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ORIGINAL RESEARCH ARTICLE

Impact of sublethal doses of thiamethoxam and *Nosema ceranae* inoculation on the hepato-nephrocitic system in young Africanized *Apis mellifera*

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We analyzed the morphological changes on the hepato-nephrocitic system (HNS) of Africanized honey bees exposed to thiamethoxam (TMX) and to *Nosema ceranae* (NOS) in isolation and co-exposure. We also analyzed the relative amount of hemocytes in TMX and/or Nosema-exposed bees. Newly emerged bees were exposed individually *per os* to TMX, at doses of 0.0856 ng/bee or 0.00856 ng/bee, or to TMX doses and 60,000 spores of *N. ceranae* per bee. Eight days after the single exposure, the results showed that TMX was extremely deleterious to the HNS at 0.00856 ng/bee, dramatically inducing morphological changes in HNS cells (trophocytes and oenocytes) and increasing hemocyte number. However, the treatment to TMX at 0.00856 ng/bee with additional NOS inoculation provoked the highest critical damage observed on HNS, collapsing the trophocytes, reducing the surface area of oenocytes, and inducing cell death in pericardial cells. Therefore, a sublethal dose of TMX, applied alone or simultaneously with NOS inoculation, triggered the disruption of the HNS and induced progressive damage on worker bees. The results demonstrated an interaction between biotic and abiotic agents in Africanized bees at individual level, whose impact at the colonial level remains to be assessed under field conditions.

Keywords: *Apis mellifera*; thiamethoxam; *Nosema ceranae*; ecotoxicology; biomarkers

Introduction

Despite the incontestable economic and ecological importance of honey bees, their populations are seriously endangered worldwide by multiple stressors, which can compromise the health of honey bees and increase colony mortality (Goulson, Nicholls, Botías, & Rotheray, 2015; Li et al., 2018). Among the biotic stresses imposed over these animals, it is worthy to mention those caused by fungi, bacteria, viruses (Evans & Schwarz, 2011), and parasites, mainly *Varroa destructor*, the most important economic honey bee pest worldwide and, therefore, being responsible for most of the reported global colony collapses. In addition to the biotic stressor agents, abiotic stressors can also negatively impact honey bee colonies, alone or in association with biotics (Genersch et al., 2010; Jacques et al., 2017; Rosenkranz, Aumeier, & Ziegelmann, 2010). Abiotic stress in honey bees such as pesticides (Sanchez-Bayo et al., 2016; Simon-Delso et al., 2015) and nutrition (Naug, 2009), as well as habitat loss associated with reduction of abundance and diversity of floral resources (Goulson et al., 2015), potentially contribute to the reduced survival and abundance of bee populations (Ferreira et al., 2015). According to Li et al. (2018), the interaction between different abiotic stresses and different biotic stressors and/or their combination may increase the severity of their effects on the health and survival of honey bees.

A combination between abiotic and biotic stresses represents a more field-realistic approach and needs further studies. Interactions between pesticides (neonicotinoid thiacloprid and miticide tau-fluvalinate) and the endoparasite *N. ceranae* were evaluated under *in vivo* colony conditions of *A. mellifera* (Retschnig et al., 2015). However, synergistic effects between *N. ceranae* and pesticides on honey bee mortality were observed only in experiments carried out under laboratory conditions (Alaux et al., 2010; Aufauvre et al., 2014; Vidau et al., 2011). Nevertheless, sublethal effects of the interaction between the microsporidium parasite and pesticides are more difficult to be detected under field conditions, so that laboratory studies still provide an important contribution to the understanding these effects in *A. mellifera* at individual level, considering that honey bees can develop many defense mechanisms to decrease the damage from the external environmental stress.

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Laboratory studies performed by Alaux et al. (2010), for example, demonstrated a significant decrease in the activity of glucose oxidase only in the group co-exposed to Nosema and imidacloprid, which affects the capacity of bees to sterilize the colony and brood food, and can potentially induce to a higher susceptibility of the colony to pathogens.

Gregorc et al. (2016) showed that the exposure to thiamethoxam (TMX), a neonicotinoid insecticide (Maienfisch et al., 2001; Tomizawa & Casida, 2005) present in pollen and nectar of treated plants (Pohorecka, Szczęsna, Witek, Miszczak, & Sikorski, 2017; Rondeau et al., 2015; Sánchez-Hernández et al., 2016), had no impact on infection cycle by *N. ceranae* in the honey bee midgut under experimental conditions, but inhibited midgut cell death when honey bees were exposed to both TMX and *N. ceranae* simultaneously. The authors also found that TMX had no impact on midgut epithelial cells, even in bees simultaneously exposed to *N. ceranae*. Histopathological alterations on midgut were observed only in response to the co-exposure of honey bees to a very low dose of TMX (500-fold below the LC50) and *N. ceranae*, but did not result in a negative impact on survival. Thus, we proposed to evaluate a novel morphological biomarker developed by Abdalla and Domingues (2015) in Africanized honey bees at the same experimental conditions studied by Gregorc et al. (2016) in order to understand the response of honey bees to the co-exposure of a biotic (parasite microsporidium, *N. ceranae*) and an abiotic (neonicotinoid insecticide, TMX) stressor.

According to Abdalla and Domingues (2015), the fat body, pericardial cells, and hemocytes, which are closely associated with the dorsal vessel, compose the hepatonephrocitic system (HNS) of bees. HNS disorders following exposure to sublethal doses of xenobiotics are not necessarily associated with an increase in mortality rates, since exposed bees can respond against xenobiotics through the HNS and their survival is not affected. Thus, hypothetically, bees can maintain their homeostasis through HNS filtration, detoxification, and bioavailability of the xenobiotics to the Malpighian tubules. For this reason, exposure of bees to a xenobiotic, or to a biotic stressor, may not demonstrate acute mortality (Domingues et al., 2017; Gregorc et al., 2016), considering that the HNS can minimize the toxic effects of such stressors.

