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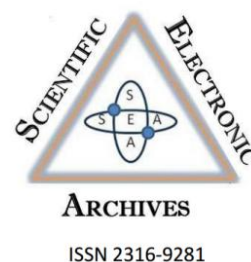
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Analysis of antioxidant enzymes and total antioxidant capacity in the midgut of Africanized *Apis mellifera* selected for tolerance to the neonicotinoid thiamethoxam

W. C. S. Pizzaia^{1*}, T. O. Diniz¹, N. C. Pereira¹, B. G. Silva², A. S. Khatlab³, E. Gasparino³, A. A. Sinópolis-Giglioli¹, V. A. A. Toledo³, M. C. C. Ruvoletto-Takasusuki¹

¹ Universidade Estadual de Maringá – Departamento de Biotecnologia, Genética e Biologia Celular

² Escola Superior de Agricultura "Luiz de Queiroz" / USP – Departamento de Ciências Exatas

³ Universidade Estadual de Maringá – Departamento de Zootecnia

* Author for correspondence: wilbiologia@hotmail.com

Abstract. Agrochemicals are considered one of the factors responsible for the decline of bee population in the world, causing a huge amount of losses. The organism of these insects seeks an alternative for their survival and adaptive factors can be triggered, such as the action of antioxidant substances, which can promote protection via the digestive system. This study aimed to evaluate the enzymatic activity and total antioxidant capacity in the midgut of adult *Apis mellifera* workers that had been selected since 2015 to be tolerant to the neonicotinoid insecticide thiamethoxam. For this, tolerant and non-tolerant honeybees were contaminated with thiamethoxam for 24 hours. Then the midgut was dissected for the enzymatic analysis. The results obtained showed that tolerant bees presented a significant result regarding the enzymatic activity and total antioxidant capacity for the reduction of damage caused by thiamethoxam when compared to the non-tolerant group.

Keywords: selection pressure, enzymes, agrochemicals

Introduction

Apis mellifera is an important insect due to their pollination activity, a process that ensures biodiversity maintenance and contributes to increasing crops productivity. Also honeybees are responsible for the production of propolis, honey, wax and royal jelly, which guarantee income to the beekeepers (Stein et al., 2017).

Pollination work in Brazil is an essential factor for development of several crops. Giannini et al. (2015) analyzed 141 crops and identified that 85 had some type of dependence on pollinators, with almost one-third of the analyzed crops considered greatly dependent or essentially dependent on pollinators. The pollination services provided by honeybees accounts for 30% of the annual production value of pollinator-dependent crops.

However, the relationship between bees and agriculture raises concern. The use of agrochemicals to minimize pest damage in crops ends up aggressively affecting foragers bees that are exposed to these agrochemicals and bring the substance to the colony, spreading the it and

ultimately leading to their decline (Williamson et al., 2014; Sanchez-Bayo et al., 2016).

The effects related to the action of agrochemicals can be manifested in morphophysiological ways, and also through behavioral changes, when in sublethal doses (Tavares et al., 2015). By ingesting harmful substances, bees can undergo drastic changes in enzyme expression, especially in the ones involved in detoxification processes (Abou-Donia, 2014).

The reaction and the protective effect of the honeybee's gut can be revealed in different ways, as these processes involve quite a lot of different structures and compounds. The midgut lumen has a peritrophic membrane that surrounds the food and separates the contents into two compartments, one inside the membrane or endoperitrophic space and the other one outside the membrane, called ectoperitrophic space (Snodgrass, 1956; Landim, 2009; Zhong et al., 2014).

There is integration in processes involving important enzymes during digestion and are involved in a synchronized and complex yet efficient

way, meeting the needs of organisms, such as digestion, detoxification process, absorption of nutrients, action against the release of free radicals and transport of nutrients (Gilbert and Wilkinson, 1974). Thus, some enzymes act to eliminate harmful compounds. Superoxide dismutase (SOD) is one of the enzymes that play a significant role, removing the superoxide radical, catalyzing the dismutation of superoxide into oxygen and hydrogen peroxide (Claudianos et al., 2006; Cui et al., 2012; Simone-Finstrom, 2016).

As in other animals, insects have effective defense mechanisms, which can be expressed by a set of enzymes that seek to promote the removal of reactive oxygen species (ROS) generated in large quantities when the insect is under stress and causing oxidative stress. ROS harm insects by affecting proteins, carbohydrates and nucleic acids (Felton and Summers, 1995; Dandekar et al., 2002; Halliwell and Gutteridge, 2007).

