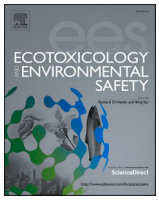
Ecotoxicology and Environmental Safety 223 (2021) 112581

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv



Enzymatic responses in the head and midgut of Africanized *Apis mellifera* contaminated with a sublethal concentration of thiamethoxam

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ARTICLE INFO

Edited by Paul Sibley

Keywords:
Honeybees
Neonicotinoid
Biomarkers
Oxidative stress

ABSTRACT

The increasing use of insecticides, promoted by the intensification of agriculture, has raised concerns about their influence on the decline of bee colonies, which play a fundamental role in pollination. Thus, it is fundamental to elucidate the effects of insecticides on bees. This study investigated the damage caused by a sublethal concentration of thiamethoxam - TMX (0.0227 ng/μL of feed) in the head and midgut of Africanized *Apis mellifera,* by analyzing the enzymatic biomarkers, oxidative stress, and occurrence of lipid peroxidation. The data showed that the insecticide increased acetylcholinesterase activity (AChE) and glutathione-S-transferase (GST), whereas carboxylesterase (CaE3) activity decreased in the heads. Our results indicate that the antioxidant enzymes were less active in the head because only glutathione peroxidase (GPX) showed alterations. In the midgut, there were no alkaline phosphatase (ALP) or superoxide dismutase (SOD) responses and a decrease in the activity of CaE was observed. Otherwise, there was an increase in GPX, and the TBARS (thiobarbituric acid reactive substances) assay also showed differences in the midgut. The TBARS (thiobarbituric acid reactive substances) assay also showed differences in the midgut. The results showed enzymes such as CaE3, GST, AChE, ALP, SOD, and GPX, as well as the TBARS assay, are useful biomarkers on bees. They may be used in combination as a promising tool for characterizing bee exposure to insecticides.

1. Introduction

Among the main pollinator insects, bees play a key role in the reproduction of most angiosperms, and are thus essential for the majority of agricultural crops (Boyle et al., 2019). In Brazil, Africanized *Apis mellifera* Linnaeus, 1758 is of great importance to the country's economy, as many crops such as coffee, citrus, soybean, sunflower, and tomato depend on them for pollination to boost agricultural production (Klein et al., 2020). At the same time, it is one of the countries with the largest commercialization of pesticides in the world. The increasing use of insecticides in modern agriculture has raised concerns about their influence on the decline of bee colonies in several countries, resulting in great economic losses (Brodschneider et al., 2018; Kulhanek et al., 2017; Oldroyd, 2007). Considering that bees are not pesticide-targets (Catae et al., 2014), there are different routes in which bees can come into contact with them, such as pollen, nectar, wax, mud, soil, particles in the air, water, and others (Boyle et al., 2019).

Neonicotinoids, such as thiamethoxam, represent one of the classes of insecticides most widely used in crops outside of the European Union (Bass and Field, 2018). They have neurotoxic actions and act as agonists of acetylcholine, competing with it for its cholinergic receptors that mediate nerve impulses (Ishaaya et al., 2007). As they have systemic actions on plants, they end up contaminating the plant resources utilized by bees, and thus expose the entire hive to the contaminated material (Bass and Field, 2018). Although the European Union has banned the use of thiamethoxam (Bass and Field, 2018), in Brazil this insecticide is still frequently used on crops. However, in 2017 the Brazilian Institute for the Environment and Renewable Natural Resources (IBAMA) published a Normative Instruction establishing the risk assessment for pollinators in Brazil (IBAMA, 2017). Furthermore, IBAMA emphasizes the importance of studying three neonicotinoid insecticides (thiamethoxam, imidacloprid, and clothianidin). So, there are knowledge gaps that need to be filled to provide data that should support regulatory decisions about the conservation of bee biodiversity (Cham et al., 2018; Rortais

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https://doi.org/10.1016/j.ecoenv.2021.112581
Received 15 April 2021; Received in revised form 8 July 2021; Accepted 28 July 2021
Available online 2 August 2021

et al., 2017).

One of the ways to analyze the toxicity of compounds, such as thiamethoxam, is to investigate the altered activity of detoxification-related enzymes called biomarkers. Ecotoxicological studies (Badiou-Bénéteau et al., 2012) have shown that the pattern of enzyme responses in *A. mellifera* can be altered under exposure to various types of insecticides. According to Hyne and Maher (2003), several enzymes, such as acetylcholinesterase (AChE), carboxylesterase (CaE3), glutathione-S-transferase (GST), metallothioneins, and Cyp450, have the potential for use as biomarkers in environmental monitoring programs. Biomarker enzymes are included in the biotransformation process of xenobiotics, a phase-divided process that aims to generate polar and water-soluble derivatives, resulting in decreased toxicity and facilitating excretion (Almazroo et al., 2017). The CaE3, enzymes used as exposure biomarkers, are Phase I enzymes that react with non-polar compounds by hydrolysis. The resulting metabolites are further processed by Phase II enzymes or excreted (Grein and Snyder, 2008; Stone et al., 2002). While GST belongs to a family of Phase II enzymes responsible for catalyzing the conjugation of reduced glutathione, playing a central role in the cellular detoxification process and reduction of the deleterious effects of xenobiotic compounds (Badiou-Bénéteau et al., 2012; Stone et al., 2002; Weirich et al., 2002).

