values after 24 hours of exposure and analyzing changes in the activity of esterase isoenzymes and the chromatin in brain cells. The LC_{50} values showed that all three insecticides were highly toxic through contact and ingestion. Electrophoretic analysis revealed that the relative EST-4 (carboxylesterase) activity in *T. fiebrigi* was partially inhibited by malathion and fipronil ingestion. Moreover, the EST-4 band intensity was increased following high-concentration thiamethoxam (contact) exposure, indicating the increased relative activity of this isoenzyme to detoxify the compound. In the cytochemical analysis of brain cells, the critical electrolyte concentration (CEC) points for the control stingless bees and malathion ingestion-exposed and thiamethoxam-exposed (contact and ingestion) stingless bees were in the range of 0.20-0.30 M $MgCl_2$, whereas that for malathion contact-exposed bees was 0.15 M $MgCl_2$, indicating chromatin relaxation and suggesting an increase in gene expression. In conclusion, *T. fiebrigi* stingless bees are susceptible to the insecticides tested, and the parameters analyzed may be used as biomarkers to detect the presence of these compounds.

Keywords: chromatin; esterases; LC_{50}; Meliponini; pesticide.

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Introduction

The majority of flowering plants require animal and insect pollination to survive (Ollerton, Winfree, & Tarrant, 2011) and the reduction in such pollination services can damage distinct plant communities in addition to primordial ecosystem functions (Stanley, Sah, Jain, Bhatt, & Sushil, 2015). Stingless bees are relevant pollinators for wild and cultivated plants in tropical regions (Nunes-Silva, Hrncir, Silva, Roldão, & Imperatriz-Fonseca, 2013; Rodrigues, Ferasso, Mossi, & Coelho, 2020).

Recent studies have shown that bees are being exposed to different pesticides used on crops (Kasiotis, Anagnostopoulos, Anastasiadou, & Machera, 2014; Van der Zee, Gray, Pisa, & Rijk, 2015; Calatayud-Vernich, Calatayud, Simó, Suarez-Varela, & Picó, 2016; Wegener et al., 2016; Daniele, Giroud, Jabot, & Vuillet, 2017; Tsvetkov et al., 2017), such as organophosphate, the phenylpyrazole fipronil, and the neonicotinoid thiamethoxam, which may be related to the decline of these pollinators (Woodcock et al., 2017). Therefore, there are many studies that evaluated the exposure of bees to insecticides (Li et al., 2017; Sánchez-Bayo, Belzunces, & Bonmatin, 2017; Shi, Wang, Liu, Qi, & Yu, 2017; Yue, Luo, Liu, & Wu, 2017; Fisher, Colman, Hoffmann, Fritz, & Rangel, 2018; Overmyer, Feken, Ruddle, Bocksch, & Thompson, 2018; Potts, Clarke, Oldfield, Wood, & Ibarra, 2018; Wood, Kozii, Koziy, Epp, & Simko, 2018; Zaluski et al., 2020). Although *Apis mellifera* is considered a model species for toxicity tests (Jacob, Soares, Nocelli, & Malaspina, 2015), effects of pesticides on stingless bees are important to know because these native species are responsible for the pollination of most of the plants that make up the Brazilian ecosystem (Wolowski et al., 2019).

The native stingless bee *Tetragonisca fiebrigi* is distributed in the Neotropical region (Moure, Urban, & Melo, 2007) and adapts easily to different nesting sites, such as hollow walls, rocks, and tree trunks, facilitating the spread of their beekeeping (Venturieri et al., 2012).

Despite the many studies assessing insecticide damage to bees, data on the toxicity of most pesticides to stingless bees are not comprehensive. Enzymes such as esterases have been employed as indicators of changes promoted by insecticides (Hashimoto, Ruvolo-Takasusuki, & Toledo, 2003; Győri et al., 2017; Julio et al., 2017; Codling, Naggar, Giesy, & Robertson, 2018; Moreira et al., 2018) because of their ability to metabolize or degrade substances in advance so that they do not affect the organism (Aïzoun et al., 2013).

