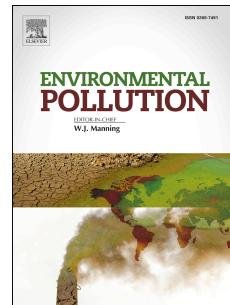


# Journal Pre-proof

*Apis mellifera* and *Melipona scutellaris* exhibit differential sensitivity to thiamethoxam

Lucas Miotelo, Ana Luiza Mendes dos Reis, José Bruno Malaquias, Osmar Malaspina, Thaisa Cristina Roat



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## **AUTHORSHIP STATEMENT**

**Manuscript title: *Apis mellifera* and *Melipona scutellaris* exhibit differential sensitivity to thiamethoxam**

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication before its appearance in the Hong Kong Journal of Occupational Therapy.

### **Authorship contributions**

Please indicate the specific contributions made by each author (list the authors' initials followed by their surnames, e.g., Y.L. Cheung). The name of each author must appear at least once in each of the three categories below.

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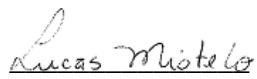
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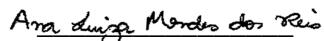
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Rio Claro, 28 Sep., 2020.

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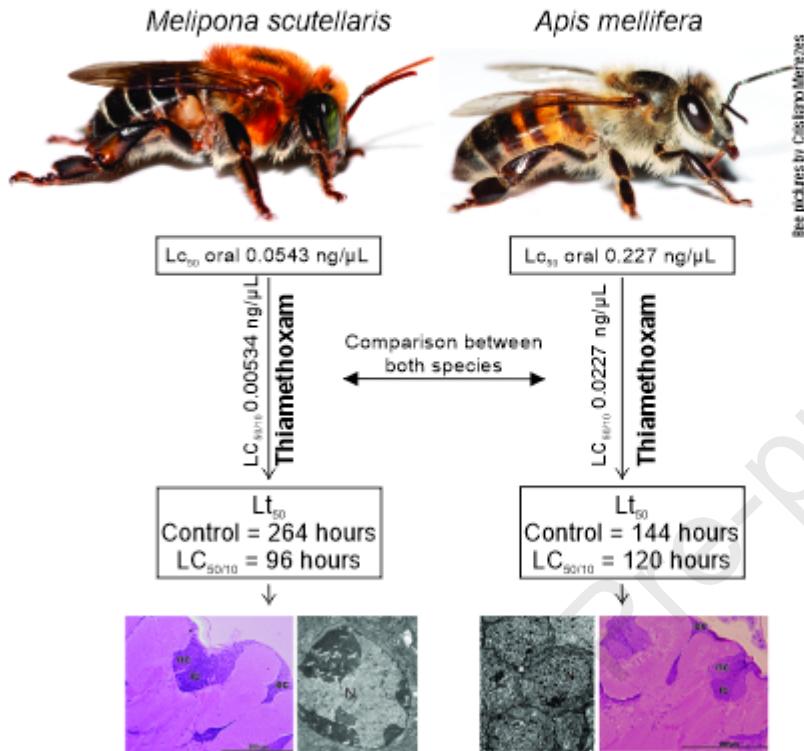


Rio Claro, 28 Sep., 2020.

Thaisa Cristina Roat



Rio Claro, 28 Sep., 2020.



1    **Research article**

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5        ***Apis mellifera* and *Melipona scutellaris* exhibit differential sensitivity to**  
6        **thiamethoxam**

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25

26 **Abstract**

27 *Apis mellifera* is a pollinator insect model in pesticide risk assessment tests for bees.  
28 However, given the economic and ecological importance of stingless bees such as  
29 *Melipona scutellaris* in the Neotropical region, as well as the lack of studies on the  
30 effect of insecticides on these bees, toxicity tests for stingless bees should be carried out  
31 to understand whether insecticides affect both species of bees in the same manner. Thus,  
32 the present study quantified the differential sensitivity of the bees *M. scutellaris* and *A.*  
33 *mellifera* to the oral ingestion of the insecticide thiamethoxam by determining the mean  
34 lethal concentration ( $LC_{50}$ ), mean lethal time ( $LT_{50}$ ), and their effect on the insecticide  
35 target organ, the brain. The results showed that the stingless bee is more sensitive to the  
36 insecticide than *A. mellifera*, with a lower  $LC_{50}$  of 0.0543 ng active ingredient (a.i.)/ $\mu$ L  
37 for the stingless bee compared to 0.227 ng a.i./ $\mu$ L for *A. mellifera*. When exposed to a  
38 sublethal concentration, morphological and ultrastructural analyses were performed and  
39 evidenced a significant increase in spaces between nerve cells of both species. Thus, *A.*  
40 *mellifera* is not the most appropriate or unique model to determine the toxicity of  
41 insecticides to stingless bees.

42

43 By comparing  $LC_{50}$ ,  $LT_{50}$ , and morphological analysis, *M. scutellaris* is more sensitive  
44 to thiamethoxam than *A. mellifera*.

45

46 **Keywords:** Stingless bees; Mushroom body; Neonicotinoid; Ultrastructure; Model  
47 organism; Toxicity tests.

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51        **1. Introduction**

52              Approximately 20,000 bee species have been described worldwide (Michener,  
53              2007). In Brazil, there are 1,678 bee species described (Michener, 2007; Pedro, 2014);  
54              among them, the Africanized honeybee *Apis mellifera* Linnaeus, 1758 (Kent, 1988;  
55              Tautz, 2008) is considered a pollinating agent of great agricultural importance because  
56              it represents a generalist species (Boyle et al., 2019; Cham et al., 2018). However, 244  
57              species are stingless bees (Pedro, 2014), such as “Uruçu Nordestina” - *Melipona*  
58              *scutellaris* Latreille, 1811 (Hymenoptera: Apidae, Meliponini), an important Brazilian  
59              native bee species from Brazil’s northeast region (Nogueira-neto, 1997). Although *A.*  
60              *mellifera* is important for the economy because it pollinates many crops, such as coffee,  
61              citrus, soybean, canola, and sunflower, this species is not able to buzz pollinate (Klein  
62              et al., 2020). Buzz pollination occurs when bees curl their bodies around anthers from  
63              flowers with poricidal anthers and contract the body's muscles, resulting in vibrations  
64              that are transmitted to the anthers, and the pollen grains are released through the apical  
65              pores (Buchmann and Hurley, 1978; De Luca and Vallejo-Marín, 2013). There are more  
66              than 50 genera of bees with the ability to sonicate; however, *A. mellifera* is not able to  
67              buzz pollinate (De Luca and Vallejo-Marín, 2013). On the other hand, *M. scutellaris*  
68              can perform this type of pollination, which is considered an essential mechanism for  
69              plants with porous anthers, thus affecting the production and quality of annatto, sweet  
70              pepper, tomato, and other crops (Caro et al., 2016; Klein et al., 2020). Thus, the  
71              economy and environmental importance of *M. scutellaris* rely on the fact that they can  
72              pollinate different plant species (crops and natives) when compared to *A. mellifera*  
73              (Klein et al., 2020).

74              During the last decade, the decrease in pollinators has attracted growing concern,  
75              and there is evidence that this decline is global; thus, several studies have sought to

76 understand the causes of this problem (Potts et al., 2010). Among the contributing  
77 factors cited are habitat fragmentation, climate change, disease (parasites/viruses), and  
78 agricultural intensification. The latter is related to the indiscriminate use of pesticides  
79 (Decourtye and Devillers, 2010; Goulson et al., 2015).

