Bulletin of Insectology **75** *(1): 27-31, 2022*
ISSN 1721-8861 eISSN 2283-0332

High doses of inorganic zinc modulate the hypopharyngeal glands of *Apis mellifera* but promote abandonment of colonies when offered over long periods

Marcela Pedraza CARILLO[1], Aline ASTOLFI[1], Edgar Junio Damasceno RODRIGUES[1], Isabella Cristina de Castro LIPPI[1], Letícia Garbin Ribeiro da SILVA[1], Matheus Gardim GUIMARÃES[1], Luiz Antônio JUSTULIN[2], Ricardo de Oliveira ORSI[1]

[1]*Center of Education, Science and Technology in Rational Beekeeping (NECTAR), College of Veterinary Medicine and Animal Sciences, UNESP - São Paulo State University, São Paulo, Botucatu, Brazil*

[2]*Institute of Biosciences, Department of Morphology, UNESP - São Paulo State University, São Paulo, Botucatu, Brazil*

Abstract

Previous studies by our research group verified the beneficial effects of zinc supplementation on honey bee colonies during off-season. Here, we used high concentrations of inorganic zinc to evaluate the effects of high levels of zinc supplementation on hypopharyngeal gland (HPG) development of *Apis mellifera* L. during off-season. Twelve colonies were randomly assigned to one of four experimental diets (0 ppm Zn, 500 ppm Zn, 1000 ppm Zn, and 1500 ppm Zn) with three replicates. Honey bees received 500 mL of diet supplement with or without an inorganic source of zinc (sulfate heptahydrate, 20% zinc) once a week for 60 days. After the experimental period, 20 worker bees (6-days-old nurse bees) were sampled from each colony for HPG development analysis. Zinc, regardless of the level, increased the average area of acini compared to the non-supplemented group. However, gradual colony population reduction was visually observed after approximately 90 days, suggesting that Zn supplementation at high concentrations may exert a toxic effect. Therefore, although zinc can positively modulate HPG, the zinc supplements must be administered with caution.

Key words: beekeeping, mineral supplementation, nutrition, protein.

Introduction

Pollen composition directly impacts the nutrition of honey bee colonies because it is the main protein, amino acid, mineral, and lipids source. These nutrients found in the pollen could affect the development of the mandibular (MG) and hypopharyngeal glands (HPGs) (Degrandi-Hoffman *et al*., 2010), located in the head of worker nurse bees and responsible for royal jelly (RJ) production (Mokaya *et al*., 2020). The HPGs are responsible for producing a secretion rich in protein, while the MG synthetizes the lipid fraction (Fujita *et al*., 2013, Milone *et al*., 2021). The mixed secretion of both glands results in RJ containing amino acids, cholesterol, lipids, sugars, and vitamins, which are fed to the larvae (Degrandi-Hoffman *et al*., 2010; Ramanathan *et al*., 2018).

HPG development is influenced by the nutritional status of the bees, season factors, geographical origin and pesticide exposure (Corby-Harris and Snyder, 2018; Ahmad *et al*., 2020). Thus, nurse worker bees that do not consume optimal amounts of protein have impaired HPG development, reducing royal jelly production (Standifer, 1980) and potentially impairing larval growth and colony maintenance (Heylen *et al*., 2011).

Apart from protein levels, minerals such as zinc may influence HPG development in *Apis mellifera* L. (Hymenoptera Apidae) because it participates in important physiological processes. Minerals are involved in several metabolic pathways, physiological processes, structural components, and enzymatic cofactors (Moraes and Almeida, 2020). For instance, zinc is an indispensable micronutrient (Zhang *et al*., 2015) associated with the modulation of biochemical processes, transcription factors, membrane integrity, cellular respiration, reproduction, and other biological functions, as well as metalloproteins and antioxidant enzymes (Sloup *et al*., 2017; Longuini *et al*., 2021).

