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Thiamethoxam and picoxystrobin reduce the survival and overload the hepato-nephrocytic system of the Africanized honeybee

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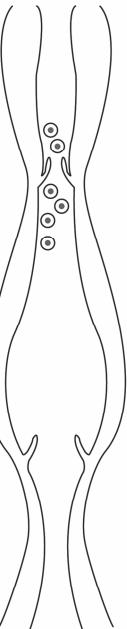
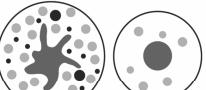
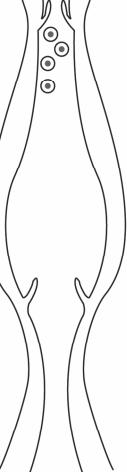
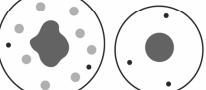
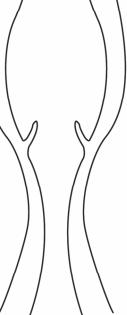
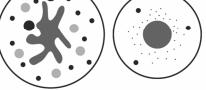
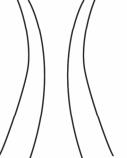
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GROUPS	HEMOCTES	FAT BODY	PERICARDIAL CELLS	DORSAL VESSEL
CTL				
TXT				
PXT				
TXT + PXT/2				

1 **Thiamethoxam and picoxystrobin reduce the survival and overload the**
2 **hepato-nephroocitic system of the Africanized honeybee**

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14 **ABSTRACT**

15 *Apis mellifera* perform important pollination roles in agroecosystems. However, there is often
16 intensive use of systemic pesticides in crops, which can be carried to the colony by forage
17 bees through the collection of contaminated pollen and nectar. Inside the colony, pollen loads
18 are stored by bees that add honey and several enzymes to this pollen. Nevertheless, intra-
19 colonial chronic exposure could induce sublethal effects in young bees exposed to a wide
20 range of pesticides present in these pollen loads. This study was aimed to both determine the
21 survival rate and evaluate the sublethal effects on the hepato-nephrocytic system in response to
22 continuous oral exposure to lower concentrations of neonicotinoid thiamethoxam (TXT) and
23 picoxystrobin fungicide (PXT). Exposure to a single chemical and co-exposure to both
24 pesticides were performed in newly emerged honeybee workers. A significant decrease in the
25 bee survival rates was observed following exposure to TXT (0.001 ng a.i./ μ L) and PXT
26 (0.018 ng a.i./ μ L), as well as following co-exposure to TXT+PXT/2. After five days of
27 continuous exposure, TXT induced sub-lethal effects in the organs involved in the
28 detoxification of xenobiotics, such as the fat body and pericardial cells, and it also induced a
29 significant increase in the hemocyte number. Thus, the hepato-nephrocytic system (HNS)
30 reached the greatest level of activity of pericardial cells as an attempt to eliminate this toxic
31 compound from hemolymph. The HNS was activated at low levels by PXT without an
32 increase in the hemocyte number; however, the mobilization of neutral glycoconjugates from
33 the trophocytes of the fat body was prominent only in this group. TXT and PXT co-exposure
34 induced intermediary morphological effects in trophocytes and pericardial cells, but oenocytes
35 from the fat body presented with atypical cytoplasm granulation only in this group. These
36 data showed that the realistic concentrations of these pesticides are harmful to newly emerged
37 Africanized honeybees, indicating that intra-colonial chronic exposure drastically reduces the
38 longevity of bees exposed to neonicotinoid insecticide (TXT) and the fungicide strobilurin
39 (PXT) as in single and co-exposure. Additionally, the sublethal effects observed in the organs
40 constituting the HNS suggest that the activation of this system, even during exposure to low
41 concentrations of these pesticides, is an attempt to maintain homeostasis of the bees. These
42 data together are alarming because these pesticides can affect the performance of the entire
43 colony.

44

45 **Keywords:** *Apis mellifera*. Ecotoxicology. Morphophysiology. Pesticides. Sublethal effects.

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55 **1. Introduction**

56 In addition to the high economic value of marketed products from the honeybee (*Apis*
57 *mellifera*), the ecosystem services provided by bees include the pollination of both native
58 plant species in natural ecosystems and crops in agricultural areas (Imperatriz-Fonseca and
59 Nunes-Silva, 2010). Nevertheless, especially in recent decades, multiple environmental
60 stressors are causing the decline of bee populations (Goulson et al., 2015). The loss and
61 fragmentation of habitats, which are associated with the increase in urbanization and intensive
62 agriculture, have decreased the nesting and floral resources for bees (Viana et al., 2012).
63 Additionally, bees foraging in the agroecosystem are exposed to pesticides used in the
64 agricultural field, and their residues could be found in different matrices of *A. mellifera*
65 colonies (Chauzat et al., 2006; David et al., 2015; David et al., 2016; Desneux et al., 2007;
66 Dolezal et al., 2016; Smodis Skerl et al., 2009; Smodis Skerl et al., 2010; Wiest et al., 2011).

67 The pesticides with systemic action in plants are translocated to the pollen and nectar,
68 representing a potential risk to pollinators (Decourtye and Devillers, 2010; Dicks et al., 2016;
69 Rondeau et al., 2014; Sánchez-Hernández et al., 2016). The residues of neonicotinoid
70 insecticides and fungicides of the strobilurin group, both with systemic action in plants, were
71 detected in high concentrations in pollen samples collected from different crops in the United
72 States (Pettis et al., 2013) and UK (David et al., 2015).

73 The toxicological effects of neonicotinoids are widely studied in *A. mellifera*
74 (Almeida-Rossi et al., 2013; Desneux et al., 2007; Giroud et al., 2013; Henry et al., 2012;
75 Krupke et al., 2012; Lawrence et al., 2016; Rondeau et al., 2014; Tavares et al., 2015;
76 Tremolada et al., 2010; Wu et al., 2011). However, there are only some studies about the
77 exposure of *A. mellifera* to fungicides (Campbell et al., 2016; Degrandi-Hoffman et al., 2015;
78 Sánchez-Bayo et al., 2016; Sprayberry et al., 2013; Zaluski and Orsi, 2015).

79 Under field conditions, there are residues of different pesticides in crops. Thus, the
80 residues of these chemical compounds are also common in *A. mellifera* hives whose workers
81 forage in crops (Chauzat et al., 2006; David et al., 2015; David et al., 2016; Dolezal et al.,
82 2016; Smodis Skerl et al., 2009; Smodis Skerl et al., 2010; Wiest et al., 2011). In this way, the
83 study of insecticide and fungicide mixtures, which have different mechanisms of action, is
84 important for the knowledge of the risk assessment of these important pollinating insects
85 (Decourtye et al., 2013).

86 Thiamethoxam belongs to the group of neonicotinoids that bind to the cholinergic
87 receptors of the central nervous system of insects (Tomizawa and Casida, 2005). The
88 neurotoxic action of thiamethoxam occurs specifically in nicotinic acetylcholine receptors

89 (nAChRs) of neurons (Maienfisch, 2012). The high affinity of the neonicotinoid molecule to
90 the to nAChRs leads to neuronal hyperexcitation in arthropods (Buckingham et al., 1997;
91 Zhang et al., 2000). At sublethal concentrations, thiamethoxam impaired homing and
92 consequently decreased foraging success and survival in honeybees (Henry et al., 2012).
93 Despite being a neurotoxic insecticides, thiamethoxam and other neonicotinoids, at sublethal
94 concentrations, induce morphological and physiological changes in different internal organs,
95 such as the midgut and Malpighian tubules (Catae et al., 2014; Gregorc et al., 2016).

96 The picoxystrobin is a systemic and broad-spectrum fungicide from the strobilurin
97 family (Agrofit, 2017) that is generally applied on crops visited by honeybees, such as
98 soybean (Chiari et al., 2008; Imperatriz-Fonseca and Nunes-Silva, 2010), corn (Malerbo-
99 Souza, 2011), and canola crops (Sabbahi et al., 2005), and also cereal grains (no rice) and
100 dried peas. This quinone oxidase inhibitor (QoI) fungicide acts by inhibiting mitochondrial
101 respiration by binding to the Qo site in cytochrome b, which is part of the complex
102 cytochrome bc1 that is located in the inner mitochondrial membrane of fungi. When
103 inhibition occurs, there is a blockade in electron transfer between cytochrome b and
104 cytochrome c1 that interrupts the energy cycle into fungi by inactivating ATP synthesis
105 (Bartlett et al., 2002; Parreira et al., 2009). However, this fungicide also inhibits *in vitro*
106 mitochondrial function in *A. mellifera* (Campbell et al., 2016) and therefore can affect the
107 synthesis of ATP in the organs of the bees that contact with it.

108 In agricultural environments, bees are often exposed to multiple pesticides, and
109 fungicides/insecticide mixtures have been frequently found in tank mixing applied on crops
110 and these mixtures have potential hazard to honeybees in the field (Colin and Belzunces,
111 1992; Pilling and Jepson, 1993). The combination of a neonicotinoid insecticide (clothianidin)
112 and a fungicide (propiconazole) induced mortality in *A. mellifera*, *Bombus terrestris* and
113 *Osmia bicornis* (Sgolastra et al., 2016). Mixture of neonicotinoid insecticides and strobilurin
114 fungicides have been detected in particle phase atmospheric samples collected from tree fruit
115 crops (apples, pears, cherries, peaches, apricots) and vineyards in Canada (Raina-Fulton,
116 2015). However, strobilurin fungicide, specifically picoxystrobin, not been yet studied in
117 bees.