Other enzymes can also be used as a biomarker in bees, such as alkaline phosphatase (ALP), which consists of a digestive enzyme involved in the adsorption and transport mechanisms, through the hydrolysis of phosphate groups (Moss, 1992). AChE is another enzyme, mainly present in the heads of bees (Badiou et al., 2007), responsible for mediating the transmission of the nerve impulse and hydrolyzing acetylcholine in cholinergic synapses, has also previously been investigated (Almasri et al., 2020; Badiou-Bénéteau et al., 2012; Badiou et al., 2008; Tavares et al., 2019). Since thiamethoxam acts on acetylcholine and nicotine receptors in the nervous system, the role of this enzyme may be compromised due to the effects of this insecticide, which may adversely affect the viability of bees (Badiou et al., 2008).

The study of enzymatic activities during oxidative stress processes offers another indication of the cellular responses of bees to the damage caused by insecticide exposure (Balieira et al., 2018; Dussaubat et al., 2012). The occurrence of oxidative stress processes arises due to an imbalance between oxidant compounds and antioxidants, in favor of the excessive generation of free radicals (reactive oxygen species - ROS) or to the detriment of the speed that they are removed. This process leads to the oxidation of biomolecules with consequent loss of their biological functions, causing potential oxidative damage to the cells and tissues (Farooqui, 2014; Halliwell and Whiteman, 2004). Some of the enzymes responsible for antioxidant defenses, such as superoxide dismutase (SOD) and glutathione peroxidase (GPX), act by means of prevention mechanisms, avoiding and/or controlling the free radicals formation (Ighodaro and Akinloye, 2019). The enzyme SOD, an excellent marker of oxidative stress, acts through the dismutation reaction, catalyzing the generation of hydrogen peroxide (H_2O_2) from the superoxide radical O_2. Concurrently, enzymes such as GPX and catalase act in an integrated manner to prevent the accumulation of H_2O_2, which, although not a free radical, is also reactive and capable of promoting potential damage (Kurutas, 2016). Finally, when the organism cannot combat the imbalance caused by ROS, the damage can extend to the cell membranes, leading to lipid peroxidation, a process that can cause changes to the structure and permeability of membranes, compromising cellular metabolism (Gaschler and Stockwell, 2017). One of the consequences of lipid peroxidation is the formation of cytotoxic products, such as malondialdehyde (MDA), a substance that can be used as an indirect measure of cellular damage, by using the widely used TBARS assay (thiobarbituric acid reactive substances) (Ghani et al., 2017).

The use of a combination of different biomarkers to describe the toxicological effects of neonicotinoids on bees can be an important pathway to understand how the insecticide works in the bee's organism (Badiou-Bénéteau et al., 2012). Given the above, this study aimed to evaluate the effects of a sublethal concentration of TMX on the physiology of Africanized *A. mellifera* bees. For this purpose, the activity of the following enzymes was evaluated: exposure biomarkers (CaE3 and GST), antioxidant enzymes (SOD and GPX), analysis of AChE in the head, ALP in the midgut, and the determination of lipid peroxidation using the TBARS assay in both organs. In this way, the evaluation of this set of enzymes can be used for biomonitoring some effects on bees such as the detoxification process and oxidative stress combat. Furthermore, this study is the first to provide an overview using six enzymes, besides lipid peroxidation, evaluated in two different organs, as biomarkers in bees exposed to a neonicotinoid.

2. Materials and methods

2.1. Obtaining biological materials

The Africanized bees, *A. mellifera* were collected from the apiary of the Biosciences Institute, Department of Biology, São Paulo State University (UNESP), at Rio Claro, SP, Brazil. Brood frames were removed from three different colonies and kept for 24 h in an incubator at 34 °C and relative humidity of 80 ± 10% (Aupinel et al., 2009). The newly emerged bees (aged 0–24 h) were marked with special non-toxic ink and returned to their respective colonies until they were collected after 20 days. All experiments followed the recommendations of the guidelines for xenobiotic assessments on bees (OECD, 1998) and for the oral exposure test, we used the insecticide thiamethoxam PESTANAL® ($C_8H_{10}ClN_5O_3S$), Sigma-Aldrich analytical standard, soluble in water, and purity ≥ 98.0%.