Thus, zinc deficiency in periods of scarcity can adversely affect bees and colony survival (Barros *et al*., 2021a). Therefore, evaluating the effects of dietary zinc levels on the development of HPG is important for formulating adequate artificial supplements for honey bees. However, studies on the effects of dietary zinc levels on the development of HPG are scarce. Hence, this study aimed to evaluate HPG development in *A. mellifera* supplemented with different levels of zinc.

Materials and methods

Treatments

Prior to the field tests, laboratory tests were conducted with worker forager bees of Africanized *A. mellifera* to verify the consumption and acceptance of sugar syrup (water and sugar in a 1:1 ratio) containing inorganic zinc (zinc sulfate heptahydrate containing 20% zinc; dynamic) at different concentrations (500, 1000, 1500, 2500, 5000, 7500, 10000, 12500, 15000, 30000, 40000 and 50000 ppm). Zn concentrations above 1500 ppm led to a viscous sugar syrup which was difficult for the bees to consume; thus, we used concentrations between 500 and 1500 ppm Zn.

The beehives used were standardized for the number of broods and food frames, with a homogenous queen posture. Sugar syrup (1:1 water and sugar) with and without zinc was offered for 60 days, and the bees were collected at the end of the experimental period.

The beehives were divided into the following treatments (three hives per treatment):

- Control treatment: no supplementation with Zn;
- Treatment 1: Zn supplementation at a concentration of 500 ppm;
- Treatment 2: Zn supplementation at a concentration of 1000 ppm;
- Treatment 3: Zn supplementation at a concentration of 1500 ppm.

The inorganic zinc source was administered every seven days through a Boardman-type feeder (500 mL) during the off-season in the study region (June and July 2015).

Development of hypopharyngeal glands

Bee sampling was performed based on the methodology described by Camilli *et al*. (2020). In June and July, 20 worker bees at 6 days of age were harvested from a random frame of a hive from each treatment each month. A frame with pupae was removed from each treatment and individually wrapped in a tissue. The frames were placed in an incubator at approximately 30 °C and relative humidity of approximately 60% until the emergence of individuals. The worker bees that emerged were marked in the thorax with a non-toxic pen, and reintroduced into their mother colonies.

At 6 days of age, nursing bees were harvested with entomological forceps and immediately stored in a plastic tube with holes to facilitate the entry of air. Thereafter, they were euthanized (decapitated with a scalpel after being anesthetized with CO_2), and their heads were fixed in 4% formaldehyde (dissolved in 0.1 M phosphate buffer, pH 7.3) for 24 hours to increase exoskeleton permeability. Subsequently, the heads of the bees were washed in running water for 24 hours and then placed in 70% ethyl alcohol until they were processed for histological analysis (Zaluski *et al*., 2017).

The procedures were based on, and adapted from, Smodiš Škerl and Gregorc (2010). The samples were processed in a graded series of ethanol (70%, 80%, 90%, and 95%) and then embedded in methacrylate resin (Historesin-Leica). Samples were placed in plastic moulds with assembly resin, forming blocks for blade production. In each block, two heads were placed and dried for a period of 24 hours. Then, the blocks were released from the moulds and glued onto the wood with Araldite glue.

Blocks were sliced into 3 µm sequential sections using an automatic rotary microtome (Leica RM2155, Germany). The slices were harvested and immediately placed on the blades for staining. From each block, 130 slices were produced in series, which were immediately arranged on glass slides, and 10 slices were placed per slat, totalling 13 slats per block. Thus, for each month, five blocks were analysed (two heads in each block), which comprised a total of 650 slices for analysis. The slides containing these sections were stained with hematoxylin-eosin, oven-dried, and coverslips were placed over the sections. Entellan® was used for mounting.