According to Porrini et al. (2003), classic studies of mortality and survival rates are not sufficient to evaluate the real toxicological effects of xenobiotic exposures on the individual, especially at sublethal doses, because they typically do not induce death of the entire experimental population. Thus, the study of the HNS allows to infer the mechanisms employed by the bee to maintain their homeostasis. This approach can be used to determine the most likely implications caused by a particular stressor, considering that the fat body cells that compose the HNS have multiple functions (Adamczyk et al., 1996; Chapman, 2013; Roma, Bueno, & Camargo-Mathias, 2010), acting on several organs and systems, such as the nervous system, intermediary metabolism, ovary development, immune responses, anti-oxidative, and metalloprotein protective system (Abdalla & Domingues, 2015; Domingues et al., 2017; Skaldina & Sorvari, 2017).

In order to examine the response of the HNS in young workers exposed to TMX and *N. ceranae* spores, we simulated an intranidal stress condition. Thus, our aim was also to study the extent of the response of hemocytes and immune cells in the hemolymph in young Africanized workers exposed *per os,* separately or combined, to TMX and Nosema spores.

Material and methods

Material collection

Comb frames with capped worker brood were obtained from three healthy managed colonies of Africanized honey bees in an apiary in a rural area of Piedade, São Paulo, Southeast Brazil (lat. 23° 42' 43" S; long. 47° 25' 40" W), and transferred to the Laboratory of Structural and Functional Biology of the Federal University of São Carlos-*Campus* Sorocaba, municipality of Sorocaba. In an incubator, emergence was monitored daily. After emergence, one-day-old worker bees were confined to cages for toxicological bioassays and they were starved for 2 h before starting the bioassay.

Insecticide

TMX was purchased from Sigma-Aldrich (analytical standard, CAS Number 153719-23-4, 98.0% purity). A TMX stock solution (1000 ng a.i./μL) was prepared using autoclaved distilled water as solvent. Next, cascade dilutions were performed to obtain the working solutions with the nominal TMX concentrations in syrup offered as food to honey bees. Before use, working solutions were stirred and offered to bees (individual feeding).

Nosema ceranae spores

N. ceranae spores used for experimental infection in bioassays were obtained from infected colonies maintained by APTA (Agência Paulista de Tecnologia dos Agronegócios), located in the municipality of Pindamonhangaba, São Paulo State, Brazil. Molecular characterization by PCR (Higes, Garcia-Palencia, Martin-Hernandez, & Meana, 2007) was performed in spore inoculums by LASA (Beekeeping Health Laboratory) – APTA, to confirm the species *N. ceranae*.

Midguts isolated from infected forager worker bees were macerated and washed three times by centrifugation in insect Ringer solution. Concentrations of spores were determined by counting the spores in a Neubauer chamber (Marienfield, Lauda-Königshofen, Germany)

under light microscopy (Cantwell, 1970). The infective dose of 60,000 spores per bee was applied, as in our previous study (Gregorc et al., 2016). Even though the lowest infection dose for European *A. mellifera* is 10,000 spores/bee (Forsgren & Fries, 2010), a sixfold higher inoculum was applied in Africanized honey bee to ensure the *N. ceranae* infection. Spore inoculums were prepared fresh for bioassays from frozen honey bees collected one day before. Sterile filter pipette tips were used for individual bees.

Exposure to thiamethoxam and/or Nosema ceranae spores

Two doses of TMX were selected based on the median lethal concentration (LC50 = 4.28 ng a.i./μL of diet) described for Africanized *A. mellifera* (Oliveira, Roat, Carvalho, & Malaspina, 2014). Doses were 50-fold and 500-fold below the LC50 that correspond, respectively, to 0.0856 ng of TMX per bee (TMX 1/50) and 0.00856 ng of TMX per bee (TMX 1/500). Both doses can be considered realistic because residue levels in pollen range from 1.1 to 127 ng/g, as well as the average contamination of pollen/beebread with TMX is around 29 ng/g (Sanchez-Bayo & Goka, 2014). Pohorecka et al. (2012) detected 4.2 and 3.8 ng/g of TMX residues in the nectar and pollen samples and calculated the overall TMX residue intake for a nurse bee to be between 0.4 and 1.4 ng/bee/day for this concentration range that has been detected in the samples.

Six experimental groups were assayed in triplicate, with 20 bees per cage (60 bees per experimental group): I) TMX 1/50 – 0.0856 ng/bee of TMX; II) TMX 1/500 – 0.00856 ng/bee of TMX; III) TMX 1/50 + NOS – 0.0856 ng/bee of TMX plus 60,000 Nosema spores; IV) TMX 1/500 + NOS – 0.00856 ng/bee of TMX plus 60,000 Nosema spores; V) NOS – 60,000 Nosema spores; and VI) CTRL – Control group that received a solution of sucrose in water (1:1).

During *per os* feeding, treatment solutions consisted of the insecticide and/or Nosema inoculums in a 1:1 sucrose to water solution, as well as vehicle treatment (control group). The volume of the treatment solution for TMX was 2 μL and the total volume of treatment solution with TMX and *N. ceranae* spores was 4 μL, which included 2 μL of TMX solution and 2 μL of *N. ceranae* spore solution.

Toxicological bioassays

The experimental procedures used in this study were performed according to the methodology proposed by Aupinel et al. (2005) and Aupinel et al. (2007) with some modifications described in toxicological guideline for honey bees (OECD, 1998).

Newly emerged worker bees were divided into groups (20 bees per cage in triplicate per experimental group), and individually exposed to the insecticide (TMX) and/or inoculated with the pathogen spores. Workers were kept in cages, i.e., transparent plastic pots (530 mL, 11 cm × 7 cm) lined with filter paper (11 cm in diameter), which were maintained in a Biochemical Oxygen Demand (BOD) incubator at 33 ± 1 °C with 70 ± 10% relative humidity, and under darkness conditions.