2.2. Oral intoxication bioassay

Three different colonies were used, from which 80 bees were collected per colony and grouped as follows: control group (120 bees) and TMX group (120 bees). For collection, 500 mL plastic cages were used with holes drilled on their sides, and 20 bees were kept per cage. The experimental cages containing forager bees were kept in an incubator (biochemical oxygen demand - D.B.O.) at 32 ± 2 °C and relative humidity of 70 ± 10% in the absence of light. For oral exposure, a stock solution of thiamethoxam was prepared using deionized water (final concentration = 1000 ng of active ingredient/μL). Serial dilutions were carried out from the stock solution until the desired sublethal concentration ($LC_{50/10}$ = 0.0227 ng thiamethoxam/mL^{-1}). The sublethal concentration was based on the average lethal concentration (LC_{50} = 0.227 ng a.i./μL) of *A. mellifera* determined Miotelo et al. (2021). The concentration presented in this study is equivalent to 22.7 ppb and, it is comparable to residues of thiamethoxam described in the literature for nectar (range from 1 to 12 ppb) and pollen (1–53 ppb) (Krupke et al., 2012; Mullin et al., 2010; Pilling et al., 2013; Stoner and Eitzer, 2012). The TMX group received food containing this sublethal concentration and the control group received a sucrose solution without contamination (1:1 water and sugar) supplied in 2 mL microtubes containing holes drilled on their sides. All groups received food *ad libitum* for 5 days. For the enzyme activity assay, collection times were determined based on the mean lethal time (LT_{50}) of *A. mellifera* exposed to $LC_{50/10}$ (Miotelo et al., 2021). The LT_{50} represents the period in which 50% of the bees die after contact with the insecticide (Moncharmont et al., 2003). Since the LT for *A. mellifera* is 120 h, were chosen 1, 3, and 5 days after the beginning of exposure to insecticide to evaluate the enzymatic activity.

2.3. Enzyme activity assay

For each experimental group (control and TMX group), 18 bees had their heads removed with the aid of ophthalmic scissors and grouped in 6 microtubes (3 heads per sample). The midgut of each of the 18 bees was collected and separated in the same way as the heads. This procedure was repeated for each exposure time. The procedure for

extracting the proteins was done according to Dussaubat et al. (2012). Each sample was added to a specific amount of enzymatic extraction buffer solution (40 mM sodium phosphate buffer, pH = 7.4, 10 mM sodium chloride, and 1% Triton X100) containing 10% (m/v) for the maceration. In the extraction buffer also was added a cocktail of protease inhibitors (2 mg/mL leupeptin, antipain and pepstatin A, 0.1 mg/mL soybean trypsin inhibitor, and 25 units/mL aprotinin) (Belzunces et al., 1992). The organs were macerated with the aid of an electric sample homogenizer (Omni) for 1 min. The crude extracts were centrifuged at 15,000 × *g* for 20 min at 4 °C. After centrifugation, the supernatants containing the protein extracts were collected for analysis. The experiments were performed in 96-well microplates and the samples were applied in triplicate, in a final reaction volume of 200 μL. All analyses were performed using a spectrophotometer Infinite® 200 pro microplate readers (TECAN, Mannedorf, Switzerland) at 25 °C (supplementary table 1).

The enzyme AChE was analyzed in the head and ALP in the midgut. CaE3, GST, SOD, GPX, and TBARS were evaluated in both organs. The protocol used for AChE, ALP, CaE3, and GST was based on Badiou-Bénéteau (Badiou-Bénéteau et al., 2012). The determination of AChE activity was done in a dosage solution composed of 100 mM phosphate buffer pH 7.0, 0.3 mM acetylthiocholine iodide, and 1.5 mM of 5, 5′-dithiobis (2-nitrobenzoic acid); readings were performed at 412 nm. ALP activity was determined at 410 nm in a reaction containing 20 μM $MgCl_2$, 2 mM ρ-NPP (nitrophenyl phosphate) as a substrate, and 100 mM Tris-HCl, pH 8.5. The determination of CaE3 activity was made in a reaction medium containing 0.1 mM p-nitrophenyl acetate, 0.01 mM 1, 5-bis (4-allyldimethylammonium-phenyl) pentan-3-one-dibromide (BW284C51), and sodium phosphate buffer 100 mM (pH = 7.0). The optical densities were read in the 412 nm wavelength range. For GST, the readings were taken at 340 nm in a medium containing 1 mM EDTA (ethylenediaminetetraacetic acid), 2.5 mM GSH (reduced glutathione), 1 mM 1-chloro-2,4-dinitrobenzene as substrate, and 100 mM of sodium phosphate at pH 7.4. The method, developed by Dussaubat et al. (2012), was used for the SOD reaction. The medium was composed of 50 mM sodium carbonate, diluted in 50 mM sodium phosphate (pH 7.8), 0.1 mM EDTA, 0.1 mM xanthine, 0.025 mM nitro blue tetrazolium chloride (NBT), and 0.008 U/mL xanthine oxidase. The spectrophotometric reading was performed at 560 nm, 10 min after the start of the reaction. For the determination of GPX, the protein extracts were submitted to the enzymatic kit Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, cat. A22188), according to the manufacturer's instructions. The readings were taken using a fluorescence method at 560 nm, after 30 min incubation of the reaction. The procedures for the determination of TBARS followed the TBARS Assay Kit protocol (Cayman Chemical, cat. 10009050), following the manufacturer's guidelines. The reagent colorimetrically quantifies lipid peroxidation and the formation of groups such as malondialdehyde (MDA). The reading was performed in a spectrophotometer at 540 nm.

2.4. Analysis of results

Among the 18 bees collected, the organs were divided into six microtubes (3 organs per microtube). During dosing, each microtube was applied in triplicate (totaling 18 applications). The averages obtained from the plate reader were analyzed with software R version 3.5.0 (R Core Team, 2018). The homogeneity of the data was verified using the Bartlett test. The *car* (Fox and Weisberg, 2019) and *nortest* (Gross and Ligges, 2015) packages were used to ascertain normality, for this purpose, the following test was used: *Shapiro test*. In the case of normal distribution (p-value> 0.05) the analysis was performed with the *T-test*. However, when non-normal distribution prevailed (p-value <0.05), the *Wilcoxon test* found in the *dplyr* package was used (Wickham et al., 2020). The graphical representations were created with the aid of the software SigmaPlot version 14.0 from the averages and the standard error obtained through the statistical analyzes.