Using a digital camera attached to the microscope, the sections were photomicrographed and analysed using the Leica Q-win Plus® image analysis software (figure 1). To quantify the mean number and mean areas of acini in the hypopharyngeal glands, 20 histological images of each bee (n = 10) were analysed for a total of 200 images. Digitized images were analysed morphometrically using the bundled software, Leica Q-win Plus® version 3.1, for Windows™ (Leica, Heidelberg, Germany). The number of acini per honey bee in each experimental group was counted. Additionally, using the same software, the acini were circumvented, and the average area (µm^2) of the 100 largest acini for each experimental group was determined. This was done because after performing the tests, it was observed that the 10 largest were representative of the whole. All measurements were saved in Microsoft Excel spreadsheets for analysis.

Statistical analysis

The results obtained were compared by ANOVA, followed by Tukey's test to verify the differences between the means. Differences were considered statistically significant when $p < 0.05$ (Zar, 1996).

Results

The colonies that received the diet with or without zinc supplementation consumed all of the food offered. However, after 30 days of experiments, we recorded a gradual reduction in the brood area and adult population of all colonies that received the diet supplemented with the mineral at all concentrations, with beehive abandonment observed at 90 days.

There was no significant difference in the number of acini from the hypopharyngeal gland between the control and zinc supplementation treatments during the off-season (table 1). Relating to the media acini area, the control group had a smaller average area compared to treatments in which zinc supplementation was performed during the off-season. The T1, T2, and T3 treatments did not differ significantly (table 2 and figure 1).

Discussion

Many studies have evaluated the effects of nutrients on individual honey bees caged but not on the colony level under natural conditions (Zheng *et al*., 2014), which could reflect different results. Therefore, in this study, zinc supplementation was realized in beehives allocated to the field, simulating the natural reality of beekeeping. The field tests were conducted in the off-season because there is a natural reduction of food resources in the environment and it is recommended that beekeepers offer artificial food to the colonies. However, there is an information gap regarding a balanced diet for bees, such as a real mineral requirement. In this study, we observed that zinc modulated HPG development by increasing the acini area compared to the unsupplemented group, regardless of the level used.