118 Toxicological studies of the mixture of pesticides on bees have great relevance. In the
119 hive, the concentration and other properties of pesticide residues present in stored food could
120 be modified under intracolonial conditions (Chauzat et al., 2006; Rondeau et al., 2014). This
121 contaminated food, when ingested by individuals inside the colony, can impact negatively the

122 colony's fitness (Desneux et al., 2007; Palmer et al., 2013; Rondeau et al. 2014; Wu et al.,
123 2011).

124 The sublethal effects of pesticides can be detected at the cellular level. Morphological
125 analysis of bee internal organs, associated with immunohistochemistry and histochemistry, is
126 an important tool to evaluate cytotoxicity or adaptive responses to cell stress induced by
127 chemical compounds on bees (Malaspina and Silva-Zacarin, 2006). In the present study, the
128 hepato-nephrocytic system (HNS), which was described by (Abdalla and Domingues, 2015),
129 was chosen for the analysis of the sub-lethal effects with the aim of evaluating the integrated
130 response of different cell types belonging to HNS after exposure to an insecticide and a
131 fungicide with different modes of action. In the HNS, there is an association among fat body
132 cells (oenocytes and trophocytes), pericardial cells and immune cells along the myogenic
133 region of the dorsal heart, which use an integrated approach in response to chemical
134 compounds. Such effect biomarker constitutes a new and sensitive tool to study the impact of
135 xenobiotics even at very low concentrations (Skaldina and Sorvari, 2017) and it can predict
136 intoxication in bees much earlier than abnormal behavior and mortality of bees (Abdalla and
137 Domingues, 2015). We also analyzed the effects of exposure to low concentrations of
138 thiamethoxam and picoxytrobion to a single chemical and co-exposure to both pesticides at
139 too low concentrations, on the survival of newly emerged workers of Africanized *Apis*
140 *mellifera*.

141

142 **2. Material and Methods**

143 Three healthy colonies of Africanized *A. mellifera* were utilized in this study. The
144 colonies were located in an apiary in the rural area of Piedade-SP (Latitude: -23.618196;
145 Longitude: -47.485535). The bioassays using the newly emerged bees were carried out at the
146 Laboratório de Biologia Estrutural e Funcional (LABEF), where bee organs were also
147 processed and sectioned. Morphological and histochemical analyses of organs were carried
148 out at LABEF and Laboratório de Ecotoxicologia e Biomarcadores em Abelhas (LEBA).
149 Immunohistochemical analyses were carried out at LEBA and Laboratório de Fisiologia da
150 Conservação (LAFISC). All mentioned laboratory are located at the Federal University of
151 São Carlos at the Sorocaba Campus, in the municipality of Sorocaba, São Paulo State, Brazil.

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154

155 2.1. Chronic exposure toxicological bioassays in newly emerged workers

156 Operculated brood combs from the three colonies were collected in the apiary and
157 were transported in specific wooden boxes for transportation to the laboratory, where they
158 were transferred to a Biochemical Oxygen Demand (BOD) incubator at 34 °C and a relative
159 humidity of 70% to monitor the emergence of Africanized *A. mellifera* workers.

160 Following emergence, one-day-old bees were transferred to transparent plastic pots
161 (530 mL, 11 cm × 7 cm) lined with filter paper (11 cm), and they were fed *ad libitum* with
162 syrup (50% water + 50% inverted sugar) for acclimation 24 hours before the beginning of
163 bioassays.

164 Next, these newly emerged bees were divided into the following experimental groups:
165 CTL (control), ingestion of syrup free of pesticides; TXT (thiamethoxam), exposed to the
166 syrup containing thiamethoxam at the concentration of 0.001 ng a.i./µL (1 ppb); PXT
167 (picoxystrobin), exposed to the syrup containing picoxystrobin at a concentration of 0.018 ng
168 a.i./µL (18 ppb); TXT+PXT/2, exposed simultaneously to the syrup containing the combined
169 concentrations of thiamethoxam, 0.0005 ng a.i./µL (0.5 ppb), and picoxystrobin, 0.009 ng
170 a.i./µL (9 ppb). In addition, a acetone control group was performed. The concentration of the
171 vehicle was the same for all concentrations of pesticides tested, so that the acetone
172 concentration in the sugar solution not exceed 1%, as recommended by OECD (2013).

173 Each experimental group was assayed in quadruplicate (four pots) and each pot
174 contained 20 bees from three different colonies. Three pots from each experimental group
175 (triplicate) were used for survival analysis, and the fourth pot of each group was used for the
176 collection of the individuals for the other analyses (2.2, 2.3, 2.4, 2.5 items).

177 In the first five days of the bioassay, the newly emerged bees were exposed to chronic
178 oral exposure *ad libitum*. The exposure time selected for organ analysis was the 5th day after
179 the beginning of the bioassay based on the time that showed a higher decay of the survival
180 curve of the bees exposed to the PXT.

181 The mortality data from each pot of experimental groups were recorded daily until the
182 last bee died, which indicated the end of the bioassay. Log-rank Kaplan-Meier with pairwise
183 multi comparison procedures (Holm-Sidak method) were conducted to compare the survival
184 among the treatments (different groups). Survival analyses were conducted using SigmaPlot
185 12.3.

186 After five days of exposure, specifically at the beginning of the sixth day of the
187 bioassay, the bees were submitted to a total count of hemocytes (N= 20 bees per group).

188 Organs removed from abdomen of 10 bees, randomly selected from a total of 20 bees used
189 previously for hemolymph analysis, were also dissected for the removal of the dorsal vessel
190 and associated cells (pericardial cells and parietal fat body), both of which were processed for
191 morphometric, morphological, histochemical and immunohistochemical analysis.

192 In the present study, the selected concentrations were based on those described in
193 pollen load collected in honeybee hives. A field-realistic concentration (“low-dose”) of
194 thiamethoxam treatment, consisting of 0.001 ng a.i./ μ L (1 μ g/kg = 1 ppb) in food, was based
195 on the exposure data reviewed in EFSA (2012), as a default value for systemic pesticide.
196 Considering the daily intake of 10 μ L of syrup per bee, after five days of continuous
197 exposure, the honeybee can have consumed around 0.05 ng of thiametoxam, similar to 53.3
198 ppb (Mullin et al., 2010) that is the highest concentration of thiamethoxam found in apiaries.
199 Pettis et al. (2013) detected extremely high concentrations of fungicides of the strobilurin
200 family in pollen samples, from 891 to 4,677 ppb (concentration mean = $2,787.1 \pm 1,890.1$
201 ppb). Considering the daily intake of 10 μ L of syrup containing 0.018 ng i.a./ μ L (18 ppb) of
202 picoxystrobin, after five days of continuous exposure, the honeybee can have consumed
203 around 900 ng that is similar to the lowest concentration described in pollen.

204 Co-exposure of thiamethoxam and picoxystrobin (TXT+PXT/2), was performed in
205 order to check if the hepato-nephrocytic system (HNS) of honeybee has responsiveness to very
206 low concentrations in combination, which can be found on field conditions. Thus, the first
207 (TXT) and the second (PXT) treatments evaluated the hazard of environmental concentrations
208 of these pesticides on honeybees. The third treatment (TXT+PXT/2), based in a commum
209 scenario in a complex landscape mosaics where honeybees forage, can add information about
210 risk assessment in an exposure dilution condition. Exposure dilution is caused by foraging of
211 honeybees on other resources than a treated resource when these other resources are not or to
212 a lesser extent contaminated (Baveco et al., 2016).

213

214 2.2. Quantitative analysis of hemocytes

215 For the quantification of hemocytes, 20 workers from each experimental group were
216 collected. They were anesthetized by cold exposure (4 °C) and had 5 μ L of their hemolymph
217 collected with a micropipette in the thorax region. This volume was transferred to a
218 polypropylene microtube containing 5 μ L of 0.2% aqueous methylene blue solution, resulting
219 in a 1:1 dilution (v:v).

220 For the determination of the total hemocyte count, 10 μ L of the hemolymph diluted in
221 0.2% methylene blue was placed in a Neubauer chamber, and cell counts were performed per

222 mL of hemolymph (in five quadrants of the chamber) under a bright field light microscope
223 with a 40 \times objective. Two total counts per individual were performed (where the value of
224 each count is the result of the means of the five quadrants), resulting in 40 counts per group.

225 To compare the means of the groups, the counts' data were submitted to Kruskal-
226 Wallis ($P < 0.0001$), a non-parametric test, and post-test of Dunn's for multiple comparisons of
227 the groups using the program GraphPad Prism 6.0.

228

229 *2.3. Morphological, histochemical and immunohistochemical analyses of organs*

230

231 *2.3.1. Obtaining and processing organs*

232

233 To obtain the dorsal vessel with pericardial cells and the parietal fat body, 10 workers
234 from each experimental group were collected, anesthetized by cold exposure (4 °C) and
235 dissected under a stereomicroscope at room temperature for the removal of organs. The
236 organs were immersed in fixative solution (paraformaldehyde 4% in sodium phosphate buffer
237 0.1 M, pH7.4) for 24 hours at 4 °C.

238 The fixed organs were then washed in the same fixative buffer and were submitted to
239 slow dehydration in increasing ethanol solutions (30%, 50%, 60%, 70%, 85%, 90%, 95%, and
240 100%), at a low temperature (4 °C) in order to improve posterior histochemical staining and
241 immunofluorescence labelling, according to the methodology described by Silva-Zacarin et
242 al. (2012).

243 Five of the fixed and dehydrated organs were embedded in historesin without the
244 catalyst for a period of 24 hours and then were included in the resin containing the catalyst.
245 The historesin blocks were submitted to microtomy to obtain histological sections of the
246 organs of 2- μ m thicknesses for morphological and histochemical analyses.

247 The other five fixed and dehydrated organs were diaphanized in xylol and were
248 embedded in ultra-pure liquid paraffin (three baths of one hour each) followed by inclusion in
249 ultra-pure paraffin (Paraplast). The paraffin blocks were submitted to microtomy to obtain
250 histological sections of organs of 6- μ m thicknesses for the analysis of cell death by the
251 TUNEL method.