The enzymatic processes present in the honeybee's digestion perform metabolic activities and act on products or substances that are not recognized by the organism, changing it into substances that will be excreted. The establishment of metabolites, such as peroxides and free radicals, can compromise and alter the insect organism when there is an accumulation of the substance (Felton and Summers, 1995; Wu et al., 2011; Cui et al., 2012).

After the dismutation of the superoxide, hydrogen peroxide (H_2O_2) is produced and it needs to be removed to mitigate the damage caused by its accumulation. The action of catalase (CAT) is important to eliminate hydrogen peroxide, creating water and molecular oxygen (Claudianos et al., 2006; Halliwell and Gutteridge, 2007; Cui et al., 2012; Simone-Finstrom, 2016).

The insecticide, as an exogenous factor, compromises the performance of molecules such as proteins, which act as enzymes, hormones, neurotransmitters, and transporters, operating in most biological processes and impacting honeybee's life (Wyatt and Pan, 1978; Rossi et al., 2013; Thompson et al., 2015).

Oxidative stress caused by insecticides such as thiamethoxam has negative effects and impacts on the insect's lives, especially because it needs to resume its balance and remove the compounds that caused damage. The insecticides action on bees directly affects the release of free radicals, which accelerates cell death, lipoperoxidation and the generation of oxidizing compounds that compromise the antioxidant defense system (Claudianos et al., 2006; Badiou-Bénéteau et al., 2012; Chakrabarti et al., 2015).

Aiming to analyze the enzymatic and antioxidant activity on the midgut of honeybees, this study used worker tolerant to the neonicotinoid insecticide thiamethoxam and non-tolerant honeybees. The tolerance has been selected since 2015, where the colony was exposed to thiamethoxam by contact. Changes in behavior were

observed during the agrochemical induction process. During the management of the nuclei, it was possible to observe difficulty in locomotion and food storage, but after the emergence of new workers and drones, the behavior and activities were resumed and the population and organizational condition of the nucleus were balanced (Pizzaia, 2016).

Methods

Biological material

The bioassay was performed with adult workers of *A. mellifera* collected from commercial hives at the Experimental Farm of Iguatemi, from Universidade Estadual de Maringá (UEM) (23° 25' S, 51° 57' O, UEM, Maringá, PR, Brazil) and taken to the Laboratory of Genetics Animal from the Department of Biotechnology, Genetics and Cell Biology of UEM.

There were two types of workers collected for the bioassay. One of them was from hives that had been exposed to thiamethoxam since 2015, creating a hive considered tolerant to the agrochemical. The other workers were collected from normal colonies and used as the control group.

Bioassays

To perform the bioassay with thiamethoxam and verify its toxicity, a dilution of the commercial agrochemical Actara 250WG containing 250 grams of active ingredient per kilogram, as recommended in the bull leaflet for *Citrus*, was used. The final solution used for the bioassays had 5×10^{-5} grams of active ingredient per milliliter (g a.i./mL) of the agrochemical's active ingredient.

The bioassay was performed in 24 hours, divided into 4 groups with 5 replicates each and 30 bees per replicate. Groups 1 and 2 were composed of tolerant honeybees and groups for 3 and 4 non-tolerant worker honeybees were used. Groups 1 and 3 were feed with candy and groups 2 and 4 were fed with candy plus 2 μ L of the agrochemical solution. The bioassays were maintained in 33 ± 2 °C and relative humidity de $80 \pm 2\%$.

Honeybees were kept in glass flasks (diameter 14.71 cm x 18.5 cm high) containing filter paper on the bottom, a container with a water-soaked cotton swab and another container with candy. The experimental design was subdivided plot and the analysis of variance was performed.

Enzymatic analysis and total antioxidant capacity

After 24 hours surviving honeybees were sacrificed at low temperatures and dissected in a saline solution using a Carl Zeiss stereoscopic microscope. 10 bees were dissected, totaling 100 mg of midgut samples. After dissection, samples was stored in microtubes, frozen in liquid nitrogen and stored in the -80 °C freezer.