Both TMX exposure and Nosema spores' inoculation procedures of caged bees were performed using a micropipette, i.e., bees were individually fed with specific solution according to each experimental group.

Following *per os* treatments, all bees in the cages were fed *ad libitum* with xenobiotic-free sugar syrup (sugar: water; 1:1; w:w) offered in wax feeder until the eighth day after the exposure, when they were collected for morphological and histochemical analyses. The feeder has an average capacity of 3 mL, and it was made with beeswax and shaped in the rectangular format with an internal channel to contain the syrup provided to the bees. Although this channel was narrow, it was wide enough to let in the bees' proboscis. Feeders were inspected and completed twice a day along the bioassay. During inspection, dead bees were removed from pots.

Morphological and histochemical analysis

Eight days after the inoculations, bees were randomly sampled from each experimental group ($N = 10$ per group). The bees were anesthetized by cold exposure (4 °C) and dissected to remove the dorsal vessel together with the parietal fat body. These organs were immersed in a fixative solution (4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4) for 24 h at 4 °C. Upon fixation, the material was embedded in JB-4 resin (Leica Biosystems Nussloch GmbH, Heidelberg, Germany) according to the manufacturer recommendations.

Histological sections of 1.5 μm thickness were cut with a Leica microtome (RM 2255) and stained with hematoxylin and eosin for qualitative analysis to establish a morphological pattern per experimental group for the studied organs. In order to determine a histochemical pattern per experimental group, one slide with 20 non-consecutive sections were analyzed per individual ($N = 10$ individuals per experimental group). Additionally, these histological sections were submitted to quantitative morphometric analysis of oenocytes and pericardial cells. Trophocytes are irregular shaped cells, making it very difficult to measure their areas; thus, these fat body cells were qualitatively evaluated by already well-established morphological parameters, such as nuclear and cytoplasm delimitations, and the presence or absence of cytoplasm vacuolization (Paes De Oliveira & Cruz-Landim, 2003).

Histological sections of the fat body were also stained with the periodic acid-Schiff (PAS) histochemical technique for the detection of neutral glycoconjugates (Mcmanus, 1946, 1948). A qualitative analysis of cells

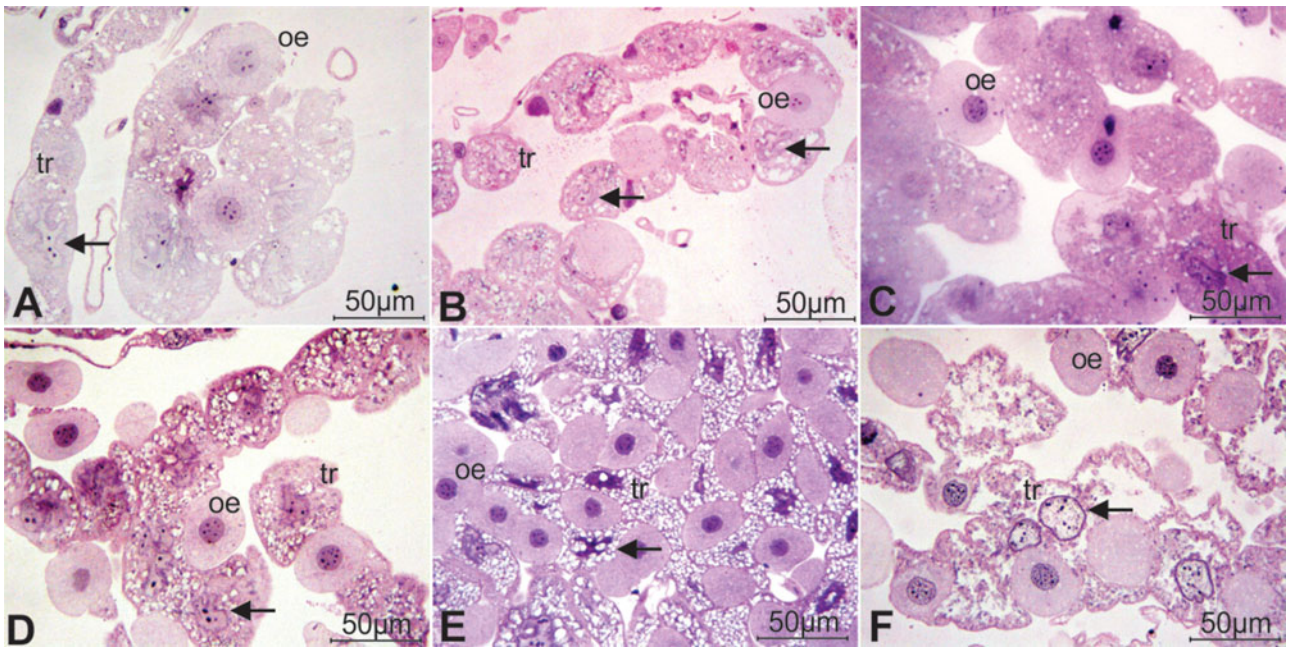

Figure 1. Photomicrographs of the fat body of the treatment groups. A: CTRL; B: NOS; C: TMX 1/50; D: TMX 1/50 + NOS; E: TMX 1/500; F: TMX 1/500 + NOS. The sections were stained with hematoxylin and eosin and feature trophocytes (tr) and oenocytes (oe) of the fat body of Africanized *A. mellifera*. The arrows indicate the nuclei of trophocytes (A–F) and their morphological changes (B–F).

was performed in order to establish a histochemical pattern per experimental group for the studied organs by the application of well-established parameters that classify the intensity levels of histochemical reaction described by Domingues et al. (2017).

