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Zinc (organic and inorganic) modulates the expression of catalase and glutathione peroxidase genes related to the antioxidant system of *Apis mellifera* forager bees

Iloran do Rosário Corrêa MOREIRA, Samir Moura KADRI, Ricardo de Oliveira ORSI
Center of Education, Science and Technology in Rational Beekeeping (NECTAR), College of Veterinary Medicine and Animal Sciences, UNESP – São Paulo State University, Botucatu, São Paulo, Brazil

Abstract

In *Apis mellifera* the activity performed by individual worker bees as brood nutrition, defence and foraging, is regulated by physiological age or colony needs, a phenomenon referred to as polyethism. Bees forage long distances in search of resources for colony maintenance, which increases their metabolism levels due to the expenditure of energy during the activity. After two weeks of foraging activities, proteins affected by oxidative stress accumulate in the brain, and cognitive capacity declines. Although bee diet is focused on high energy and protein foods, other nutrients, such as vitamins and minerals, are essential for colony development. Zinc (Zn) is a vital structural and functional component of organisms. Oxidative stress can occur under Zn deficiency, and Zn supplementation can help prevent oxidative stress-induced damage in different cells and tissues. Therefore, the aims of the present study were to verify if Zn supplementation (inorganic and organic forms) can modulate the antioxidant system of forager bees. This was assessed by analysing catalase (CAT) and glutathione peroxidase (GPX) gene expression. Following organic Zn supplementation, increased expression of GPX was observed, with significant difference from those in the control and inorganic Zn treatments. Conversely, there was an increase in CAT expression under inorganic Zn supplementation, with a significant difference from those in the control and organic Zn treatments. Zn, regardless of the source, did not have harmful effects on the colonies and could modulate the expression of antioxidant system genes.

Key words: gene expression, longevity, mineral, nutrition.

Introduction

In *Apis mellifera* L., workers are known to alter functions based on physiological age or colony requirements; a phenomenon known as polyethism (Sagona *et al*., 2021). Forager bees fly over long distances in search of resources for colony maintenance as nectar, pollen, resin and water, and their metabolic ratio increases 10-100-folds (Suarez *et al*., 1996). After two weeks of foraging activities, proteins affected by oxidative stress accumulate in the brain, resulting in a decline in cognitive capacity (Krishnan and Kodrík, 2012). The increased levels of highly reactive compounds, such as reactive oxygen species (ROS), can be caused by environmental pollutants, which include some synthetic biochemical compounds used in industry and agriculture, with deleterious effects on tissues, including premature aging (Margotta *et al*., 2018). ROS increases cause oxidative damage to proteins, lipids and nucleic acids, and an efficient system is required to combat the effects of free radicals and avoid metabolic imbalance in tissues (Dmochowska-Ślęzak *et al*., 2015). The enzymes of the antioxidant system, including catalase (CAT) and glutathione peroxidase (GPX) (Margotta *et al*., 2018), perform the regulation of oxidative processes.

Although bee nutrition is primarily associated with high energy and protein foods, other nutrients, such as vitamins and minerals, are also essential for colony development (Haydak, 1970; Brodschneider and Crailsheim, 2010). Bees obtain minerals from collected pollen, nectar, and water. Minerals such as Zn are vital structural and functional components of living organisms; structurally, it is present in metalloproteins, and functionally, it plays a catalytic role in enzyme systems (Haragushi, 2004; Faa *et al*., 2008; Kafel *et al*., 2014). In addition, Zn can influence the antioxidant system of honey bees (Zhang *et al.*, 2015). Oxidative stress can occur under Zn deficiency, and Zn supplementation can minimize or prevent oxidative damage in cells and tissues (Oiteza *et al*., 2012).

The mineral source influences its bioavailability; minerals bound to organic molecules, called chelated minerals, exhibit better absorption and reduced competition than other minerals for binding sites with other minerals (Rider *et al*., 2010). In livestock production, studies with different mineral sources are common; however, in honey bees are scarce.

Therefore, the aims of the study were to evaluate the effects of Zn supplementation (organic and inorganic) in diet and evaluate the survival, population development, and antioxidant system gene expression in *A. mellifera* forager bees.

Materials and methods

The present study was carried out within the following geographical coordinates: 22°49'14.9"S 48°23'23.8"W. Fifteen colonies of Africanized *A. mellifera* bees kept in standard Langstroth model hives, equalized according to the number of brood and food frames, were used. Each treatment contained five randomly distributed colonies, according to the following treatments: Control (no Zn supplementation), ZnO50 (organic Zn source 50 ppm), and ZnI50 (inorganic Zn source 50 ppm).