252

253 *2.3.2. Organ analysis by bright field light microscopy*

254 Slides containing the histological sections of organs of each experimental group were
255 stained with hematoxylin and eosin for morphological analysis (Junqueira and Junqueira,
256

257 1983). The other slides containing the histological sections of the parietal fat body were
258 submitted to the following histochemical techniques: bromophenol blue for the detection of
259 total proteins (Junqueira and Junqueira, 1983), Periodic acid-Schiff (PAS) for the detection of
260 neutral glycoconjugates and glycogen (Mcmanus, 1946), and Sudan Black for the detection of
261 total lipids (Pearse, 1985). At the end of these techniques, all slides were mounted in ERV-
262 Mount. The slides were analyzed and photodocumented under a bright field light microscope
263 (LEICA DM1000) using the program *Leica Application Suite V3.8* (LAS V3.8).

264 The fat body from five individuals from each experimental group was evaluated
265 qualitatively, and the description of the results was based on the analysis of the staining or
266 reaction patterns for each cell type (trophocyte and oenocyte) in each group. The
267 histochemical analysis was performed using the double-blind method based on the intensity of
268 the histochemical reactions in the cells: (-) absence of reaction/staining, (+) reaction/staining
269 with weak intensity, (++) reaction/staining with medium intensity, (+++) reaction/staining
270 with strong intensity, and (++++) reaction/staining with very strong intensity. All histological
271 sections of each individual were evaluated by this parameter for the determination of the
272 pattern for each experimental group.

273

274 *2.4. Incidence of different stages of pericardial cell activity*

275

276 The incidence of different stages of pericardial cell activity was performed using the
277 random analysis of non-sequential histological sections. Five individuals were analyzed for
278 each experimental group. Four sections at different tissue depths were analyzed for each
279 individual. For each histological section, three view fields were analyzed. The area of each
280 view field used for analysis was $12.739,5 \mu\text{m}^2$. Thus, 20 view fields were evaluated at random
281 for each experimental group.

282 The isolated cells and cells that form the cord from two to six cells were included in
283 the analyses and were classified into the stages of activity according to Mills and King (1965)
284 as follows: Stage 1 (central nucleus and peripheral small vacuoles); Stage 2 (small peripheral
285 central vacuoles and increase of coalescence peripheral vacuoles); Stage 3 (increase of
286 coalescence central vacuoles and small peripheral vacuoles); and Stage 4 (large irregular
287 vacuoles with displacement of the nucleus toward the periphery and small peripheral
288 vacuoles).

289 The incidence of different stages of pericardial cells activity was expressed as a
290 percentage by group. The total numbers of pericardial cells (isolated cells and cells that form

291 the cord) by stage of activity in each experimental group were submitted to the Chi-square
292 statistical analysis, using the R program, with a finality to distinguish significant differences
293 between groups. All incidence analyses were performed using LEICA DME microscope
294 equipped with the camera LEICA DFC295 and using the program *Image Manager* (LEICA
295 IM50).

296

297 *2.5. Immunohistochemical detection of cell death by the TUNEL method*

298 Slides containing the histological sections of the paraffin-embedded organs were
299 deparaffinized and hydrated. Next, they were submitted to the TUNEL (Terminal
300 deoxynucleotidyl transferase - mediated dUTP nick end labeling) method, through the
301 application of In Situ Cell Death Detection Kit (ISCDDK) according to the manufacturer's
302 instructions (Roche, Sigma-Aldrich).

303 The ISCDDK is based on the detection of single- and double-stranded DNA breaks
304 that occur at the early stages of cell death. After application of the TUNEL reaction mixture,
305 which contains TdT and fluorescein-dUTP, histological sections were washed with
306 phosphate-buffered saline (PBS) and were mounted with aqueous medium. A positive control
307 was performed by incubation of the slide with histological sections in recombinant DNase I,
308 which induces DNA strand breaks, prior to the labeling procedures.

309 For the evaluation by Laser Scanning Confocal Microscopy, we used an excitation
310 wavelength of 488 nm and detection in the range of 515–565 nm (green pseudo-color).

311

312 **3. Results**

313
314 *3.1. Toxicological bioassays*

315 After five days of chronic exposure to pesticides, there was a marked decrease in the
316 survival of the bees of the PXT group and TXT+PXT/2 group compared with that in the other
317 groups (Fig. 1).

318 Survival analysis throughout the bioassay allowed the comparison between the
319 survival curves of the experimental groups and determination of the Median Lethal Time
320 (LT_{50}) for each curve (Fig. 1). A significant difference was observed between the survival
321 curves of groups ($P<0.001$).

322 The control group presented an LT_{50} value equivalent to 14.133 ± 0.509 days
323 (confidence limit 95% = 13.136 – 15.131 days), similar statistically to that observed in the

324 acetone control group, which presented a value of LT₅₀ of 11±0.829 days (95% confidence
325 limit = 9.376 – 12.624 days).

326 The pesticide-exposed groups showed a significant decrease compared with that in the
327 control groups. The PXT group presented the LT₅₀ equivalent to 6.817±0.597 days (95%
328 confidence interval = 5.646 – 7.987 days), characterizing a reduction of 51.76% in the life
329 time of these bees relative to those in the control group and 38% relative to those in the
330 acetone control group. The TXT group presented the LT₅₀ equivalent to 7.517±0.633 days
331 (95% confidence interval = 6.276 – 8.758 days), characterizing a reduction of 46.81% in the
332 life time of these bees relative to those in the control group and 31.6% relative to those in the
333 acetone control group. The TXT+PXT/2 group presented the LT₅₀ equivalent to 7.367±0.619
334 days (95% confidence interval = 6.154 – 8.580 days), characterizing a reduction of 47.8% in
335 the life time of the bees relative to those in the control group and 33% relative to those in the
336 acetone control group.

337

338

339 **Fig. 1** - Survival curves of adult workers of Africanized *A. mellifera* exposed continuously to
340 pesticides by *ad libitum* ingestion. SigmaPlot 11.0 (Systat). Log-Rank Test: 61.720, Df: 4. *Statistical
341 difference ($P<0.001$), Kaplan-Meier with the statistical test Log-Rank. N= 60 individuals per group.

342

343

344 3.2. Morphological analysis

345

346 Figure 2 highlights the oenocytes and trophocytes of the fat body of the control group
347 (CTL). Trophocytes presented slightly branched nuclei, and oenocytes presented an ellipsoid
348 shape, a centralized nucleus, well-defined cellular limits and homogeneous cytoplasm (Fig.
349 2A). The PXT group presented trophocytes and oenocytes like those in the CTL group (Fig.
350 2C). Meanwhile, in the TXT group, trophocytes with more branched nuclei and oenocytes
351 with the presence of cytoplasmic vacuolization were observed. In addition, the occurrence of
352 small oenocytes with atypical morphology — i.e., much irregular cellular contour with
353 peripheral nuclei — were observed in the TXT group (Fig. 2B). In the TXT group, and
354 TXT+PXT/2 group, trophocytes also presented branched nuclei and intense cytoplasm
355 vacuolization, and oenocytes presented with cytoplasmic granulations concentrated close to
356 nuclei, and some of them presented atypical morphology, i.e., loss of shape and decrease of
357 size (Fig. 2D). In all experimental groups, the oenocytes presented very acidophilic
358 cytoplasm.

359

360

361 **Fig. 2** - Micrographs of the oenocytes and trophocytes of the parietal fat body of newly emerged
362 workers of Africanized *A. mellifera* after five days of oral continuous exposure to pesticides.
363 Histological sections were stained with hematoxylin and eosin. N= 5 individuals per experimental
364 group. (A) Control; (B) Thiamethoxam, 0.001 ng a.i./ μ L; (C) Picoxystrobin 0.018 ng a.i./ μ L; (D)
365 (Thiamethoxam + Picoxystrobin)/2. oe = oenocyte, n = nucleus, tr = trophocyte, asterisks in (B) and
366 (D) = oenocytes with atypical morphology, white arrow in (D) = cytoplasmic granulations in the
367 oenocytes, and black arrow in (B) = cytoplasmic vacuoles in the oenocytes.

368

369 3.3. Quantitative analysis of hemocytes

370

371 The results of the quantitative analysis of hemocytes demonstrated that the TXT group
372 presented a higher incidence of hemocytes ($P<0.0001$) than the other experimental groups,
373 which presented no statistically significant difference in relationship to the control. A higher
374 incidence of hemocytes, mainly pro-hemocytes of 7.5 μ m of diameter, located in the lumen of
375 the dorsal vessel and in its surroundings, was observed only in the group exposed to TXT
376 (Fig. 3), these morphological data were in concordance with the hemocyte quantification data.

377

378

379 **Fig. 3** - Total hemocyte count from newly emerged workers of Africanized *A. mellifera* after five days
380 of oral continuous exposure to pesticides. *Significant difference ($P<0.0001$), Kruskal-Wallis
381 followed by post-test of Dunn's. N= 20 individuals per group and micrographs of the hemocytes of
382 newly emerged workers of Africanized *A. mellifera* after five days of oral continuous exposure to the
383 insecticide thiamethoxam. Histological sections stained with hematoxylin and eosin. (A-D)
384 Thiamethoxam, 0.001 ng a.i./ μ L. l = lumen, black arrow = agglomeration of hemocytes, and dv =
385 dorsal vessel

386

387 3.4. Determination of the stage of pericardial cell activity

388

389 The results obtained on the level of activity of the pericardial cells demonstrated
390 significant differences among all groups (P value = 2.2×10^{-16}).