Chromatin modification can also be used to identify the effects of external factors (Catae, Roat, Oliveira, Nocelli, & Malaspina, 2014; Santos et al., 2014; Moreira et al., 2018). This is achieved by analyzing the critical electrolyte concentration (CEC), using a technique developed by Vidal and Mello (1989). This analysis allows for the verification of whether changes in gene expression have occurred after exposure to an insecticide, as the activation or deactivation of genes after exposure leads to changes in the CEC value (Fermino, Falco, Toledo, & Ruvolo-Takasusuki 2011; Santos et al., 2014).

Because stingless bees are more susceptible to pesticides than other bee species, including *Apis*, the objective of this study was to evaluate the toxicity of three insecticides with different modes of action (the organophosphate malathion, phenylpyrazole fipronil, and the neonicotinoid thiamethoxam) to *T. fiebrigi*. The relative activities of several esterase isoenzymes and alterations in chromatin in brain cells were used as biomarkers of exposure. Studies of this nature are necessary for meliponiculture as they indicate appropriate strategies for the management of stingless bees. This is especially important when considering that the widespread development of agriculture and the inappropriate use of pesticides may lead to the decline of bee species that are fundamental for the pollination of native plants and the preservation of the Brazilian flora.

Material and methods

Biological material

Tetragonisca fiebrigi adult stingless bees from five nests located on the campus of the *Universidade Estadual de Maringá*, state of Paraná (23°24′40″S; 51°56′23″W) were collected in cages (18 cm width × 15 cm height) and taken to the Laboratory of Biotechnology and Animal Genetics, *Departamento de Biotecnologia, Genética e Biologia Celular*. Bees were kept at 28 ± 2°C and 70 ± 10% relative humidity and were fed with candi (sugar and honey).

Bioassays

The commercial insecticides were diluted as described on the manufacturer package inserts for application on crops. The concentration of fipronil 800 WG (Nortox S/A MAPA Register 10412) stock solution was 2.625×10^5 µg active ingredient (a.i.) per 1,000 mL^{-1} water, based on the dilution to be used to control the soybean pest *Sternechus susignatus*. For malathion 500 EC (Cheminova Brasil Ltda/MAPA Register 01598705), the concentration of the stock solution was 2.5×10^4 µg a.i. per 1,000 mL^{-1} water, as recommended for the tomato (borer) pest *Myzus persicae*. For thiamethoxam contained in Actara 250 WG (Syngenta S/A MAPA Register 10098), the concentration of the stock solution was 1.2×10^6 µg a.i. per 1,000 mL^{-1} water, as recommended for the sugarcane pest *Heterotermes tenuis*. Subsequently, dilutions of the stock solutions were made to obtain the sublethal concentrations (Tables 1 and 2) to which bees were exposed through contact or ingestion.

For exposure through contact, 20 bees were anesthetized at a low temperature (-5°C) and then placed in Petri dishes (150 × 20 mm) containing food (candi) and a filter paper (15 cm diameter) soaked in 1 mL^{-1} insecticide (treatment group) or water only (control group). Five concentrations of each insecticide were evaluated (Table 1). Experiments were carried out in triplicate on a total of 1,080 stingless bees.

Table 1. Insecticide concentrations used in bioassays for contact exposure.

Insecticides	Concentrations µg a.i. L^{-1}				
Fipronil	1.06	1.27	1.49	2.13	2.34
Malathion	21.5	22.5	23.7	25	50
Thiamethoxam	4.2×10^3	4.8×10^3	5.1×10^3	5.4×10^3	6×10^3

a.i.: active ingredient

For exposure through ingestion, 20 stingless bees were anesthetized at a low temperature (-5°C) and then placed in Petri dishes (150 × 20 mm) containing a filter paper soaked in water and a container with food (candi)

mixed with the insecticide for the treatment group or without insecticide for the control group. Five concentrations were tested for each insecticide, as listed in Table 2. The experiments were carried out in triplicate on a total of 1,080 bees.

