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Can the exposure of *Apis mellifera* (Hymenoptera, Apiadae) larvae to a field concentration of thiamethoxam affect newly emerged bees?



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27 **Abstract**

28 The use of insecticides on crops can affect non-target insects, such as bees. In addition to the adult
29 bees, larvae can be exposed to the insecticide through contaminated floral resources. Therefore, this
30 study aimed to investigate the possible effects of the exposure of *A. mellifera* larvae to a field
31 concentration of thiamethoxam (0.001 ng/µL thiamethoxam) on larval and pupal survival and on
32 the percentage of adult emergence. Additionally, its cytotoxic effects on the digestive cells of
33 midgut, Malpighian tubules cells and Kenyon cells of the brain of newly emerged *A. mellifera* bees
34 were analyzed. The results showed that larval exposure to this concentration of thiamethoxam did
35 not influence larval and pupal survival or the percentage of adult bee emergence. However, this
36 exposure caused ultra-structural alterations in the target and non-target organs of newly emerged
37 bees. The digestive cell of bees that were exposed to the insecticide exhibited a basal labyrinth
38 without long and thin channels and compromised mitochondria. In Malpighian tubules cells,
39 disorganized basal labyrinth, dilated mitochondria with a deformed shape and a loss of cristae, and
40 disorganized microvilli were observed. The results showed that the exposed bees presented Kenyon
41 cells with alterations in the nucleus and mitochondria. These alterations indicate possible tissue
42 degeneration, demonstrating the cytotoxicity of thiamethoxam in the target and non-target organs of
43 newly emerged bees. Such results suggest cellular organelle impairment that can compromise
44 cellular function of the midgut cells, Malpighian tubules cells and Kenyon cells, and, consequently,
45 can compromise the longevity of the bees of the whole colony.

46

47 **Keywords:** Bees; neonicotinoid; ulstrastrucuture; midgut; Malpighian tubules; mushroom body.

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53 **1. Introduction**

54 Over the past decade, there has been increasing concern about the global decline of
55 pollinators. Many studies have shown the decline of honeybee populations, and the protection of
56 these pollinators is being implemented in many countries (Potts et al., 2016). Colony losses
57 observed in some parts of the world can be due to pathogens (Neumann and Carreck, 2010),
58 insecticides (Henry et al., 2012), weather, habitat loss (Potts et al., 2010; vanEngelsdorp and
59 Meixner, 2010), or interactions among these factors (Goulson et al., 2015). At the same time,
60 growing evidence shows that insecticides have inevitably caused adverse behavioral and
61 physiological effects on individual bees and colonies (Henry et al., 2012; Di Prisco et al., 2013).

62 Not only do forager bees come into contact with toxic substances that are present in the
63 environment, but also those who perform intracolonial activities and larvae that can feed on pollen
64 and nectar that have been contaminated with insecticides. Therefore, it is necessary to understand
65 the possible consequences of the exposure of larvae to xenobiotics and how it can affect bee
66 development (Rortais et al., 2005; Desneux et al., 2007; Blacquière et al., 2012).

67 Neurotoxic insecticides such as neonicotinoid stand out among the most used pesticides
68 today (Tomizawa; Casida; 2003). A member of this class of insecticides is thiamethoxam, which is
69 used on various crops, including sugarcane, citrus, coffee, rice and pineapple, via an aerial or
70 terrestrial application (Nondillo et al., 2007).

71 Neonicotinoids acts systemically in the plant and can contaminate the pollen and nectar that
72 are used by bees as food resources (Bonmatin et al., 2003). Thus, through the metabolism route,
73 the insecticide is ingested along with the food and comes into contact with non-target organs
74 (midgut and Malpighian tubules) besides reaching the target organ (brain).

75 The midgut is a non-target organ of thiamethoxan whose functions include the digestion and
76 absorption of food (Kakamand et al., 2008; Cruz et al., 2010; Oliveira et al., 2013). The digestive
77 system of the bees is divided into three regions: anterior intestine; midgut, and posterior intestine.
78 The midgut is composed of three cell types: digestive, endocrine and regenerative cells. Digestive

79 cells are the most abundant and produce digestive enzymes as well as absorb digestion products
80 (Neves et al., 2003; Cruz-Landim, 2009).

81 After the passage of the contaminated food through the intestine, the active ingredient
82 reaches the hemolymph and can be transported until to target organ (the brain) as well as be
83 absorbed by the Malpighi tubules for excretion. The excretory system is primarily responsible for
84 maintaining homeostasis. In insects, it is most often composed of a variable number of Malpighi
85 tubules, which play an important role in the detoxification process since it actively promotes the
86 elimination of substances that have not yet been metabolized and/or are in excess in the body
87 (Chapman, 1998).

88 Insecticides absorbed in midgut by oral administration will be translocated for hemolymph,
89 which is immediately bombed to head by dorsal heart and the great amount of absorbed insecticide
90 reach very fast the brain. According to Antunes-Kenyon and Kennedy (2001), thiamethoxam acts as
91 an agonist for nicotinic acetylcholine receptors. Thus, acetylcholine is mimicked by the molecule,
92 which binds to the nicotinic receptor site and block it. Due to this blockage, there is a buildup of
93 acetylcholine, thus causing paralysis and death (Rancan et al., 2006).