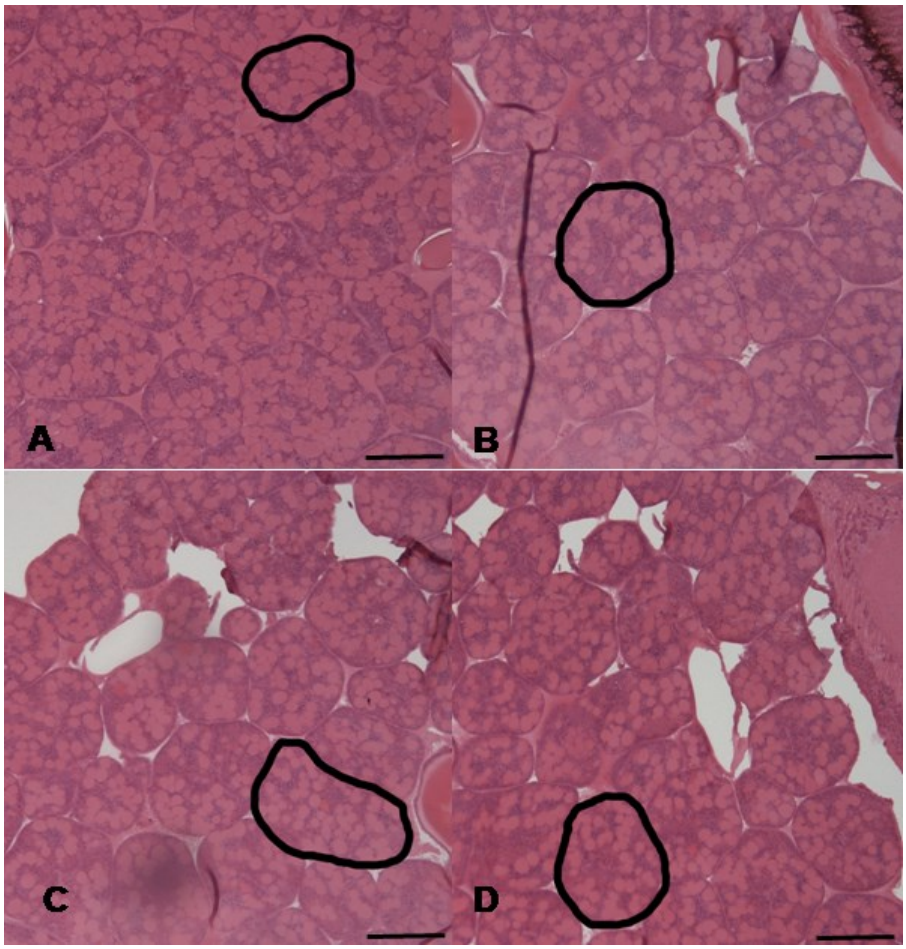

Figure 1. Representative photomicrographs of the hypopharyngeal gland of Africanized honey bees (*A. mellifera*) at six days of age stained by hematoxylin and eosin. **A)** Control (without addition of Zn), **B)** T1 treatment (0.5 mg mL^{-1} Zn), **C)** T2 treatment (1.0 mg mL^{-1} Zn) and **D)** T3 treatment (1.5 mg mL^{-1} Zn). Scale bars: 10 µm.

Table 1. Number of acini from the hypopharyngeal glands of Africanized honey bees (*A. mellifera)* harvested in the off-season. The values expressed are the means followed by their standard deviations.

Diets	Number of acini
Control	16.6 ± 2.7
T1	15.0 ± 1.4
T2	16.3 ± 0.5
T3	19.3 ± 3.8

Table 2. Average area of the 10 largest acini (μm^2) of the pituitary glands of Africanized honey bees (*A. mellifera)* harvested during the off-season. The values expressed are the means followed by their standard deviations.

Diets	Average area of acini (μm^2)
Control	26.9 ± 4.8 A
T1	28.9 ± 5.2 B
T2	29.0 ± 4.0 B
T3	28.5 ± 5.2 B

Different capital case letters in the same column indicate a significant difference among the means ($p \leq 0.05$).

HPG development may be associated with colony development, longevity, larval growth, and royal jelly production (Deseyn and Billen, 2005). HPG size is significantly plastic and is affected by nutritional factors, such as dietary protein levels (Camilli *et al*., 2020), pollen flavonoid content (Bovi *et al*., 2017), iron supplementation levels (Barros *et al*., 2021b), and lipid and protein sources (Corby-Harris *et al*., 2016).

The royal jelly component produced by these glands serves as a food source for different castes of bees and the queen throughout her life (Ji *et al*., 2014) and the nutrients of the diet could modulate its size and function, which are important tools for the development of bee colonies. Previous studies by our research group comparing the organic and inorganic zinc source showed better effects in the mandibular glands (MG) when the honey bee colonies received the organic zinc (zinc-methionine) source; the inorganic zinc source promoted negative effects in the MG area at concentrations of 50 and 75 ppm (Barros *et al*., 2021a), in field tests. In another study (data not published), it was observed that the organic source zinc showed better results in the HPG development than the inorganic source, at concentrations of 50 and 75 ppm; however, the inorganic zinc source was similar to the control treatment, suggesting that higher doses could have beneficial effects.

Few studies have assessed the efficiency of different mineral sources in the glandular development of *A. mellifera* honey bees. Even though organic zinc sources have higher bioavailability (linkage to organic molecules) compared to inorganic sources (Rider *et al*., 2010), it is more expensive when added to animal nutrition (Zhao *et al*., 2014) and could not be used in practice by beekeepers. Thus, we conducted this research with high dosages of inorganic zinc to test different concentrations and find the highest proportion of zinc and sugar syrup that the bee consumed, which is 1500 ppm.