80 Currently, neonicotinoids are the most widely used insecticides in crop areas.  
81 The second generation of this group includes compounds such as thiamethoxam,  
82 derived from the nicotine molecule and chemically classified as N-nitroguanidines  
83 (Bass and Field, 2018; Maienfisch et al., 2001; Tomizawa and Casida, 2005).  
84 Thiamethoxam is highly specific for insect nicotinic acetylcholine receptors (nAChR),  
85 an important ion signaling pathway. Additionally, it acts as an agonist of acetylcholine,  
86 mimicking its action in contacting acetylcholinesterase enzymatic substrates.  
87 Consequently, there is competition for cholinergic receptors that mediate nerve  
88 impulses (Bass and Field, 2018; Chapman, 2013; El Hassani et al., 2008). However,  
89 because of the presence of thiamethoxam, the insecticide molecules are not degraded by  
90 acetylcholinesterase, which leads to the buildup of acetylcholine and continuous  
91 transmission of nerve impulses leading to hyperexcitability and death (El Hassani et al.,  
92 2008). Additionally, thiamethoxam has systemic properties, which means that it can be  
93 distributed to all plant tissues, potentially reaching pollen and nectar (Bass and Field,  
94 2018). Hence, bees can be exposed to sublethal concentrations during foraging activity  
95 (Aliouane et al., 2009; Bass and Field, 2018; Yang et al., 2008). Although the European  
96 Union has banned the use of thiamethoxam (Bass and Field, 2018), in Brazil, it is often  
97 used in the main agricultural plantations, such as soybean, coffee, cotton, and citrus  
98 (Klein et al., 2020). In addition, thiamethoxam is listed by the Brazilian Institute of  
99 Environment and Natural Resources (IBAMA) to carry out a reassessment of the  
100 environmental risk of pesticides for pollinators (IBAMA, 2017).

101        The insect brain is the target of neonicotinoid insecticides. However, in oral  
102    exposures, other organs can be affected, such as the midgut (Catae et al., 2017; Friol et  
103    al., 2017) and Malpighian tubules (Catae et al., 2014). In the brain, the best-studied  
104    region is the mushroom bodies (Heisenberg, 2003). The mushroom bodies are present at  
105    the protocerebrum and are mainly formed by Kenyon cells (neurons), approximately  
106    170,000 Kenyon cells (Fahrbach, 2006; Farris and Sinakevitch, 2003; Heisenberg,  
107    2003). This specific region in the bee brain is responsible for learning and memory  
108    processes (Daly et al., 1998; Heisenberg, 2003).

109        To register these compounds, the submission of the results from toxicity tests in  
110    various agents, including bees, is required. However, in Brazil, the bee used in toxicity  
111    tests is the *A. mellifera* species, which is a non-native bee. Questions have been raised  
112    regarding whether this species should be used as a model in pesticide risk assessments  
113    (Boyle et al., 2019; Cham et al., 2018). Therefore, IBAMA (Brazilian Institute for the  
114    Environment and Renewable Natural Resources) has emphasized the need to study the  
115    possible effects caused by three active ingredients of the neonicotinoid class  
116    (thiamethoxam, imidacloprid, and clothianidin) in stingless bees. Additionally, there are  
117    knowledge gaps that need to be filled (Cham et al., 2018; Rortais et al., 2017). For this  
118    purpose, IBAMA has listed five species of bees, including *M. scutellaris*, for further  
119    studies, which may contribute to risk assessment tests in the future (IBAMA, 2017).  
120    This initiative occurred because specific protection measures for a single model species  
121    may not be sufficient to protect all existing bee species (Cham et al., 2018).

122        Studies of the susceptibility of Neotropical bee species have demonstrated the  
123    high toxicity of different active ingredients, including the following: spinosad,  
124    malathion, phosmet, and acetamiprid in *Plebeia emerina* Friese (1900) and  
125    *Tetragonisca fiebrigi* Schwarz (1938) (Padilha et al., 2020); glyphosate in

126 *Nannotrigona testaceicornis* Lepeletier (1836) and *Tetragonisca angustula* Latreille  
127 (1811) (Guimarães-Cestaro et al., 2020); deltamethrin and methamidophos in *Melipona*  
128 *quadrifasciata* Lepeletier (1836) (Del Sarto et al., 2014); fipronil, acetamiprid,  
129 imidaclorpid, thiacloprid, and dimethoate in *Scaptotrigona postica* Latreille (1807)  
130 (Jacob et al., 2014, 2019, 2013) and imidaclorpid, fipronil, abamectin, and  
131 difenoconazole in *M. scutellaris* (Costa et al., 2015; de Moraes et al., 2018; Lourenço et  
132 al., 2012a, 2012b; Prado et al., 2020). However, only Del Sarto (2014) and Jacob (Jacob  
133 et al., 2019) carried out studies comparing the toxicity of stingless bees with *A.*  
134 *mellifera*, despite this fact, none of the previous studies made comparisons between *A.*  
135 *mellifera* and *M. scutellaris*. Due to the lack of information available on toxicological  
136 protocols for stingless bees, there is a clear need for studies to determine the effects of  
137 pesticides on these bees (Boyle et al., 2019; Cham et al., 2018; Padilha et al., 2020).

138 One way to assess the effects of pollutants on organisms is to perform acute  
139 toxicity tests, which are used to measure the toxicity of agricultural pesticides and other  
140 chemical substances by the mean lethal dose ( $LD_{50}$ ) or average lethal concentration  
141 ( $LC_{50}$ ) (Rortais et al., 2017). The  $LC_{50}$  represents the concentration of a substance that is  
142 required to kill 50% of the experimental population over a given time period (Rortais et  
143 al., 2005). Thus, an interesting approach involving bees and agrochemicals is to  
144 establish the toxicity in different species to verify whether there are differences in the  
145 sensitivity to these compounds among bees. Survival assays to obtain the mean lethal  
146 time ( $LT_{50}$ ) measure the time period in which 50% of the bees die after contact with the  
147 insecticide.  $LC_{50}$  and  $LT_{50}$  are important in determining and standardizing a period of  
148 exposure (Desneux et al., 2007; Moncharmont et al., 2003). Additionally, it is possible  
149 to observe how sublethal effects can act on the survival of bees since they do not cause

150 the immediate death of the individual and result in prolonged exposure of the brain  
151 (Rortais et al., 2005).

152 Thus, the current challenge is to establish the maximum concentrations or limits  
153 of environmental contaminants that protect the diversity of bee species in Neotropical  
154 regions, compare the data obtained for *A. mellifera* to those obtained for stingless bees  
155 and verify whether toxicity tests for a model species are safe and effective at inferring  
156 effects on the ecosystem as a whole (Boyle et al., 2019; Cham et al., 2018).  
157 Accordingly, this work has determined the acute toxicity of thiamethoxam on *A.*  
158 *mellifera* and *M. scutellaris* by obtaining LC<sub>50</sub> values and analyzing the effects of  
159 sublethal concentrations of thiamethoxam on the brain in both species to generate data  
160 to ensure biodiversity protection of the bees in Brazil.

161 **2. Materials and Methods**

162 **2.1 Acute toxicity test**

163 All experiments to determine oral LC<sub>50</sub> values followed recommendations of the  
164 Organization for Economic Cooperation and Development for the evaluation of  
165 pesticides in bees (OECD, 1998a, 1998b). The bees were obtained from adequately fed,  
166 healthy, disease-free, and queen-right colonies with a history and physiological status  
167 already known. The pesticide used was thiamethoxam PESTANAL® (C<sub>8</sub>H<sub>10</sub>ClN<sub>5</sub>O<sub>3</sub>S),  
168 Sigma-Aldrich analytical standard, soluble in water, and purity ≥ 98.0%. For both  
169 species, a stock solution of 1000 ng a.i. of thiamethoxam per µL in deionized water was  
170 prepared. From this solution, cascade dilutions were carried out until they reached the  
171 required concentrations. The food used in the control group and to perform the cascade  
172 dilution was composed of water and sucrose (50% v/v). The two species received an *ad*  
173 *libitum* diet, and the only contact with the insecticide was through oral ingestion via  
174 contaminated food. To ensure that the bees only had oral contact with the contaminated

175 food, the Eppendorf's that were used as feeders were previously tested to avoid a leak.  
176 Therefore, only Eppendorf's in which the food did not leak was used, ensuring that the  
177 bees did not have topical contact with the insecticide. Furthermore, all assays were  
178 carried out in triplicate.

179 **2.1.1 *A. mellifera***

180 Forager bees were collected from three different colonies in the apiary of the  
181 Institute of Bioscience, Universidade Estadual Paulista (UNESP), Campus Rio Claro,  
182 São Paulo, Brazil. Three hundred and sixty bees were divided into two groups: control  
183 (60 bees) and exposed to thiamethoxam (300 bees). The bioassays were carried out by  
184 oral exposure. The group exposed to the insecticide received 5 different concentrations,  
185 and the bees were randomly allocated to each one. The experiments consisted of three  
186 replicate cages per treatment (60 individuals/concentration). Twenty bees in one cage  
187 represent one replicate. The individuals were kept in 500 mL plastic cages (20 bees per  
188 cage) with holes drilled on the sides to allow air to pass. The bees were fed through  
189 microtubes (feeders) (2 mL) punched in extremities and kept in biochemical oxygen  
190 demand (D.B.O.) chamber at  $32 \pm 2$  °C and relative humidity of  $60 \pm 10\%$  in constant  
191 darkness. A cascade dilution was carried out until the following thiamethoxam  
192 concentrations were reached: 1.0, 0.47, 0.22, 0.10 and 0.05 ng a.i./ $\mu$ L of food. The bees  
193 were then fed *ad libitum* through the feeders, and the number of dead bees was recorded  
194 after 24 h of initial exposure. For validation, only assays in which the mortality of the  
195 control group was <10% across all tests were considered valid (OECD, 1998a).