3. Results

AChE activity was exclusively analyzed in the head because it is involved with the thiamethoxam mechanisms of action. The AChE activity at 1, 3, and 5 days of exposure was 86.09 ± 5.48, 109.22 ± 5.25, and 117.07 ± 3.19 mUA x min^{-1}/mg protein in the control group, respectively. For the TMX group, the activity was 119.66 ± 5.53, 122.42 ± 3.55 and 130.00 ± 6.98 mUA x min^{-1}/mg protein. The analysis showed that only on 1 and 3 days of contamination incurred a significant increase in the enzymatic activity (Fig. 1A). In contrast, ALP, analyzed on midgut, did not show any statistical differences in activity in any of the evaluated periods (1B). The ALP activity at 1, 3, and 5 days of exposure was 9.97 ± 0.2, 6.84 ± 0.55, and 8.96 ± 0.76 mUA x min^{-1}/mg protein in the control group, respectively. For the TMX group, the activity was 10.45 ± 0.9, 6.27 ± 0.27, and 8.83 ± 1.25 mUA x min^{-1}/mg protein.

In the case of CaE3, there was a decrease in activity after the first day of exposure to thiamethoxam (Fig. 2A), both in the head and in the midgut, and the activity further decreased in the midgut after 5 days of exposure (Fig. 2A). In the head, the CaE3 activity at 1, 3, and 5 days of exposure was 56.33 ± 2.15, 40.40 ± 2.28, and 41.72 ± 2.68 mUA x min^{-1}/mg protein in the control group, respectively. For the TMX group, the activity was 50.25 ± 2.04, 42.81 ± 2.70, and 42.44 ± 2.24 mUA x min^{-1}/mg protein. In the midguts, the CaE3 activity at 1, 3, and 5 days of exposure was 85.80 ± 3.25, 73.81 ± 4.09, and 63.21 ± 3.10 mUA x min^{-1}/mg protein in the control group, respectively. For the TMX group, the activity was 76.07 ± 3.01, 77.20 ± 4.62, and 52.90 ± 3.01 mUA x min^{-1}/mg protein. The GST enzyme was less affected in the presence of the insecticide, as it showed a significant increase in activity in the head after five days of exposure (Fig. 2B). In the head, the GST activity at 1, 3, and 5 days of exposure was 49.11 ± 2.54, 39.89 ± 1.36, and 35.26 ± 2.43 mUA x min^{-1}/mg protein in the control group, respectively. For the TMX group, the activity was 43.37 ± 2.34, 39.71 ± 2.55, and 53.20 ± 2.03 mUA x min^{-1}/mg protein. In the midguts, the GST activity at 1, 3, and 5 days of exposure was 52.35 ± 2.35, 49.46 ± 3.05, and 46.77 ± 3.09 mUA x min^{-1}/mg protein in the control group, respectively. For the TMX group, the activity was 54.38 ± 3.0, 46.56 ± 3.06, and 47.90 ± 4.1 mUA x min^{-1}/mg protein.

In the head, the SOD activity at 1, 3, and 5 days of exposure was 46.80 ± 2.74, 48.66 ± 1.38, and 45.59 ± 2.31 mUA/mg protein in the control group, respectively. For the TMX group, the activity was 46.92 ± 3.00, 44.53 ± 1.81, and 42.00 ± 1.58 mUA/mg protein. In the midguts, the SOD activity at 1, 3, and 5 days of exposure was 90.89 ± 2.02, 149.4 ± 22.7, and 127.4 ± 21.9 mUA/mg protein in the control group, respectively. For the TMX group, the activity was 93.76 ± 5.01, 127.4 ± 21.9, and 171.1 ± 33.5 mUA/mg protein. In relation to the oxidative stress-related enzymes, SOD activity was not affected in either organ on the evaluated days (Fig. 2C). However, GPX increased in the head after 1 day, but there was a decrease in activity after 3 days of exposure, and in the midgut, GPX activity increased after 3 days of exposure (Fig. 2D). In the head, the GPX activity at 1, 3, and 5 days of exposure was 0.30 ± 0.01, 0.35 ± 0.01, and 0.24 ± 0.02 mUA of HRP/mg protein in the control group, respectively. For the TMX group, the activity was 0.34 ± 0.02, 0.35 ± 0.01, and 0.24 ± 0.02 mUA of HRP/mg protein. In the midguts, the GPX activity at 1, 3, and 5 days of exposure was 0.15 ± 0.01, 0.08 ± 0.009, and 0.13 ± 0.02 mUA of HRP/mg protein in the control group, respectively. For the TMX group, the activity was 0.16 ± 0.04, 0.11 ± 0.01, and 0.16 ± 0.01 mUA of HRP/mg protein. On the other hand, the TBARS assay was not significantly altered in the head after the three exposure times assessed, but there was an increase in the midgut after 5 days of exposure to neonicotinoid (Fig. 2E). In the head, the TBARS activity at 1, 3, and 5 days of exposure was 6.70 ± 0.48, 6.86 ± 0.21, and 8.79 ± 0.66 μM MDA/mg in the control group, respectively. For the TMX group, the activity was 8.55 ± 0.96, 7.27 ± 0.36, and 7.52 ± 0.31 μM MDA/mg. In the midguts, the TBARS activity at 1, 3, and 5 days of exposure was 13.4 ± 1.25,

