

Effect of organic and inorganic zinc supplementation on the development of mandibular glands in *Apis mellifera*

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Abstract

We investigated the effects of organic and inorganic zinc (Zn) supplementation on the morphology of mandibular glands in *Apis mellifera* L.. We randomly assigned 28 beehives to seven treatment groups (four beehives in each group) as follows: control (no Zn) and Zn organic or inorganic supplementation (25, 50, and 75 ppm organic or inorganic Zn, respectively). The inorganic source was Zn sulfate monohydrate (37% Zn) and the organic source was Zn-methionine (16% Zn), which were diluted in sugar syrup 1:1 (50% water and 50% sugar) and provided to the honey bees for 36 days. The morphology of the mandibular glands collected from 6-day-old nurse honey bees from each group was analysed after sectioning and visualizing the sections under a microscope. The results were compared using analysis of variance followed by Tukey's test. The area of the mandibular glands increased significantly in the treatments containing the organic Zn source and was higher in the 75 ppm treatment. The results from the inorganic Zn sources of 25 and 50 ppm were similar to those of the control; however, the 75 ppm treatment showed the worst glands development. Therefore, organic Zn supplementation in the feeding of honey bees, in the concentrations used in this study, positively modulated development of the mandibular glands.

Key words: beekeeping, mandibular glands, mineral supplementation, morphology, nutrition.

Introduction

Honey bees (*Apis mellifera* L.), similar to all living organisms, require nutrients in a quantity and quality that is adequate for their developmental require and routine activities. Their nutritional needs are exclusively obtained via the floral resources in the form of nectar (amino acids and phytochemicals) and pollen (which provides proteins, vitamins, lipids, and minerals) (Imdorf *et al.*, 1998; Gupta, 2014; Omar *et al.*, 2017).

Larval feeding plays a fundamental role in caste differentiation by promoting changes in the hormone profile and maintaining the integrity of ovaries in the queens. This developmental difference is attributed to their exclusive diet that is rich in royal jelly produced by nurse worker honey bees, aged 4 to 12 days, from the secretions of the mandibular glands located in the head region (Cruz-Landim and Abdalla, 2009; Feng *et al.*, 2009; Kamakura, 2011). Deseyn and Billen (2005) found that the amount of royal jelly secreted was directly related to the size of the glands and demonstrated that well-developed glands produced more royal jelly. Thus, the mandibular glands secretes a protein-based liquid substance from pollen digestion, which is used to feed both larvae and queen, and are therefore extremely important in the nutrition of the colony members (Li *et al.*, 2010).

Although the foods of *A. mellifera* mainly consists of protein and energy-rich foods, other nutrients such as vitamins and minerals are important for colony development (Brodschneider and Crailsheim, 2010). Under natural conditions, the mineral requirements of *A. mellifera* are exclusively supplied by floral resources (e.g., pollen and nectar) at concentrations that may vary depending on the region and plant variety in the vicinity of the apiary (Zhang *et al.*, 2015).

Herbert and Shimanuki (1978) suggested a diet with 1000 ppm of potassium, 500 ppm of calcium, 300 ppm of magnesium, and 50 ppm each of sodium, manganese, copper, iron, and zinc (Zn). Among the essential elements required to the complete development of honey bees, Zn is extremely important. The mineral Zn contributes to normal cellular functioning, acting as an enzymatic cofactor and participating in metabolism, the regulation of gene expression, the structural maintenance of biomembranes, immunity, and protection against free radicals and protein synthesis (Faa *et al.*, 2008).

Zn is one of the main protectors of the immune system, participating in several enzymatic activities and collaborating to the production of deoxyribonucleic acids and ribonucleic acids with the formation of so-called Zn fingers that allowing the adhesion between proteins and acids for good regulation and gene expression (Zhang *et al.*, 2015; Maret, 2017).

The source in which the mineral is offered (i.e., organic or inorganic) might interfere with its bioavailability (Hara-guchi, 2014; Maret, 2017). However, for *A. mellifera*, no studies have evaluated the best source of Zn to date.