391 CTL group presented a higher percentage of stage 1 pericardial cells (91.80%) than the
392 other exposed groups (Fig. 4A). The percentage values of stage 1 pericardial cells in the
393 pesticide-exposed groups were 56.77% (PXT), 47.96% (TXT+PXT/2), and 15.61% (TXT).
394 Based on the highest percentage of pericardial cells in stage 2, the group more representative
395 was the PXT group with 28.36%. The TXT group presented 22.50%, the TXT+PXT/2 group
396 presented 22.22%, and the CTL group presented 5.62% of pericardial cells in stage 2 (Fig.
397 4B). The determination of the level of activity of the pericardial cells in stage 3 demonstrated
398 the highest percentage in the TXT group with 30.77%. The TXT+PXT/2 group presented
399 22.00%, the PXT group presented 12.48%, and the CTL group presented 2.50% of the

400 pericardial cells in stage 3 (Fig. 4C). Regarding stage 4 activity, the group that presented the
 401 highest percentage was the TXT group with 31.12%. The TXT+PXT/2 group presented
 402 7.28%, the PXT group presented 2.39%, and the CTL group presented 0.08% of the
 403 pericardial cells in stage 4 (Figure 4D).

404

405 **Fig. 4** - Activity stages of pericardial cells from newly emerged workers of Africanized *A. mellifera*
 406 after five days of oral continuous exposure to pesticides. (A) Pericardial cells in stage 1; (B)
 407 Pericardial cells in stage 2; (C) Pericardial cells in stage 3; (D) Pericardial cells in stage 4. The graphs
 408 represent the box-plot of the total numbers of pericardial cells (isolated cells and cells that form the
 409 cord) per activity stage (axis y) in each experimental group (axis x).

410

411 3.5. Histochemical analysis of the fat body

412

413 Table 1 lists the results regarding the intensity of staining or reaction observed in the
 414 fat body.

415

Group	Bromophenol blue				Periodic acid-Schiff			Sudan Black	
	Oenocyte		Trophocyte		Cytoplasm		Cytoplasm		
	C	N	C	Gr	N	GE	GT	GE	GT
CTL	+	++	+	-	+	-	+++	+	++
TXT	+	++	+	-	++	-	++	+	++
PXT	+	++	+	-	++	-	+	+	++
TXT+PXT/2	+	++	+	-	++	-	++	+	+

416 **Table 1** - Histochemical analyses of the parietal fat body from newly emerged workers of Africanized
 417 *A. mellifera* after five days of oral continuous exposure to pesticides.

418 Legend: (-) Absence of reaction/staining; (+) Reaction/staining with weak intensity; (++)
 419 Reaction/staining with median intensity; (+++) Reaction/staining with strong intensity. C= cytoplasm,
 420 GE= granules in the oenocytes, Gr= granules, GT= granules in the trophocytes, N= nucleus.

421

422 All groups showed negative labeling for neutral glycoconjugate granules in the
 423 oenocytes. PAS-positive cytoplasmic granules were observed in trophocytes, but the intensity
 424 of labeling was completely different between the groups (Fig. 5). The CTL group presented
 425 the highest incidence of PAS-positive granules in the trophocyte compared with the other
 426 groups (Fig. 5A), whereas the TXT group and the TXT+PXT/2 group presented a reduction of

427 the glycoconjugates granules (Fig. 5B and D). The PXT group showed the small incidence of
428 glycoconjugate granules in trophocyte cytoplasm (Fig. 5C).

429

430

431 **Fig. 5** - Periodic Acid-Schiff technique (PAS reaction) in oenocytes and trophocytes of the parietal fat
432 body from newly emerged workers of Africanized *A. mellifera* after five days of oral continuous
433 exposure to pesticides. (A) Control; (B) Thiamethoxam, 0.001 ng a.i./ μ L; (C) Picoxystrobin, 0.018
434 ng a.i./ μ L; (D) (Thiamethoxam + Picoxystrobin)/2. oe = oenocytes, n = nucleus, tr = trophocyte,
435 black arrow in (A), (B), (C) and (D) = neutral glycoconjugate accumulation in trophocytes.

436

437 Cytotoxicity analysis of pesticides performed in the fat body and pericardial cells
438 using the TUNEL method (ISCDDK) did not show DNA fragmentation in these cells. Thus,
439 the absence of DNA fragmentation in the fat body or pericardial cells, in all experimental
440 groups, indicated the absence of cell death triggered by TXT or PXT after five days of
441 continuous oral exposure (Fig. 6).

442

443 **Fig. 6** - TUNEL reaction (ISCDDK). Laser Confocal Microscopy. Oenocytes, trophocytes and
444 pericardial cells from newly emerged workers of Africanized *A. mellifera* after five days of continuous
445 oral exposure to pesticides. (A-C) Positive control of reaction (DNase-I) evidencing positive nuclear
446 fragmentation of the DNA (white arrows); (D-F) Thiamethoxam, 0.001 ng a.i./ μ L. oe = oenocytes, n
447 = nucleus, tr = trophocytes, pc = pericardial cells.

448

449 Summarizing the several responses exhibited by HNS from Africanized honeybees
450 after exposure to pesticides: alterations of the normal morphological and histochemical
451 patterns were more pronounced in the fat body, pericardial cells and hemocytes from bees
452 exposed to thiamethoxam followed by the co-exposure of thiamethoxam and picoxystrobin.
453 The intense decrease in glycogen deposits in trophocytes was an exclusive feature observed in
454 the picoxystrobin-exposed group.

455

456 **4. Discussion**

457 The observed decrease in the survival of *A. mellifera* workers exposed to
458 thiamethoxam and picoxystrobin in the present study showed that very low concentrations of
459 these pesticides negatively impact these bees. Individual responses of bees to stress, such as
460 the elevation of worker death rates, can impact the colony performance because of the
461 breakdown in the division of labor of workers according to the demographic model described

462 by Perry et al. (2015), therefore a rapid loss of the adult population in the hive can impose a
463 whole colony collapse.

464 Considering that fungicides are generally categorized as less lethal to bees than
465 insecticides and that the exposure of bees to fungicides in agricultural fields is higher than that
466 to other pesticides (Pettis et al., 2013), the risk of bee exposure to fungicides is increased
467 under realistic conditions. Regarding picoxystrobin, there are no published data regarding the
468 ecotoxicological bioassays using this fungicide in bees. Therefore the present study is the first
469 on toxicological effects of picoxystrobin in honeybees at realistic concentrations.

470 In addition, this study also showed that too low concentration of thiamethoxam (0.001
471 ng a.i./ μ L) significantly reduced the survival of young honeybee workers. Significant increase
472 in the mortality rate of forager workers has been described in European *A. mellifera* submitted
473 to not realistic concentrations of thiamethoxam by acute exposure (Kakamand, Mahmoud and
474 Amim, 2008). Thus, the present study emphasizes the importance of the evaluation of the
475 chronic exposure of the honeybees to the realistic concentrations of neonicotinoid
476 insecticides.

477 Additionally, sublethal effects on bees exposed to low concentrations of pesticides can
478 be observed at the cellular and tissue levels (Malaspina and Silva-Zacarin, 2006). The
479 sublethal effects in the cells that comprise the hepato-nephrocytic system were evident in this
480 study, although with different responses among the tested pesticides. Such an overload of the
481 HNS demonstrate the sensibility of the effect biomarker to study the effect of xenobiotics in
482 bees, however the HNS presents xenobiotic-specific responses, what could allow or predict
483 the effect of the xenobiotic in other functions or organs of bees, since the cells of the HNS are
484 involved in a plethora of different function, usually linked and interdependent to other organs
485 of bees.

486 The exact mechanism involved in increasing the total number of hemocytes observed
487 in bees exposed to the sublethal concentration of thiamethoxam (0.001 ng a.i./ μ L) remains
488 unknown. Changes in the populations of hemocytes in bees probably reflect their
489 physiological responses to stress factors (Marringa et al., 2014; Pandey and Tiwari, 2012), as
490 would be the case with the chemical stress induced by this insecticide. However, in the co-
491 exposed group (thiamethoxam and picoxystrobin), the amount of hemocytes presented an
492 intermediate value compared with pesticides in the single-exposed group, suggesting that the
493 increase in the number of hemocytes occurs when the concentration reaches a threshold
494 concentration of thiamethoxam. In view of these observations, it is necessary to intensify the
495 studies on the mechanisms involved in modulating the number of hemocytes in bees exposed

496 to different low concentrations of the various neonicotinoid insecticides, which is an
497 unexplored area.

498 Thiamethoxam is a neurotoxic neonicotinoid insecticide (Tomizawa and Casida,
499 2005), and its molecules are rapidly absorbed in the midgut after the ingestion of
500 contaminated food and they reach the hemolymph. In turn, the hemolymph enters in the
501 dorsal vessel by ostiole and is rapidly pumped into the head from the abdominal region
502 (Winston, 2003). Through this hemolymph pathway system, the neonicotinoid and/or its
503 metabolites are transported to the brain, where the molecules will act and lead the insect to
504 death through neural hyperexcitation. In this way, the fat body, located around the dorsal
505 vessel of the abdomen, interacts intensely with the hemolymph. Because there is a great
506 exchange of metabolites between hemolymph and the fat body in this abdominal region
507 (Chapman, 2013; Cruz-Landim, 2009; Kilby, 1965; Price, 1973), morphological changes in
508 the fat body cells could represent a response to their exposure to pesticide molecules in the
509 hemolymph that have been absorbed by the intestine and will be transported by the dorsal
510 vessel to the head. Thus, the trophocytes located around the dorsal vessel altered their typical
511 morphology (Behan and Hagedorn, 1978; Keeley, 1985; Locke, 1998; Paes de Oliveira and
512 Cruz-Landim, 2003a). In the present study, the bees of the group exposed to thiamethoxam
513 presented the most pronounced changes in the fat body, followed by the group co-exposed to
514 thiamethoxam and picoxystrobin (TXT+PXT/2).