94 With the abnormally extended activation of acetylcholine receptors, the central nervous
95 system enters a state of hyperexcitability due to the continuous and uncontrolled transmission of
96 nerve impulses. The symptoms resulting from the neonicotinoid intoxication include nervous
97 system collapse, tremors, and death (Faria, 2009).

98 In the central nervous system, the mushroom body consists of innumerable neurons, such as
99 the Kenyon cells, which form the chalices with their dendrites, the peduncle and the lobes (α , β , and
100 γ) with their axons (Farris, 2005; Fahrbach, 2006). As paired structures located symmetrically on
101 each side of the protocerebrum, the mushroom bodies are described as being the center of the neural
102 basis for the storage and processing of olfactory information (Davis, 2001; Gerber; Tanimoto;
103 Heisenberg, 2004); it is also involved in other forms of learning (Liu et al., 1999; Mizunami;
104 Weibrech; Strausfeld, 1993).

105 As the brains are the target of the neonicotinoid insecticides, several studies have shown that
106 these compounds can affect the behavior of bees. Several behavioral changes are observed,
107 including changes in olfactory memory, loss of orientation, and trouble in foraging activities
108 (Decourtey et al., 2003; Decourtey et al., 2004; Colin et al., 2004; Yang et al., 2008; SchneideR et
109 al., 2012).

110 Africanized *A. mellifera* bees are important pollinators that maintain the biodiversity of the
111 ecosystem, which enabled the production of diverse cultures and is essential for human life as well
112 as for the economy of the country. However, due the increasing agricultural activities in recent
113 times, the use of pesticides has multiplied in order to control pests and have likely affected the bees,
114 which are non-target insects.

115 Most studies about the effects of pesticides on bees emphasize the adult individuals present
116 in the colony; therefore, there is currently a lack of studies regarding the larvae and the possible
117 consequences that exposure during the larval stage can have on their adult stage, because the larvae
118 is tasked with ensuring the viability and survival of the colony.

119 Fipronil is an example of an insecticide that can cause toxic effects on the organs of bees,
120 changing several structures that are important for the correct functioning of the body of the insect.
121 Cruz et al. (2010) found that sublethal doses of fipronil caused alterations at the ultrastructural level
122 in the midgut, Malpighian tubules and silk glands of *A. mellifera*. At low levels (1 ppm), dimethoate
123 decreases the foraging activity of bees (Waller et al., 1979) and may, together with malathion, lead
124 to morphogenic defects in adults that were exposed to the product during the larval phase (Atkins;
125 Kellum, 1986), thus affecting the ability of adult bees to perform their tasks, such as foraging. Jacob
126 et al. (2014) analyzed the sublethal effects of the insecticide fipronil in the mushroom bodies of
127 stingless *Scaptotrigona postica* worker bees and observed through TEM analysis the presence of
128 ultrastructural alterations in Kenyon cells from bees following both oral and topical exposure that
129 are characteristic of cellular death.

130 Therefore, it is important to determine if the exposure of Africanized honey bee larvae to a
131 field concentration of thiamethoxam can alter the survival of bees during the larval and pupal stage
132 as well as the percentage of adult bee emergence. In addition, this study aimed to evaluate the
133 cytotoxicity of thiamethoxam to the midgut, Malpighian tubules and mushroom bodies of newly
134 emerged bees after exposure during the larval stage to this pesticide. Through this analysis, it is
135 possible to report the morphological alterations in the tissues of these bees, which will contribute to
136 the understanding of the effects of thiamethoxam in non-target and target organs, and therefore, aid
137 in the creation of strategies and arguments to ensure the preservation and protection of bees.

138

139 **2. Material and methods**

140

141 **2.1. Honey bee breeding and collection**

142 Larvae of Africanized *A. mellifera* were collected from the apiary of the Institute of
143 Biosciences of Rio Claro in the Department of Biology at the University of São Paulo State
144 (UNESP) in Rio Claro, SP, Brazil. Three colonies in ideal conditions for collection were selected.
145 The honeybee larvae were reared *in vitro* using the method described by Aupinel et al (2005; 2007)
146 and the recommendations of the Organisation for Economic Co-operation and Development
147 (OECD, 2013).

148 A 48-well microplate was filled with sterile dental cotton impregnated with 500 µL of an
149 aqueous solution containing 15% glycerol and 0.2% sodium dichloroisocyanurate, with the purpose
150 of maintaining humidity and avoiding possible contamination. A plastic queen-starter-cell was
151 placed in the interior of each well, which had been previously sterilized for 30 minutes with the
152 same sodium dichloroisocyanurate solution and dried in ultraviolet light. To obtain first instar
153 larvae, empty combs were placed inside a beehive for queen laying. A total of 96 larvae from each
154 of the 3 different colonies were used, totaling 288 larvae for each group (exposed and control).
155 Thus, 576 first instar larvae (no more than 24 h old) were collected and transferred to the plastic

156 queen-starter-cell containing 20 µL of diet A (see below). Microplates containing the plastic queen
157 starter cells were kept throughout the experiment in a sealed box at a temperature of 34 ± 2°C and a
158 relative humidity of 90 ± 5% in the dark. The larvae were fed for 6 days.