Little is known about the actual requirement of Zn and its influence on the physiology of honey bees (Brodschneider and Crailsheim, 2010). It is, therefore, important to study this mineral that could affect the development of mandibular glands in nurse honey bees.

Materials and methods

Treatments

A total of 28 beehives of *A. mellifera*, standardized for the number of food frames, were segregated into the following seven treatment groups, with four experimental

replicates per group: Zn0 (control treatment) - sugar syrup without Zn supplementation; ZnI25 - sugar syrup supplemented with 25 ppm inorganic Zn; ZnI50 - sugar syrup supplemented with inorganic 50 ppm Zn; ZnI75 - sugar syrup supplemented with 75 ppm inorganic Zn; ZnO25 - sugar syrup supplemented with 25 ppm organic Zn; ZnO50 - sugar syrup supplemented with 50 ppm organic Zn; and ZnO75 - sugar syrup supplemented with 75 ppm organic Zn.

The inorganic source was Zn sulfate monohydrate (containing 37% Zn) and the organic source was Zn-methionine (containing 16% Zn). These were diluted in sugar syrup 1:1 ratio (50% water and 50% crystal sugar), vortexed and administered using a Boardman feeder in all treatments (500 mL per week for 30 days). The feed was consumed within 24 hours by the experimental colonies, thereby ensuring the stability of the Zn (Ingraham, 1963; Labadi *et al.*, 1993). Zn sulfate is an inorganic source and Zn-methionine is an organic source that are both used in animal research on nutrition (Legg and Sears, 1960; Maage *et al.*, 2001; Yu *et al.*, 2005).

Herbert and Shimanuki (1978) suggested the inclusion of 50 ppm Zn in the diet of honey bees; therefore, we tested the effects of the inclusion of Zn at concentrations above and below the recommended value. In this study, we supplemented the feed of honey bees in the colonies with inorganic and organic Zn to verify its effects on the glandular physiology of nurse honey bees.

Zn levels were determined using an atomic absorption spectrometry (FAAS) and were 23.57, 49.44, and 75.09 ppm in the ZnI25, ZnI50, and ZnI75 groups, respectively. In the ZnO25, ZnO50, and ZnO75 groups, these concentrations were 23.16, 48.80, and 74.44 ppm, respectively.

Collection and storage of bees

The supply of sugar syrup containing the different Zn sources and concentrations was provided weekly for 36 days and the honey bee collections for morphological analyses were performed at age 36 days (6 days of adult life) ensuring the receipt of Zn supplementation during the larval and adult phase until the nursing phase.

Two combs by each colony (eight per treatment) were removed from each treatment and were individually wrapped in tissue. The frames were placed 24 hours in an incubator at approximately 34 °C and 60% relative humidity until the emergence of adult workers. On hatching, fifty workers were marked with non-toxic dye and introduced in their mother colony. At 6 days of age, the nurse honey bees were collected with entomological forceps and immediately stored in a plastic tube with holes to allow air circulation. Ten 6-day-old worker honey bees per treatment were collected (two to three honey bees per colony), totalling 70 honey bees in the present study. These honey bees were decapitated with a scalpel after being anesthetized with CO₂ 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 were washed in running water for 24 hours and then placed in 70% ethyl alcohol until processing for histological analysis (Zaluski *et al.*, 2017).

Histological analysis

Histological analyses were performed, according to an adaptation of the method described by Smodiš-Škerland (2010). The samples were processed in a graded series of ethanol (70, 80, 90 and 95%) and cuts were made on the anterior and posterior portions of the heads (eliminating an approximately 1-mm thick layer of chitin); thereafter, the heads were embedded in methacrylate resin (HistoResin®, Leica, Heidelberg, Germany) and included in plastic molds with the assembly resin to form blocks for slide production. The blocks were glued in wood and were then sliced into 3-µm sections using an automatic rotary microtome (Leica RM2155, Germany).