Table 2. Insecticide concentrations used in bioassays for ingestion exposure.

Insecticides	Concentrations µg a.i. L^{-1}				
Fipronil	2.55	53.2	1.25×10^3	2.5×10^3	2.5×10^4
Malathion	2.5×10^3	3.75×10^3	5×10^3	5.62×10^3	6.25×10^3
Thiamethoxam	9×10^2	1.2×10^3	1.32×10^3	1.44×10^3	3×10^3

a.i.: active ingredient

For both bioassays, bees were kept in the dark at 28 ± 2°C and 70 ± 10% relative humidity, for 24 hours. Thereafter, the number of dead bees was counted and the surviving bees were sacrificed and stored in properly identified and numbered flasks at -20°C until the electrophoretic and CEC analyses.

Electrophoretic analysis of esterases

Ten insecticide-exposed and 10 control bees from each of the contact and ingestion bioassays were analyzed for esterase activities. As the bee abdomen contains proteases that can degrade esterases, only the heads and thoraxes of the insects were used for analysis. Samples were individually homogenized in 1.5 mL^{-1} polypropylene tubes containing 35 μL^{-1} 2-mercaptoethanol solution plus 10% glycerol and centrifuged at 10,000 *g*, 4°C, for 10 min.

Esterases were analyzed by polyacrylamide gel electrophoresis (PAGE) using the standard methods described by Davis (1964) and Laemmli (1970). Vertical electrophoresis was performed using 8% PAGE gels and 5% stacking gels for esterase detection, with 0.1 M Tris-glycine (pH 8.3) used as running buffer. Gels were electrophoresed at a voltage of approximately 200 V at 5°C for 0.5 h.

For identification of esterase isozymes, gels were pre-incubated in 0.1 M sodium phosphate buffer (pH 6.2) for 30 min. The buffer was then discarded and a staining solution consisting of 50 mL^{-1} 0.1 M sodium phosphate buffer (pH 6.2), 0.03 g α-naphthyl acetate, 0.04 g β-naphthyl acetate, and 0.06 g Fast Blue RR Salt was added. Isozymes were visualized on the gels as brown (α-esterases) or red (β-esterases) bands, and their relative activities were qualitatively determined from the staining intensity of the bands (Zhu & Gao, 1999).

Cytochemical analysis

Owing to the high mortality rate obtained with fipronil, it was not possible to evaluate the CEC for this insecticide. Therefore, the CEC was determined for thiamethoxam and malathion only, for both contact and ingestion administrations. The concentrations of malathion used for the CEC analysis were 23.7 µg a.i. L-1 (contact), 5 × 103 µg a.i. L-1 (ingestion), and 5.62 × 103 µg a.i. L-1 (ingestion). The concentrations of thiamethoxam used were 4.8 × 103 µg a.i. L-1 (contact), 5.1 × 103 µg a.i. L-1 (contact), and 1.2 × 103 and 1.32 × 103 µg a.i. L-1 (ingestion).

For the bioassay, surviving stingless bees were dissected under a stereomicroscope (Karl Zeiss) and the brain was removed and placed on slides containing 45% acetic acid. A coverslip was placed over the specimen and pressed down hard to crush the tissue. The slide was held with tweezers and frozen in liquid nitrogen, following which the coverslip was removed and the tissue was fixed in ethanol: acetic acid solution (3:1, v v-1), for 2 min. Thereafter, it was soaked in 70% ethanol for 5 min.

Cells were stained for 20 min. in a solution of McIlvaine buffer (pH 4.0) containing 0.025% toluidine blue (Merck, Germany) in the absence or presence of various concentrations (0.02, 0.05, 0.08, 0.10, 0.12, 0.15, 0.20, and 0.30 M) of MgCl2 (Merck). After staining, slides were placed in a solution of 0.05 M $MgCl_2$ (Merck) and withdrawn after 5, 10, and 15 min. Then, slides were washed in distilled water for 5 s, dried at 28 ± 2°C, incubated in xylene for 15 min., mounted with Entellan (Merck), and analyzed under a Zeiss microscope (Mello, Vidal, Dantas, & Monteiro, 1993).