Morphometric analyses of pericardial cells and oenocytes

Slides containing sections stained with hematoxylin and eosin were submitted to morphometric analysis of oenocytes and pericardial cells by means of the program Leica Application Suite (LAS V3.8) coupled to a photomicroscope. One slide containing 20 non-consecutive sections were analyzed per individual ($N = 10$ individuals per experimental group). Thus, 10 slides were analyzed per experimental group. In each slide, 20 measurements from histological photomicrographs were performed for each cell type (oenocytes or pericardial cells), thus obtaining 200 morphometric measurements of the pericardial cells and 200 morphometrics of the oenocytes.

At first, to evaluate the distribution of the dataset, we performed D'Agostino & Pearson omnibus normality test. Since the data did not present normal distribution and for comparison between the groups, the Kruskal–Wallis nonparametric test ($p < 0.05$) was followed by the Dunn's post-test for multiple comparisons using the statistical program GraphPad Prism 8 (GraphPad Prism Software, Inc., San Diego, CA, USA).

Quantitative analysis of hemocytes

For the quantification of hemocytes, 20 workers from each experimental group were collected. They were anesthetized by cold exposure (4 °C), and 5 µL of hemolymph was collected from the thoracic region with a micropipette. This volume was transferred to a polypropylene microtube containing 5 µL of 0.2% aqueous methylene blue solution, resulting in a 1:1 dilution (v:v). For the determination of the total hemocyte count, 10 µL of the hemolymph diluted in 0.2% methylene blue was placed in a Neubauer chamber, and cell counts were performed per mL of hemolymph (in five quadrants of the chamber) under a bright field light microscope at a magnification of 400x. Two total counts per individual were performed (the value of each count is the result of the means of the five quadrants), resulting in 40 counts per group. To compare the data obtained for each group, the nonparametric one-way ANOVA ($p < 0.05$) followed by Bonferroni's post-test for multiple comparisons were performed using the program GraphPad Prism 8.0 (GraphPad Prism Software, Inc.).

Results

Morphological and histochemical analysis

In the CTRL group, oenocytes appeared as ellipsoidal cells with acidophilic cytoplasm and central, round shaped nuclei distributed in the fat body in close association with trophocytes (Figure 1A). Trophocytes are polygonal cells with acidophilic cytoplasm containing small acidophilic and basophilic granules, facultative vacuoles, and a large, irregular, and branched nucleus (Figure 1A). Pericardial cells presented as columnar-like cells, with central nuclei and cordonal arrangement (Figure 2A).

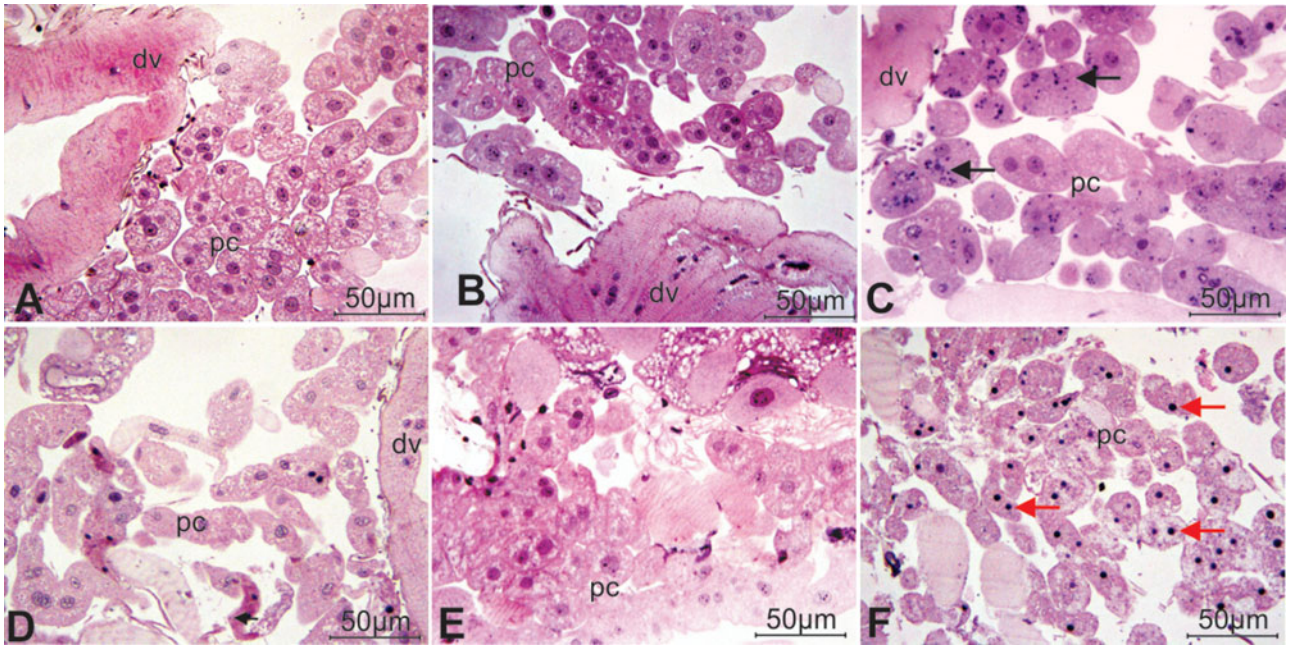

Figure 2. Photomicrographs of the dorsal vessel (dv) and pericardial cells (pc) of Africanized *A. mellifera*. Treatment groups: A: CTRL; B: NOS; C: TMX 1/50; D: TMX 1/50 + NOS; E: TMX 1/500; F: TMX 1/500 + NOS. The sections were stained with hematoxylin and eosin. The red arrows point out the pyknotic nuclei of pericardial cells in F, and the black arrows indicate granules in the cytoplasm of pericardial cells in C.