196 **2.1.2 *M. scutellaris***

197 Forager bees were collected from three different colonies in the meliponary of  
198 the Institute of Bioscience, Universidade Estadual Paulista (UNESP), Campus Rio  
199 Claro, São Paulo, Brazil, for toxicity tests. It is worth noting that it is necessary to

200 respect 15 days between collections to avoid weakening the colony. To estimate the  
201 interval between the collections, we based on the developmental differences between *A.*  
202 *mellifera* and *M.-scutellaris*, as shown by Cham (2018). One hundred eighty forager  
203 bees were divided into two groups: control (30 bees) and exposed to thiamethoxam (150  
204 bees). The bioassays were carried out by oral exposure. The group exposed to the  
205 insecticide received 5 different concentrations, and the bees were randomly allocated to  
206 each one. The experiments consisted of three replicate cages per treatment (30  
207 individuals/concentration). Ten bees in one cage represent one replicate. The individuals  
208 were kept in 250 mL plastic cages (10 bees per cage) with holes drilled on the sides to  
209 allow air to pass. The bees were fed through microtubes (feeders) (2 mL) punched in  
210 extremities and kept in biochemical oxygen demand (D.B.O.) chamber at  $28 \pm 2$  °C and  
211 relative humidity of  $70 \pm 10\%$  in constant darkness. A cascade dilution was carried out  
212 until the following thiamethoxam concentrations were reached: 0.2, 0.1, 0.05, 0.025 and  
213 0.0125 ng a.i./ $\mu$ L of food. The bees were then fed *ad libitum* through the feeders, and  
214 the number of dead bees was recorded after 24 h of initial exposure. For validation, only  
215 assays in which the mortality of the control group was <10% across all tests were  
216 considered valid (OECD, 1998a).

## 217 **2.2 Statistical analysis for determination of LC<sub>50</sub>**

218 After 24 h of exposure, mortality was counted and subjected to statistical dose-  
219 response analysis. For that, they were analyzed with generalized binomial models. The  
220 link function logit, probit complementary log-log, and cauchit were tested (Moral et al.,  
221 2017; Venables and Ripley, 2002). The binomial logistic model with the cauchit link  
222 function was the best model that fitted the 24-hour mortality data. The goodness-of-fit  
223 was evaluated using half-normal plots (Moral et al., 2017) with a half normal plot  
224 employing the *hnp* package of software R (R Core Team, 2018).

225 **2.3 Survival test to obtain the LT<sub>50</sub>**

226 Three replicates were used for each treatment. Thus, 60 bees of the species *A.*  
227 *mellifera* were used for the control group, and 60 bees were used for the group exposed  
228 to thiamethoxam. The species *M. scutellaris* was represented by 30 bees of the control  
229 group and 30 bees of the group exposed to the insecticide. The control groups did not  
230 receive contaminated food, while the experimental groups received an *ad libitum* diet  
231 contaminated with thiamethoxam at a sublethal concentration equivalent to LC<sub>50/10</sub> (*A.*  
232 *mellifera* - 0.0227 ng a.i./μL and *M. scutellaris* - 0.00534 ng a.i./μL). Bees were  
233 evaluated until the last one died. The experiment continued until 100% mortality in the  
234 experimental group or 51% of the mortality in the control group was reached. Mortality  
235 results from the survival trial were analyzed using SigmaPlot 11.0 software (Systat)  
236 using the log-rank test to compare the survival curves of each experimental group  
237 (Kaplan-Meier method).

238 **2.4 Procedures for morphological analysis and quantification of the cell spaces  
239 between Kenyon cells and the area of strong cell staining**

240 After exposure to LC<sub>50/10</sub> (*A. mellifera* - 0.0227 ng a.i./μL and *M. scutellaris* -  
241 0.00534 ng a.i./μL), only live bees were selected for dissection, but they were  
242 previously submitted to anesthesia at -20°C. The anaesthesia time for *A. mellifera* was  
243 95 seconds, and that for *M. scutellaris* was 70 seconds. The anesthesia time was used  
244 only to immobilize the bees, thus avoiding the freezing of the bees. Six brains (each one  
245 has four mushroom bodies) were dissected in 4% paraformaldehyde fixative solution on  
246 the first and eighth days of exposure to thiamethoxam and were kept in 0.1 M sodium  
247 phosphate buffer solution (pH 7.4) until dehydration in an ethanol gradient (15% to  
248 100%) at 4 °C for 2 hours per bath. At the end of the dehydration, they were immersed  
249 in resin (Leica) and polymerized in historesin with the addition of catalyst at 37 °C.

250 Histological sections of 5 µm thickness were sectioned on an RM2255 microtome  
251 (Leica) and stained with HE for morphological analysis using light microscopy  
252 (Junqueira and Carneiro, 2012). The other six brains that were not exposed to  
253 thiamethoxam were used as the control group.

254 Images were acquired through a digital camera inserted on an Olympus BX51  
255 microscope with the aid of DP Controller® software. Twenty-four mushroom bodies  
256 from each experimental group were analyzed. The measurements were adapted from the  
257 method described by Azevedo et al. (2020). In each mushroom body, ten measurements  
258 of the space between Kenyon cells were obtained. However, an average per brain was  
259 determined. Additionally, the area of strong cell staining in the mushroom body was  
260 analyzed, and the average per brain was determined. To perform these measurements,  
261 ImageJ v1.52a software was used (Collins, 2007). A three-way ANOVA model was  
262 used to test the significance of the effects of the interactions of time (without repeated  
263 measures over time) *versus* treatment *versus* species of bee in the following variables:  
264 spaces between Kenyon cells and condensation of nuclear chromatin area. In these  
265 analyses, the Tukey test ( $p = 0.05$ ) was used to compare the variables between the  
266 factors. The data did not show normality or homogeneity of variance. Therefore, we  
267 transformed the data using the “boxcox” function of the “MASS” package. Lambda  
268 values were extracted that maximized the function and were substituted in the formula:  
269  $yT = y\lambda - 1/\lambda$  (Box and Cox, 1964).

270 **2.5 Transmission electron microscopy**

271 Considering that the staining technique previously analyzed showed spacing  
272 between Kenyon cells and heavily stained nuclei, an ultrastructural analysis using  
273 transmission electron microscopy (TEM) was performed to observe putative cell  
274 damage caused by the insecticide. The aim was to search for additional evidence of cell

death that could not be visualized by light microscopy. For this purpose, using the same sublethal concentrations, brains were collected on the first and eighth days after the beginning of the bioassay, totaling six samples per treatment/day. The brains of the control groups and insecticide-exposed groups were dissected in fixative solution with the aid of an EZ4 stereomicroscope (Leica). Organs were fixed in 2.5% glutaraldehyde and maintained in sodium cacodylate buffer (0.1 M, pH = 7.4). The washes were performed with the same buffer solution, and the brains were postfixed in 1% osmium tetroxide for 2 hours. Afterwards, the organs were again washed in the same buffer solution, contrasted in 1% uranyl acetate dissolved in 10% alcohol for 2 hours, and dehydrated in 70% alcohol and then 100% alcohol. After dehydration, the organs were embedded in Epon-Araldite resin and resin-polymerized with catalyst at 60 °C. The obtained blocks were sectioned in an Ultranova Microtome Supernova (Reichert) to obtain ultrafine cuts and were subjected to contrast in uranyl acetate and lead citrate. Finally, photographs were taken by transmission electron microscopy (JOEL-JEM1011) at the Nucleus of Support for Research in Electron Microscopy Applied to Agriculture (NAP/MEPA) of the "Luiz de Queiroz" School of Agriculture (ESALQ), Piracicaba.