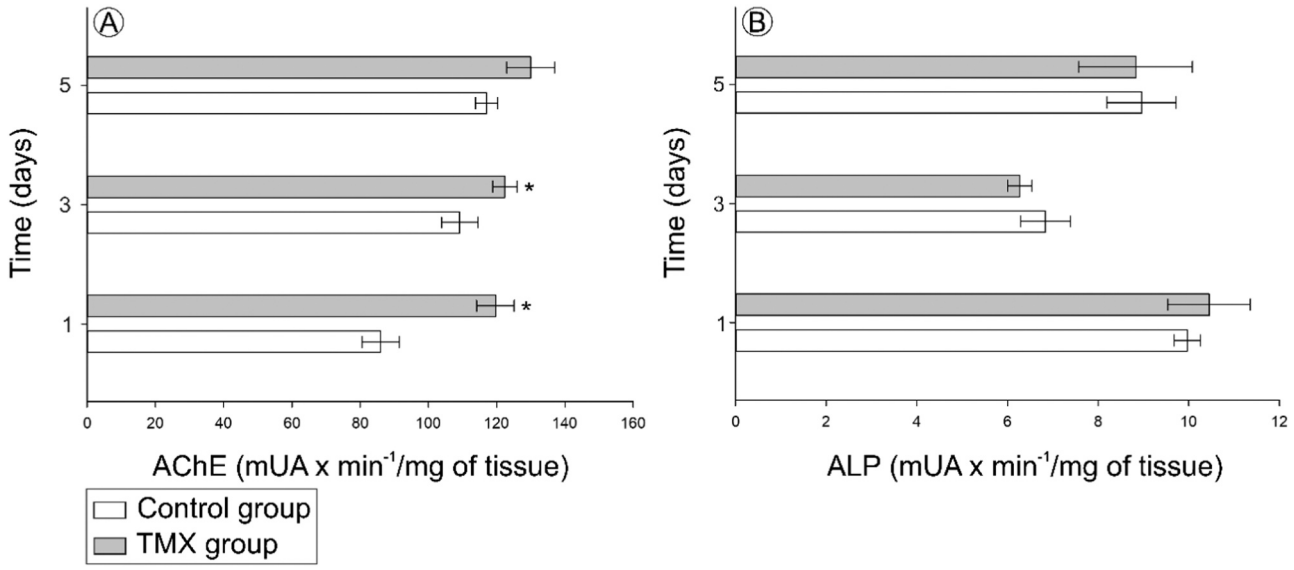

Fig. 1. Effects of thiamethoxam on activity of (A) Acetylcholinesterase (AChE) in the heads and (B) Alkaline Phosphatase (ALP) in the midgut of Africanized *Apis mellifera*. Adult bees were exposed, through contaminated food, to a sublethal concentration of 0.0227 ng thiamethoxam / μL^{-1} diet, and evaluated after 1, 3, and 5 days of exposure. Data showing in bars represent the mean values ± SD of 6 replicates (3 bees/replicate) performed in triplicate. The asterisk (*) indicates a significant difference ($p < 0.05$) in the enzymatic activity between the control group and the group exposed to thiamethoxam.

9.07 ± 1.22, and 7.04 ± 0.670 μM MDA/mg in the control group, respectively. For the TMX group, the activity was 14.3 ± 0.77, 7.78 ± 0.81, and 9.49 ± 0.56 μM MDA/mg. The Table 1 summarizes the results of enzymatic activities and determination of the TBARS in the bee heads and midguts. Also, as supplementary table 2, all the statistical results were summarized.

Adult bees were exposed, through contaminated food, to a sublethal concentration of 0.0227 ng thiamethoxam / μL^{-1} diet, and evaluated after 1, 3, and 5 days of exposure. Arrows indicate increased (up) or decreased (down) statistically ($p < 0.05$) enzymatic activity in the group exposed to thiamethoxam in relation to the control group. (-): indicates that there was no statistically significant difference; Ø indicates that the enzyme was not evaluated in the organ.

4. Discussion

Research on the effects of insecticides on pollinators has become mandatory in many countries, with specific tools and protocols being developed for this purpose (Boyle et al., 2019). The results obtained in this study showed that the insecticide was capable of inducing inhibition and increased activity for some of the analyzed enzymes, depending on the duration of the exposure and the evaluated organ. However, it was also possible to verify that thiamethoxam did not affect some of the analyzed enzymes. Despite that, this study is the first to report this group of six enzymes as biomarkers showing their potential in risk assessment.

