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ARTÍCULO ORIGINAL

Effects of dietary supplementation with abscisic acid on *Apis mellifera* colonies confined in overwintering nucleus: studies on the adult honey bee population, nosemosis, and expression of nutrition- and immune-related genes

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Abstract

Due to the complex interactions between pathogens and environmental and anthropogenic stressors, in temperate climates, honey bee populations suffer depopulation and/or colony death, mainly during overwintering. *Nosema* spp. comprises one of the primary pathogens affecting *Apis mellifera* colonies, and cold ambient temperature promotes its intensity in worker bees. Additionally, the drastic reduction of plants decreases the diversity and quality of pollen and nectar diet for bees, leading to malnutrition of honey bee colonies and compromising the immunity of individuals, increasingly vulnerable to parasites and pathogens. Previous studies showed that abscisic acid (ABA) stimulates the health of honey bees at individual and colony levels against different stress conditions. This study aimed at evaluating the effect of supplementing the diet with ABA on overwintering nuclei, considering the population growth of honey bee colonies, nosemosis dynamics, and the nutrition- and immune-related gene expression of honey bees. Results in this work showed that ABA had no significant effect on any of the parameters studied. However, taking into account the results observed in the variables measured, we encourage the development of more studies focused on this topic, using more colonies per group and measuring the blooming period and the plant species involved.

Key words: *Apis mellifera*; abscisic acid; nosemosis; gene expression

Efectos de una dieta suplementaria con ácido abscísico en núcleos de *Apis mellifera* durante el invierno: estudios sobre la población de abejas adultas, nosemosis y la expresión de genes relacionados con la nutrición y el sistema inmunológico

Resumen

Debido a las complejas interacciones entre diversos estresores, en climas templados las poblaciones de abejas melíferas sufren despoblaciones y/o mortalidad, principalmente durante el invierno. *Nosema* spp. se encuentra entre los principales patógenos que afectan a las colonias de *Apis mellifera* y es durante el invierno donde se han registrado valores altos del desarrollo de esta enfermedad en las abejas obreras. Además, la reducción de la diversidad vegetal disminuye la cantidad y la calidad de la dieta de polen y néctar, lo que lleva a la malnutrición de las colonias de abejas. Estudios anteriores demostraron que el ácido abscísico (ABA) estimula la salud

de las abejas melíferas frente a diferentes condiciones de estrés. En este contexto, el objetivo principal de este estudio fue evaluar el efecto de la suplementación del alimento con ABA durante el invierno en núcleos de abejas melíferas considerando el desarrollo de la población, la dinámica de la nosemosis y la expresión génica relacionada con la nutrición y la inmunidad. Los resultados obtenidos en este trabajo mostraron que ABA no tuvo un efecto significativo en ninguno de los parámetros estudiados. Sin embargo, teniendo en cuenta los resultados observados, consideramos profundizar los estudios enfocados en esta temática, utilizando un mayor número de colonias por grupo y midiendo los periodos de floración y las especies de plantas involucradas.

Palabras clave: *Apis mellifera*; ácido abscísico; nosemosis; expresión génica

INTRODUCTION

The conventional agricultural production system adopted as a standard practice worldwide is currently leading to the loss of biodiversity (Eardley *et al*., 2016) due to changes in land management, land cover and configuration, and agrochemical use (such as herbicides, fungicides, and insecticides) linked to these intensive production models (Dicks *et al*., 2021). Consequently, a decline in some wild and managed pollinators in several world regions is evident (Potts *et al*., 2010; Koh *et al*., 2016; Requier *et al*., 2018). Bee populations comprise around 20,000 species with diverse life forms and habits, fulfilling a fundamental role in ecosystems through plant pollination (Eardley *et al*., 2016).