Statistical analysis of the mortality and LC_{50} data

The bioassay results were analyzed in statistical software (SPSS 22.0, IBM, USA) for mortality rates and lethal concentration 50% of the test animals (LC50) values by probit regression as well as the respective 95% confidence interval (95% C.I.) values. The graph of percentage mortality rates was constructed using GraphPad Prism version 5.00.

Results

The results demonstrated that fipronil was the most toxic insecticide to *T. fiebrigi*, with LC_{50} values of 2 µg a.i. L^{-1} through contact (95% C.I. = 0.000001–0.000003) and 107 µg a.i. L^{-1} through ingestion exposure (95% C.I. = 0.000322–0.002). Increases in fipronil concentration for both modes of exposure resulted in higher mortality rates (Figures 1A and B).

The LC_{50} of malathion was 22 µg a.i. L^{-1} (95% C.I. = 0.000010–0.000037) through the contact exposure and 5×10^3 µg a.i. L^{-1} (95% C.I. = 0.001–0.005) through the ingestion exposure. At concentrations of 25 and 50 µg a.i. L^{-1} per contact exposure, this insecticide caused the death of all the insects tested (Figures 1C and D). However, there was a stronger relationship between the concentrations of malathion applied by ingestion and increased mortality (Figures 1C and D).

With thiamethoxam, the LC_{50} was 5×10^3 µg a.i. L^{-1} through contact (95% C.I. = 0.004–0.005) and 1×10^3 µg a.i. L^{-1} through ingestion exposure (95% C.I. = 0.001–0.002). Thiamethoxam was highly toxic at the concentrations of 5.4×10^3 and 6×10^3 µg a.i. L^{-1} through contact exposure (Figure 1E) and highly lethal at 3×10^3 µg a.i. L^{-1} through ingestion exposure (Figure 1F).