For both organs analyzed, there was a decreased activity of CaE3 with 1 day of exposure, and further decrease after 5 days of exposure in the midgut. This enzyme inhibition was also reported by Yu et al. (1984), Li et al. (2017), and Badiou-Bénéteau et al. (2012). In the detoxification process, CaE3 are phase I enzymes that catalyze the hydrolysis of non-polar compounds, after that, the resulting metabolites are transformed by phase II enzymes or directly excreted (Grein and Snyder, 2008; Stone et al., 2002). The results of the present study suggests that initially other metabolic pathways were been used for detoxification. According to Badiou-Bénéteau et al. (2012), lower concentration of pesticides inhibits the activity of CaE3 and CaE1, while increase the activity of CaE2 (not evaluated in this study). In addition, considering that in the treated groups the activity of CaE3 was never higher than the control, is possible that CaE3 didn't contributed to the detoxification process.

However, no changes in GST (a phase II enzyme) were observed in the midgut. Despite that, other studies report that GST activity has increased in the midguts of *A. mellifera* exposed to thiamethoxam (Badiou-Bénéteau et al., 2012), to the spinosad (Carvalho et al., 2013), permethrin (Yu et al., 1984), and imidacloprid (Li et al., 2017). The GST is responsible for catalyzing the conjugation of electrophilic compounds with reduced glutathione (GSH), playing an important role in detoxification and contributing to cell protection against oxidative damage (Barata et al., 2005). Although the concentration of thiamethoxam used in this study was relatively low, causing no GST response to the midgut intoxication, 5 days of constant exposure of the head to the neurotoxic insecticide was sufficient to increase enzyme activity, suggesting an attempt by the nervous system to protect itself or of the degradation of the insecticide molecules. Thus, showing that chronic exposure to neonicotinoids may be a risk to bee health as discussed by Sanchez-Bayo and Goka (2014).

The ALP analysis presented in this study showed that the sublethal concentration of TMX did not show toxic effects on the midgut. This enzyme plays a key role in the maintenance of the midgut homeostasis of bees, and is representative of a family of enzymes responsible for hydrolysis in digestive processes, cell signaling, and the transport of metabolites (Lallès, 2010; Moss, 1992). The literature reports an increase in ALP activity in bees exposed to higher doses of thiamethoxam (Badiou-Bénéteau et al., 2012). Carvalho et al. (2013) showed that in bees treated with fipronil, there was a significant decrease in ALP activity, while a low dose of spinosad was able to induce an increase in the activity of this enzyme. Although the use of ALP as a biomarker for the effects of insecticides is relevant to assess the damage caused, no significant changes were observed in the evaluated concentration and time conditions, demonstrating that this enzyme was not required to regulate essential cell processes.

Conversely, AChE activity showed a significant increase in relation to the control in the first and third day of exposure. However, Li et al. (2017) observed that AChE activity was reduced in the brains of *A. mellifera* bees exposed to LD_{50} of imidacloprid and clothianidin. In contrast, Badiou-Bénéteau et al. (2012) found no change in AChE activity in bees chronically exposed to thiamethoxam; similar results were obtained by Gauthier et al. (2018) for thiamethoxam and imidacloprid. The AChE enzyme is directly related to the mode of action of neonicotinoids, since it is responsible for rapid hydrolysis of acetylcholine in