Serial slices were produced from each block and were immediately mounted on glass slides. A total of 12 slices were cut per blade and an average of 13 slides were made from each block. The slides containing the sections were stained with hematoxylin-eosin, oven-dried, and coverslips were placed over the sections. Entellan® was used for mounting.

Morphometry of mandibular glands

The sections were photographed using a digital camera (Leica DC300FX) coupled to a microscope (Leica DMLB80) and the images were analysed using image analysis software (Leica Q-win version 3 for Windows). The mean area of the reservoir in the mandibular glands was determined for honey bees in each of the experimental groups.

For determining the area of the mandibular glands reservoir, 10 honey bees (five blocks) were analysed per treatment and 200 images of the mandibular glands were analysed for each treatment (20 images per bee, 40 images per block).

The results were compared using analysis of variance, followed by Tukey's test to verify the differences among means, the data were normal and homoscedastic. The differences were considered statistically significant at $P < 0.05$ (Zar, 1996).

Results

The ZnO75 group showed higher area glands development compared with the other treatments. ZnI75 showed the smallest development of the mandibular glands of all treatments. Average mandibular glands area for every experimental condition is reported in table 1 and figure 1.

Discussion

The objective of the present study was to conduct an experiment under field conditions to reflect the practice of beekeeping activity and simulate the reality because honey bees are free animals. However, during the off-season period, there is a natural reduction in the availability of food near the apiary, requiring intervention by a beekeeper via the provision of supplementary feed (Butler, 1946; Penick and Crofton, 2016; Eşanu *et al.*, 2018). Thus, the purpose of the present study was to evaluate the effect of organic and inorganic Zn supplementation in the

Table 1. Area of the mandibular gland (μm^2) means \pm standard deviations. Zn0 (without zinc addition), ZnI25 (supplemented with 25 ppm zinc inorganic), ZnI50 (50 ppm zinc inorganic), ZnI75 (75 ppm zinc inorganic), ZnO25 (25 ppm zinc organic), ZnO50 (50 ppm zinc organic), ZnO75 (75 ppm zinc organic).

Average area ($\mu\text{m}^2 \times 10^4$) of mandibular gland in <i>A. mellifera</i>						
Zn0	ZnI25	ZnI50	ZnI75	ZnO25	ZnO50	ZnO75
4.7 \pm 1.4cd	5.6 \pm 2.1c	3.9 \pm 1.1de	3.7 \pm 1.0e	7.3 \pm 2.0b	7.6 \pm 2.1b	8.8 \pm 2.4a

Values followed by different letters indicate a significant difference in the mean area between treatments ($P > 0.05$).