Thus, the concentrations of 500, 1000, and 1500 ppm resulted in positive effects were after the addition of inorganic zinc (IZn) for 60 days, at all concentrations used, with an increase in the HPG area. The need for mineral zinc by bees is met by harvesting nectar and pollen; however, its concentration can vary according to the grazing area available to bees around the apiary (0.012 ppm to 173.77 ppm for honey and from 5.1 to 340.0 ppm for bee pollen) (Zhang *et al*., 2015; Wright *et al*., 2018). When bees do not find the proper amount of a mineral in the collected nectar and pollen, malnutrition can occur, and inadequate nutrition can reduce the size of the HPG, especially during the first week of adult development (Corby-Harris and Snyder, 2018). Therefore, dietary factors directly affect the development of HPG, as nutrition, longevity, and colony maintenance are intertwined. Therefore, providing a proper balance of nutrients is favorable not only for growth and honey production but also for improving the immune status. Nutrition can modulate the gut microbiome and positively affect immune responses, metabolism, and maintenance (Pegoraro *et al*., 2013; Ricigliano and Simone-Finstrom, 2020).

However, during the supplementation period, a gradual decrease in the beehive's population and brood (visual assessment) was observed for all Zn inclusion levels. Thirty days after the start of the experiment, a reduction in sealed brood was observed in the colonies that received zinc and continued until 60 days, when the bees were collected for HPG analysis. At the end of the experiment, the colonies had queens and honey bees, but the posture rate and brood area were reduced, and up to 90 days, the colonies were swarming, abandoning their hives. This effect was not measured, and only visual observations were possible.

This effect could be related to a possible toxic effect of zinc minerals, probably due to the higher concentrations used in this study. Zinc sulfate (500 to 10000 ppm) could interfere with the physiological development of endocrine glands, changing their structure and activity, as observed in *Spodoptera littoralis* (Boisduval) (Sharaby *et al*., 2013). Furthermore, zinc supplementation has been associated with increased levels of biomarkers of oxidative damage (malonaldehyde) in honey bees (Zhang *et al*., 2015). Previous studies have suggested that 60 ppm of Zn in collected pollen would be sufficient to meet the nutritional needs of colonies in the presence of broods. However, the study was conducted under laboratory conditions using fewer bees and a shorter supplementation period. In this study, higher doses of zinc were chosen to determine an adequate inorganic zinc level to supplement bee diets under field conditions during the off-season, which may explain the gradual abandonment of the colony and the potential toxic effect when used for a long time.

Conclusions

We found that zinc can affect HPG development. When Zn supplementation levels exceeded the nutritional requirements of *A. mellifera*, a toxic effect was exerted in the long term.

Acknowledgements

This study was funded by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), process 2014/14699-9.

References

AHMAD S., KHAN S. A., KHAN K. A., LI J., 2020.- Novel insight into the development and function of hypopharyngeal glands in honey bees.- *Frontiers in Physiology*, 11: 615830.

BARROS D. C. B., CAMILLI M. P., MARTINELI G. M., LONGUINI A. A., KADRI S. M., JUSTULIN L. A., ORSI R. O., 2021a.- Effect of organic and inorganic zinc supplementation on the development of mandibular glands in *Apis mellifera*.- *Bulletin of Insectology*, 74: 209-212.

BARROS D. C. B., SHINOHARA A. J., CAMILLI M. P., SANTOS S. A., JUSTULIN L. A., ORSI R. O., 2021b.- Mineral iron affects the development of mandibular gland in *Apis mellifera*.- *Journal of Apicultural Research*, 60: 439-444.

BOVI T. D., CAEIRO A., SANTOS S. A., ZALUSKI R., SHINOHARA A. J., LIMA G. P., CAMPOS M. D., JUNIOR L. A., ORSI R. O., 2017.- Seasonal variation of flavonoid content in bee bread: potential impact on hypopharyngeal gland development in *Apis mellifera* honey bees.- *Journal of Apicultural Research*, 59: 170-177.