159

160 2.2 Larval feeding

161 Three types of diets (A, B and C) containing different concentrations of nutrients were used
162 to meet the nutritional requirements of each larval stage.

163 On the first day of *in vitro* rearing, each larva was fed with 20 µL of diet A containing 50%
164 (w/w) royal jelly and 50% (w/w) of an aqueous solution (distilled water) containing 24% sugar (D-
165 glucose and D-fructose) and 2% yeast extract. On the 2nd day, the larvae were not fed, in
166 accordance with the method adopted and validated by Aupinel et al (2005, 2007). This period was
167 necessary in order to acclimate the larvae to laboratory conditions. On the third day, the larvae were
168 fed with 20 µL of diet B containing 30% sugar (D-glucose and D-fructose) and 3% yeast extract; or
169 36% sugar (D-glucose and D-fructose) and 4% yeast extract. For the fourth, fifth and sixth days, the
170 larvae were fed, respectively, 30 µL, 40 µL and 50 µL of diet C, which contained 36% sugar (D-
171 glucose and D-fructose) and 4% yeast extract.

172 After the sixth day (d-6), during the pupation period (d-7 to d-15), the temperature was
173 maintained by the RH was altered to 80% to simulate the conditions of the colony. On d-15, the
174 queen cells were removed from the plates and placed inside plastic pots containing food that
175 consisted of sugar and honey 1:1 (w/w) and water. The pots were conditioned in a BOD incubator at
176 an RH of 70%. Emergent bees were defined as the bees that left the interior of the queen cells.

177

178 2.3. Acute sublethal exposure to thiamethoxam

179 For the sublethal exposure of bees, a concentration of 0.001 ng/µL of thiamethoxam was
180 chosen. This concentration was selected based on the residual amounts found in the field; for
181 example, on the nectar and pollen, which have values of approximately 1-53 µg/kg (Mullin et al.,

182 2010; Stoner; Eitzer, 2012; Pilling et al., 2013; Krupke et al., 2012). On d-4 of the experiment, the
183 larvae were exposed to thiamethoxam through 30 µL of contaminated diet. For contamination, a
184 stock solution of 1.000 ng of thiamethoxam/µL of acetone + water in the ratio of 1:9 was made.
185 Subsequently, from the stock solution, a cascade dilution was done using the previously prepared
186 diet C as the solvent; a 3 x concentrated solution was achieved, which was directly added to the
187 larval food (diet C) to obtain a concentration of 0.001 ng/µL. The larval intake was 0.03 ng/µL.
188 Larval and pupal mortality was observed daily with the aid of a stereomicroscope.

189

190 2.4. Analisys of larval and pupal survival and porcentage of adult emergence

191 After the acute sublethal exposure to thiamethoxam during the larval phase and
192 metamorphosis, the larval and pupal mortality was observed daily with the aid of a
193 stereomicroscope. The percentage of emergence was also observed. The data were analyzed using
194 the statistical R Development Core Team software (2017). To evaluate the influence of
195 thiamethoxam on larval and pupal survival, the data were analyzed using the Cox proportional
196 hazards regression model (survival package), and a significant difference was considered when $P <$
197 0.05.

198 The influence of thiamethoxam on emergence success was analyzed by a chi-square test R
199 function (Chisq.test) that makes pair-wise comparisons between the exposed and control groups,
200 with 1 df and $P < 0.001$.

201

202 2.5. Transmission Electron Microscopy (TEM) of organs of newly emerged bees

203 After the acute sublethal exposure to thiamethoxam during the larval phase and at the end of
204 the post-embryonic development, 12 newly emerged worker bees of Africanized *A. mellifera* were
205 collected from the exposed and the control groups to ultrastructural analysis of the organs.

206 Midguts, Malpighian tubules and brains of exposed and control bees were removed in buffer
207 solution containing 20 mM Na₂HPO₄/ KH₂PO₄, pH 7.4 1 130 mM of NaCl (modified of Dade,

208 2009) and fixed in modified Karnovsky (4% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M
209 sodium cacodylate buffer, pH 7.2), during 2 h at 4C. Once fixed, the organs were washed in the
210 same buffer and postfixed in 1% osmium tetroxide in the same buffer for 2 h at room temperature
211 and again washed in the buffer. The post-fixed organs were contrasted in 2% uranyl acetate in 10%
212 acetone for 2 h and then dehydrated in a standard acetone series. The material was embedded in
213 Epon–Araldite resin. Ultrathin sections cut on a Porter Blum ultramicrotome MT2 were stained
214 with lead citrate for 15 min. The material was examined under the transmission electron microscopy
215 (JEOL-JEM1011), no NAP/MEPA - Núcleo de Apoio à Pesquisa em Microscopia Eletrônica
216 Aplicada a Agricultura - ESALq em Piracicaba-SP.