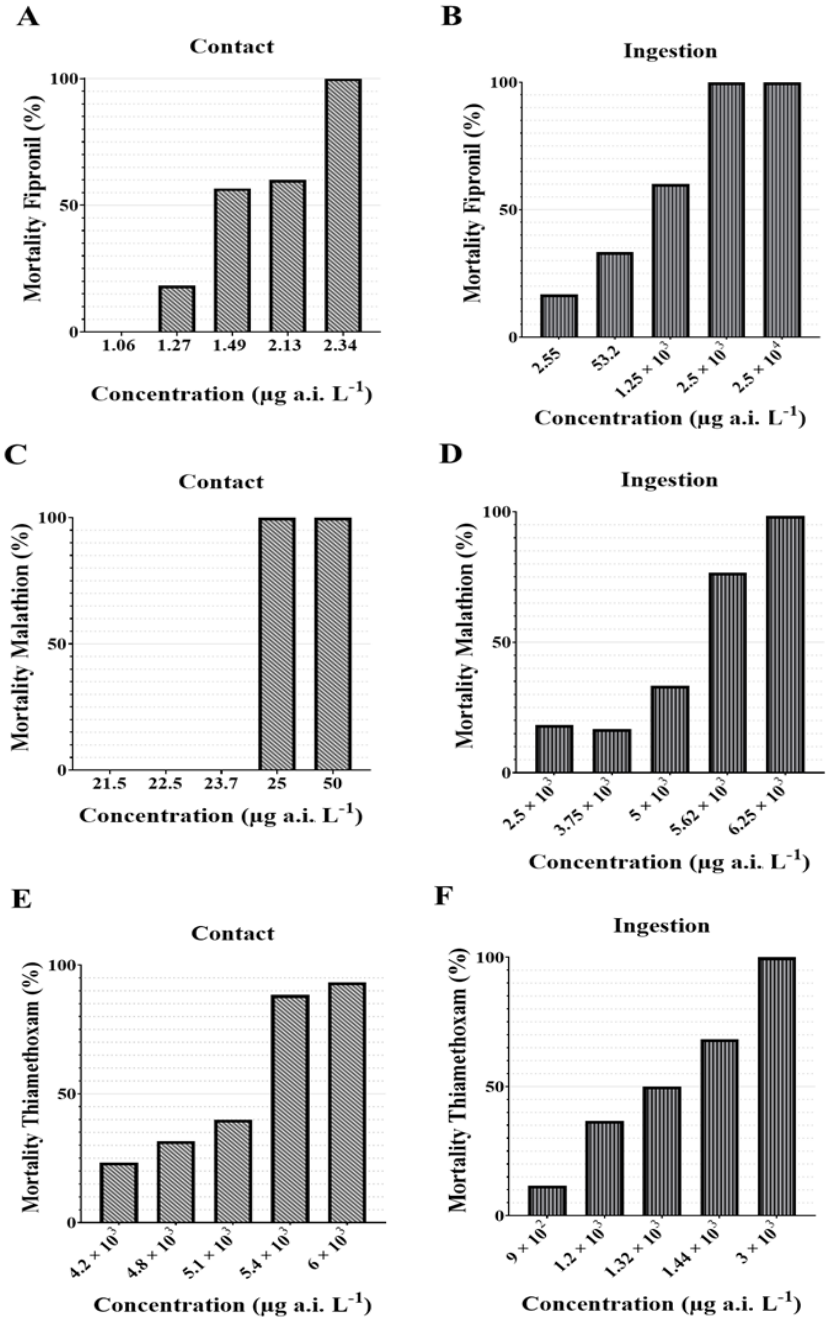

Figure 1. Mortality rates of *Tetragonisca fiebrigi* after 24 hours of exposure to fipronil, malathion, and thiamethoxam through contact or ingestion.

According to electrophoresis of protein extracts from the head and thorax of *T. fiebrigi* for the contact bioassays (Table 3), although thiamethoxam increased the activity of EST-4 (carboxylesterase) in one treatment only, none of the other treatments resulted in any changes for all esterase isozymes. For the ingestion bioassay (Table 3), only one treatment with fipronil and two treatments with malathion seemed to partially inhibit the activity of EST-4.

In cytochemical analysis of *T. fiebrigi* brain cells, CEC values for stingless bees exposed to malathion (ingestion) (Table 4) and thiamethoxam (contact and ingestion) (Table 5) remained in the range 0.20–0.30 M $MgCl_2$, similar to that observed for control bees (Tables 4 and 5). The CEC value was reduced to 0.15 M $MgCl_2$ only for stingless bees exposed to malathion through contact (Table 4).

Table 3. Inhibition of the relative esterase activity in *Tetragonisca fiebrigi* bees after exposure to fipronil, malathion, and thiamethoxam through contact or ingestion. (-) Lack of inhibition; (+) Increased intensity.

Concentrations Contact	*EST-1 (CHE)*	*EST-2*	*EST-4 (CbE)*	Concentration Ingestion	*EST-1 (CHE)*	*EST-2*	*EST-4 (CbE)*
Fipronil µg a.i. L^{-1}				*Fipronil* µg a.i. L^{-1}			
1.06	-	-	-	2.55	-	-	-
1.27	-	-	-	53.2	+	-	+
1.49	-	-	-	1.25×10^3	-	-	-
2.13	-	-	-	2.5×10^3	-	-	-
2.34	-	-	-	2.5×10^4	-	-	-
Malathion µg a.i. L^{-1}				*Malathion* µg a.i. L^{-1}			
21.5	-	-	-	2.5×10^3	-	-	+
22.5	-	-	-	3.75×10^3	-	-	-
23.7	-	-	-	5×10^3	-	-	-
25	-	-	-	5.62×10^3	-	-	+
50	-	-	-	6.25×10^3	-	-	-
Thiamethoxam µg a.i. L^{-1}				*Thiamethoxam* µg a.i. L^{-1}			
4.2×10^3	-	-	-	9×10^2	-	-	-
4.8×10^3	-	-	-	1.2×10^3	-	-	-
5.1×10^3	-	-	-	1.32×10^3	-	-	-
5.4×10^3	-	-	+	1.44×10^3	-	-	-
6×10^3	-	-	-	3×10^3	-	-	-