515 The response of the trophocytes from bees exposed to the neonicotinoid molecules
516 present in the hemolymph was the intensification of nuclear branching and increased number
517 of vacuoles present in their cytoplasm. According to Locke (1984), these characteristics are of
518 cells in high metabolic activity, in which the nucleus branches to increase the contact surface
519 area. According to Paes de Oliveira and Cruz-Landim (2003b), in the vacuoles present in
520 trophocytes, there are glycogen deposits free or conjugated to lipids and proteins, allowing
521 mobilization that will address the energy demand of the organism. Histochemical data
522 reinforced the presence of neutral glycoconjugates and lipids in the vacuoles of trophocytes of
523 bees exposed to thiamethoxam (TXT or TXT+PXT/2).

524 Another cell type of the fat body analyzed was the oenocytes, which are distributed
525 among the trophocytes (Dean et al., 1985). Oenocytes of the bees from the control and
526 picoxystrobin groups showed a characteristic morphology of this cell type — that is, an
527 ellipsoid shape with well-delimited cytoplasmic contours, the absence of cytoplasmic granules
528 and a centralized, spherical nucleus with a regular contour (Cruz-Landim, 1983; Dean et al.,
529 1985; Martins and Ramalho-Ortigão, 2012).

530 However, in the oenocytes of bees from the group exposed to thiamethoxam, the
531 presence of vacuoles in the cytoplasm and irregularly shaped nuclei may indicate an increase
532 in the degree of cellular activity. The oenocytes express (NADPH)-cytochrome P450
533 reductase enzyme, which is required for transfer of electrons from NADPH to P450
534 (cytochrome P450 monooxygenase) that is closely associated with the major mechanism of
535 xenobiotic biotransformation (Chung et al., 2009; Lycett et al., 2006).

536 In the oenocytes of bees co-exposed to thiamethoxam and picoxystrobin, the
537 cytoplasmic granulations may be related to the storage of substances. Martins et al. (2011)
538 carried out the isolation of oenocytes from the pupae of mosquitoes and their maintenance in
539 primary culture for the morphological characterization of these cells. The authors observed
540 irregular granular clusters near the nucleus of the oenocytes, similar to those observed in the
541 present study, but they have not characterized the chemical nature of these granulations.

542 Although most of the oenocytes of bees exposed to thiamethoxam showed
543 morphological characteristics that indicate an increase in their activity, the presence of some
544 atypical oenocytes in the TXT and TXT+PXT/2 groups was also observed. One hypothesis
545 for these atypical cells is that they are inactive cells. Cousin et al. (2013) observed the
546 presence of oenocytes with reduced cytoplasmic volume compared with the nuclear volume in
547 *A. mellifera* larvae that were exposed to Paraquat and suggest that this morphological
548 alteration was the result of the oxidative stress caused by exposure to the herbicide. The
549 authors emphasized the high degree of sensitivity of oenocytes from larvae exposed to very
550 low concentrations of the herbicide, which was also observed in the present study with
551 oenocytes from adult bees exposed to two different pesticides. Together, these data indicate
552 the importance of the oenocyte evaluation in toxicological studies in *A. mellifera* or other
553 bees.

554 The evaluation of the different stages of activity of pericardial cells from bees exposed
555 to thiamethoxam, which presented major alterations, corroborated the hypothesis of an
556 increase in the activity of most oenocytes in this experimental group.

557 Thiamethoxam induced an increased amount of pericardial cells in stages 3 and 4 of
558 activity, and these stages indicate a high cellular capacity in the collection of hemolymph
559 substances by pinocytosis (Mills and King, 1965). By removing molecules by exocytosis and
560 absorbing others by endocytosis, as proteins (Fife et al., 1987), the pericardial cells contribute
561 to the maintenance of hemolymph homeostasis (Crossley, 1972). In the present study, the
562 presence of pericardial cells with condensed chromatin could indicate a decrease in nuclear
563 metabolic activity observed in stage 4 cells. The absence of positive labeling for nuclear

564 fragmentation by the TUNEL method demonstrated that nuclear condensation of stage 4 cells
565 is not related to the cell death process, at least over five days of continuous exposure to the
566 tested pesticides. If this stressful condition continues for a long time, these 4 stage cells likely
567 will collapse.

568 Contrary to that observed in the group exposed to thiamethoxam, bees exposed to
569 picoxystrobin presented a higher percentage of pericardial cells in stages 1 and 2, indicating
570 that the fungicide increased the activity level of these cells in a less exacerbated form than the
571 insecticide.

572 A possible explanation for this response can be found in the mechanism of action of
573 the fungicide, which inhibits the ATP synthesis of fungi and other eukaryotes (Bartlett et al.,
574 2002; Parreira et al., 2009). The pericardial cells perform the endocytic-exocytic cycle that is
575 dependent on ATP, continuously capturing fluids and solutes by pinocytosis (Crosley, 1972;
576 Cruz-Landim, 2009; Das et al., 2008; Mills and King, 1965; Poiani and Cruz-Landim, 2006)
577 and suggesting that the fungicide would reduce this pinocytic process and keep the cells at
578 level 2 of activity. In the CTL group, the highest percentage of cells was in stage 1 activity,
579 which is found in epithelial arrangements characteristic of this stage (Fife et al., 1987).

580 In addition, slight changes in the organs of bees of the PXT group (pericardial cells
581 and fat body) may be a reflection of the initial effect of picoxystrobin on the first organ that
582 comes into contact with the fungicide—that is, the intestine of the bees—that would decrease
583 the concentration of its bioavailable molecules in the hemolymph. Campbell et al. (2016)
584 suggested that the negative effects of fungicides are likely to be observed in the midgut,
585 where mitochondria would be exposed to higher concentrations of these chemicals present in
586 ingested food than in other organs. The authors report that the fungicide pyraclostrobin, which
587 is from the strobilurin group, as well as picoxystrobin, induced a negative effect on the
588 feeding rate and protein digestion in *A. mellifera*. If we consider this hypothesis of
589 picoxystrobin to have induced morphophysiological changes in the midgut epithelium of the
590 bees, the absorption of nutrients would be compromised, which may have caused starvation in
591 these bees.

592 Regarding the hypothesis elaborated in the present study, based on the conclusions
593 obtained by Campbell et al. (2016), the fungicide would affect the absorption of nutrients in
594 the intestine and, therefore, it would be necessary for the bee to mobilize the nutrient source
595 from the trophocytes, which are specialized cells in nutrient storage, i.e., intermediate
596 metabolism (Grzelak and Kumaran, 1986; Locke, 1984). In the vacuoles present in the
597 trophocytes, there is a presence of glycogen-free or glycogen-conjugated lipids and proteins,

598 which allow mobilization that will address the body's energy demand (Paes de Oliveira; Cruz-
599 Landim, 2003b). The weak reaction to neutral glycoconjugates in the trophocytes of the bees
600 exposed to picoxystrobin probably indicates the mobilization of these glycoconjugates present
601 in the trophocyte vacuoles (Behan and Hagedorn, 1978; Cruz-Landim, 2009; Keeley, 1985;
602 Locke, 1998). On the other hand, although there are no studies on the relationship between
603 strobilurin fungicides and glycogenolysis in insects, this glycogen deposit mobilization is
604 probably occurring in the fat body (trophocytes) of exposed bees as a compensatory
605 mechanism to maintain cellular ATP. This compensatory mechanism is discussed by Felser et
606 al. (2014) in human hepatocytes exposed to a known mitochondrial toxicant (benzbromarone).

607 According to Arrese and Soulages (2010), glycogen and triglycerides are the main
608 energy reserves in animal cells. The authors relate the mobilization of neutral glycogen and
609 glycogen of the fat body trophocytes in situations of thermal stress and lack of humidity.
610 However, the data obtained in this work indicate the mobilization of these polysaccharides in
611 response to chemical stress.

612 The weak labeling of lipids in the trophocytes of the TXT+PXT/2 group may be
613 related to their mobilization because the PAS reaction was inversely proportional to that in the
614 PXT group, where there was more mobilization of neutral glycoconjugates than lipids. Thus,
615 for this group, the preference by mobilization of lipids (triglycerides) is suggested in response
616 exposure to pesticide. Lipid reserve mobilization may occur during periods of starvation, as
617 well as during immunological responses (Arrese and Soulages, 2010), which could be
618 occurring in the TXT+PXT/2 group.

619 Regarding the bromophenol blue reaction, the lack of protein in the granules of
620 trophocytes is comprehensive because protein granules are typical of pre-metamorphic phase
621 trophocytes and are mobilized during metamorphosis until the insect reaches adulthood (Tojo
622 et al., 1978).

623 The results obtained in this study corroborate the mechanisms of action of HNS,
624 described by Abdalla and Domingues (2015) for *Bombus morio* that was chronically exposed
625 to cadmium (1 ppb) because the HNS was activated by pesticides. This activation was more
626 intense in the bees exposed to thiamethoxam, which affected both the first barrier (fat body
627 cells) and second barrier (pericardial cells), accompanied by the increase in the number of
628 hemocytes of the immune system but with the maintenance of cell viability because the dorsal
629 vessel and other cells that constitute the HNS were negative to the cell death test. The
630 activation of HNS by picoxystrobin was more subtle but also affected the first barrier (fat
631 body cells) and second barrier (pericardial cells) with no immune cell response. Co-exposure

632 with thiamethoxam and picoxystrobin induced the HNS at an intermediary level, indicating
633 that this system is sensitive to very low concentrations of pesticides.