217

218 3. Results

219

220 3.1 Analysis of larval and pupal survival as well as the percentage of emergence

221 It was observed that there was no significant difference in larval survival between the group
222 exposed to thiamethoxam and the control group, as shown in Figure 1 (Cox model $P > 0.05$).

223 Figure 2 shows the pupal mortality during the bioassays. The group exposed to
224 thiamethoxan during the larval phase did not show a significant difference in pupal survival
225 compared to the pupae from the control group (Cox model $P > 0.05$).

226 The percentage of adult bee emergence was calculated from the number of pupae in each
227 experimental group. According to the results, it was observed that the exposed group and control
228 group showed a similar percentage of adult emergence, and there was no significant difference in
229 this parameter between the exposed group and the control group, as shown in Figure 3 Chi-square
230 test with 1 df * $P \leq 0.001$.

231

232 3.2 Transmission electron microscopy

233

234 3.2.1 Midgut

235 The analysis performed in the digestive cells of midgut of newly emerged *A. mellifera* bees
236 reared *in vitro* showed that the control group exhibited a midgut with the typical ultrastructural
237 characteristics of this organ (Figure 4A). The digestive cells showed nuclei with a shape from oval
238 to spherical, the interior of the nucleus showed decondensed chromatin (euchromatin) while some
239 regions presented condensed chromatin (heterochromatin) around the nuclear envelope, and an
240 evident nucleolus was observed (Figure 4A). The mitochondria possessed cristae, and its
241 morphology remained preserved (Figure 4C); additionally, the basal labyrinth exhibited a typical
242 morphology with long and thin channels (Figure 4E).

243 However, the ultrastructure of the digestive cells of newly emerged *A. mellifera* bees after
244 exposure to thiamethoxam during the larval phase showed alterations in the ultrastructure of this
245 organ when compared to the control group (Figure 4B). The nucleus became irregular (Figure 4B),
246 the mitochondria were dilated with a loss of cristae and matrix (Figure 4D), and the basal labyrinth
247 showed the absence of long and thin channels (Figure 4F).

248

249 3.2.2 Malpighian tubules

250 The Malpighian tubules of bees from the control group showed structural integrity (Figure
251 5A) and spherical-shaped nuclei; the interior of the nucleus was filled with euchromatin with some
252 regions presenting heterochromatin around the nuclear envelope, and the presence of a nucleolus
253 was observed (Figure 5A). Mitochondria exhibited cristae and were found throughout the cell, with
254 a higher concentration in the apical region and within the microvilli (Figure 5E); additionally, intact
255 microvilli were observed (Figure 5G).

256 The Malpighian tubules of bees from the exposed group showed nuclei with similar
257 morphology as that observed in the control group, (Figure 5B). The basal labyrinth was
258 disorganized with a loss of folds in the plasma membrane when compared to the control group, and
259 there were mitochondria observed in the region (Figure 5D). The mitochondria were very dilated

260 and exhibited a loss of cristae (Figure 5F). Parts of the microvillus were lost, showing structural
261 disorganization in this structure (Figure 5H).

262

263 **3.2.3 Mushroom bodies**

264 The mushroom body of newly emerged *A. mellifera* bees reared *in vitro* presented Kenyon
265 cells with typical ultrastructural characteristics (Figure 6A), such as a spherical-shaped nucleus that
266 occupied a large part of the cell; a perinuclear space without expansion; the interior of the nucleus
267 was filled with decondensed chromatin (euchromatin) while some regions presented condensed
268 chromatin (heterochromatin) around the nuclear envelope; the presence of an evident nucleolus
269 (Figure 6C); and elongated mitochondria throughout the cytoplasm with visible cristae (Figure 6E).

270 Newly emerged *A. mellifera* bees after exposure to thiamethoxam during the larval stage
271 showed evident alterations in the ultrastructure of the Kenyon cells when compared to the control
272 group (Figure 6B). The nucleus of these cells showed an irregular shape and small size compared to
273 the control group. The perinuclear space was dilated; however, the interior of the nucleus remained
274 filled by decondensed chromatin (euchromatin) while some regions around the nuclear envelope
275 presented condensed chromatin (heterochromatin), which was similar to the control group (Figure
276 6D). Mitochondrial alterations were also observed, with varied morphology and the loss of some
277 cristae (Figure 6F).

278

279 **4. Discussion**

280

281 During foraging activities, bees may come into contact with insecticides in the field.
282 Through the collection of contaminated floral resources, bees are, thus able to carry insecticide
283 residues into the hive and contaminate the larvae. The results found in the present study showed that
284 during the *in vitro* larval development of Africanized *A. mellifera*, the field concentration of 0.001

285 ng/µL thiamethoxam did not interfere with the survival of larvae and pupae or with the percentage
286 of emerged adult bees.