To perform the analysis of the enzymatic activity of catalase (CAT), superoxide dismutase (SOD), total antioxidant capacity via sequestering activity of the stable free radical 2,2-diphenyl-1-

picrilhidrazil (DPPH) and determination of thiobarbituric acid reactive substances (TBARS), 100 mg of midgut samples were added in 1,000 μ L potassium phosphate buffer 0.1 M, pH 7.4, and homogenized with Dounce homogenizer until its complete dissociation. After that, it was centrifuged at 10,000 x g for 10 minutes at 4°C. The supernatant was collected in a clean microtube and used as a sample.

Analysis of antioxidant enzymes CAT and SOD

CAT activity was based on the ability of the enzyme to convert hydrogen peroxide (H_2O_2) into water and oxygen. For this, 20 μ L of the supernatant obtained from the midgut of the bees were added in 980 μ L of the reactive mixture (tris buffer 1 M containing 5 mM of EDTA pH 8.0 and H_2O_2). Enzyme activity was monitored via spectrophotometer EvolutionTM 300 UV-VIS (Thermo Fisher ScientificTM) at a wavelength of 240 nm, for 60 seconds. CAT activity was expressed as the amount of H_2O_2 consumed/min/mg of protein ($\epsilon = 33.33 M^{-1} \times cm^{-1}$) (Aebi, 1984).

SOD activity was measured according to its ability to inhibit the autoxidation of the pyrogallol that generates the anion superoxide (O_2^-). SOD present in the sample competes for the O_2 radical. The increase in absorbance of the samples was verified at the wavelength of 420 nm, for 180 seconds in a microplate reader (VersaMaxTM, Molecular Devices). The supernatant containing the enzyme SOD was added to Tris-HCl 200 mM buffer containing EDTA at 2 mM, pH 8.2, and pyrogallol 15 mM. The analysis was performed at room temperature in duplicate. A unit of SOD (U) was defined as the amount of enzyme needed to inhibit the autoxidation rate of pyrogallol by 50%. Thus, the enzymatic activity was expressed as U of SOD/mg of protein (Marklund and Marklund, 1974).

Biomarkers of oxidative stress and total antioxidant capacity

The determination of lipid peroxidation was performed based on the capacity of thiobarbituric acid to bind to oxidized lipids, this analysis was performed as described by Buege and Aust (1978). Samples were read at a wavelength of 535 nm, via spectrophotometer EvolutionTM 300 UV-VIS (Thermo Fisher ScientificTM). The content of TBARS was determined using the molar extinction coefficient $\epsilon = 1.56 \times 10^5 mol^{-1} cm^{-1}$, according to the Lambert Berr Law. The results were expressed as nmol of TBARS/mg of protein.

Total antioxidant capacity via stable free radical sequestering activity DPPH was performed according to the method described by Brand-Williams et al. (1995), with modifications. 100 mg of midgut was added in 1,000 μ L of methanol, homogenized with Dounce homogenizer and then centrifuged at 10,000 x g for 10 min at 4°C. Then, 180 μ L of the DPPH solution at 0.06 mM was added to the wells of a microplate containing 20 μ L of the

supernatant obtained from the samples. These reactions were maintained in the dark for 30 min. After this, samples were read in the microplate reader (VersaMaxTM, Molecular Devices) at a wavelength of 515 nm. The antioxidant capacity of each sample (% antioxidant activity) was determined as follows: % antioxidant activity = $(1 - (\text{sample absorbance} / \text{absorbance of the DPPH})) \times 100$.

The analysis of CAT, SOD, and TBARS was adjusted by quantifying total proteins of the samples by the Bradford method (Bradford, 1976).

Data analysis

Statistical analysis was performed through the software Statistical Analysis System (SAS, 2012). Regarding the assumption of normality and homogeneity of variances, the Shapiro-Wilk test and the Bartlett test were used, respectively. When the normality hypothesis was satisfied, variance analysis (ANOVA) was used to determine whether, on average, there are significant differences between the groups. Tukey test was used to verify statically differences between the groups, in which we considered the significance level at 5% in all tests.

Results and discussion

The assumption of normality and homogeneity of variances were met (p -value $p > 0.05$). Significant expression was observed in the interaction of tolerant and non-tolerant honeybees (p -value < 0.05) and in the total antioxidant capacity by the method via the free radical sequestering activity of DPPH (Table 1). Tolerant honeybees showed a higher average when they received the food with the agrochemical, differing from the bees that received only food, which showed a lower value, similar, to the group of non-tolerant bees, regardless of the type of food received.