634

635 **5. Conclusion**

636 The morphophysiological evaluation of the hepato-nephrocytic system, associated
637 with the evaluation of the survival rate of bees chronically exposed to a single chemical and
638 co-exposure to thiamethoxam and picoxystrobin, indicated that low concentrations of these
639 pesticides are harmful to Africanized *A. mellifera* workers because they may affect the
640 performance of these bees. This study highlights the relevance of the intra-colonial exposure
641 of worker honeybees through food-containing residues of pesticides that can comprise
642 negatively the whole colony.

643

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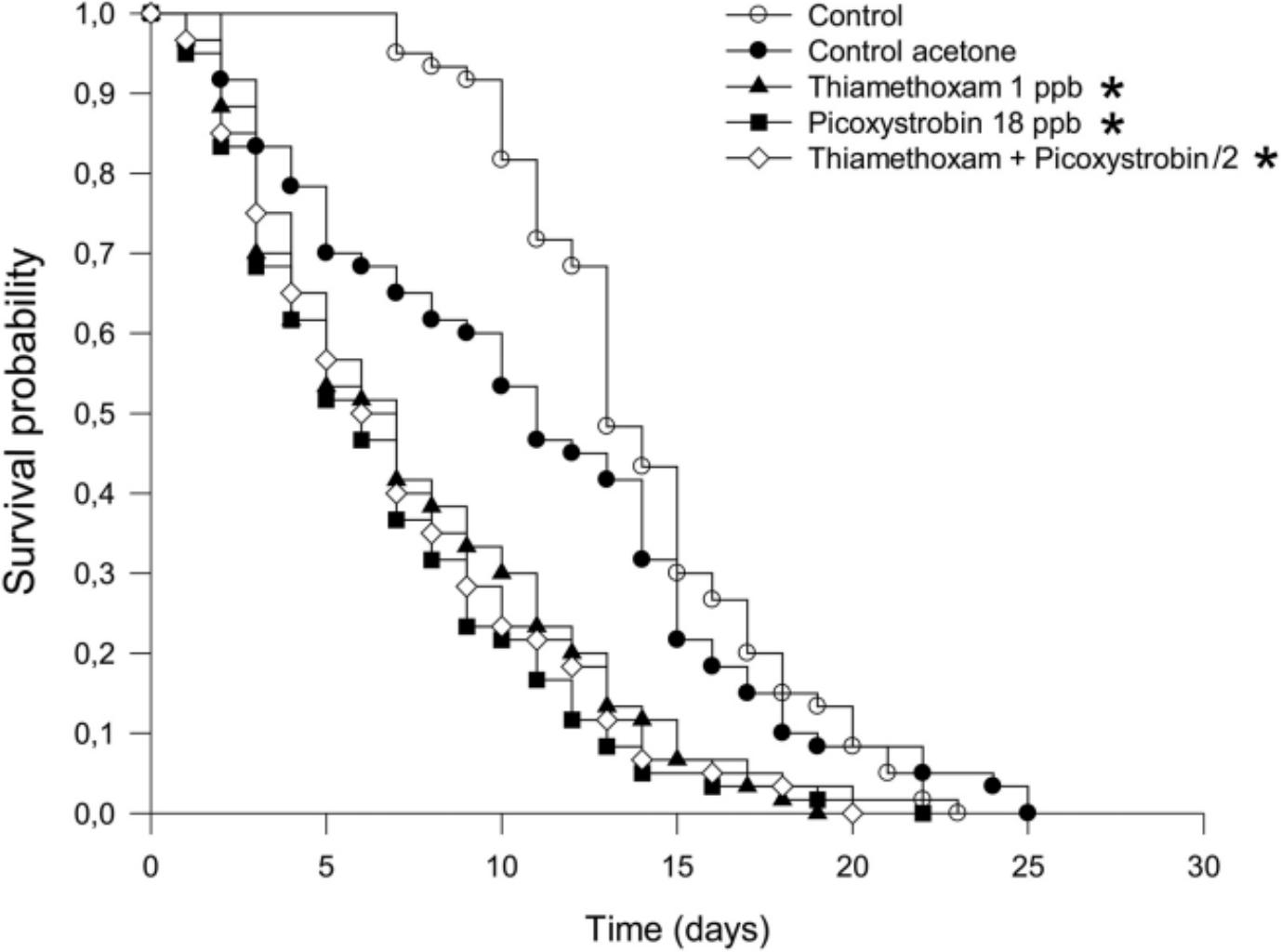