287 A possible reason for this result is that the larvae were exposed to a field concentration that
288 was low (intake of 0.03 ng/µL per larva), and higher concentrations are required to cause mortality.
289 Tavares et al. (2015) evaluated the sublethal effects of thiamethoxam in larvae, pupae and newly
290 emerged workers of *A. mellifera* exposed to this insecticide during the larval stage. It was observed
291 that the sublethal concentration of 1.44 ng/µL thiamethoxam decreased larval survival, and the
292 concentrations of 1.44 and 0.01 ng/µL thiamethoxam decreased pupae survival and influenced the
293 percentage of emerging bees.

294 There are several studies in the literature regarding the effects of thiamethoxam on adult *A.*
295 *mellifera* bees, but there is still a lack of studies on the effects of thiamethoxam or other insecticides
296 on the development of *A. mellifera* (Cousin et al., 2013; Desneux et al., 2007, Yang et al., 2012).
297 Thus, new studies on this subject are necessary in order to understand the mechanisms involved in
298 larval survival and their tolerance to thiamethoxam.

299 Although there was no interference of the insecticide in the survival of larvae and pupae or
300 in the percentage of adults that emerged, ultrastructural alterations were observed in target and non-
301 target organs of the newly emerged bees after thiamethoxam exposure in the larval stage. These
302 alterations indicate possible tissue degeneration, demonstrating the cytotoxicity of thiamethoxam in
303 the target and non-target organs of newly emerged bees that can compromise cellular function,
304 showing that even though it does not cause changes in survival, field concentrations of this
305 insecticide causes cellular alterations in the bees' organs, which may compromise their viability.

306 It could be that the concentration of 0.001 ng/µL thiamethoxam that was administered
307 during the larval development *in vitro* was low and could not interfere with larval survival and
308 metamorphosis, or larvae may be more tolerant to the insecticide than adult bees. However, in the
309 newly emerged phase, ultrastructural alterations were observed in the organs of these bees, possibly

310 indicating that the transition stages may be vulnerable to insecticides, thus leading to alterations in
311 adulthood.

312 Zhu et al. (2014) studied the effects of chronic exposure of *A. mellifera* larvae to
313 environmental doses of fluvalinate, coumafos, chlorothalonil, and chlorpyrifos. They showed that
314 the unconventional response to toxicity may be a consequence of the time required to accumulate
315 internal concentrations of the insecticide that are sufficient to exert its actions on the targets present
316 in the central nervous system of bees.

317 Thus, although the concentration of 0.001 ng/µL did not alter the survival of the larvae, they
318 may have accumulated the insecticide to levels that were able to cause alteration in the bees' organs
319 in the newly emerged phase.

320 Yang et al. (2012) exposed *A. mellifera* larvae to 0.04 ng of imidacloprid and found that
321 although this dose did not cause larval mortality, it affected memory and learning when they
322 emerged as adult bees. Thus, although larvae appear to be more tolerant to neonicotinoids than adult
323 bees, adverse effects may occur in later stages of development as a result of exposure during the
324 larval period.

325 Thus, it is important to analyze the cytotoxicity of thiamethoxam in target and non-target
326 organs of newly emerged bees after exposure to this compound during the larval phase.

327 Considering that neonicotinoids have a systemic action, all parts of the plant as well as
328 nectar and pollen contain residual traces of these products (Blacquiere et al., 2012); hence, bees can
329 be contaminated through food collection. When the contaminated food is ingested, it comes into
330 contact with the midgut.

331 This organ, even though it is a non-target organ, can also be affected by the insecticide since
332 it is involved in the metabolism of the compound. The midgut, according to Cruz-Landim (2009), is
333 the portion of the digestive tract that is responsible for most of the digestion and absorption of food
334 and is considered the functional stomach in insects.

335 Ultrastructural alterations could be observed in the digestive cells of midgut of newly
336 emerged *A. mellifera* bees after exposure to thiamethoxam during the larval phase. Bees are
337 holometabolous insects and therefore undergo a complete reorganization of organs during
338 metamorphosis, i. e., during pupae phase. In Hymenoptera changes in the midgut begin in the
339 prepupa with the larval epithelium degenerating, leaving only the basement membrane and the
340 regenerative cells. At metamorphosis the larval midgut epithelium is reabsorbed and substituted for
341 cells from regenerative cells (Cruz Landim, 2009). So, the insecticide intake during the larval phase
342 probably is cytotoxic to the regenerative cells in the larval phase. This toxicity reflected in alteration
343 in their differentiation in digestive cells during the metamorphosis. As a consequence, newly
344 emerged bees, showed digestive cells with ultrastructural alteration.

345 In this way, bees of the exposed group presented a basal labyrinth without long and thin
346 channels and compromised mitochondria with a loss of cristae and matrix.

347 It is known that in the digestive cells of the midgut of the insects, the folds of the basolateral
348 membranes form fine and long channels with adhered particles on their cytoplasmic side (Terra,
349 1988); these characteristics were observed in the control group (Santos et al., 1984). These folds of
350 membranes contribute to the concentration of solutes, thus generating an osmotic gradient between
351 the lumen and the compartment and favoring the water absorption (Terra et al., 2006). The
352 basolateral membranes, in addition to their involvement with the trans-epithelial transport of water
353 and solutes, may also possess other functions such as hemolymph trehalose digestion (Azuma and
354 Yamashita, 1985).