### 3. Results

#### 3.1 Mean lethal concentration ( $LC_{50}$ ) of thiamethoxam

We compared median lethal concentration values using the confidence interval overlap method. There was a significant difference between  $LC_{50}$  levels between *A. mellifera* and *M. scutellaris* ( $P<0.05$ ). The  $LC_{50}$  oral value for thiamethoxam insecticide obtained for female workers of the species *A. mellifera* was 0.227 ng a.i./ $\mu$ L (ppm) syrup at 24 hours (Fig. 1A). For *M. scutellaris* foragers, it was 0.0543 ng a.i./ $\mu$ L (ppm) syrup at 24 hours (Fig. 1B).

#### 3.2 Average lethal time ( $LT_{50}$ )

300 When we compared the average lethal time ( $LT_{50}$ ) in the control and insecticide  
301 groups for *A. mellifera*, there was no difference ( $\chi^2= 1.7$ ;  $df= 1$ ;  $p= 0.2$ ); however, for *M.*  
302 *scutellaris*, we found a difference between these groups ( $\chi^2= 21.7$ ;  $df= 1$ ;  $p= 3e-06$ ). The  
303  $LT_{50}$  differed between *A. mellifera* and *M. scutellaris* in the control group ( $\chi^2= 23.7$ ,  $df=$   
304 1,  $p= 1e-06$ ) and when exposed to insecticide ( $\chi^2= 4.4$ ,  $df= 1$ ,  $p= 0.04$ ). The values  
305 obtained for the average  $LT_{50}$  for *A. mellifera* were 144 hours in the control groups and  
306 120 hours at an  $LC_{50/10}$  of 0.0227 ng a.i./ $\mu$ L syrup (Fig. 2A). Those for *M. scutellaris*  
307 were 264 hours in the control groups and 72 hours at  $LC_{50/10}$  0.00543 ng a.i./ $\mu$ L syrup  
308 (Fig. 2B).

### 309 **3.3 Morphological analyses in the brain via HE staining**

310 The results of the morphological analysis of the control group *A. mellifera* after  
311 1 day (Fig. 3A, B and C) and 8 days (Fig. 3G, H and I) of the experiment indicated  
312 intact structures without morphological alterations with standard staining. The  $LC_{50/10}$   
313 groups exposed to thiamethoxam for 1 day showed an increase in the spaces between  
314 the Kenyon cells of the mushroom body (Fig. 3D, E and F). After eight days of  
315 exposure, the group exposed to the insecticide had a higher number of strongly stained  
316 cells than the control group, suggesting condensation of nuclear chromatin.  
317 Furthermore, the Kenyon cells were also disorganized in the exposed group (Fig. 3J, K  
318 and L).

319 The morphological analyses carried out in *M. scutellaris* showed that the 1-day  
320 (Fig. 4A, B and C) and 8-day (Fig. 4G, H and I) control groups had mushroom bodies  
321 without morphological alterations, with Kenyon cells that were intact and normal  
322 staining, while the groups exposed to the insecticide for 1 day (Fig. 4D, E and F) and 8  
323 days (Fig. 4J, K and L) presented spacing between the Kenyon cells.

324 **3.4 Quantification of the cell spaces between Kenyon cells and condensation of**  
325 **nuclear chromatin**

326 The results of this study demonstrate that the interaction of time versus treatment  
327 versus bee species was significant for spaces between Kenyon cells ( $F_{3,40} = 4.80$ ;  $p =$   
328 0.0060) (table 1) and for condensation of nuclear chromatin area ( $F_{3,40} = 9.56$ ;  $p =$   
329 0.0001) (table 2). Therefore, significant differences were found between species  
330 according to the control and exposed groups and time of exposure for both variables.  
331 When we compared bee species in relation to spaces between Kenyon cells, *A. mellifera*  
332 had smaller spaces than *M. scutellaris* on the first day of exposure with the control  
333 group, and these spaces were larger in *A. mellifera* with exposure to thiamethoxam on  
334 the eighth day than in *M. scutellaris*. In general, *A. mellifera* and *M. scutellaris* showed  
335 a significant increase in the spaces between Kenyon cells and more condensation of  
336 nuclear chromatin when exposed to thiamethoxam, mainly on the eighth day. Only on  
337 the first day of exposure did *A. mellifera* have more condensation of nuclear chromatin  
338 than *M. scutellaris*. No difference between these species was observed when they were  
339 exposed to thiamethoxam.

340 **3.5 Ultrastructural analysis of the mushroom body (TEM)**

341 The results obtained for *A. mellifera* showed that the ultrastructural  
342 characteristics of the 1-day (Fig. 5A and B) and 8-day (Fig. 5E and F) control groups  
343 remained typical with intact cytoplasm, including the presence of mitochondria, which  
344 had typical and homogeneous electron-dense matrices with visible crests, the recurrent  
345 presence of Golgi complexes and free ribosomes widely and homogenously distributed,  
346 and the nucleus with a spherical shape, filled mainly with decondensed chromatin with  
347 some heterochromatin points. In this group, the cells were united and had axon and

348 dendrite extensions, showing that the tissue had good connectivity, which is important  
349 for cell-cell communication and the transmission of nerve impulses.

350 When exposed to LC<sub>50/10</sub> for 1 day (Fig. 5C and D), the Kenyon cells from *A.*  
351 *mellifera* developed a more elongated, almost oval change in their shape and a greater  
352 compaction of chromatin close to the periphery of the nuclear envelope, which was also  
353 elongated (Fig. 5C). In the group exposed to LC<sub>50/10</sub> for 8 days (Fig. 5G and H), some  
354 alterations were observed, such as changes in cellular shape, dilation of mitochondria,  
355 rupture of the mitochondrial membrane (Fig. 5H), and an increase in irregularity in the  
356 nuclear envelope format (Fig. 5G).

357 The *M. scutellaris* control group showed typical ultrastructural Kenyon cell  
358 features, both after 1 day (Fig. 6A and B) and 8 days (Fig. 6E and F) of exposure, such  
359 as spherical nuclei, the presence of decondensed chromatin and heterochromatin regions  
360 occupying the periphery of the nuclei, and cytoplasm containing elongated  
361 mitochondria throughout it, with visible crests remaining intact. The same morphology  
362 was observed in the Kenyon cells of bees exposed to thiamethoxam LC<sub>50/10</sub> for 1 day  
363 (Fig. 6C and D). However, in the group exposed to LC<sub>50/10</sub> for 8 days (Fig. 6G and H),  
364 some changes were observed in the mitochondria, such as loss of crests and the  
365 presence of lucid electron regions in the mitochondrial matrix (Fig. 6H). The nuclei,  
366 however, exhibited an irregular shape and showed an increase in the presence of  
367 heterochromatin (Fig. 6H), mainly with an 8-day exposure to the insecticide.

368 **4. Discussion**

369 In the last few years, researchers have tried to understand whether *A. mellifera* is  
370 a good model to represent all bee species since it is used in current pesticide risk  
371 assessments (Bireley et al., 2018; Boyle et al., 2019; Cham et al., 2018; Dietzsch and  
372 Jütte, 2020). The results reported in this study show that the stingless bee is more

373 sensitive to thiamethoxam in terms of LC<sub>50</sub> than the species used as a model in risk  
374 assessments. However, morphological analyses showed that the *A. mellifera* brain  
375 underwent more changes when exposed to thiamethoxam. Although *A. mellifera* is  
376 generally described as more sensitive to pesticides than other bees (Arena and Sgolastra,  
377 2014) or other insect species (Porrini et al., 2003), this does not apply to stingless bees.  
378 Few studies have reported the effects of LC<sub>50</sub> for *M. scutellaris* (Costa et al., 2015;  
379 Lourenço et al., 2012a); however, if we compare these studies with *A. mellifera*'s LC<sub>50</sub>  
380 (Catae et al., 2017; Roat et al., 2013), *M. scutellaris* was more sensitive in all cases.

381 Despite the recent emphasis on non-*Apis* bees, studies that compare *A. mellifera*  
382 with stingless bees are rare (Arena and Sgolastra, 2014; Del Sarto et al., 2014; Jacob et  
383 al., 2019), and none of them compared *A. mellifera* with *M. scutellaris*. In this sense, the  
384 literature has not yet presented a consensus on whether *A. mellifera* is a good model for  
385 representing pollinators in risk assessments. Some authors showed that *A. mellifera* can  
386 be used as a model species for several compounds (Heard et al., 2016) but emphasize  
387 that for some of them, the species is not applicable, and high pesticide concentrations  
388 must be avoided in field applications (Heard et al., 2016; Jacob et al., 2019). However,  
389 the present study demonstrates that lower concentrations can affect stingless bees from  
390 the *Melipona* genus, and the same was found by Del Sarto et al. (2014). Using a meta-  
391 analysis approach, Arena and Sgolastra (2014) reinforce that stingless bees present the  
392 highest sensitivity compared to several other species, even when compared to solitary  
393 bees and bumblebees.