CAMILLI M. P., DE BARROS D. C., JUSTULIN L. A., TSE M. L. P., ORSI R. O., 2020.- Protein feed stimulates the development of mandibular glands of honey bees (*Apis mellifera*).- *Journal of Apicultural Research*, 60: 165-171.

CORBY-HARRIS V., SNYDER L. A., 2018.- Measuring hypopharyngeal gland acinus size in honeybee (*Apis mellifera*) workers.- *Journal of Visualized Experiments*, 139: e58261.

CORBY-HARRIS V., MEADOR C. A., SNYDER L. A., SCHWAN M. R., MAES P., JONES B. M., WALTON A., ANDERSON K. E., 2016.- Transcriptional, translational, and physiological signatures of undernourished honey bees (*Apis mellifera*) suggest a role for hormonal factors in hypopharyngeal gland degradation.- *Journal of Insect Physiology*, 85: 65-75.

DEGRANDI-HOFFMAN G., CHEN Y., HUANG E., HUANG M. H., 2010.- The effect of diet on protein concentration, hypopharyngeal gland development and virus load in worker honey bees (*Apis mellifera* L.).- *Journal of Insect Physiology*, 56: 1184-1191.

DESEYN J., BILLEN J., 2005.- Age-dependent morphology and ultrastructure of the hypopharyngeal gland of *Apis mellifera* workers (*Hymenoptera, Apidae*).- *Apidologie*, 36: 49-57.

FUJITA T., KOZUZA-HATA H., AO-KONDO H., KUNIEDA T., OYAMA M., KUBO T., 2013.- Proteomic analysis of the royal jelly and characterization of the functions of its derivation glands in the honeybee.- *Journal of Proteome Research*, 12: 404-411.

HEYLEN K., GOBIN B., ARCKENS L., HUYBRECHTS R., BILLEN J., 2011.- The effects of four crop protection products on the morphology and ultrastructure of the hypopharyngeal gland of the European honeybee, *Apis mellifera*.- *Apidologie*, 42: 103-116.

JI T., LIU Z., SHEN J., SHEN F., LIANG Q., WU L., CHEN G., CORONA M., 2014.- Proteomics analysis reveals protein expression differences for hypopharyngeal gland activity in the honeybee, *Apis mellifera carnica* Pollmann.- *BMC Genomics*, 15: 665.

LONGUINI A. A., MARTINELI G. M., CAMILLI M. P., DE BARROS D. C. B., VIEIRA J. C. S., DE MAGALHÃES PADILHA P., DE OLIVEIRA ORSI R., 2021.- Supplementation with an inorganic zinc source in the metalloproteomic profile of royal jelly in *Apis mellifera* L.- *Biological Trace Element Research*, 199: 4308-4318.

MILONE J. P., CHAKRABARTI P., SAGILI R. R., TARPY D. R., 2021.- Colony-level pesticide exposure affects honey bee (*Apis mellifera* L.) royal jelly production and nutritional composition.- *Chemosphere*, 263: 128183.

MOKAYA H. O., NJERU L. K., LATTORFF M. G., 2020.- African honeybee royal jelly: phytochemical contents, free radical scavenging activity, and phsycochemical properties.- *Food Bioscience*, 37: 100733.

MORAES G., ALMEIDA L. C., 2020.- Nutrition and functional aspects of digestion in fish, pp. 251-271. In: *Biology and physiology of freshwater neotropical fish* (BALDISSEROTTO B., URBINATI E., CYRINO J., Eds).- Academic Press, London, UK.

PEGORARO A., NUNES F. L., PEREIRA F. F., TEIXEIRA R. A., KRUGER E., SERMANN K. C., 2013.- Perdas de colônias de *Apis mellifera* no inverno suplementadas com alimentação artificial com pólen e favos de mel.- *Revista Agrarian*, 6: 67-74.