355 Thus, these membrane folds are of great importance to the physiology of digestive cells in
356 the midgut. The absence of these long and thin channels in the basal labyrinth, which was observed
357 in the present study within the digestive cells of midgut of exposed bees, may interfere with water
358 absorption, the trans-epithelial transport of water and solutes, and hemolymph trehalose digestion.
359 The absence of long and thin channels can occur due to a loss or reduction of the basal labyrinth

360 and may indicate possible tissue degeneration, thus demonstrating the cytotoxicity of thiamethoxam
361 to this organ.

362 In addition, the group exposed to the insecticide showed mitochondria with ultrastructural
363 alterations, which demonstrate that the metabolic ability of this organelle may be altered by
364 thiamethoxam.

365 The morphology and quantity of cristae in the mitochondria are reflective of the organelle's
366 energy demand (Scheffler, 1999, Mannella, 2006, Zick, et al., 2009). Thus, considering the
367 importance of these cellular structures, the loss of cristae and matrix in the group exposed to
368 thiamethoxam suggests a lack of energy demand and may cause compromised mitochondrial
369 functions.

370 Catae et al. (2014) observed the effects of a sublethal dose of thiamethoxam on the midgut
371 of Africanized *A. mellifera* and verified through ultrastructural analysis that bees exposed for 1 day
372 to the insecticide presented digestive cells with alterations in several organelles. The mitochondria
373 exhibited a decrease in cristae, the rough endoplasmic reticulum showed dilated cisterns that were
374 disorganized and had a smaller number of ribosomes, and the nuclei of these cells were irregular,
375 demonstrating that this neonicotinoid causes alterations in the midgut of these insects, compromise
376 the cell organelles, and alter the physiology of these cells.

377 In the body of the bee, following the metabolism of thiamethoxam, the digested products or
378 the undigested molecule will reach the hemolymph and, subsequently, the brain (target organ of the
379 insecticide) and the Malpighian tubules. This organ is responsible for the maintenance of
380 homeostasis and for the detoxification process that eliminates excess substances, even those that
381 have not been metabolized by the body (Chapman, 1998).

382 Ultrastructural alterations were also observed in the Malpighian tubules of exposed bees.
383 Bees in the group exposed to thiamethoxam exhibited tubules with a disorganized basal labyrinth.
384 According to Cruz-Landim (2009), the basal labyrinth, in the Malpighian tubules, is a structure that
385 is responsible for optimizing the uptake of metabolized substances and for promoting greater

386 contact with hemolymph. Therefore, it is evident that the function of the basal labyrinth has been
387 compromised in the group exposed to thiamethoxam, and these changes had potentially damaged
388 the excretory ability of the Malpighian tubules.

389 In the Malpighian tubules of the exposed bees, the mitochondria were dilated, exhibited a
390 loss of cristae, and possessed a distorted shape. Mitochondrial alterations may have been caused
391 "directly or indirectly" by neonicotinoid. According to Nicodemo et al. (2014) imidacloprid, other
392 insecticide of neonicotinoid class, affects the stage 3 of the cellular respiration process, inhibiting
393 the production of ATP. Mitochondrial alterations observed may have seriously compromised
394 mitochondrial function. Thus, the alterations observed in these organelles in the exposed group are
395 highly detrimental to the whole structure of the organ, interfering in the performance of its normal
396 functions, such as ATP production, and preventing them from performing the metabolic activities
397 that the cells need to survive.

398 Catae et al. (2014) found that on the first day the Africanized bees were exposed to
399 thiamethoxam, the Malpighian tubules exhibited a disorganized basal labyrinth. On the fifth day of
400 exposure, there were alterations in the basal labyrinth as well as the presence of very dilated
401 mitochondria and some alterations in the microvilli. On the eighth day of exposure, there was an
402 almost complete loss of the basal labyrinth of the Malpighian tubules and nuclei with condensed
403 chromatin; additionally, the apical portion of the microvilli was dilated.

404 Considering that bees are holometabolous insects, the insecticide intake by the larvae was
405 stored in the fat body, may be inactivated, since the larva is not affected. As the fat body cells are
406 broken during metamorphosis for release the energetic compounds used in the insect post-
407 embryonic development (Cruz Landim, 2009), the insecticide might be liberated in the
408 haemolymph. Then this compound can reach the brain and the Malpighian tubules affecting their
409 cells morphology. The larval Malpighian tubules are totally reabsorbed and new ones arise during
410 pupation to function in adult (Cruz Landim, 2009). So, during the metamorphosis when the
411 insecticide is released to the haemolymph, it can reach the Malpighian tubules affecting their

412 differentiation and cells morphology. The same occurs to the brain, which structure is reorganized
413 during metamorphosis by neurons proliferation and differentiation.

414 Ultrastructural alterations were observed in the brain; more specifically, in the mushroom
415 body of newly emerged *A. mellifera* bees that were exposed to thiamethoxam during the larval
416 stage.