Morphological results showed a significant response in oenocytes to TMX 1/500 + NOS, as cells presented pyknotic nuclei (Figure 1F).

The trophocytes of the CTRL group showed few vacuoles and the typical branched nucleus contour, like those observed in the NOS group (Figure 1B). In the other treatment groups, these cells exhibited extensive morphological changes compared to the control. In the TMX 1/500 group, trophocytes did not possess many vacuoles, but the nuclei displayed a round shape, losing their typically branched contour (Figure 1C). In the TMX 1/50 + NOS group, trophocytes showed high cytoplasm vacuolization, similar to the TMX 1/500 group, and in both treated groups, cells' nuclei lost their characteristic branched contour (Figure 1C,D), presenting a very condensed chromatin. In TMX 1/500 group, the morphology of trophocytes was typical for the cell deletion process (Figure 1E). The TMX 1/500 + NOS group had trophocytes with vacuolated cytoplasm and cells with anomalous characteristics, with a total loss of morphology. The cytoplasm was completely unstructured and vacuolated, and nuclei were large and round, but chromatin was not condensed, in contrast to other treatment groups (Figure 1F).

Neither NOS nor TMX 1/50 + NOS showed evident morphological differences in pericardial cells compared to the control group (Figure 2D). In contrast, TMX 1/50 had pericardial cells with many basophilic cytoplasmic granules (Figure 2C), and in the TMX 1/500 + NOS group, cell collapse and pyknotic nuclei were observed in pericardial cells, indicating cell death (Figure 2F). In all cases, pericardial cells apparently stayed in stage I, or basal activity.

Morphometric analysis

There was a reduction in the surface area of oenocytes in TMX groups ($p = 0.0026$) and NOS group ($p = 0.0036$) in comparison with the CRTL group (Figure 3A), but no significant difference was observed between the doses (TMX 1/50 and 1/500). TMX 1/50 + NOS ($p = 0.0412$) and TMX 1/500 + NOS ($p = p = 0.0016$) groups had a higher reduction of oenocyte surface areas with regard to the CRTL group (Figure 3A). The areas of oenocytes from co-exposed groups (TMX 1/50 + NOS and TMX 1/500 + NOS) were similar to those observed in bees exposed to high dose of TMX (Figure 3A).

The pericardial cells of the NOS group presented the highest reduction in surface area, followed by the TMX 1/50 group (Figure 3B). In co-exposed groups, only TMX 1/500 + NOS presented reductions in area compared with the CTRL group (Figure 3B).

Periodic acid-Schiff (PAS)

The HNS of CTRL showed few PAS-positive granules indicative of glycogen in the trophocytes, and a basal amount of PAS-positive granules indicative of glycogen in the oenocytes and in pericardial cells of the dorsal vessel (Figure 4A). NOS treatment induced a slight increase in the glycogen level in pericardial cells (Figure

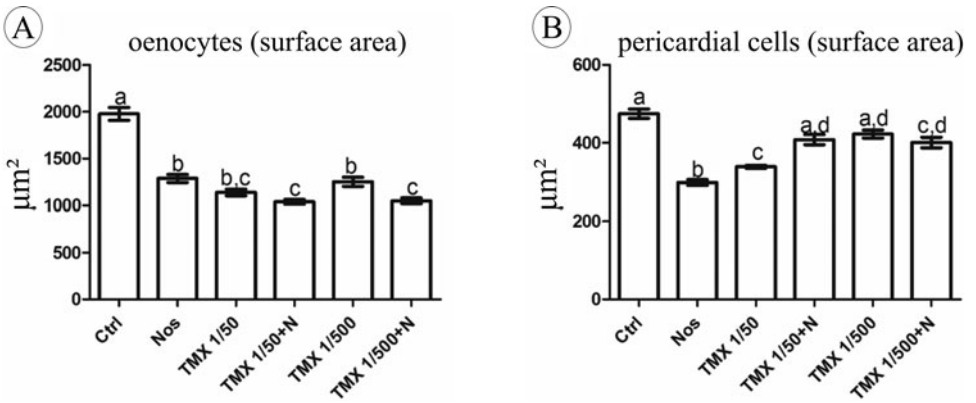

Figure 3. Morphometric analysis graph of surface area of pericardial cells (A) and oenocytes (B) of bees exposed to different treatment protocols. Different letters indicate statistically significant differences ($p < 0.05$) between columns, and the same letters indicate no significant difference. Treatment groups were as follows: A: CTRL; B: NOS; C: TMX 1/50; D: TMX 1/50 + NOS; E: TMX 1/500; F: TMX 1/500 + NOS.