Tolerant honeybees have been kept in contact with thiamethoxam since 2015, undergoing a selection pressure. This induced contact may have stimulated the adaptation of these insects, altering their action in detoxification and the increase of their total antioxidant capacity. Research related to insect defense system seeks to understand the effects of insecticides on insect's life. According to Plapp and Casida (1970), agrochemicals are inducers of various detoxifying enzymes. Field et al. (1996) and Field (2000) suggest that DNA methylation of a specific insect gene may act in insects tolerant to agrochemicals.

Pizzaia (2016) reports the behavior of bees when exposed to thiamethoxam, through a filter paper contaminated with the agrochemical, and its adaptation over time. Changes in the colony were observed, such as locomotion problems (tremors, spasms); flight difficulties; changes in the foraging, deposition of food in the cells of offspring, produced for the establishment of new bees even with the presence of the queen performing posture; reduction in the amount of food stored. With the development of new workers and drones, there was a balance

over the days, resuming normal activity in the colony.

With the prolonged exposure to thiamethoxam, tolerant bees suffered stresses that triggered the imbalance in their body. Subsequently, there was a balanced resumption in the colony, suggesting that the changes influenced the expression and increase of enzymatic activity of detoxification over the generations. Glastad et al. (2011) showed that changes influenced by the environment may occur in several insects, such as Hymenoptera, and these changes occur because honeybees have a functional system for methylation, which contributes significantly to adaptation without modifying the DNA sequence (Wang et al., 2006).

Tolerant bees that received food containing thiamethoxam displayed an abundant production of total antioxidant capacity by the DPPH radical capture method (Table 1), suggesting that, in the period in which the insecticide ingestion occurred, its body was able to act against free radicals. The tolerance of an insect is linked to genetic factors that undergo mutations on target proteins, affecting its metabolism. Because they are tolerant, they survive longer and transmit these tolerance genes to the next generations (Beauty and Marquardt, 1996; Li et al., 2007).

Studies with the herbicide Paraquat indicate that this agrochemical contributed to the expression of several genes related to antioxidant defense and can significantly affect the survival of bees. After offering a diet rich in pollen, Mattos et al. (2006) obtained a significant effect in detoxification genes expression and the oxidative stress generated by this herbicide proved to be an entry route for pathogens.

The effect of tolerant and non-tolerant populations (p -value<0.05) on SOD was also verified and tolerant honeybees showed higher production of this enzyme compared to the non-tolerant population. The adaptation of the insect over the time of exposure in the field may be the factor that stimulated the production of this detoxification compound (Table 1).

SOD is an enzyme that acts against ROS, that is harmful to the body and are generated during oxidative stress, affecting molecules such as proteins, nucleic acids, lipids and carbohydrates. Also, it catalyzes the dismutation of the superoxide anion into oxygen and hydrogen peroxide being important in antioxidant defense (Fridovich, 1995; Claudianos et al., 2006; Cui et al., 2012).

The effect of the population on CAT was significant (p -value<0.05). CAT is an antioxidant enzyme that acts on hydrogen peroxide preventing the formation of new by-products. Tolerant bees showed a significant expression of this enzyme (Table 1) which was not observed in non-tolerant honeybees, showing a lower average than tolerant bees.

TBARS activity was significant (p -value<0.05) between the tolerant and non-tolerant honeybees. When the bees were challenged by high metabolic activities, resulting from environmental stressors, the organism produces or activate antioxidant mechanisms to combat the imbalance, seeking to detoxify and repair the damage caused by ROS.

Oxidative stress can cause severe cellular damage, generating lipoperoxidation or lipid peroxidation. Lipoperoxidation occurs through oxidation of ROS, that act on membrane components such as lipoproteins, compromising all cellular structure and function, such as the action of enzymes and membrane permeability. With lipoperoxidation, due to xenobiotics, the release and formation of cytotoxic products can occur, resulting in apoptosis and death (Shan et al., 1990; Mello-Filho et al., 1983; Hershko, 1989).

The significant mean of the tolerant population regarding the enzymes SOD and CAT may suggest that these enzymatic detoxification mechanisms were able to act on after agrochemical contamination, being able to overcome the lipid peroxidation of the membranes.