417 In the present study, the mushroom bodies deserved attention for being related to learning
418 and memory in the bees (Daly; Doyen; Purcell III; 1998; Cruz-Landin, 2009). In insects, these brain
419 regions are centers of multimodal integration and are thus responsible for both receiving and
420 interpreting all of the mechanosensory, gustatory, visual and olfactory information (Zars, 2000;
421 Komischke et al., 2005; kiya et al., 2007).

422 The mushroom bodies of bees in the exposed group presented Kenyon cells with modified
423 cytoplasm, the presence of digestive vacuoles, and the impairment of mitochondria, where cristae
424 loss was observed.

425 Decourtye et al. (2004) observed an increase in the activity of the enzyme cytochrome
426 oxidase (CO) in the mushroom bodies of *A. mellifera* worker bees that were exposed to
427 imidacloprid. In the process of mitochondrial respiration, CO is the terminal enzyme in the electron
428 transport chain. Nicodemo et al. (2014) also showed that in Africanized bees, fipronil and
429 imidacloprid affect the production of ATP in the brain and are inhibitors of mitochondrial
430 bioenergetics, resulting in the failure of cellular respiration.

431 Thus, in this study, the ultrastructural alterations observed in mitochondria show that,
432 similar to imidacloprid, which is also a neonicotinoid with neurotoxic actions, thiamethoxam also
433 impairs mitochondrial functions, thus showing that this class of insecticides can change neural
434 activity by increasing cellular respiration.

435 According to Decourtye et al. (2004), changes in the activity of the central nervous system
436 occur at the same time problems appear in the memory process of the bee. To characterize the brain
437 structures involved in memory processes in invertebrates, the histochemical detection of the

438 enzymatic activity of CO is considered an important tool (Agin et al., 2001; Dégilde et al., 2003;
439 Roat et al., 2013).

440 In addition, the increase in mitochondrial activity can generate ROS, causing oxidative
441 stress in the cells. During the reduction of molecular oxygen, water and ROS are typically formed
442 during mitochondrial respiration. The accumulation of ROS causes damages to cells, carbohydrates,
443 lipids, proteins and nucleic acids (Trushina; McMurray, 2007; Halliwell; Gutteridge, 2015).

444 The cellular degeneration promoted by oxidative stress occurs because of the cytotoxic
445 effects of ROS, which include superoxide anion (O_2^-), hydroxyl radical (OH) and hydrogen peroxide
446 (H_2O_2) (Gutteridge, 1994; Barbosa; Medeiros et al., 2006; Halliwell; Gutteridge, 2015).

447 In the present study, the cytotoxic effects, both the EROs and the neurotoxic effect of
448 thiamethoxam, can also be seen in the ultrastructural alterations observed in the nucleus of Kenyon
449 cells. The bees of the exposed group presented Kenyon cells with an irregular nucleus and a dilated
450 perinuclear space. These nuclear alterations signal cell degeneration, which can, along with the
451 mitochondrial impairment, trigger cell death.

452 The process of cell death can be identified by a series of characteristic cell alterations, such
453 as mitochondrial and nuclear changes (Bowen; Bowen; Jones, 1998). According to Häcker (2000);
454 the loss of mitochondrial matrix content can be observed in cells that are undergoing the process of
455 cell death. Inductive signals of cellular death cause alterations in the permeability of the
456 mitochondrial membranes, resulting in the release of cell death-activating proteins to the cytoplasm
457 and the interruption of ATP synthesis (Grivicich; Regner; Rocha, 2007; Loeffler; Kroemer, 2000).

458 In this study, exposure of larvae to thiamethoxam caused rather evident ultrastructural changes in
459 the mitochondria of newly emerged worker bees. These evidence in addition to the observed
460 nuclear alterations indicate that these cells are in the early stages of cell death. Changes in nuclear
461 morphology are a typical feature of programmed cell death and are related to the decreased activity
462 of cells that will undergo degeneration (Kerr; Wyllie; Currie, 1972; Cruz-Landim; Cavalcante,
463 2003; Silva-Zacarin et al., 2007).

464 Thereby, it is noticeable that the use of low concentrations of the insecticide thiamethoxam
465 may cause sublethal effects in Africanized *A. mellifera*. It is worth mentioning that Yang et al.
466 (2008), through behavioral tests, were able to verify that worker bees that were exposed to sublethal
467 doses of imidacloprid also exhibited alterations in their foraging activities. According to ISHAAAYA
468 et al. (2007), it is probable that the neonicotinoids affect the behavior of the worker bees by directly
469 acting on the acetylcholine and nicotine receptors, which can subsequently cause paralysis,
470 excitement and even death. As the mushroom bodies are associated with both the processes of
471 learning and memory, the damage caused in these structures by several insecticides can lead to
472 disorientation of the bee and can harm their foraging ability, thus jeopardizing the entire colony.