CHE: cholinesterase; CbE: carboxylesterase

Table 4. Critical electrolyte concentration values for brain chromatin in *Tetragonisca fiebrigi* worker bees after exposure to malathion through ingestion and contact (stained with 0.025% toluidine blue and various concentrations (in mol L^{-1}) of $MgCl_2$).

Staining	Control	Malathion (contact) µg a.i. L^{-1}	Malathion (ingestion) µg a.i. L^{-1}	
		23.7	5×10^3	5.62×10^3
TB	Vi	Vi	Vi	Vi
TB + $MgCl_2$ 0.02 mol L^{-1}	Vi	Vi	Vi	Vi
TB + $MgCl_2$ 0.05 mol L^{-1}	Vi/Bl	Vi	Vi/Bl	Vi
TB + $MgCl_2$ 0.08 mol L^{-1}	Vi/Bl	Vi	Vi/Bl	Vi/Bl
TB + $MgCl_2$ 0.10 mol L^{-1}	Vi/Bl	Vi/Bl	Vi/Bl	Vi/Bl
TB + $MgCl_2$ 0.12 mol L^{-1}	Vi/Bl	Bl	Bl	Vi/Bl
TB + $MgCl_2$ 0.15 mol L^{-1}	Bl	Gr*	Bl	Vi/Bl
TB + $MgCl_2$ 0.20 mol L^{-1}	Bl/Gr*	Bl	Bl	Bl
TB + $MgCl_2$ 0.30 mol L^{-1}	Gr*	Bl	Gr*	Gr*
CEC value (mol L^{-1})	0.20 < CEC < 0.30	0.15	0.30	0.30

a.i.: active ingredient. CEC: critical electrolyte concentration. TB: toluidine blue. Vi: violet. Bl: Blue. Gr*: Green

Discussion

In this study, *T. fiebrigi* adult stingless bees were susceptible to the different classes of insecticides tested. Among them, fipronil presented LC_{50} values of 2 µg a.i. L^{-1} through contact exposure and 107 µg a.i. L^{-1} through ingestion exposure, and was considered the most toxic insecticide for this bee species because of high mortality rate even at low concentrations. The toxicity of this insecticide is associated with its mode of action, where it binds strongly to GABA-gated chloride channels in neurons and irreversibly blocks neurotransmission (Tennekes & Sanchez-Bayo, 2013). Physiological effects of fipronil toxicity include hyperstimulation, convulsions, and paralysis, culminating in death of bees (Holder, Jones, Tyler, & Cresswell, 2018) and consequently a decrease in bee colonies, as observed in Brazil (Castilhos, Bergamo, Gramacho, & Gonçalves, 2019).

Table 5. Critical electrolyte concentration values for brain chromatin in *Tetragonisca fiebrigi* worker bees after exposure to thiamethoxam through contact or ingestion (stained with 0.025% toluidine blue and various concentrations (in mol L^{-1}) of $MgCl_2$).