The European honey bee *Apis mellifera* is one of the few bees exploited by humans for commercial purposes. Although beekeepers are yearly multiplying bee hives to recover loss and/or increase them for productive purposes, a decrease in honey bees has been recorded in some regions (Meixner, 2010; vanEngelsdorp *et al*., 2012; Requier *et al*., 2018). Several factors have been identified as the main drivers of the loss and depopulation of honey bees (Steinhauer *et al*., 2018). Reports show that the interaction between pathogens and environmental and anthropogenic stressors are acting synergistically to negatively impact honey bee populations (DeGrandi-Hoffman & Chen, 2015; Steinhauer *et al*., 2018).

Main pathogens affecting the population dynamics of *A. mellifera* colonies include the intracellular parasite *Nosema* spp. (Higes *et al*., 2008). *Nosema apis* and *Nosema ceranae* are the etiological agents of nosemosis (Higes *et al*., 2008; Fries *et al*., 2013). Recently, the genera *Nosema* and *Vairimorpha* have been renamed as *N. ceranae* and *N. apis* as *Vairimorpha ceranae* and *Vairimorpha apis*, respectively (Tokarev *et al*., 2020). Nevertheless, in the present report, and to avoid any confusion, we will keep the traditional classification. *Nosema* infections negatively impact colony strength and productivity (Botías *et al*., 2013). However, *N. ceranae* infections have been reported to be more severe in honey bee colonies than *N. apis* infections (Higes *et al*., 2008). In Argentina, genetic studies reported that the cosmopolite microsporidia species is *N. ceranae* (Medici *et al*., 2012; Porrini *et al*., 2020). In addition, other studies have concluded that nosemosis can act synergistically with other risk factors (Steinhauer *et al*., 2018). For example, in temperate climates like that in Argentina, the cold ambient temperature promotes *Nosema* spp. intensity in honey bee workers (Retschnig *et al*., 2017).

The drastic reduction of wild and native grasslands is probably one of the most critical ecological consequences arising from conventional agriculture, decreasing the diversity and quality of pollen and nectar diet for bees (Eardley *et al*., 2016). This decrease produces malnutrition in honey bee colonies, compromising the immunity of individuals and turning bee populations more vulnerable to parasites and pathogens (DeGrandi-Hoffman & Chen, 2015; Negri *et al*., 2019). Thus, honey bee colonies growing in temperate climates with reduced flower resources are doubly challenged by cold stress and malnutrition (Döke *et al*., 2015; Steinhauer *et al*., 2018).

In Argentina, the growth of monocultures is significant, particularly soybean. Since 1996, this crop has continuously replaced natural extensions of habitats, affecting plant diversity and, consequently, pollen and nectar available to honey bees. Recently, a study also reported a reduction in honey yield of ~60 % in colonies associated with soybean crops (de Groot *et al*., 2021). The advance of monocultures, the loss of floral resources, and the impact of cold climates induce beekeepers to adopt standard practices after the honey harvest season, such as supplementary energetic feeding of their colonies (sugar syrup, usually sucrose: water 2:1) to meet the energetic requirements overwintering. However, this supplement has low nutritional value compared to that of honey (Wheeler & Robinson, 2014), providing only a carbohydrate source. By contrast, honey is a natural food mainly derived from nectar and a highly nutritious source that provides natural and diversified sugars and low levels of minerals, amino acids, proteins (Erler & Moritz, 2016), molecules acting as secondary metabolites (phenolic acids, terpenes,

alkaloids, etc.), and phytohormones (Wang *et al*., 2017; Akšić *et al*., 2020). Research shows that such natural molecules in nectar, pollen, and resins have beneficial effects on the health of honey bees, associated with the concept of self-medication (Erler & Moritz, 2016). Examples of these molecules include p-coumaric acid (phenolic acid), abscisic acid (sesquiterpenoid hormone), anabasine (alkaloid), catalpol (monoterpenoid), quercetin (flavonol), nicotine (alkaloid), and thymol (terpenoid) (Bernklau *et al*., 2019; Negri *et al*., 2019; Mitton *et al*., 2020).