473 The studies mentioned so far demonstrate that different insecticides can induce sublethal
474 effects in Kenyon cells; however, research on the effects of thiamethoxam exposure during the
475 larval stage on the brains of newly emerged bees is still scarce in the literature, making this a
476 pioneer study that showed such effects in the central nervous system and in the non-target organs of
477 Africanized *A. mellifera* bees through ultrastructural analyses. This shows that even in organs that
478 are remodeled during metamorphosis (Cruz-Landim, 2009), the effects of the insecticide are
479 prolonged and remain, allowing for the observation of its consequences in later stages of bee
480 development.

481

482 **5. Conclusions**

483

484 In general, the results obtained in the present study showed that there was no interference of
485 the insecticide thiamethoxam in the survival of both larvae and pupae as well as in the emergence of
486 adult bees. On the other hand, the digestive cells, Malpighian tubules cells and Kenyon cells of the
487 newly emerged bees after exposure to thiamethoxam during the larval phase showed ultrastructural
488 alterations, demonstrating that even the use of a field dose that is considered low (0.001 ng/µL) can

489 cause serious damages to the cells of these organs, that may interfere with the normal function of
490 them, and may alter the behavior of the insect.

491

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493

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496

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798 **Figure Legends**

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800 **Figure 1.** Effect of a field concentration of thiamethoxam on the survival of Africanized *A.*
801 *mellifera* larvae. The data indicate the survival percentage of *A. mellifera* larvae in the days after
802 exposure to thiamethoxam (Cox model $P > 0.05$).

803

804 **Figure 2.** Effect of a field concentration of thiamethoxam on the survival of Africanized *A.*
805 *mellifera* pupae. The data indicate the survival percentage of *A. mellifera* pupae in the days after
806 exposure to thiamethoxam (Cox model $P > 0.05$).

807

808 **Figure 3.** Effect of thiamethoxam on the emergence success of honeybee adults. The percentage of
809 emergence was calculated from the number of pupae in each experimental group: (i) control, $n =$
810 63; and (ii) 0.001 ng/ μ L, $n = 75$. The comparisons between the exposed groups and controls were
811 done with the Chi-square test with 1 df ($P \leq 0.001$). Bars represent the mean \pm standard deviation of
812 3 replicates.

813

814 **Figure 4.** TEM of digestive cells from the midgut of newly emerged *A. mellifera* with or without
815 exposure to thiamethoxam during the larval phase. **A** – Nucleus (n) from a cell of a honeybee in the
816 control group without ultrastructural alterations; **B** – Nucleus (n) with altered shape from a cell
817 from a honeybee in the exposed group; **C** – Well-defined mitochondria (m) with cristae of a cell
818 from a honeybee in the control group; **D** – Mitochondria (m) of a cell from a honeybee in the
819 exposed group showing decreased mitochondrial cristae; **E** – Basal labyrinth (bl) from the control
820 group without morphological alterations; and **F** – Basal labyrinth (bl) of a cell from the group
821 exposed to thiamethoxam, showing the loss of long and thin channels.

822

823 **Figure 5.** TEM of Malpighian tubules of newly emerged *A. mellifera* with or without exposure to
824 thiamethoxam during the larval phase. **A and B** – Nucleus (n) of a cell of a honeybee from the
825 control group and exposed group, respectively, showing no alterations on this structure; **C** –
826 Invaginations of the plasmatic membrane constituting the basal labyrinth of (bl) of a cell from a
827 honeybee in the control group; **D** – Basal labyrinth (bl) of a cell from the exposed group to
828 thiamethoxam, showing the disorganization of the basal labyrinth; **E** – Mitochondria (m) of a cell
829 from a honeybee of the control group; showing a typical structure; **F** – Mitochondria (m) of a cell
830 from a honeybee in the exposed group. Note dilated mitochondria (m), with loss of cristae; **G** –
831 Microvilli (mv) from the control group without morphological alterations; and **H** – Microvilli of a
832 cell from the exposed group to thiamethoxam, showing that part of the microvilli is lost (arrows).

833

834 **Figura 6.** TEM of mushroom bodies of newly emerged *A. mellifera* with or without exposure to
835 thiamethoxam during the larval phase. **A** – General view of a Kenyon cell from a honeybee in the
836 control group without ultrastructural alterations; **B** – General view of a Kenyon cell from a
837 honeybee in the exposed group; **C** – Nucleus (n) of a Kenyon cell from a honeybee in the control
838 group without ultrastructural alterations; **D** – Nucleus (n) with irregular shape of a Kenyon cell
839 from a honeybee in exposed group; **E** – Well-defined mitochondria (m) with cristae of a Kenyon
840 cell from a honeybee in the control group; and **F** – Mitochondria (m) of a Kenyon cell from a
841 honeybee in the exposed group showing the loss of some cristae.

842

Highlights

Thiamethoxam, a neonicotinoid insecticide, can affect non-target insects, as bees

It's effects on bees were investigated after exposure during the larval phase

Larval and pupal survival and percentage of emergence were not altered

Ultrastructural alterations were observed on organs of newly emerged bees

The exposure to thiamethoxam can cause cellular alterations in organs of adult bees.