Stain	Control	Thiamethoxam (contact) µg a.i. L^{-1}		Thiamethoxam (ingestion) µg a.i. L^{-1}	
		4.8×10^3	5.1×10^3	1.2×10^3	1.32×10^3
TB	Vi	Vi	Vi	Vi	Vi
TB + $MgCl_2$ 0.02 mol L^{-1}	Vi	Vi	Vi	Vi	Vi/Bl
TB + $MgCl_2$ 0.05 mol L^{-1}	Vi/Bl	Vi/Bl	Vi	Vi/Bl	Vi/Bl
TB + $MgCl_2$ 0.08 mol L^{-1}	Vi/Bl	Vi/Bl	Vi/Bl	Bl	Vi/Bl
TB + $MgCl_2$ 0.10 mol L^{-1}	Vi/Bl	Vi/Bl	Vi/Bl	Bl	Bl
TB + $MgCl_2$ 0.12 mol L^{-1}	Vi/Bl	Bl	Bl	Bl/Ve	Bl/Gr*
TB + $MgCl_2$ 0.15 mol L^{-1}	Bl	Bl	Bl/Gr	Bl/Ve	Bl/Gr*
TB + $MgCl_2$ 0.20 mol L^{-1}	*Bl/Gr**	Bl/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	0.30	0.20	0.20	0.20 ⩽ CEC < 0.30

a.i.: active ingredient. CEC: critical electrolyte concentration. TB: toluidine blue. Vi: violet. Bl: Blue. Gr*: Green

The neonicotinoid thiamethoxam acts as an agonist of nicotinic acetylcholine receptors (nAChRs) (Tennekes & Sanchez-Bayo, 2013). This compound cannot be immediately degraded, and its accumulation in insects results in hyperexcitation of the nervous system followed by paralysis and death (Tomizawa & Casida, 2005). In this study, the LC_{50} of thiamethoxam was 5×10^3 µg a.i. L^{-1} through contact exposure and 1×10^3 µg a.i. L^{-1} through ingestion exposure, indicating that it is less toxic than fipronil and malathion to *T. fiebrigi*, and higher doses and an increase in exposure period are required to cause mortality.

Even if thiamethoxam does not cause the immediate death of stingless bees, the effects resulting from exposure to these sublethal concentrations may compromise the survival of these insects over time — as demonstrated for *Scaptotrigona bipunctata* (Moreira et al., 2018), *Scaptotrigona postica* (Ferreira et al. (2013); Jacob, Soares, Carvalho, Nocelli, & Malaspina (2013); Jacob et al. (2015)), *Melipona scutellaris* (Lourenço, Carvalho, Malaspina, & Nocelli, 2012), and *Apis cerana japonica* (Yasuda, Sakamoto, Goka, Nagamitsu, & Taki, 2017) given that most insecticides are systemic and can accumulate in pollen and nectar (Aliouane, Hassani, Gary, Armengaud, & Gauthier, 2009). Therefore, bees may carry residues of these insecticides to hives, infecting their products, offspring, queen, and other workers (Tomé, Martins, Lima, Campos, & Guedes, 2012).

Insecticides can affect the behavior of bees, causing orientation problems in foraging (Tomé et al., 2012), given that at least two types of nAChRs are known to be involved in tactile and olfactory learning and memory, which are essential for foraging behavior (Thany & Gauthier, 2005). Pesticides have also been reported to cause alterations in the midgut and activity of enzymes, affecting the memory capacity (Hashimoto et al., 2003) and morphophysiology of bees (Moreira et al., 2018), which in the long term may compromise not only the survival of bee species but also limit the performance (Pettis et al., 2013) and productivity of the colonies (Sandrock et al., 2014).

Many of these alterations can be evaluated in the laboratory or field, and the results can then be used to develop strategies to minimize the death of these non-target insects, which directly influences pollination in natural forest areas and agricultural production, especially in agroecosystems (Egan, Dicks, Hokkanen, & Stenberg, 2020). The methods used to determine the effects of insecticides on bees include the analysis of the relative activity of enzymes, such as esterases, which have been used for different species, for example, *A. mellifera* (Hashimoto et al., 2003; Attencia, Ruvolo-Takasusuki, & Toledo, 2005; Yao, Zhu, Adamczyk, & Luttrell, 2018) and *S. bipunctata* (Moreira et al., 2018).