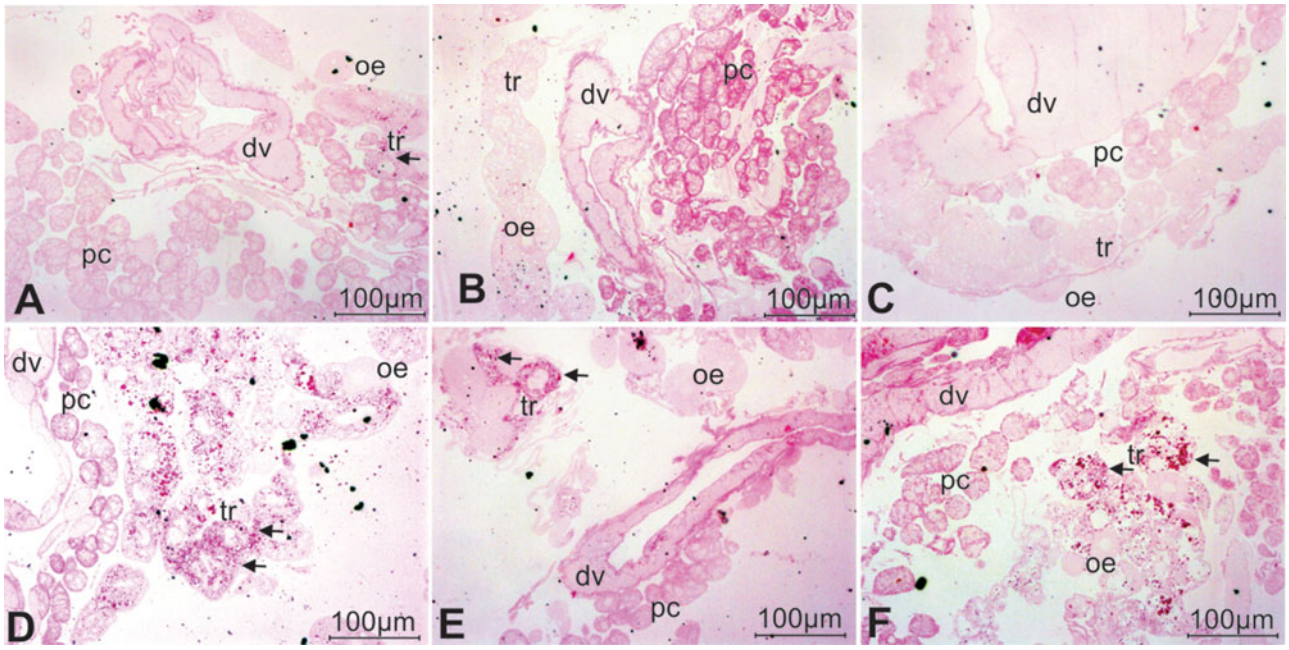

Figure 4. Photomicrographs of the dorsal vessel (dv), pericardial cells (pc), oenocytes (oe), and trophocytes (tr) of Africanized *A. mellifera* bees exposed to different treatment protocols. A: CTRL; B:NOS; C:TMX 1/50; D:TMX 1/50 + NOS; E:TMX 1/500; F:TMX 1/500 + NOS. The sections were stained with periodic acid-Schiff. The black arrows indicate the PAS-positive granules of glycogen accumulated in trophocytes.

4B), while following TMX 1/50 treatment we observed a basal level of glycogen in cells of the HNS (Figure 4C). Trophocytes from TMX 1/50 + NOS, TMX 1/500, and TMX 1/500 + NOS treatment groups had great amount of glycogen granules compared to CTRL and NOS groups (Figure 4D–F).

Quantitative analysis of hemocytes

The results of hemocytes' counts indicated that the number of hemocytes in NOS, TMX 1/500, and TMX 1/50 + NOS treatment groups were different than those observed to the CRTL (Figure 5), whereas TMX 1/500 + NOS and TMX 1/50 treatment groups were not different (Figure 5). The relative mean hemocyte number for CTRL was 2100 cells/mL, NOS had 2800 cells/mL, TMX 1/500 had 2800 cells/mL, and TMX 1/50 + NOS had 2700 cells/ml. All groups that differed from the CTRL had increased hemocytes' number.

Discussion

During the systole and diastole of the myogenic region of the dorsal vessel of bees, the hemolymph enters the dorsal vessel through small openings, taking a one-way direction from the distal portion of the abdomen to the posterior portion into the head, where the vessel opens. The hemolymph can also contact the bee's internal organs by body shake. Therefore, the hemolymph into the body cavities and/or before it enters the

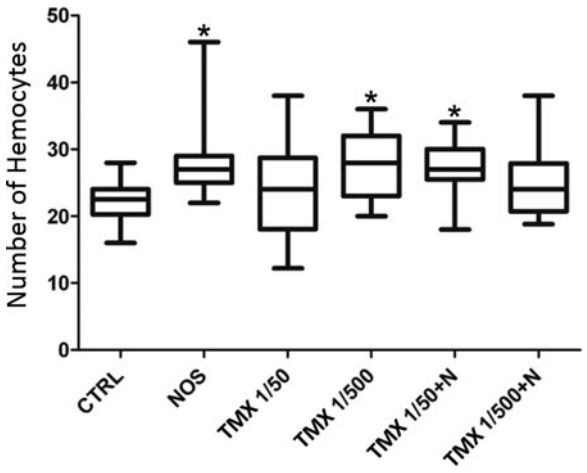

Figure 5. Boxplot of the hemocyte counts of the treatment groups studied. Asterisks indicate statistical differences ($p < 0.05$) to the control group.

dorsal vessel, it must pass through the fat body, the pericardial cells, and the hemocytes (Abdalla & Domingues, 2015). During this process, toxicants may be taken up by pericardial cells, metabolized by the trophocytes and/or modified by oenocytes by enzymes of the cytochrome P450 NADPH reductase family. Therefore, the HNS is considered a novel and precise biomarker of risk assessment of bees, being responsive to all ingested toxicants and affecting many vital systems of bees, from molecular to physiological aspects, even if the bees do not present behavioral sings of intoxication (Abdalla & Domingues, 2015; Domingues et al., 2017). Therefore, this study showed that honey bee's HNS even when exposed to trace concentrations of TMX was negatively impacted on the toxicant, in theory, damaging the intermediate metabolism, the activity of antioxidants and detoxicant enzymes, the capacity to uptake toxicants of the hemolymph, and even impairing the immune response of the bees (Abdalla & Domingues, 2015; Domingues et al., 2017).