394 Another important aspect of our study is related to the sublethal concentration.  
395 Data from the literature indicated the minimum and maximum levels of thiamethoxam  
396 residues found in the field for pollen (1 – 53 ppb; approximately 0.053 ng/µL) and  
397 nectar (1 – 11 ppb; approximately 0.011 ng/µL) (Krupke et al., 2012; Mullin et al.,

398 2010; Pilling et al., 2013; Stoner and Eitzer, 2012). Regarding the values described in  
399 the literature for nectar, our study analyzed the sublethal concentration for *A. mellifera*  
400 (0.0227 ng/µL) and *M. scutellaris* (0.00543 ng/µL) that can be accessed on the field  
401 when bees collected nectar, showing that low concentrations can be harmful to bees.  
402 Although the LC<sub>50</sub> for *M. scutellaris* is approximately four times lower than the LC<sub>50</sub> for  
403 *A. mellifera*, both species were affected by the insecticide. Sublethal effects can be very  
404 damaging to bees, affecting their neurological and cognitive systems and resulting in  
405 abnormal foraging activities and general behavior (Lu et al., 2020; Prado et al., 2020).

406 Brittain and Potts (2011) further noted that the vulnerability of different bee  
407 species to insecticides depends on some elements, such as body size, age, floral  
408 specialization, flight period, sociality, and nest behavior. Furthermore, the toxicity of  
409 many compounds is related to the exposure time, an observation that is also true for  
410 agrochemicals, which follow this species-dependent pattern. Insecticides remain for  
411 extended periods in the body of the individual, and even in sublethal doses, they are  
412 capable of promoting toxic effects (Prado et al., 2020). Molecules that present a high  
413 average LT<sub>50</sub> can cause greater risks to the animal because they allow it to return to the  
414 colony, and even if the insecticide does not cause the immediate death of the bees, the  
415 dispersion of the toxic molecule is aided by the contamination of other individuals. In  
416 the present study, no significant differences in the survival time of *A. mellifera* were  
417 found. However, exposed *M. scutellaris* had a shorter lifespan than the control group,  
418 with the survival of the exposed group being reduced by almost three times (TL<sub>50</sub> of 11-  
419 days in the control group and TL<sub>50</sub> group exposed to thiamethoxam for 4 days).

420 The morphological analysis using light microscopy allows us to visualize spaces  
421 between Kenyon cells and heavily stained nuclei in both species. Cell spaces indicate  
422 loss of contact between cells and represent tissue disorganization (Grella et al., 2019;

423 Häcker, 2000). Heavily stained nuclei represent chromatin condensation, an indicator of  
424 cell death. Insects that suffer from tissue degeneration commonly present cells with this  
425 characteristic, and when the cell death process begins, this condition is irreversible (Dai  
426 and Gilbert, 1997; Gregorc and Bowen, 1997; Grella et al., 2019; Silva-Zacarin et al.,  
427 2008). According to Häcker (2000), during apoptosis, the most striking changes are  
428 related to the reorganization of the outer edges of the cell, including changes in cell  
429 shape and the disruption of contact with neighboring cells in the tissue. Similar  
430 alterations were observed by other authors for *A. mellifera* and *S. postica* (Azevedo et  
431 al., 2020; Ferreira et al., 2013; Grella et al., 2019; Jacob et al., 2014; Oliveira et al.,  
432 2013; Tavares et al., 2015). Regarding nuclear chromatin, its high condensation may  
433 indicate low transcriptional activity for both species (Häcker, 2000), and according to  
434 Wyllie (1981) and Silva-Zacarin (2008), it can also indicate cell death. Since the  
435 condensation of nuclear chromatin and irregular nuclear shape were more significant for  
436 *A. mellifera* (ultrastructural results), perhaps the two species were in different stages of  
437 cell death (Cavalcante and Cruz-Landim, 2004; Häcker, 2000; Silva-Zacarin et al.,  
438 2007). Another important point that can justify this difference is related to the LC<sub>50</sub>  
439 because *A. mellifera* was exposed to a higher concentration than *M. scutellaris*.  
440 However, to confirm these hypotheses, further studies must be performed.

441 Another characteristic related to cell death is the ultrastructure of the  
442 mitochondria. Mitochondria may remain physically intact during the early stages of cell  
443 death, but the loss of mitochondrial matrix content can also be observed in cells that  
444 undergo this process (Häcker, 2000). When cell death-inducing signals reach the  
445 mitochondria, there are alterations in membrane permeability, resulting in the release of  
446 cell death-activating proteins into the cytoplasm and interruption of ATP synthesis  
447 (Grivicich et al., 2007; Loeffler and Kroemer, 2000). Some of these cell death traits

448 were observed in the mitochondria of *A. mellifera* (mitochondrial dilation and  
449 membrane rupture) and *M. scutellaris* (loss of crest and electrolyzed regions in the  
450 matrix), indicating that after an 8-day exposure, thiamethoxam is capable of inducing  
451 alterations in mitochondria. Moreover, the release of neurotransmitters from synapses  
452 requires large amounts of energy, so it is essential that mitochondria function normally  
453 (Chen and Chan, 2006). As there is a dependent relationship between neurons and their  
454 mitochondria, the mitochondrial alterations observed in the present study may indicate  
455 the functional impairment of these neurons.

456 The mushroom body is associated with memory and learning processes, so  
457 damage to this structure can lead to disorientation and impair the foraging activity of  
458 bees (Aliouane et al., 2009; Decourtye et al., 2004). Aliouane et al. (2009) observed that  
459 treatments with a sublethal concentration of thiamethoxam led to decreases in the  
460 performance of olfactory learning and memory 24 hours after the learning activity in *A.*  
461 *mellifera*; as a result, the learning performance of the bees decreased. Although foraging  
462 behavior was not analyzed in this study, the tissue disorganization and cellular  
463 alterations found in *M. scutellaris* could lead to the same impairment in foraging  
464 activity, affecting the pollination of crops and wild plants performed by these bees.

465 Additionally, despite having similar sizes, *A. mellifera* and *M. scutellaris* present  
466 differences from the individual to the social level that interfere in the response presented  
467 by both species when exposed to the same insecticide. In general, *A. mellifera* hives can  
468 reach up to 50,000 individuals against 2,000 individuals for *M. scutellaris* (Cham et al.,  
469 2018; Rosa-fontana et al., 2020). Furthermore, *A. mellifera* queens can oviposit  
470 approximately 1,000 eggs per day, while *M. scutellaris* queens lay approximately 22  
471 eggs (Cham et al., 2018). Thus, the sublethal effects of thiamethoxam can be more

472 harmful and result in more severe consequences at the colony level for *M. scutellaris*  
473 than for *A. mellifera*.

474 **5. Conclusions**

475 Foraging stingless bees are more sensitive to thiamethoxam because they have  
476 lower oral toxicity values (LC<sub>50</sub>). Perhaps cytological changes in *M. scutellaris* could  
477 occur in different physiological pathways when compared to *A. mellifera*. However,  
478 further analysis is necessary to understand how thiamethoxam acts in both species.  
479 Moreover, a decrease in survival time was observed in *M. scutellaris*, evidencing that  
480 the insecticide may have caused biochemical alterations that led to the death of the  
481 individuals. In addition, the lower cytotoxicity of the stingless bee can lead to greater  
482 indirect contamination of the colony when receiving contaminated workers after  
483 foraging, which could have relatively greater mortality impacts to *M. scutellaris*  
484 colonies because they are often much smaller than *A. mellifera* colonies. Therefore, our  
485 study showed that *A. mellifera* may not be the most appropriate model to determine the  
486 toxicity of insecticides in stingless bees. Therefore, reinforcing the argument for  
487 additional toxicity studies using stingless bees and including this information to support  
488 regulatory decisions is needed to support risk assessments for the conservation of bee  
489 biodiversity and the essential pollinating services it provides.