In *T. fiebrigi*, the carboxylesterase EST-4 (Stuchi, Toledo, Lopes, Cantagalli, & Ruvolo-Takasusuki, 2014) was shown to be partially inhibited after exposure of stingless bees to malathion and fipronil through ingestion. As these carboxylesterases are involved in the metabolic detoxification of xenobiotics, the low concentrations of these insecticides and their short period of exposure (only 24 hours) were probably not sufficient to allow *T. fiebrigi* to respond to them by increasing the levels of the detoxifying enzyme. Thus, the partial inhibition may be indicative of a reduction in relative activity of this enzyme and, consequently, a decrease in the metabolism of these compounds, resulting in the high toxicity observed. The effects of fipronil on the relative activity of this esterase have also been observed in the Africanized honeybees (Nahar & Ohtani, 2015; Zaluski, Kadri, Alonso, Martins, & Orsi, 2015; Roat, Carvalho, Palma, & Malaspina, 2017; Kairo et al., 2017; Lunardi, Zaluski, & Orsi, 2017; Yasuda et al., 2017).

In contrast, the band intensity of EST-4 increased only after contact exposure of *T. fiebrigi* to high concentrations of thiamethoxam, which is different to the results observed for *S. bipunctata* by Moreira et al.

(2018). In this case, the increase in EST-4 activity is indicative of a natural response to a large amount of toxin in the bee system, whereupon the insect should act to metabolize the insecticide, thereby contributing to low toxicity of thiamethoxam towards *T. fiebrigi* compared to that of the other insecticides evaluated.

In this study, CEC values fell in the range 0.20–0.30 M $MgCl_2$ for the control of stingless bees as well as the stingless bees exposed to malathion (ingestion) and thiamethoxam (contact and ingestion). Only bees exposed to malathion through contact exhibited a lower CEC value (0.15 M $MgCl_2$). The determination of the CEC point is based on the DNA–protein complexation in chromatin. When somatic cells are treated with toluidine blue (pH 4.0) in the absence of or at low concentrations of Mg^{2+} ions, chromatin exhibits metachromasia (a violet color). If staining with toluidine blue occurs in the presence of Mg^{2+} ions, there will be competition between these ions and the similarly charged dye dipole for negative charges of the polyanionic substrate. If the Mg^{2+} concentration corresponds to the CEC value, there will be no DNA metachromasia and the color displayed is green. This qualitative analysis allows for the determination of variations in chromatin compaction and, consequently, changes in gene expression under different physiological conditions, including exposure to contaminants (Moreira et al., 2018). A high CEC value means that chromatin is more compact and therefore gene expression is low. A reduction in this value indicates chromatin relaxation and consequently increased gene expression (Vidal & Mello, 1989).

Thus, an increase in the CEC value in stingless bees exposed to an insecticide compared to control bees registers greater chromatin compaction and the inactivation of gene expression as observed for *S. bipunctata* exposure to thiamethoxam by ingestion (Moreira et al., 2018). Similar results were described by Rossi, Roat, Tavares, Cintra-Socolowski, and Malaspina (2013) using the neonicotinoid imidacloprid, where chromatin condensation was found in the *A. mellifera* brain. The decreased CEC value observed in *T. fiebrigi* bees exposed to malathion through contact may be indicative of chromatin relaxation and, consequently, an increase in gene expression in response to chemical stress or contaminant metabolism.

Conclusion

In conclusion, our results suggest that *T. fiebrigi* stingless bees are susceptible to all the insecticides analyzed, whether through contact or ingestion exposure, although fipronil was the most toxic agent. Electrophoretic analysis showed an increase in band intensity of the carboxylesterase EST-4 in these stingless bees following their contact with thiamethoxam, indicating an increase in the relative activity of this isoenzyme in response to exposure. The change in the CEC point in *T. fiebrigi* bees exposed to malathion through contact was possibly due to chromatin relaxation, which may be indicative of increased gene expression. Therefore, in the determination of dose–mortality relationships, parameters such as alterations in the relative activity of esterases and modification of chromatin in brain cells may be used as biomarkers to detect the presence of insecticide compounds and their toxicity to bees.

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