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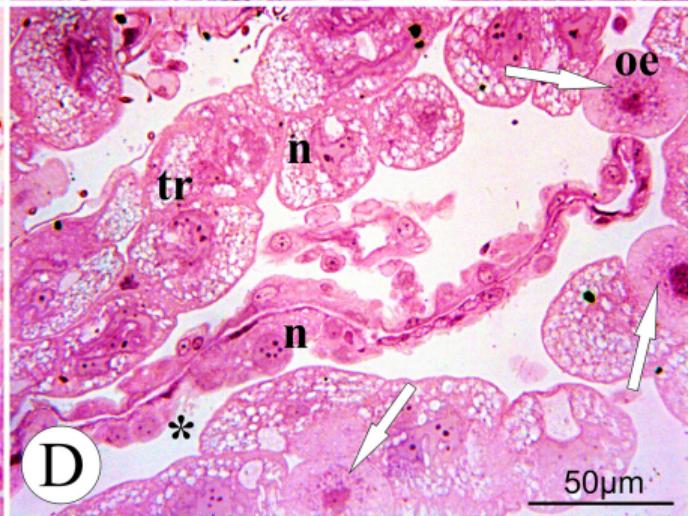
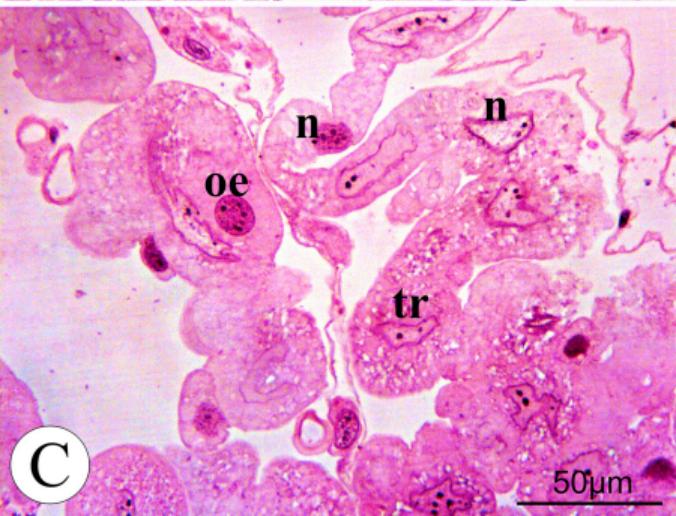
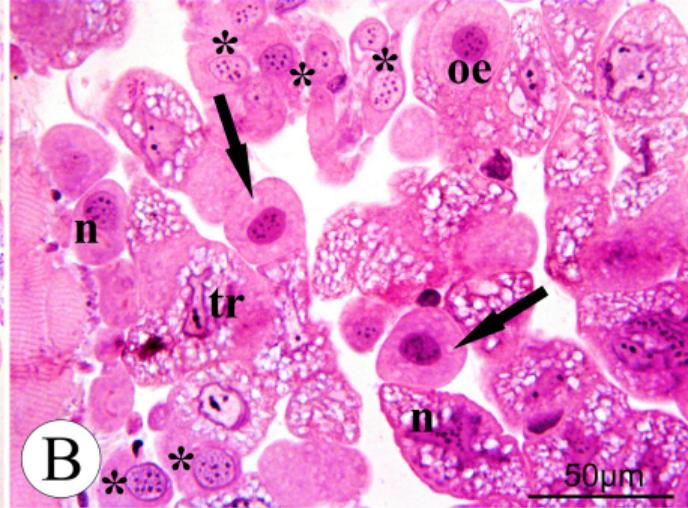
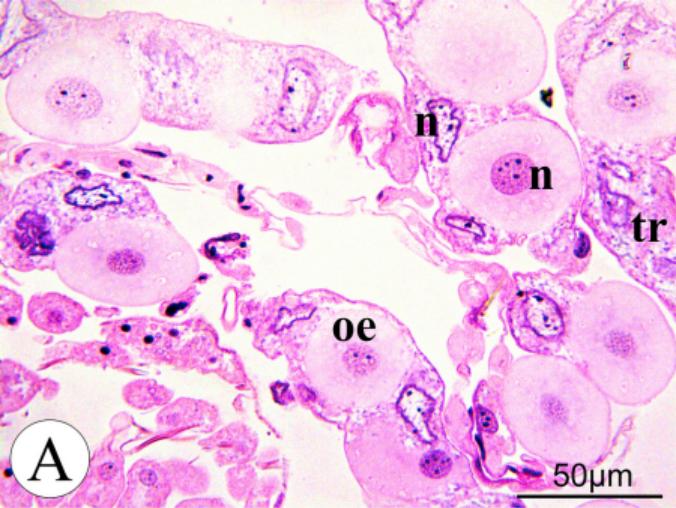
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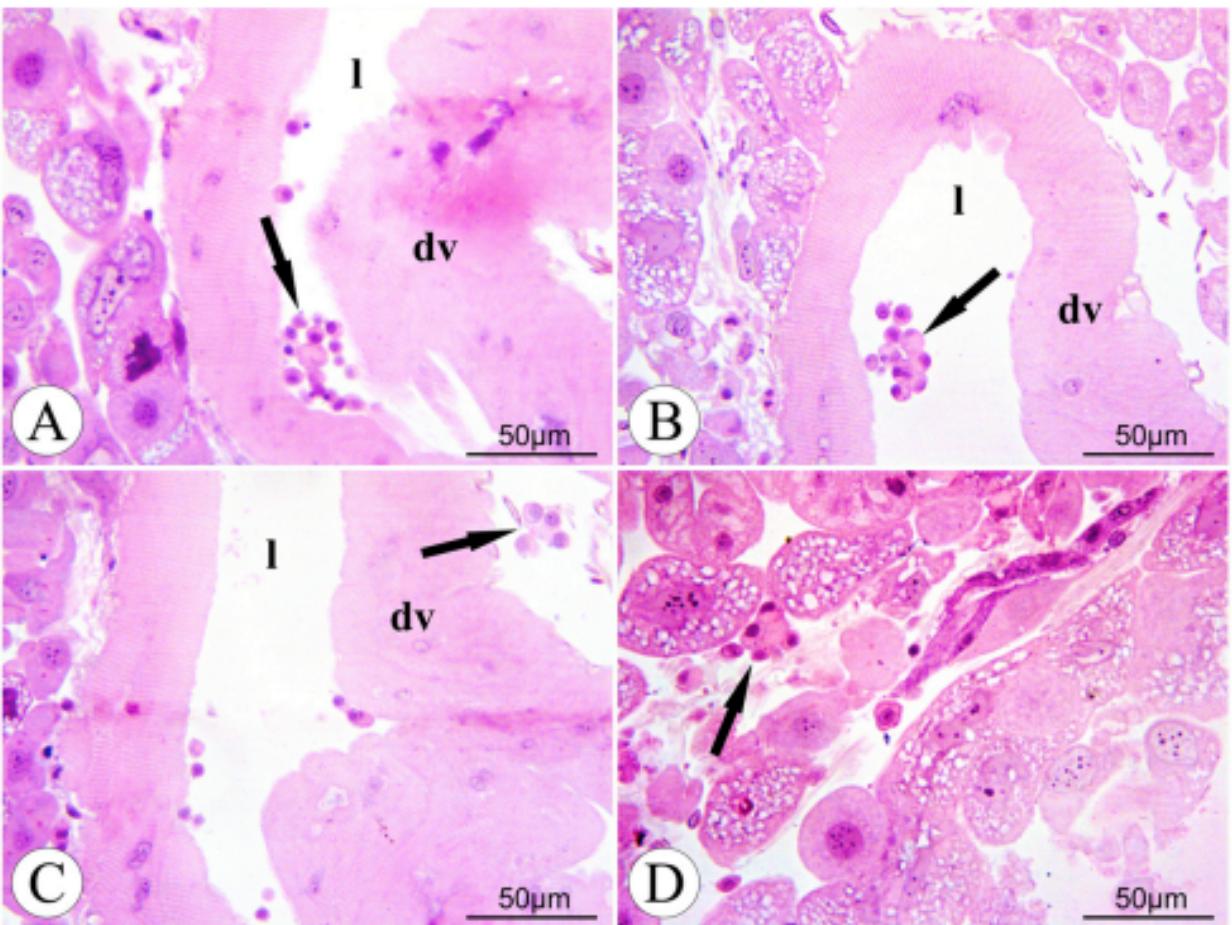
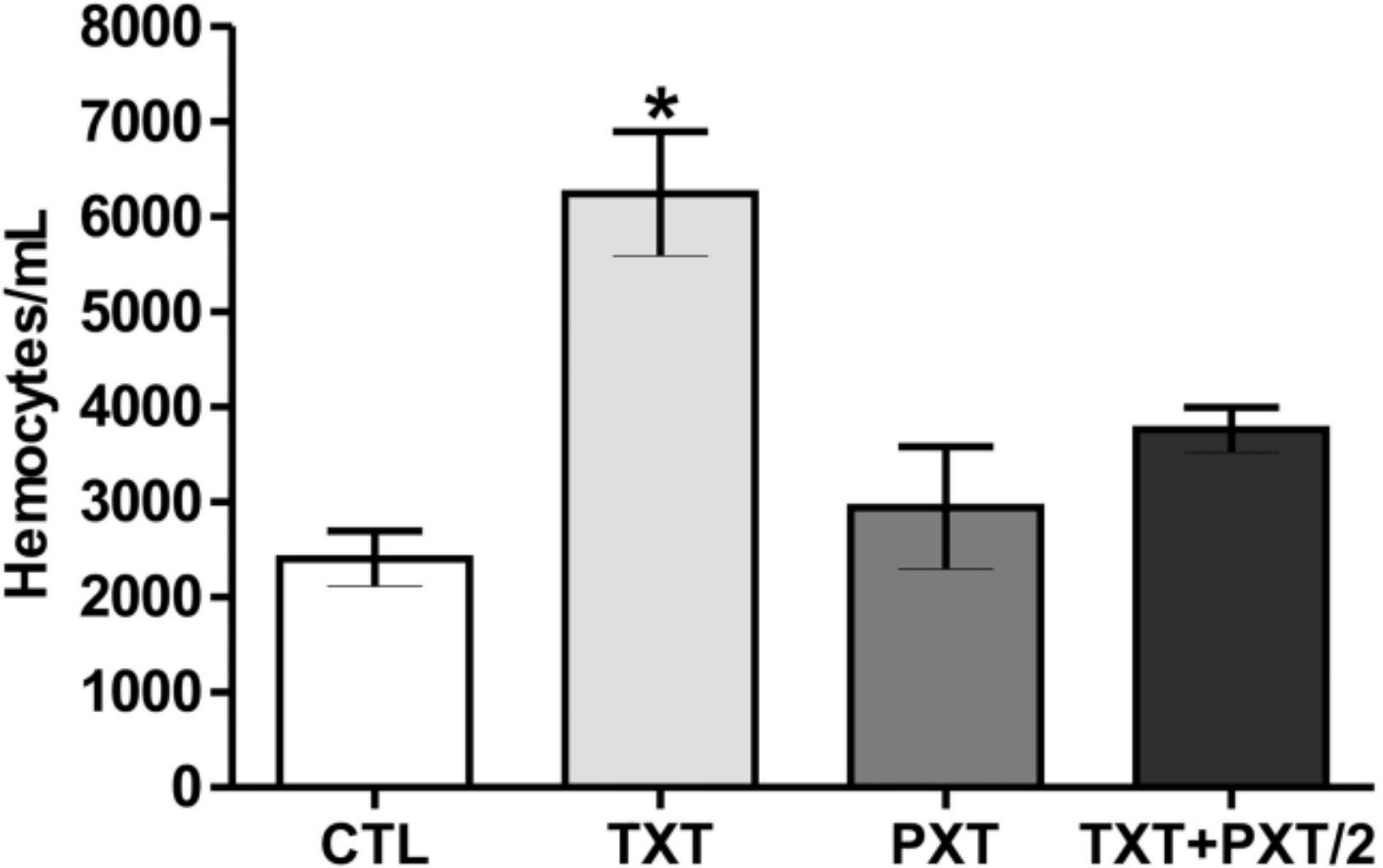
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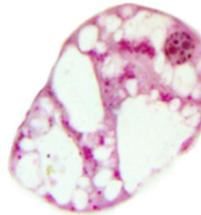
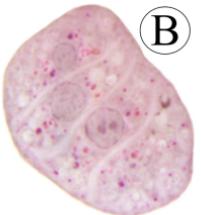
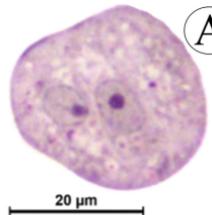
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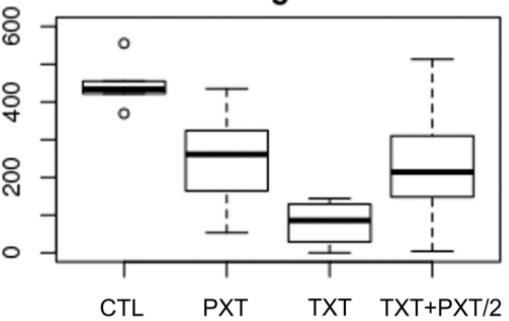