In recent years, our research group has focused on the role of phytohormone abscisic acid (ABA), reporting favourable results on the health of honey bees at different levels (individual and colony), under different stress conditions (Negri *et al*., 2015; 2017; 2020). *In vitro*-reared bee larvae exposed to cold stress and ABA supplementation revealed that the phytochemical induced the expression of several immune-related genes, mainly those of the Toll and JAK/STAT pathways, nitric oxide synthase (Negri *et al*., 2020), and nutrition-related gen vitellogenin (Ramirez *et al*., 2017). In field conditions, Negri *et al*. (2015) evaluated the effects of ABA on the immune response of honey bees and the performance of small *A. mellifera* colonies throughout winter. Results demonstrated that ABA had an effect at the individual level by stimulating innate cellular and humoral responses, and at a colony level where the group supplemented with ABA resulted in an adult population of honey bees of approximately 70% larger than the control group after winter (Negri *et al*., 2015). Similarly, other studies have shown that the ABA dietary supplementation in honey bee colonies kept in Langstroth hives in combination with two different beekeeping nutritional strategies to confront overwintering, “honey management”, and “syrup management” had positive effects on the population dynamics. In such conditions, *A. mellifera* colonies reached stable levels of adults bees, increased brood during overwintering, and tended to prevent nosemosis at the colony level (prevalence) (Szawarski *et al*., 2019). The honey bee colonies supplemented with ABA showed that the level of parasitized individuals did not increase significantly in late winter, in contrast with the control, which increased significantly (Szawarski *et al*., 2019).

However, the effect of ABA on colonies of honey bees integrated into nuclei during winter has not yet been studied. The multiplication of honey bee colonies by means of nuclei comprising three to five frames with adults, brood, pollen, and honey reserves is a common practice followed by beekeepers to replace or increase the number of productive hives. Nevertheless, considering the small population of these colonies, beekeepers choose to produce them during the warmer seasons, especially during spring. In this sense, the growth and development of these colonies throughout the summer avoid possible losses due to the cold temperatures that characterize winter in temperate climates.

Based on our previous reports, the main goal of this study was to evaluate the effect of ABA supplementation on overwintering nuclei colonies, centred on the experimental design and the use of small bee colonies (nucleus), to measure different biological variables at colony and individual levels. Specifically, we were interested in the impact of ABA supplementation on: 1) the honey bee population dynamics of nuclei from late summer that has to survive winter (colony level); 2) *Nosema* spp. population dynamics (colony level); and 3) the nutrition- and immune-related gene expression (individual level). Accordingly, we hypothesize that ABA supplementation impacts positively colony strength, *Nosema* spp. dynamics, and expression of nutrition- and immune-related genes.

MATERIALS AND METHODS

Study location and biological material

The field trial was performed in the experimental apiary belonging to the Social Bee Research Centre (CIAS-IIPROSAM-UNMdP), at the Santa Paula farm (38°10'06” S, 57°38'10” W), Mar del Plata, Buenos Aires, Argentina. This study was carried out with a local honey bee ecotype (*A. mellifera carnica* x *A. mellifera ligustica*) (Giménez-Martínez *et al*., 2017). Queens came from a mother colony at “Mar y Sierras” beekeeping hut, at the Santa Paula farm, provided by Dr. Leonardo De Feudis. Experimental units (honey bee colonies) were assembled into a nucleus; each consisted of three brood frames, one reserve frame (with honey and pollen), a newly fertilized queen, and a large adult bee population. They were prepared between February and March 2017. A total of eight nuclei were used (4 per treatment) following the Langstroth dimensions associated with the production system. Before beginning the trial, an acaricide treatment based on oxalic acid (Aluen CAP®, Cooperativa Apícola Pampero, Bahía Blanca, Argentina) was applied to the honey bee colonies for varroosis control.