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### 765 Author Contributions Statement

766 L. M – He performed all bioassays with *M. scutellaris*, prepared all figures and wrote  
767 the text.

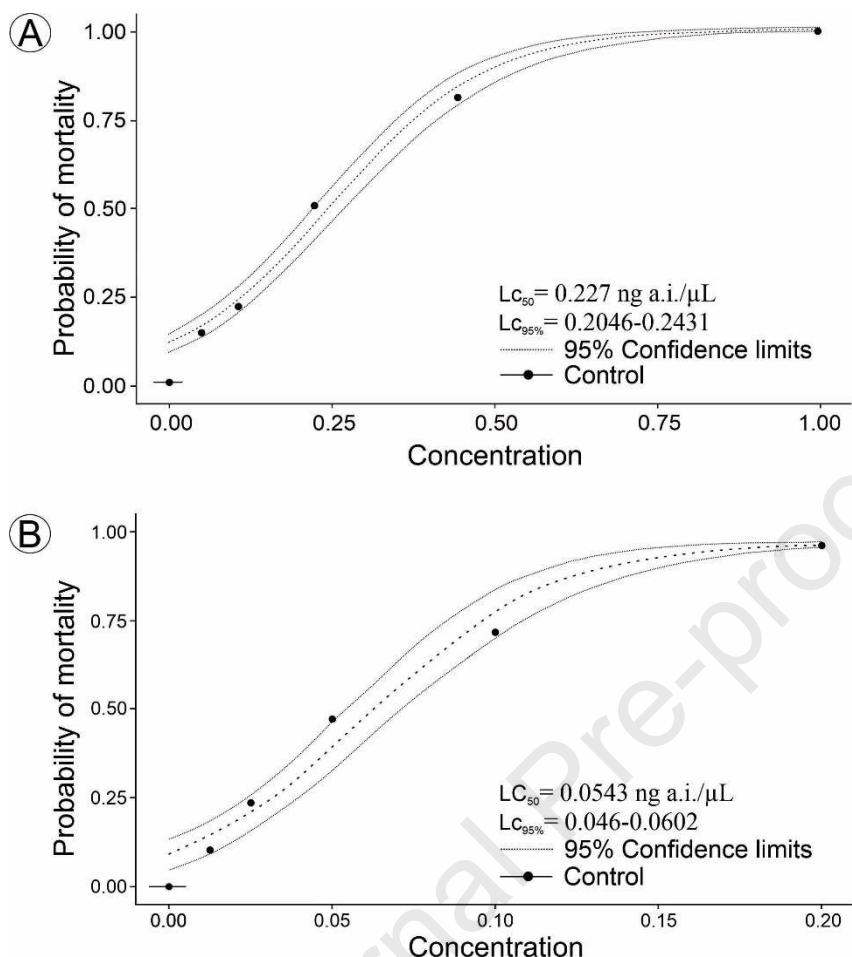
768 A. L. M. R - She performed all bioassays with *A. mellifera*.

769 O. M - Project supervisor and provided the physical structure of laboratories.

770 J.B.M – Performed statistical analysis and interpretation and discussion of results.

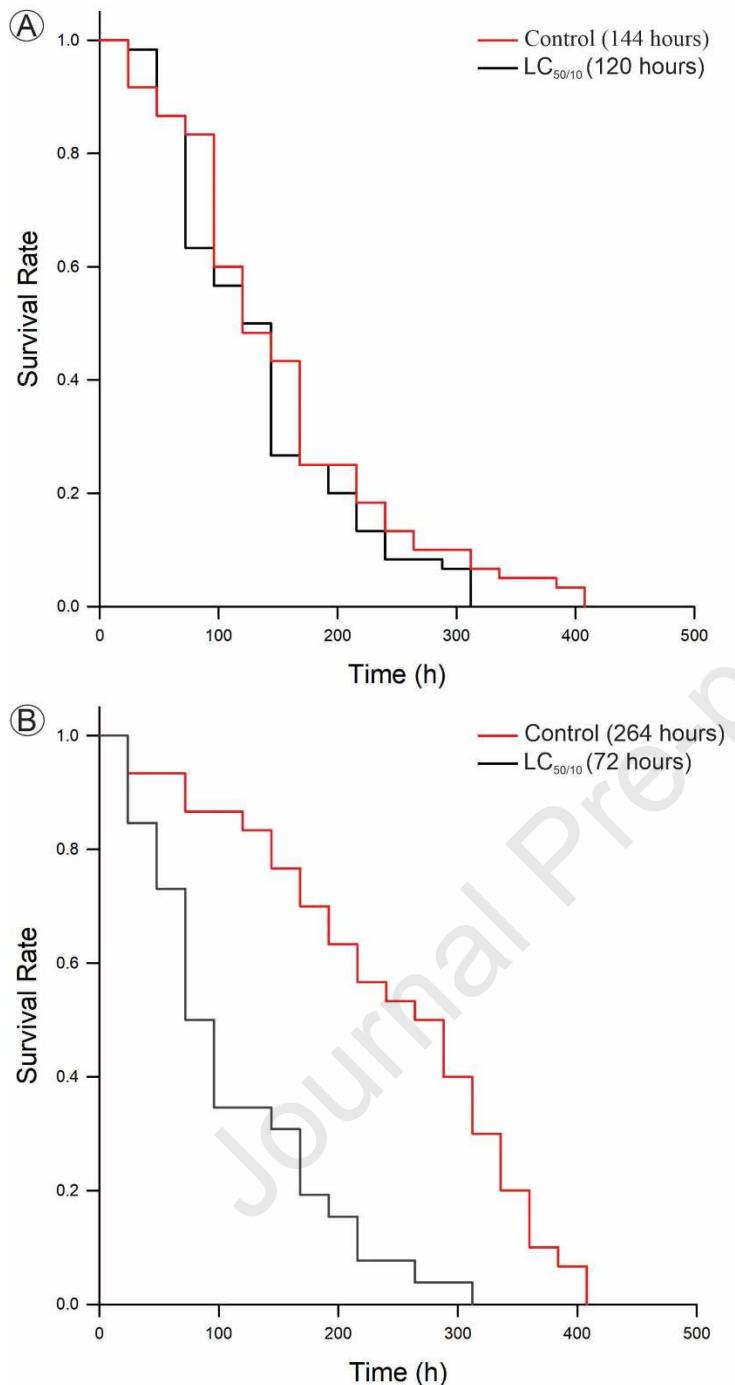
771 T. C. R - Oriented theoretical and practical studies.