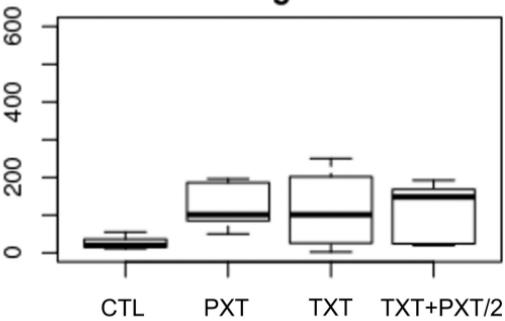




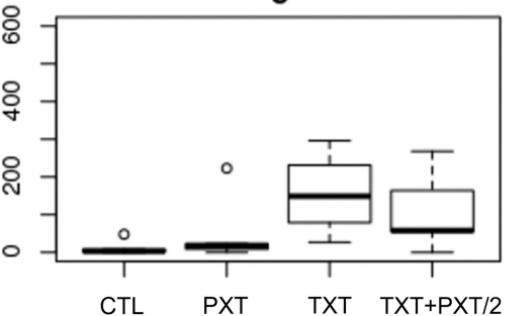
Stage 1



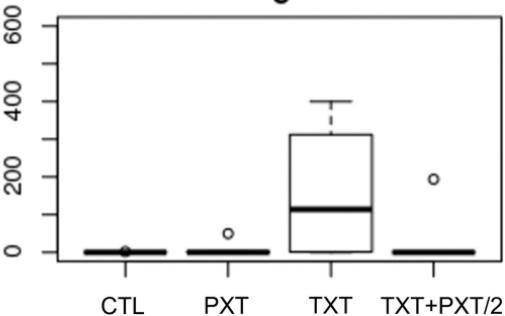
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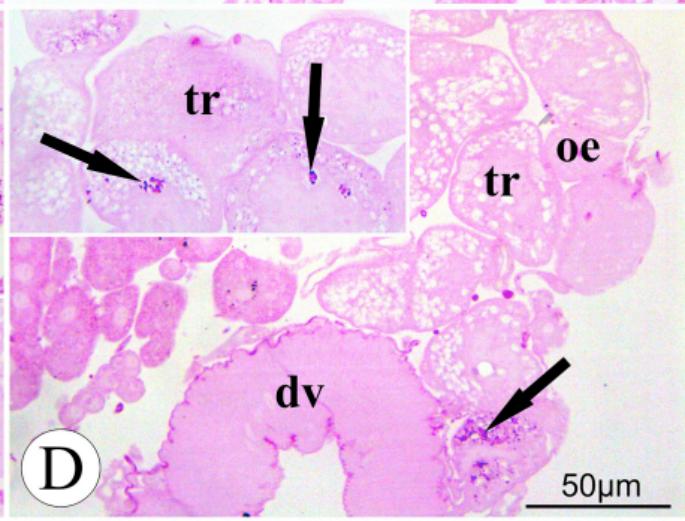
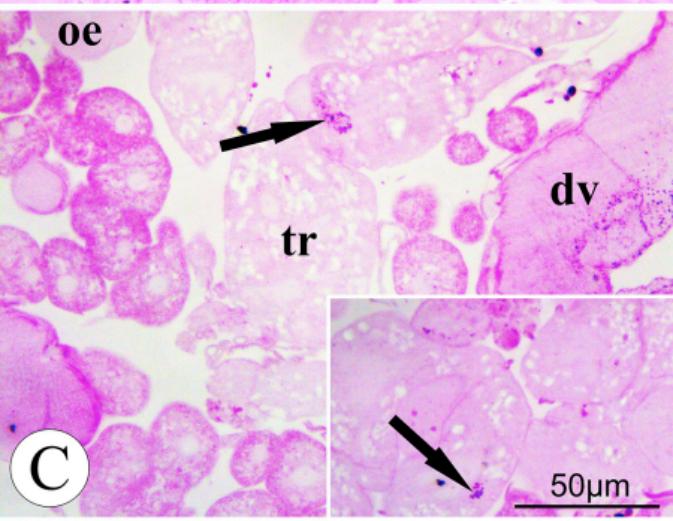
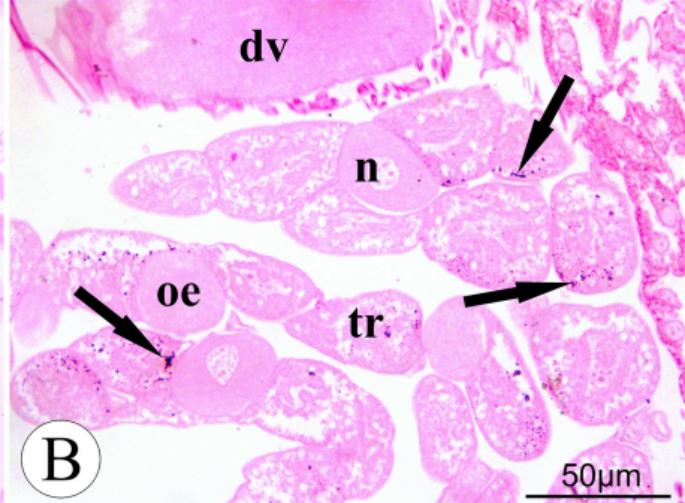
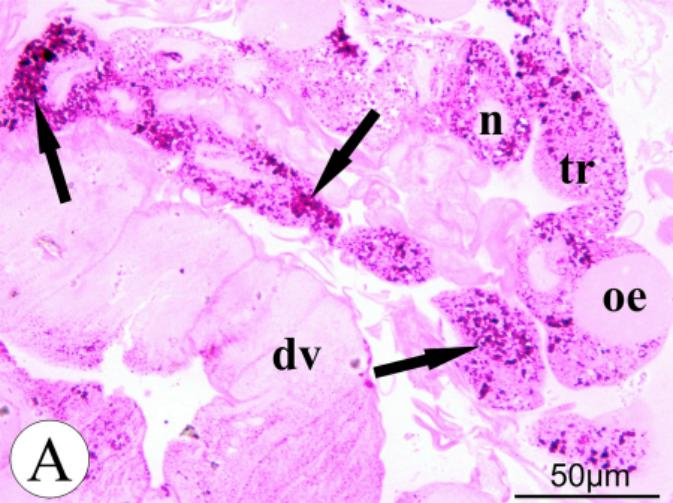


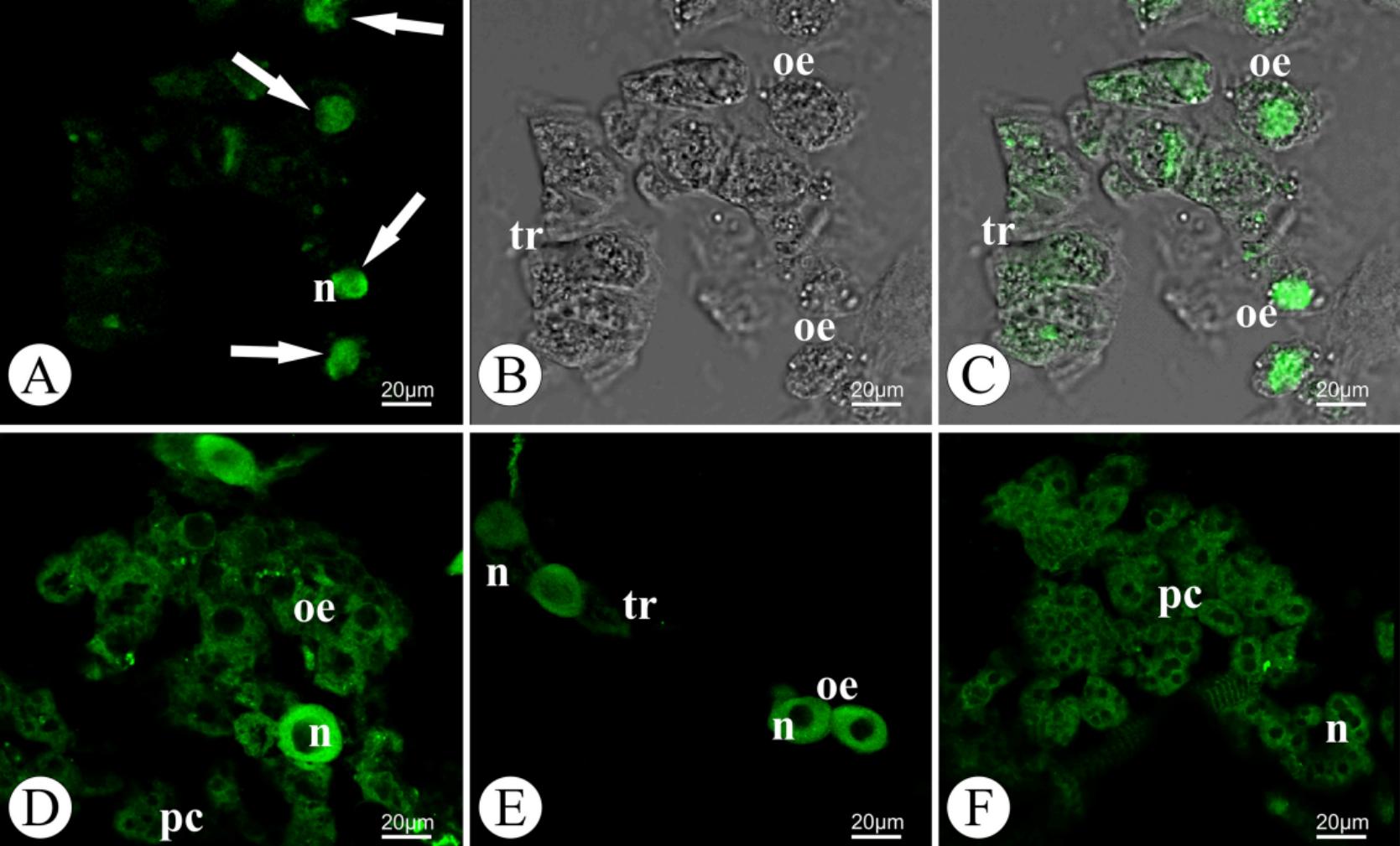
Stage 3



Stage 4







Thiamethoxam and picoxystrobin reduce the survival and overload the hepato-nephroocitic system of the Africanized honeybee**HIGHLIGHTS**

- Realistic concentrations of thiamethoxam and picoxystrobin overload the hepato-nephroocitic system in honeybee.
- Co-exposure of honeybees to low concentrations of thiamethoxam and picoxystrobin drastically overload the hepato-nephroocitic system and reduces bee longevity.
- Thiamethoxam and picoxystrobin are harmful to newly emerged Africanized honeybees at low concentrations.
- Chronic exposure to thiamethoxam and picoxystrobin activated the hepato-nephroocitic system and reduce longevity in honeybee.

GRAPHIC ABSTRACT SUBTITLE: Responses of the hepato-nephroocitic system (HNS) from newly emerged workers of Africanized *A. mellifera* after five days of oral continuous exposure to pesticides. HNS is composed of the fat body, pericardial cells and immune cells along the myogenic region of the dorsal vessel. Hemocytes = representation of the hemocyte number in each experimental group; Fat body = representation of nucleus alterations (level of nuclear branching in trophocytes) and cytoplasmic granules of trophocytes and oenocytes; Pericardial cells = representation of the position of the nucleus (in black), cytoplasmic vacuolations (in gray) and grouping of these cells; Dorsal vessel = evidence of the absence of morphological changes but highlights the accumulation of hemocytes in the its lumen in bees from the TXT group; trophocytes and oenocytes; dark droplets in trophocytes = glycogen; grew droplets in trophocytes and oenocytes= vacuoles; bigger dark droplets in oenocytes= granules; small dark spots in oenocytes= granulation