Phytochemical

(R,S)-abscisic acid (ABA) was purchased from Sigma-Aldrich® (USA). The stock of ABA was dissolved in absolute ethanol (≥ 99.5% V/V purity, Biopack®) for the preparation of mother solution (50 mM). The final concentration of ABA dissolved in syrup (2:1, sugar: water, v: v) was 50 µM. The concentration of phytochemical ABA was defined according to the concentrations found in honey, also reported as a driver of physiological effects on the health of honey bees (Ramirez *et al*., 2017; Szawarski *et al*., 2019).

Treatment

Each group consisted of four colonies integrated into a honey bee nucleus plus a Doolittle feeder. The control group was fed weekly with 500 ml of sugar syrup (2:1, sugar: water, v:v) and the ABA group was fed weekly with 50 µM ABA dissolved in 500 ml of sugar syrup (2:1, sugar: water, v:v). Both diets (control or ABA treatment) were supplied throughout the experiment (autumn-winter). The study was performed from May 11(first day of application of the control and phytochemical treatment, autumn) to September 19 (late winter) 2017.

Population dynamics and survival of honey bee colonies

As a parameter of the strength of colonies, the number of combs covered by adult honey bees (defined as "degree") was measured in each of these, according to the subjective method (Delaplane *et al*., 2013). The population degree of the honey bee colonies was used to estimate the population size of adult individuals. To measure this parameter, we quantified adult honey bees covering the frames. Therefore, this variable was defined as degree x = x comb covered by adult honey bees.

Quantification of spores of *Nosema* spp. by optical microscopy

Quantification of *Nosema* spp. was carried out according to Fries *et al*. (2013). Samples from forager bees were taken from every colony after each treatment: (a) T1 time-point (May 17), (b) T2 time-point (August 5), and (c) T3 time-point (September 19). The entrance of each hive was covered with rubber foam so that the returning forager honey bees from the flight could concentrate on entrance. For each hive, a representative group of approximately 100 individuals was sampled using a bottle with 70% ethanol. Forager bees were sampled as they showed high infection levels compared to those of younger bees. All samples were collected at noon to compare time-points between treatments (Meana *et al*., 2010). To determine the prevalence of *Nosema* spp. (number of infected honey bees/number of honey bees examined), 21 bees from each sample group (per hive) were used. Their abdomen was dissected and homogenized individually in 1 ml of distilled water. To determine the abundance of *Nosema* spp. (n° spores/bee), pools of 60 honey bees from each sample group (per hive) were used. Their abdomen was dissected, and with a mortar, they were homogenized in 60 ml of distilled water (in a ratio of 1 ml of water/abdomen). The spore count of *Nosema* spp. was performed using a Neubauer counting chamber (BOECO, Germany) and an optical microscope (LEYCA DM 500, Germany) (480X).

Molecular biology analysis

Sampling: For the determination of the gene expression related to the immune system of *A. mellifera*, samples of nurse bees were taken from the brood frames (n = 15) (Steinman *et al*., 2015) in a winter time-point (August 4). The sampling consisted of collecting nurses individually with forceps in a single bag (with zip closure, Ziploc® type), duly labelled for each colony, and stored with dry ice (~ -70°C). These were then transferred to a freezer at -80 °C until RNA extractions were started.

RNA extraction: RNA was extracted from pools of ten adult bees randomly selected from each sample, according to Reynaldi *et al*. (2010) with modifications (Brasesco *et al*., 2021). Bees were ground with a glass rod and homogenized with 3 ml of phosphate buffer saline free of RNases (1X PBS). The homogenate obtained from each sample was centrifuged for 15 min at 15,000 xg. Total RNA was extracted using 500 µl of Trizol® reagent (Invitrogen, Carlsbad, CA, USA) mixed with 500 µl of the supernatant. The mixture was extracted with 220 µl of cold chloroform. After centrifuging at 12,000 xg for 15 min, the RNA contained in the aqueous solution was precipitated by adding 750 µl of isopropanol. The precipitated RNA was collected by centrifugation at 12,000 xg for 15 min, washed with 70% ethanol, and dissolved in 50 µl of RNase-free water.