RAMANATHAN A. N. K. G., NAIR A. J., SUGUNAN V. S., 2018.- A review on royal jelly proteins and peptides.- *Journal of Functional Foods*, 44 (3): 255-264.

RICIGLIANO V. A., SIMONE-FINSTROM M., 2020.- Nutritional and prebiotic efficacy of the microalga *Arthrospira platensis* (spirulina) in honey bees.- *Apidologie*, 51: 889-910.

RIDER S. A., DAVIES S. J., JHA A. N., CLOUGH R., SWEETMAN J. W., 2010.- Bioavailability of co-supplemented organic and inorganic zinc and selenium sources in a white fishmeal-based rainbow trout (*Oncorhynchus mykiss*) diet.- *Journal of Animal Physiology and Animal Nutrition*, 94: 99-110

SHARABY A. M., EL-HAWARY F. M., MOAWAD S., 2013.- Zinc sulfate as growth disruptor for *Spodoptera littoralis* wih reference to histological changes in larval endocrine glands.- *Journal of Agricultural and Veterinary Science*, 5: 67-74.

SLOUP V., JANKOVSKÁ I., NECHYBOVÁ S., PEŘINKOVÁ P., LANGROVÁ I., 2017.- Zinc in the animal organism: a review.- *Scientia Agriculturae Bohemica*, 48: 13-21.

SMODIŠ ŠKERL M. I. S., GREGORC A., 2010.- Heat shock proteins and cell death in situ localisation in hypopharyngeal glands of honeybee (*Apis mellifera carnica*) workers after imidacloprid or coumaphos treatment.- *Apidologie*, 41: 73-86.

STANDIFER L. N., 1980. Honeybee nutrition and supplemental feeding. pp. 39-45. In: *Beekeeping in the United States*.- Agriculture Handbook 335, United States Department of Agriculture, Washington DC, USA.

WRIGHT G. A., NICOLSON S. W., SHAFIR S., 2018.- Nutritional physiology and ecology of honey bees.- *Annual Review of Entomology*, 63:327-344.

ZALUSKI R., JUSTULIN L. A. JR., ORSI R. DE O., 2017. Field-relevant doses of the systemic insecticide fipronil and fungicide pyraclostrobin impair mandibular and hypopharyngeal glands in nurse honeybees (*Apis mellifera*).- *Scientific Reports*, 7: 15217.

ZAR J. H., 1996.- *Biostatistical analysis*.- Prentice Hall, New Jersey, USA.

ZHANG G. E., ZHANG W., QUI X., BAOHUA X., 2015.- Zinc nutrition increases the antioxidant defenses of honeybees.- *Entomologia Experimentalis et Applicata*, 156: 201-210.

ZHAO C. Y., TAN S. X., XIAO X. Y., QIU X. S., PAN J. Q., TANG Z. X., 2014.- Effects of dietary zinc oxide nanoparticles on growth performance and antioxidative status in broilers.- *Biological Trace Element Research*, 160: 361-367.

ZHENG B., WU Z., XU B., 2014.- The effects of dietary protein levels on the population growth, performance, and physiology of honey bee workers during early spring.- *Journal of Insect Science*, 14: 191.

Authors' addresses: Ricardo de Oliveira ORSI (corresponding author: ricardo.orsi@unesp.br), Marcela Pedraza CARILLO, Aline ASTOLFI, Edgar Junio Damasceno RODRIGUES, Isabella Cristina de Castro LIPPI, Letícia Garbin Ribeiro da SILVA, Matheus Gardim GUIMARÃES, Animal Production and Preventive Medicine Veterinary, College of Veterinary Medicine and Animal Sciences, São Paulo State University, UNESP, Botucatu, São Paulo, Brazil; Luiz Antônio JUSTULIN, Institute of Biosciences, Department of Morphology, São Paulo State University, UNESP, Botucatu, São Paulo, Brazil.

Received June 22, 2021. Accepted November 4, 2021.