In honey bees, the effect of Nosema inoculation and xenobiotic exposition is well studied (Aguila et al., 2006; Dussaubat et al., 2012; García-Palencia et al., 2010; Higes et al., 2007; James & Green, 2002). In honey bee workers inoculated with *N. ceranae* spores, cell death was detected in midgut at the first stage of the parasite infection (Gregorc et al., 2016), i.e., five days post-inoculation, which represents a defense mechanism of the midgut to prevent the spread of infection to neighboring cells (James & Green, 2002). Following this first stage, there is a decrease in the cell death rate in bee's midgut, which is induced by microsporidium capable of interfering with the apoptosis pathways of the host cell to extend their life span (Aguila et al., 2006), allowing its life cycle inside the epithelial cells. In the later phase of infection, i.e., seven days after exposition, Nosema spores released from midgut cells to the intestinal lumen trigger cell lysis and epithelium degeneration (García-Palencia et al., 2010; Higes et al., 2007). Decreased rates of epithelial renewal in the gut (Dussaubat et al., 2012) can cause death of the bee at the end of the infection period, at day 7, probably by starvation. Thus, although midgut has been studied in honey bees infected by Nosema spores, alone or in combination with pesticides, there is a gap in studies with other bee tissues or organs that could be indirectly affected by the microsporidium infection, as HNS.

Oenocytes of the fat body are cells that play a role in detoxification and bioavailability of xenobiotics for excretion by the Malpighi tubules (Roma et al., 2010); thus, these cells are involved in protection against insecticides. Detoxification enzymes present in oenocytes, i.e., P450 reductase, are potentially induced by chemical stress (Lycett et al., 2006). Another enzyme that has been demonstrated to be very efficient to metabolize some neonicotinoids as thiacloprid was recently identified as a single cytochrome P450, CYP9Q3 (Manjon et al., 2018).

Xenobiotics, after being absorbed by midgut cells, are incorporated into hemolymph, coming in direct contact with fat body cells and pericardial cells that constitute the HNS (Abdalla & Domingues, 2015). Honey bee oenocytes present great plasticity during bee development regarding their surface area. The size of oenocytes increases gradually during pupal development and also in early days of the adult phase (Cousin et al., 2013; Martins & Ramalho-Ortigão, 2012; Ruvolo & Cruz-Landim, 1993). Besides the natural plasticity that occurs with age and polyethism of honey bees, these cells are very sensitive to chemical compounds (Cousin et al., 2013). According to Cousin et al. (2013), after 48-h exposure to the herbicide Paraquat, there was a drastic reduction in the size of oenocytes, even at concentrations as small as 0.001 μg paraquat/kg food. Therefore, the measurement of oenocyte surface area can be an important parameter to indicate the toxicological response of the organism.

According to our results, both TMX and NOS, alone or in combination, induced a decrease in oenocytes' surface area. When these stressors were administered simultaneously, they caused the greatest decrease in the surface area of the oenocytes in comparison with the control. It is well known that the fat body of honey bees is reduced in bees infested by parasites as Nosema and Varroa (Le Conte, Ellis, & Ritter, 2010). Therefore, the present results corroborate that the decrease in oenocyte surface area is a reliable parameter to indicate toxicity induced by abiotic stressors and with biotic agents.

According to Gregorc et al. (2016), TMX has no negative impact on *N. ceranae* development in experimental conditions. The authors also found that TMX had no impact on the epithelial cells of the midgut of five-day old Africanized honey bee workers. Even more, TMX seems to reduce cell death in the midgut of bees simultaneously exposed to TMX and *N. ceranae*.

Probably, this reduction of cell death rate during this first stage of Nosema infection could be explained by a decrease in spore production in a dose-dependent manner at five days post-inoculation in Africanized honey bees exposed to TMX, as observed by these authors.

If the midgut absorption of nutrients was not negatively impacted by TMX nor by *N. ceranae* in five-day old Africanized honey bees (Gregorc et al., 2016), we can suggest that the accumulation of glycogen into trophocytes was a direct consequence of TMX or NOS inoculation. Indeed, eight-day-old Africanized honey bees exposed to TMX and *N. ceranae* simultaneously (TMX 1/50 + NOS and TMX 1/500 + NOS) had more glycogen granules in trophocytes than bees separately exposed to NOS or TMX 1/50, except for the TMX 1/500 group. Interestingly, when bees were exposed to TMX 1/50, there was not an increase in glycogen granules inside trophocytes. However, when TMX 1/50 was administered simultaneously with NOS, we observed an extensive increase in glycogen level. In previous experiments, survival and mortality rates of Africanized honey bee workers were not affected by TMX and/or NOS (Gregorc et al., 2016), while in our experiment, we found an evident impact on the cells of the HNS of eight-day-old workers exposed to NO or TMX alone (TMX 1/50) or in combination with NOS (TMX 1/500 + NOS), which is probably playing a defense role against both stressors in order to keep bees' homeostasis. Midgut morphology of Nosema inoculated 8-day workers will be studied in future.

When bees were exposed to NOS, there were no evident changes in morphology of trophocytes, but these cells collapsed when bees were exposed simultaneously to TMX and NOS. This is evident in the TMX 1/500 and TMX 1/500 +NOS groups, in which the impact on trophocytes was more severe. Their nuclei had an extreme pyknosis, suggesting cell death, whereas the TMX 1/500 + NOS group had larger cytoplasmic vacuoles and round nuclei, without signs of chromatin condensation.

Bees consume large amounts of sugar to supply energy for general metabolism related to flight, cellular respiration, and physical activities, such as thermoregulation and locomotion (Chapman, 2013). However, to access the complex sugar from honey, bees first break down disaccharides into monosaccharides, since only monosaccharides can pass from the midgut epithelium to the hemolymph (Crailsheim, 1988). This physiological process depends on carbohydrate-metabolizing enzymes, present in the midgut, which convert sucrose into glucose and fructose (Kunieda et al., 2006). Therefore, the accumulation of glycogen in trophocytes may be a response to the increased bioavailability of monosaccharide absorption promoted by TMX + NOS, since this combination seems to reduce levels of cell death in midgut of honey bee workers (Gregorc et al., 2016). However, in addition to oenocyte surface reduction, the accumulation of glycogen into damaged trophocytes also seems to be an indication of toxicity because glycogen probably will not be mobilized for hemolymph due to possible inactivity of morphologically altered trophocytes.