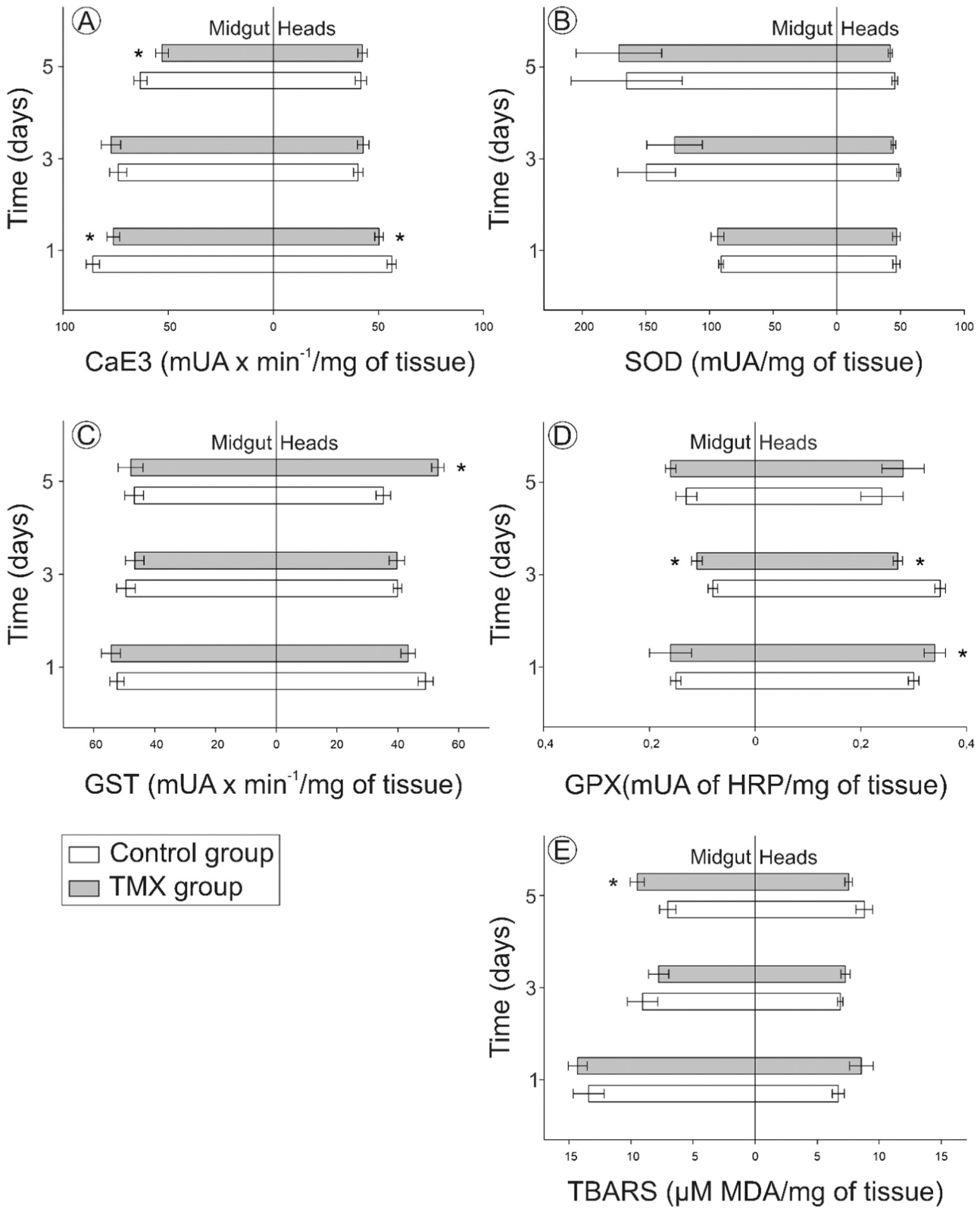

Fig. 2. Effects of thiamethoxam on activity of (A) Carboxylesterase (CaE3), (B) Glutathione-S-Transferase (GST), (C) Superoxide Dismutase (SOD), (D) Glutathione peroxidase (GPX) and (E) determination of thiobarbituric acid reactive substance (TBARS) in the heads and midgut of Africanized *Apis mellifera*. Adult bees were exposed, through contaminated food, to a sublethal concentration of 0.0227 ng thiamethoxam / μL^{-1} diet, and evaluated after 1, 3, and 5 days of exposure. Data showing in bars represent the mean values ± SD of 6 replicates (3 bees/replicate) performed in triplicate. The asterisk (*) indicates a significant difference ($p < 0.05$) in the enzymatic activity between the control group and the group exposed to thiamethoxam.

Table 1
Summarized results of the assays for Acetylcholinesterase (AChE), Alkaline Phosphatase (ALP) Carboxylesterase (CaE3), Glutathione-S-Transferase (GST), Superoxide Dismutase (SOD), Glutathione Peroxidase (GPX) and determination of Thiobarbituric Acid Reactive Substance (TBARS) in Africanized *Apis mellifera* exposed to the sublethal concentration of thiamethoxam compared to the control group.

	ASSAY	Days	ORGANS	
			Head	Midgut
EXPOSURE BIOMARKERS	AChE	1	↑	Ø
		3	↑	Ø
		5	_	Ø
	ALP	1	Ø	_
		3	Ø	_
		5	Ø	_
	CaE3	1	↓	↓
		3	_	_
		5	_	↓
	GST	1	_	_
		3	_	_
		5	↑	_
ANTIOXIDANTS	SOD	1	_	_
		3	_	_
		5	_	_
	GPX	1	↑	_
		3	↓	↑
		5	_	_
INDICATION OF LIPID PEROXIDATION	TBARS	1	_	_
		3	_	_
		5	_	↑

cholinergic synapses, facilitating precise control of neuronal transmission modulation (Badiou et al., 2007). Although on the fifth day of exposure, AChE activity stabilized, the results of the present study indicate that, with the nicotinergic receptors occupied by molecules of thiamethoxam, which mimics the acetylcholine molecule, AChE cannot act because it does not recognize the insecticide. Consequently, there is an increase in enzymatic activity in an attempt to degrade the binding to the receptor and thereby restore the nerve impulse. The nervous system is thus affected, because upon reaching its target organ, neonicotinoids cause abnormally prolonged activation of postsynaptic receptors with the impaired function of AChE (Ishaaya et al., 2007). Based on the reports made by Li et al. (2017), Badiou-Bénéteau et al. (2012), Gauthier et al. (2018), and our results, it has also been concluded that AChE activity is modulated in different ways according to the specific insecticides used and their concentrations.

For the antioxidant enzymes, the sublethal concentration of thiamethoxam increased GPX activity in the head after 1 day of exposure. However, there was a significant decrease after 3 days, indicating the enzymatic activity against the insecticide action is beginning to be exhausted at this point. Thus, in 5 days, there were no significant differences in the GPX activity between the experimental groups, showing that the balance of the antioxidant defense was restored. This hypothesis is confirmed because the SOD activity and the TBARS assay did not show significant differences in any of the three evaluation periods, showing that the nervous tissue was less affected by the oxidative stress. There are few studies using GPX to evaluate the impact of insecticides on bee heads. With the exception of Nielsen et al. (2000) that observed the activity of GPX and GST in different larval stages and in adult *A. mellifera* bees exposed to an insecticide of the pyrethroid class. In the literature, studies that apply GPX analysis do not assess the nervous system of the target organism, for example: the effects of the presence of the microsporidium Nosema in the gut (Dussaubat et al., 2012), effects of a neonicotinoids in the honey bee drone semen (Abdelkader et al., 2019), and differences between oxidative stress in the trophocytes and fat cells during aging (Hsu and Hsieh, 2014). Thus, the mechanism accessed to combat TMX in the heads was the action of AChE and the exposure biomarker enzymes (CaE3 and GST) instead of the antioxidant enzymes.