772 All authors reviewed the manuscript.

773 **Figures**

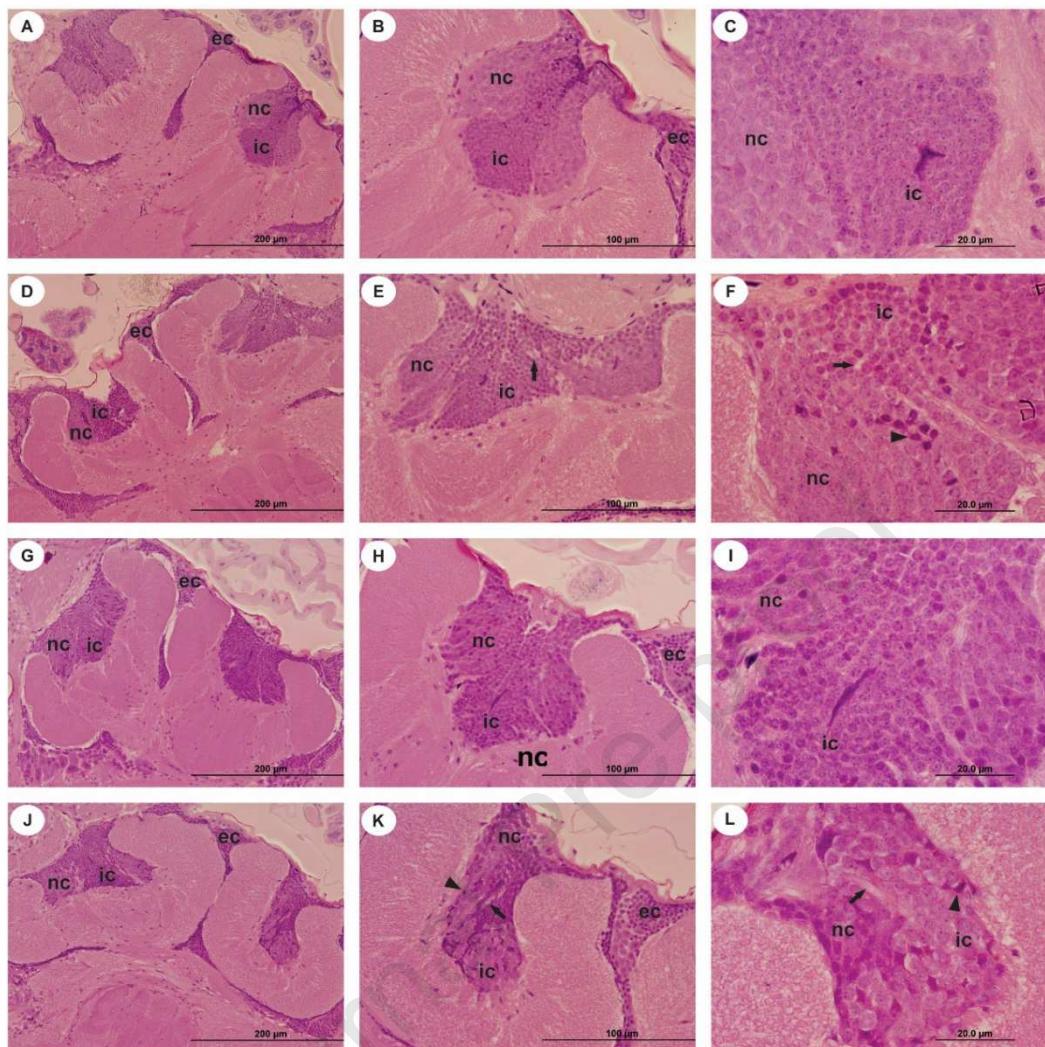
774

775 Figure 1 – Mortality of *A. mellifera* and *M. scutellaris* after the ingestion of food  
 776 contaminated with thiamethoxam. A - The assay was carried out with a completely  
 777 randomized design using five concentrations of the insecticide, dividing each group into  
 778 3 replicates of 20 forager bees per concentration. n = 360 forager bees of *A. mellifera*  
 779 ( $LC_{50} = 0.227 \text{ ng a.i./}\mu\text{L of diet (24 h); 95\% confidence interval} = 0.1460-0.3211 \text{ ng}$   
 780 a.i./  $\mu\text{L of diet; degree of freedom} = 4$ ; Thu - Square = 0.5498). B - The assay was  
 781 carried out with a completely randomized design using five concentrations of the  
 782 insecticide, dividing each group into 3 replicates of 10 forager bees per concentration. n  
 783 = 180 forager bees of *M. scutellaris* ( $LC_{50} = 0.0543 \text{ ng a.i./}\mu\text{L of diet (24 h); 95\%}$   
 784 confidence interval = 0.0425–0.0696 ng a.i./  $\mu\text{L of diet; degree of freedom} = 4$ ; Thu -  
 785 Square = 1.3148).



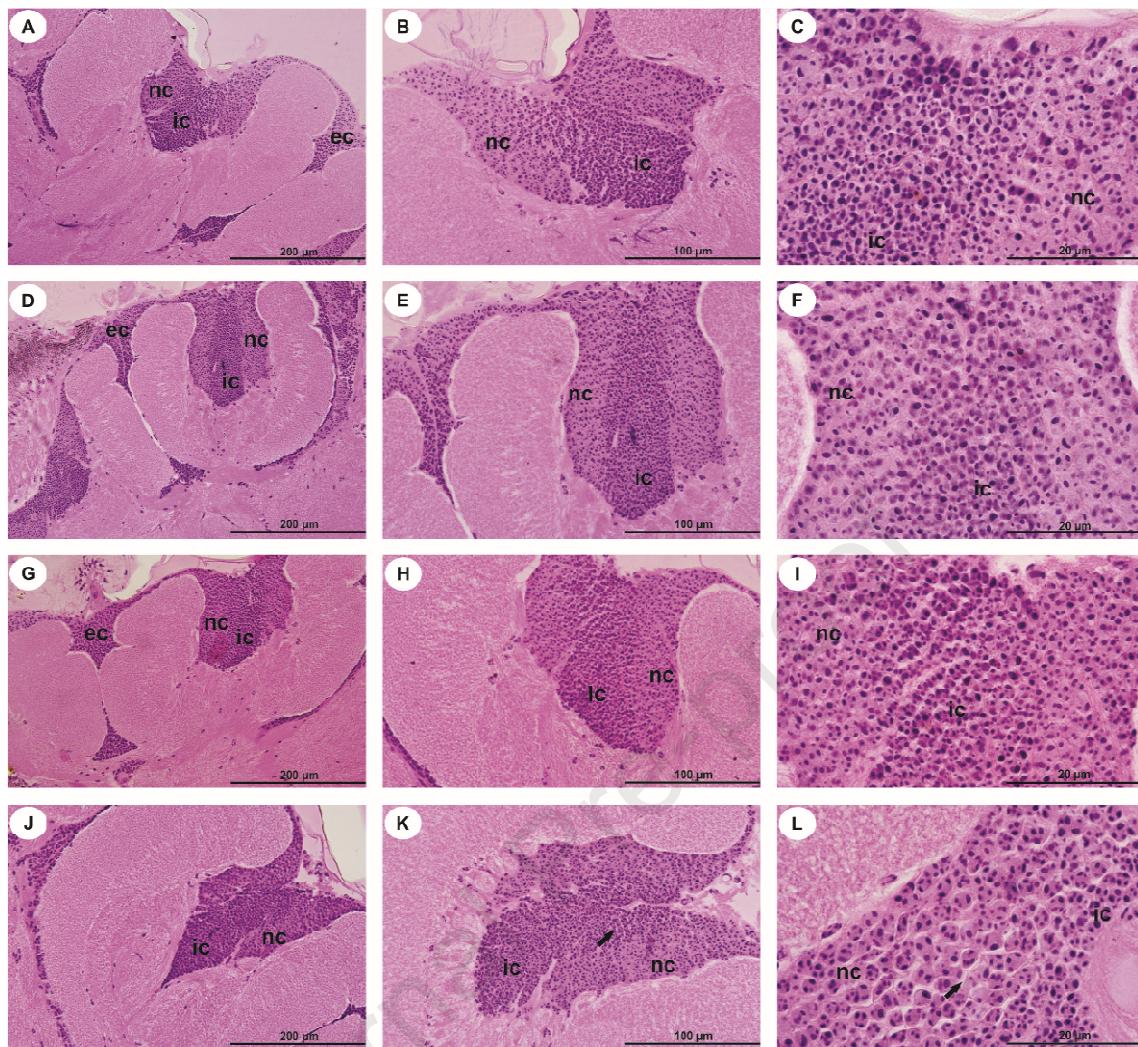
786

787 Figure 2 - Survival rates of *A. mellifera* and *M. scutellaris* forager bees after the  
 788 ingestion of food contaminated with thiamethoxam at a sublethal concentration. A- The  
 789 average lethal time for *A. mellifera* was 144 hours in the control groups and 120 hours  
 790 at an LC<sub>50/10</sub> of 0.0227 ng a.i./μL syrup. B - Average lethal time for *M. scutellaris* was  
 791 264 hours in the control groups and 72 hours at LC<sub>50/10</sub> 0.00543 ng a.i./μL syrup.



792

793 Figure 3 - Histological sections of the mushroom body of *A. mellifera* stained with HE,  
 794 with and without exposure to thiamethoxam. A, B and C – The mushroom body of a  
 795 honeybee from the control group showing no alterations to this structure on day one. D,  
 796 E and F – The LC<sub>50/10</sub> groups exposed to thiamethoxam for 1 day showed an increase in  
 797 the spacing between the Kenyon cells of the mushroom body (arrows). G, H and I - The  
 798 mushroom body of a honeybee from the control group showing no alterations in this  
 799 structure on day eight. J, K and L - The LC<sub>50/10</sub> groups exposed to thiamethoxam for 8  
 800 days with disorganized Kenyon cells (arrowheads). **nc** = non-compact cells; **ic** = inner cells  
 801 and **ec** = compact external cells; arrowheads = strongly stained cells.