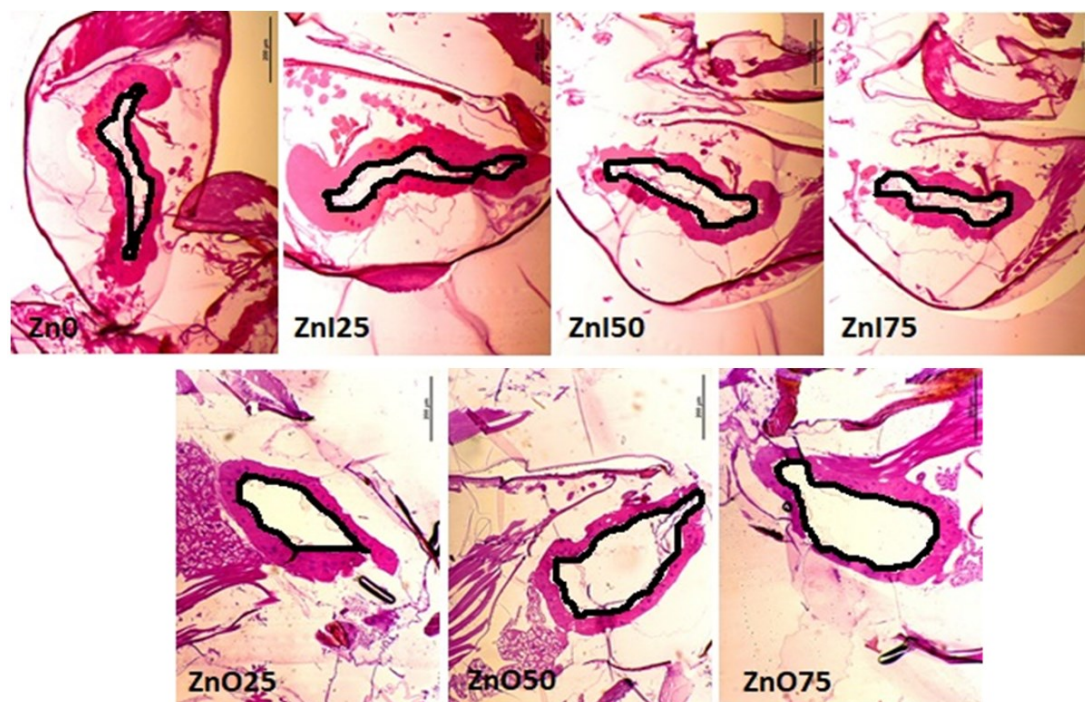


Figure 1. Representative photomicrographs of the area of the mandibular gland of *A. mellifera* bees. Scale bars 200 μm .

honey bee diet on the development of the mandibular glands, under field conditions, with the honey bees having access to foraging activities.

The mandibular glands are important for colony development in honey bees and their size is an indication of their activity, reflecting the amount of protein secreted. Thus, the mandibular glands has been used as an indicator of development and activity under different conditions of management and nutrition (Smodiš-Škerl and Gregorc, 2010). Several factors may affect the development of the mandibular glands, such as the caste and physiological age of bees, contact with pesticides, juvenile hormone concentration that is increased in queen bees, and swarm nutrition (Pankiw *et al.*, 1998; Salles and Cruz-Landim, 2004; Zaluski *et al.*, 2017).

We observed that organic Zn treatments (i.e., ZnO25, ZnO50, and ZnO75) positively altered the development of the mandibular glands in the different treatments studied. Zn is fundamentally important as a structural and functional component of living beings, is present in metalloproteins, and plays a catalytic role in enzymatic systems (Haraguchi, 2014; Maret, 2017). Zn is the mineral that is present in the largest number of cells and plays a fundamental role in the function of more than 300 classes of enzymes. Of all the minerals that bind to proteins,

Zn is the most abundant (Shi and Chance, 2011).

Supplementation with organic Zn can modulate the nutritional and physiological status of worker bees. The amount of royal jelly secretion is directly related to the size of the mandibular glands, showing that well-developed glands produce more royal jelly, a hypothesis that would directly influence colony nutrition and development (Deseyn and Billen, 2005; Peters *et al.*, 2010).

Longuini *et al.* (2021) evaluated the quality of royal jelly through proteomic analyzes, and it was found that vital proteins were impaired in supplementing honey bees exposed to the mineral zinc in its inorganic form in all concentrations (zinc sulfate monohydrate: 0 - 25 - 50 and 75 ppm).

The honey bees in the control and inorganic Zn groups did not positively modulate the glands, showing less development of the mandibular glands compared to the organic Zn groups.

These results can be explained based on the hypothesis that the source (organic or inorganic) in which the mineral is offered may interfere with its bioavailability; minerals linked to organic molecules, called chelates, feature advantages over the inorganic form, with better absorption and less competition for binding sites with other minerals, suggesting that the supplementation of organic

Zn positively modulated the nutrition of the swarms (Haraguchi, 2014; Maret, 2017). We concluded from the present study that the supplementation of organic Zn in the diet of *A. mellifera* positively modulated the development of the mandibular glands.

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The authors declare that they have no potential conflict of interest concerning the study in this paper.

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