As in the head, there was no difference in the activity of SOD in the midgut. Although, the enzyme GPX showed an increase after 3 days of exposure in this organ. Furthermore, the TBARS assay showed a significant increase in MDA, the cytotoxic product released under lipid peroxidation, in the exposed group after 5 days. Antioxidant enzymes are the first mechanisms of cellular defense against reactive oxygen species (ROS) (Ighodaro and Akinloye, 2019). SOD, which specializes in the removal of the superoxide radical (O_2-), catalyzes the dismutation of superoxide, converting it into hydrogen peroxide (H_2O_2), which is less reactive and can be degraded by CAT and GPX enzymes. CAT will degrade the H_2O_2 into H_2O and O_2. The other antioxidant pathway to break down H_2O_2 is formed by glutathione (GSH), which operates by alternating its oxidized form and its reduced form through the action of glutathione peroxidase (GPX) and glutathione reductase (GR) (Halliwell and Gutteridge, 2015). Although TMX has not modulated the action of SOD, it is possible to see action of GPX and change in the TBARS assay because there are other sources of hydrogen peroxide in the cell in which superoxide is not the precursor (Birben et al., 2012; Kurutas, 2016).

Thus, we can infer that, although the midgut is not the target organ of the neonicotinoid insecticides, the activation of the antioxidant defense system was higher in this organ than in the brain, suggesting that oral contamination left the midgut more susceptible to the effects of oxidative stress. Since antioxidant enzymes cannot reverse the damage caused by thiamethoxam, the TBARS assay showed that after 5 days of exposure, the damage had reached the cell membranes. Although all cellular components are sensitive to the actions of ROS lipid peroxidation, when it occurs in the cell membrane, it leads to changes in the structure and selectivity, that can lead to cell death (Gaschler and Stockwell, 2017). Gauthier et al. (2018) also report a pro-oxidant effect of thiamethoxam and imidacloprid in *A. mellifera* bees. However, Yu et al. (1984) showed that exposure to a sublethal concentration of thiamethoxam had no effect on some of the analyzed enzymes (SOD and TBARS in the head, ALP and GST in the midgut). Although the literature does not show a pattern of enzymatic expression when bees are exposed to insecticides, we emphasize that this fact may be related to physiological differences at the time of organ collection for enzymatic activity.

The TMX sublethal concentration used in the present study was able to modulate the activity of AChE, CaE3, GST, and GPX in the head, while in the midgut, CaE3, GPX, and TBARS were altered in some periods evaluated (Table 1). The modulation of AChE demonstrates the neurotoxic effect of the insecticide, as well as the presence of exposure biomarker enzymes (CaE3 and GST) in the heads. On the other hand, in the midgut, the antioxidant system stands out in defense against the possible effects of TMX. Furthermore, this enzymes group and the TBARS assay have the potential to be used as biomarkers in bees. A combination of these enzymatic analyses could be a promising tool to evaluate bee health and to characterize their exposure to insecticides. Additional studies using other markers for cell stress, such as enzyme cytochrome P450, heat shock proteins (HSP 70 and 90), gene expression of genes involved in detoxification, and the immune system can help to understand how different metabolic pathways can influence the enzymatic response or assist with other cellular defense mechanisms.

Authorship statement

Conception and design of study: P. Decio, F.D. Campos Pereira, T.C. Roat, M.A. Marin-morales and O. Malaspina. Acquisition of data: P. Decio and F.D. Campos Pereira L. Analysis and/or interpretation of data: P. Decio, F.D. Campos Pereira, L. Miotelo and T.C. Roat. Drafting the manuscript: P. Decio and L. Miotelo. Revising the manuscript critically for important intellectual content: P. Decio, F.D. Campos Pereira, L. Miotelo, T.C. Roat, M.A. Marin-morales and O. Malaspina. Approval of the version of the manuscript to be published: P. Decio, F.D. Campos Pereira, L. Miotelo, T.C. Roat, M.A. Marin-morales and O. Malaspina.

Declaration of Competing Interest

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

Acknowledgments

The authors would like to thank the São Paulo Research Foundation (FAPESP) from Brazil for supporting this study with grants 2012/13370-8 (TCR), 2017/21097-3 (OM) and scholarships 2014/23197-7 (PD) and 2013/21833-0 (LM). We also would like to thank Manuela de Oliveira Ramalho Sanchez and Igor Vinicius Ramos Otero for proofreading the manuscript and fruitful discussion. Lastly, we would like to thank Ana Claudia Marcato, Thais Guedes, Rodrigo Barbosa and Daiana Tavares for helping and sharing tips during the experimental testing phase.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.112581.

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