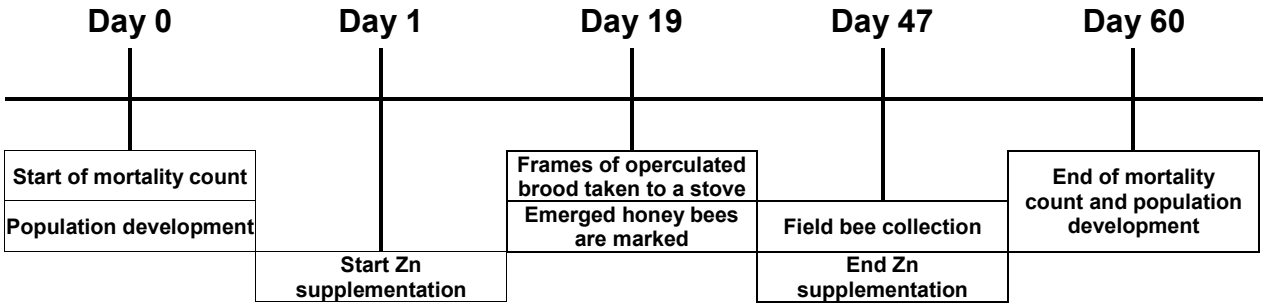

Figure 1. Time line of experimental protocol.

The inorganic Zn source was zinc sulfate monohydrate (37.4% Zn), and the organic source was zinc methionine (16.0% Zn). The mineral was supplied in sugar syrup (water and commercial sugar in a 1:1 ratio) and each colony received 500 mL of syrup per week via a Boardman feeder, containing 50 ppm of each zinc source. The experiments were conducted during the off-season period, and the colonies were supplemented with Zn for 60 days.

To evaluate bee mortality, underbasket bee collectors were installed (Accorti *et al*., 1991). The collectors were placed seven days before and removed 14 days after the beginning of foraging activities, and the mortality was evaluated daily. The data were expressed in absolute number.

To evaluate population development, a central frame from each experimental colony was selected, and the areas of open and sealed brood; and the amount of stored food were evaluated weekly, starting one week before the beginning of the experiment, and ending after four weeks, using the method described by Al-Tikrity *et al*. (1971).

Queens of each experimental colony were confined in a nest frame with combs containing empty cells, for the standardization of laying. After laying, the queen was released and Zn supplementation was initiated. Nineteen days later, two frames containing combs with sealed brood were removed from each experimental colony. The frames were wrapped in perforated tissue and kept in a temperature and humidity-controlled incubator (± 34 °C and 60% RH). After, approximately 500 newly emerged bees were marked on the dorsal part of the thorax (pronotum) with a non-toxic pen Uni posca (Mitsubishi Pencil, Tokyo, Japan) (Barros *et al*., 2021), and reintroduced to their colonies of origin. Twenty-eight days after reintroduction, 10 marked forager bees from each experimental colony (totalizing 50 marked bees per treatment), were collected and immediately frozen at −80 °C for use in later gene expression analyses (figure 1).

RNA was extracted from the head's pools (five heads per pool with four repetitions, totalizing 20 heads to treatment) using 500 µL TRIzol® Reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions (ThermoFisher Scientific, Waltham, MA, USA) (Chomczynski, 1993). The extraction product was visualized on 1% agarose gel and quantified on a NanoDrop ND-1000 UV/VIS Spectrophotometer (Thermo Scientific, Waltham, MA, USA). The samples were then stored at −80 °C. Afterward, a solution including 0.75 mM dT oligos (N = 18), 0.15 mM random oligos (N = 8), 0.75 mM dNTPs, and 11 µL RNA treated with DNAse, was prepared, incubated at 65 °C for 5 minutes, and placed on ice for 1 minute. 1 × DTT 0.005 buffer, RNAse out 40 U, and 100 U of the SuperScript enzyme® III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) were added to the above preparation. The preparation was incubated at 50 °C for 1 hour and then at 70 °C for 15 minutes. The real-time PCR reactions were performed in an ABI 7300 apparatus (Applied Biosystems, Foster City, CA, USA) using the SYBR® Green PCR Master Mix kit (Applied Biosystems) under the following conditions: one cycle at 50 °C for 2 minutes; one cycle at 94 °C for 10 minutes, followed by 40 cycles of 94 °C for 15 seconds and 60 °C for 1 minute. The dissociation curve was obtained as follows: 95 °C for 15 seconds, 60 °C for 30 seconds, and 95 °C for 15 seconds. CAT (Kadri *et al*., 2021) and GPX expression levels were determined using real-time PCR, in triplicates, using ribosomal protein 49 (*rp49*) as the control (Lourenço *et al*., 2008). Primers for GPX gene were designed using the NCBI Primer Design tool for each reaction, a negative control was used, consisting of the mixture of reagents and water. The sequences and information of the oligonucleotides used are listed in table 1.