TMX in isolation, also simultaneously to *N. ceranae*, altered the quantity of hemocytes, except in bees exposed to TMX 1/50 and TMX 1/500 + NOS. It is evident that both biotic and abiotic stressors, in isolated or combined exposures, caused an increase in the amount of circulating hemocytes in the hemocoel. We would like to emphasize that more studies are still needed to better understand the processes involved in hemocyte counts when insects are exposed to pesticides. It was found that *Rhynocoris kumarii* (Hemiptera) exposed to organophosphate insecticides, such as dimethoate and methylparation, had an increase in hemocytes' counts after 10 days of continuous exposure (George & Ambrose, 2004). TMX also increased the hemocyte counts in workers of Africanized *A. mellifera* after five days of continuous exposure (Domingues et al., 2017). On the contrary, the total number of hemocytes decreased in young queens of Western honey bees exposed to field-realistic concentrations of neonicotinoid pesticides (thiacloprid and clothianidin), suggesting differences between castes in response to neonicotinoid exposure (Brandt et al., 2017).

In bees, as well as in vertebrates, two types of immune responses have been described, cellular and humoral. Neonicotinoids, such as clothianidin, negatively modulate immune signaling (Di Prisco et al., 2013). The antimicrobial peptides present in insect hemolymph are key elements to promote their innate immunity. Not only the fat body, but also the hemocytes, are responsible for synthetizing and releasing peptides; however, hemocytes are able to release antimicrobials more rapidly than the fat body cells (Yakovlev et al., 2016). Alterations in the immunocompetence of honey bees induced by changes in total hemocyte counts could possibly lead to an impaired disease resistance capacity, enhancing the progression of pathogens in honey bees (Brandt et al., 2017; Di Prisco et al., 2013).

In our study, both *N. ceranae* and/or TMX induced alterations in oenocytes, trophocytes, and pericardial cells. Meanwhile, there are no data on the toxicokinetics of TMX in honey bees. In some insects, TMX is rapidly converted to clothianidin after oral administration (Benzidane et al., 2010). In *Spodoptera frugiperda* (Lepidoptera), after 4-h administration of 1 μg of TMX, 30% of neonicotinoid-equivalents in larvae hemolymph were clothianidin, reaching 90% after 24 h. When 5 μg of TMX was administered, clothianidin represented 10% after 4 h, and 60% after 24 h of neonicotinoid-equivalents in hemolymph (Nauen, Ebbinghaus-Kintscher, Salgado, & Kaussmann, 2003). According to Benzidane et al. (2010), in *Periplaneta americana* (Blattodea) TMX was metabolized to clothianidin 1 h after its application,

and TMX levels remained persistent after 48 h. Domingues et al. (2017) continuously exposed newly emerged honey bees workers to very low doses of thiamethoxam (0.001 ng) and after five days described that the neonicotinoid induced sublethal effects in the HNS, altering the morphology of trophocytes and pericardial cells. Taken together, it is evident that TMX and/or Nosema spores induced effects at the cellular level in honey bees and these changes in cell biomarkers can be considered as defense response at individual level to TMX exposure or Nosema infection whether applied alone or in combination.

The relevance of extrapolating the here-obtained data at sub-individual level to population level still needs to be evaluated through long-term studies at field conditions, since classic field studies did not demonstrate a synergistic effect between Nosema infection and neonicotinoid exposure on the bee mortality at the colony level (Odemer & Rosenkranz, 2019; Retschnig et al., 2015).

Neonicotinoids can impair the ability of young workers to perform the tasks and activity of foragers, i.e., affecting age polyethism of workers (Perry, Sovik, Myerscough, & Barron, 2015). In addition, assuming Nosema infection accelerates age polyethism in young honey bees (Lecocq, Jensen, Kryger, & Nieh, 2016), it becomes urgent to develop long-term field studies of population dynamics taking into consideration the age polyethism changes in bee colonies exposed to Nosema in combination with neonicotenoids, not only bee mortality at colony level, since bees are not only exposed to a single stressor in the environment, but to a plethora of them.

According Di Pasquale et al. (2013), the quality of pollen affects both physiology and the survival of honey bees inoculated and non-inoculated with *N. ceranae*. The quality of pollen may have no beneficial consequences on the physiology of healthy bees, but it can affect their capacity to tolerate an external stress such as Nosema infection. Nurse bees infected by *N. ceranae* fed with polyfloral blend lived longer than bees fed with monofloral pollens. According to the authors, a polyfloral blend can supply a variety of pollen from different vegetal species, improving the tolerance of infected bees with Nosema. In our research, we used sucrose to avoid such influence on the bioassays (OECD/OCDE, 1998).

Concerning countries such as Brazil that depend on agricultural commodities exportation, areas of intensive agriculture greatly reduce the supply of pollen diversity and increase the exposure of bees to various types of pesticides, and according to the findings of Di Pasquale et al. (2013) that could affect the susceptibility of the bees to Nosema infection by the lack of pollen variety in monoculture areas. In 2019, the Brazilian government released more than 300 pesticides (ANVISA, 2019), most of them harmful to bees and banned in developed countries, as the sulfoximine sulfoxaflor (Siviter, Brown, & Leadbeater, 2018). In light of our findings, the results of Di Pasquale et al. (2013) provide potential for future research of combinatorial effects in terms of nutritional stress, pathogen infection, and pesticide exposure.

Conclusions

Thiamethoxam, alone or associated with *N. ceranae*, induced responses in the HNS of caged honey bees, namely surface area reduction of oenocytes and pericardial cells, morphological alterations in trophocytes with glycogen accumulation, increases of hemocyte number. The pericardial cells remained in stage I, indicating the maintenance of filtration capacity of the neonicotinoid present in the hemolymph.

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Disclosure statement

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