The increase in detoxification enzymatic activity may be linked to amino acid substitution, modifying target sites generating adaptation to the agrochemical, which increases tolerance (Hemingway, 2000; Perry et al., 2011).

SOD activity of on honeybees fed with thiamethoxam was significative (p -value<0,05). Both groups showed a lower mean on SOD activity when compared to the groups that were fed with candy only. This result indicates that the bees produced and used these enzymes significantly, while the bees with higher media did not need to use this enzyme for detoxification purposes.

The performance of the antioxidant system is vital in honeybees and because it is an intracellular enzyme, SOD performs its activity against oxidative stress, regardless of its origin, acting against ROS, being functional within a system composed of several antioxidant agents (Jovanovic-Galovic et al., 2004; Korayem et al., 2012; Dmochowska-Ślęzak et al., 2014).

SOD showed high activity in thiamethoxam tolerant bees during the 24-hour bioassay. The honeybees sought to adapt over time, so the tolerance mechanisms showed in some individuals also adapted due to selection pressure. In this case, SOD is important in eliminating free radicals from the bee's metabolism (Bianchi and Antunes, 1999; Nemec et al., 2000; Erel, 2004; Mamidala et al., 2011; Badiou-Bénéteau et al., 2012).

Changes caused thiamethoxam in the midgut enzymatic activities of honeybees were evident, as well as in the total antioxidant capacity of insects, since the agrochemical input route was the digestive system, where the first contact with contaminated food occurs.

Table 1. The activity of superoxide dismutase (SOD), catalase (CAT), total antioxidant capacity (DPPH) and lipid peroxidation (TBARS) in *A. mellifera* workers tolerant and non-tolerant to thiamethoxam

Population Diet		SOD		CAT		DPPH		TBAR	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
F4	Candy + Thiamethoxam	19,86 ^b	4,56	1050,80 ^a	59,33	69,45 ^a	8,60	5,84 ^a	1,39
	Candy	31,68 ^a	9,39	1035,30 ^a	136,66	39,19 ^b	6,36	5,76 ^a	0,91
Normal	Candy + Thiamethoxam	12,74 ^b	1,73	682,54 ^b	114,90	40,30 ^b	7,17	12,58 ^a	2,13
	Candy	15,96 ^a	3,66	712,52 ^a	75,13	39,68 ^b	5,27	11,07 ^a	0,55
Main effects									
Population	F4	25,77 ^a	9,34	1043,10 ^a	99,66	54,32 ^a	17,47	5,80 ^a	1,11
	Normal	14,35 ^b	3,18	697,53 ^b	92,87	39,99 ^b	5,94	11,82 ^b	1,67
Diet	Candy + Thiamethoxam	16,30 ^b	4,96	866,66 ^a	212,37	54,87 ^a	17,08	9,21 ^a	3,93
	Candy	23,82 ^a	10,66	873,92 ^a	199,38	39,43 ^b	5,51	8,42 ^a	2,88
<i>p-value</i>									
Population		<0,05 [*]		<0,05 [*]		<0,05 [*]		<0,05 [*]	
Diet		<0,05 [*]		0,87		<0,05 [*]		0,22	
Interaction		0,11		0,62		<0,05 [*]		0,27	

*Means followed by distinct letters in the column differ from each other by the Tukey test at 5% significance.

The results demonstrated a positive regulation in antioxidant activity when honeybees were exposed to thiamethoxam in the laboratory bioassay, increasing its defense against the agrochemical. Besides, bees that are already tolerant, in the apiary, developed adaptation and tolerance, triggering the enzymatic responses and maintaining this mechanism throughout the generations.

Higher enzymatic activity of tolerant honeybees was observed when compared to non-tolerant honeybees, even though they did not show significant values in all responses of enzymatic activity. These data suggest that the intensity of agrochemical use contributes to the increase of adaptive enzymatic defense as a way to avoid oxidative stress.

Conclusion

The analysis of enzymatic activity and total antioxidant capacity of thiamethoxam tolerant bees showed that they were able to develop an adaptation related to detoxification and that in 24

hours the action of enzymes was able to mitigate the damage of oxidative stress such as lipoperoxidation in the midgut after ingestion of contaminated food. Also, the pressure of selection with honeybees through thiamethoxam induction allowed to observe the tolerance to the insecticide after four generations and its adaptation in detoxification processes when compared to honeybees that were not tolerant, and the defense mechanism was passed over the generations.

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