Table 1. Oligonucleotides used in the gene expression study of Africanized *A. mellifera* for ribosomal protein 49 (*rp49*), glutathione peroxidase (GPX), and catalase (CAT).

Gene	Gene accession number	*Primer* sequence 5'-3'	References
rp49	AF441189	CGTCATATGTTGCCAACTGGT TTGAGCACGTTCAACAATGG	Lourenço *et al*., 2008
GPX	NC_037638.1	TGGTCAAGAAGAACCGGGAAATA CCATAAAGGATGCGCAGAAT	This study
CAT	XM_003699011.1	CCTGGTATTGGAGCAAGTCC GGCCATCACGTTGGTAGTTT	Kadri *et al*., 2021

Table 2. Open and sealed brood areas (cm^2) of the colonies of Africanized *A. mellifera*, for control (sugar syrup), organic Zn (ZnO), and inorganic Zn (ZnI) supplementation.

	Open brood	Sealed brood
Control	136.0 ± 147.2	568.0 ± 411.2
ZnO	174.4 ± 201.6	540.8 ± 380.8
ZnI	160.0 ± 152.0	561.6 ± 348.8

Table 3. Mortality (absolute number) of Africanized *A. mellifera*, for control (sugar syrup), organic Zn (ZnO), and inorganic Zn (ZnI) supplementation.

	Mortality
Control	10.2 ± 13.9
ZnO	14.5 ± 14.4
ZnI	11.3 ± 9.7

The relative quantification of genes (R) was determined according to Pfaffl (2001), in which CP (crossing point) was defined as the point at which the detected fluorescence was appreciably above the background fluorescence using the following formula: R = E target ΔCP target (control – sample) / ΔCP endogenous (control – sample) E endogenous.

The results of mortality and population development were analysed using analysis of variance followed by Tukey's test for comparison of means. The results of gene expression analysis were compared using the Kruskal-Wallis test, with Dunn's post-hoc test. In all tests, the results were considered statistically different at $P < 0.05$ (Zar, 1996).

Results

All experimental colonies consumed 100% of the sugar syrup from all treatments. Organic and inorganic zinc supplementation did not influence population development (table 2). Similarly, supplementation with ZnO or ZnI did not influence bee mortality in the experimental colonies, compared with the control treatment (table 3).

Regarding gene expression, there was a significant increase in GPX expression with the ZnO supplementation, when compared with the control and ZnI. There was a significant increase in CAT expression with the ZnI supplementation, when compared with the control and ZnO treatments (figure 2).

Discussion

In this study colonies that received the diet supplemented with Zn, organic or inorganic form, consumed all syrup provided. No differences were observed in population development among experimental colonies, suggesting that energy substitute provided (sugar syrup) was able to maintain an adequate brood area in the off-season period.

In literature, there are several reports of Zn toxicity to bees (Eisler, 1993; Chang *et al*., 2012), with potential neurotoxic effects, reduced longevity, reduced brain weight, and loss of protein mass (Milivojevic *et al*., 2015). Zn can be toxic at high concentrations, leading to low reproductive activity and high mortality (Walker *et al*., 2012). Previously studies of our research group evaluated high zinc inorganic concentrations (500, 1000 and 1500 ppm) in the colonies diet showing toxic effects with drastic population reduction and colonies losses (Carillo *et al*., 2022). In this experiment, organic and inorganic Zn consumption by the experimental colonies did not cause bee mortality or affect the population of the colonies, suggesting that 50 ppm of zinc did not exhibit toxic effects by the time evaluated, probably because this concentration is the nutritional requirements for bees (Herbert and Shimanhuki, 1978; Zhang *et al*., 2015). Other result shows that zinc organic was better than inorganic source in the hypopharyngeal and mandibular glands and royal jelly production, suggesting positive effects in the development of colonies (Barros *et al*., 2021; Longuini *et al*., 2021).

In nature, bees obtain zinc from food resources such as nectar and pollen (Potts *et al*., 2016; Ghosh *et al*., 2017), and this mineral is important to participate in some biochemical processes associated with cell membrane integrity maintenance, respiration, cell reproduction, and other essential functions (Eisler, 1993; Zhang *et al*., 2015; Longuini *et al*., 2021).