802

803 Figure 4 - Histological sections of the mushroom body of *M. scutellaris* stained with  
 804 HE, with and without exposure to thiamethoxam. A, B and C - The mushroom body of a  
 805 honeybee from the control group showing no alterations to this structure on day one. D,  
 806 E and F – The LC<sub>50/10</sub> groups exposed to thiamethoxam for 1 day showed no evident  
 807 alterations. G, H and I – The mushroom body of a honeybee from the control group  
 808 showing no alterations to this structure on day eight. J, K and L - The groups exposed to  
 809 LC<sub>50/10</sub> for 8 days with spacing between the Kenyon cells (arrows). **nc** = non-compact  
 810 cells; **ic** = inner cells and **ec** = compact external cells.

811

812

813

814   **Table 1-** Spaces between Kenyon cells in *A. mellifera* and *M. scutellaris* when exposed to  
 815   thiamethoxam.

Day	Treatment	<i>Apis mellifera</i>	<i>Melipona scutellaris</i>
1st	Control	0.59±0.01 a B	0.50±0.01 b C
	Thiamethoxam	0.86±0.02 a A	0.77±0.01 a AB
8th	Control	0.63±0.03 a B	0.68±0.03 a B
	Thiamethoxam	1.02±0.07 a A	0.81±0.02 b A

816   Means followed by the same capital letter (comparing columns) and lower case (comparing  
 817   rows) do not differ according to the Tukey test (p = 0.05).

818

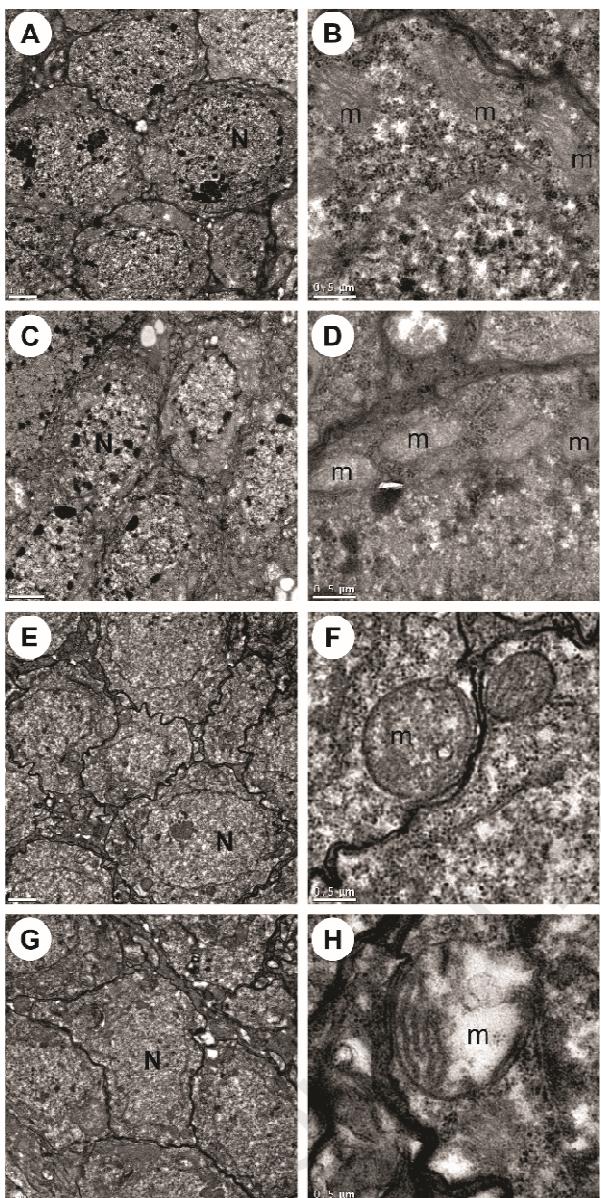
819   **Table 2 -** Condensation of nuclear chromatin area in *A. mellifera* and *M. scutellaris* when  
 820   exposed to thiamethoxam.

Day	Treatment	<i>Apis mellifera</i>	<i>Melipona scutellaris</i>
1st	Control	0.21±0.02 a C	0.13±0.01 b C
	Thiamethoxam	0.33±0.02 a BC	0.33±0.01 a B
8th	Control	0.27±0.01 a AB	0.28±0.01 a B
	Thiamethoxam	0.47±0.06 a A	0.67±0.09 a A

821   Means followed by the same capital letter (comparing columns) and lower case (comparing  
 822   rows) do not differ according to the Tukey test (p = 0.05).

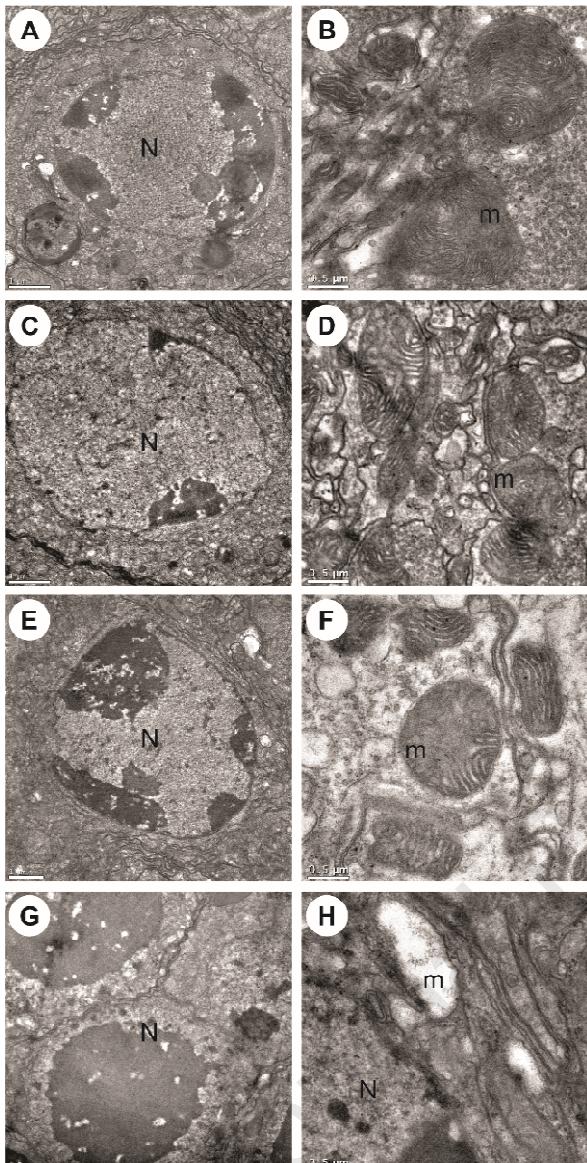
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825

826 Figure 5 - TEM of the mushroom body of *A. mellifera* with and without exposure to  
 827 thiamethoxam. A and B - Ultrastructural characteristics of the 1-day control groups  
 828 remained typical, with the presence of mitochondria (m) and nuclei (N) with a spherical  
 829 shape. C and D – 1-day LC<sub>50/10</sub> group, Kenyon cells with a change in the cellular shape  
 830 (N). E and F - Ultrastructural characteristics of the 8-day control groups remained  
 831 typical, with the presence of mitochondria (m) and nuclei (N) with a spherical shape. G  
 832 and H – 8-day LC<sub>50/10</sub> group with alteration to the cellular shape (N), dilation of  
 833 mitochondria (m), and rupture of the membranes to the cytoplasm.



834

835 Figure 6 - TEM of the mushroom body of *M. scutellaris* with and without exposure to  
 836 thiamethoxam. A and B - Ultrastructural characteristics of the 1-day control groups  
 837 remained typical, with the presence of mitochondria (m) and nuclei (N) with a spherical  
 838 shape. C and D – Kenyon cells of bees exposed to thiamethoxam LC<sub>50/10</sub> for 1 day with  
 839 no alterations. E and F - Ultrastructural characteristics of the 8-day control groups  
 840 remained typical, with the presence of mitochondria (m) and nuclei (N) with a spherical  
 841 shape. G and H – 8-day LC<sub>50/10</sub> group with mitochondria (m) with loss of crest and  
 842 presence of electrolytic regions in the matrix, nuclei (N) with an irregular shape and an  
 843 increase in the presence of heterochromatin.

## Highlights

- M. scutellaris* is more sensitive to thiamethoxam than the *A. mellifera*
- Survival time reaffirms differences in sensitivity among species
- Sublethal doses were able to cause several cellular alterations on both species
- Cellular alterations take longer to appear in *M. scutellaris* than in *A. mellifera*

**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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