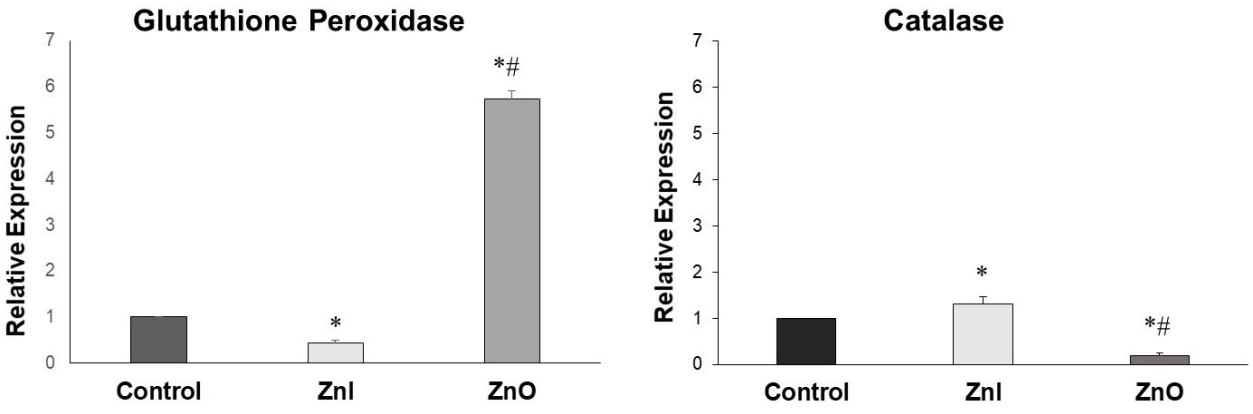

Figure 2. Relative gene expression of glutathione peroxidase (GPX) and catalase (CAT) in foragers Africanized *A. mellifera*, feed with organic (ZnO) and inorganic (ZnI) zinc supplementation. *Indicates significant difference in relation to the control. #Indicates a significant difference between Zn sources.

Bees, especially the foragers, have an antioxidant system for combatting the damaging effects of reactive oxygen species (Birben *et al*., 2012), which includes a set of enzymes, such as CAT and GPX (Krishnan and Kodric, 2012), and can facilitate bee longevity (Munch *et al*., 2008). However, the system could be impaired by the absence or reduction in nutrients in the bee diet, with detrimental effects such as reduced longevity of the forager bees. According to Sun *et al*. (2005) Zn influences the activities of enzymes in the antioxidant system, which are responsible for suppressing free radicals and controlling peroxidation.

In this experiment, was observed that zinc (organic or inorganic source) increased the CAT and GPX expression, suggesting better antioxidant maintenance (Strachecka *et al*., 2014; Nikolenko *et al*., 2012). Thus, as the mineral Zn cannot be stored in the body, it requires regular intake of foods, supplements, or diets that contain this nutrient (Camilli *et al*., 2022) can be important handling practices for beekeeping.

The source of supplemented minerals in diets could influence their bioavailability; minerals bound to organic molecules have better absorption characteristics and decreased competition than other minerals for binding sites with other minerals of interest (Peixoto *et al*., 2005), being used in livestock production. However, at this study, higher CAT expression was observed in colonies that received a diet supplemented with inorganic than organic Zn. On the other hand, when colonies were supplemented with a diet containing organic Zn, higher GPX expression was observed than in colonies supplied with inorganic Zn.

Camilli *et al*. (2022) evaluated organic and inorganic zinc supplementation in honey bees gene expression by transcriptomic analysis. The results observed showed 371 genes expression altered with inorganic source, 28 genes with organic source and 63 genes common both sources, affecting some pathways as metabolic process. Camilli *et al*. (2022) suggest that the zinc influence can interfere with glycolysis and the oxidation of pyruvate and ascorbate, related to antioxidant metabolism pathways where Zinc is directly involved. Brain tissue is more vulnerable to oxidative stress than other insect tissues, as they consume much oxygen and contain large amounts of polyunsaturated fatty acids available for lipid peroxidation. So, zinc supplementation is important to bees for antioxidant system maintenance.

The results suggest that Zn source can influence antioxidant genes expression, and further studies are required to elucidate the effects and the underlying mechanisms.

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Authors' addresses: Ricardo de Oliveira ORSI (corresponding author: ricardo.orsi@unesp.br), Iloran do Rosário Corrêa MOREIRA (iloran.rosario@unesp.br), Samir Moura KADRI (samirkbr@yahoo.com), Animal Production and Preventive Medicine Veterinary, College of Veterinary Medicine and Animal Sciences, São Paulo State University, UNESP, Botucatu, São Paulo, Brazil.

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