DNAse treatment and reverse transcription (RT-PCR): DNAse I Amplification Grade (Invitrogen, Carlsbad, CA, USA) was used to remove traces of

Table 1. Primer sequences, annealing temperatures (AT), melting temperatures (MT), and references.

Primers	Primers sequence (5'-3')	A T (°C)	M T (°C)	Reference
β-actin- Fw *β-actin-Rv*	ATGCCAACACTGTCCTTTCTGG GACCCACCAATCCATACGGA	60	83.2	Yang & Cox-Foster, 2005
domeless- Fw *domeless-Rv*	TTGTGCTCCTGAAAATGCTG AACCTCCAAATCGCTCTGTG	53	78.5	Evans *et al*., 2006
nos- Fw *nos-RV*	TCCACTCGCAGGTACTTTCC TCTGGAGGATCACCATTTCC	54	76.7	Gregorc *et al*., 2012
toll18w- Fw *toll18w-Rv*	CTCGGTAGCAATCCGTGGTC CAATCGCCGCCTGTAAGC	59	91.2	Negri *et al*., 2020
vitellogenin- Fw *vitellogenin-Rv*	AGTTCCGACCGACGACGA TTCCCTCCCACGGAGTCC	54	80.5	Corona *et al*., 2007

entrained genomic DNA in RNA extraction by digestion at 37 °C for 30 min. Complementary DNA (cDNA) was synthesized using a reaction mixture containing 1 µg of total RNA, random hexamers (12 ng / µl), and the reverse transcriptase enzyme from Moloney murine leukemia virus (Invitrogen, USA), following the manufacturer's suggested procedures. Negative controls were performed, omitting RNA or reverse transcriptase. Then the cDNA was stored in a freezer at -20 °C for use in qPCR amplifications.

Quantitative PCR Amplification (qPCR) and studied genes: The cycling programs for DNA amplifications consisted of an initial denaturation of 2 min at 95 °C and 45 cycles of 10 s at 95 °C, 15 s at the specific hybridization temperature and 15 s at 72 °C. All qPCR reactions were carried out in a Rotor-Gene 6000 thermal cycler (Qiagen, Hilden, Germany) in a final volume of 20 µl using EvaGreen as an intercalating fluorescent dye (KAPA FAST, Biosystems, Woburn, USA). All determinations were made with a primer concentration between 448 to 900 mM.

Table 1 summarizes the primers (synthesized by Eurofins MWG Operon, USA) used in the qPCRs of this study, with their respective specific annealing and melting temperatures. Amplifications were normalized by analysing the constitutively expressed β-actin of *A. mellifera* (Yang & Cox-Foster, 2005). Specific primers for β-actin amplification are found in Table 1. The cycle program consisted of an initial denaturation of 2 min at 95 °C and 40 cycles of 10 s at 95 °C, 15 s at 60 °C and 15 s at 72 °C. After amplification, a melting curve analysis was performed, which resulted in an individual product-specific melting curve.

Relative expression (RE) values were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Each of the four targets was analysed separately and normalized to *A. mellifera* β-actin. The amplification efficiency was determined for each gene using 10-fold dilutions of cDNA. In all assays, standard curves for each gene had a slope between -3.2 and -3.5, and the standard deviation between duplicates was in all cases <0.167, as recommended for qPCR (Bustin, 2002).

Statistical analysis

The effects of the treatments with ABA on the population degree of adult bees (number of combs covered by bees during overwinter) were evaluated with generalised linear mixed models (GLMM). We also assumed normal distribution of the variable "population grade" given the nature of the response variables and the structure of the sampling design (repeated measures of bees per colony) (Pinheiro & Bates, 2000; Gelman & Hill, 2006). The diet treatment, a categorical variable with two levels, and time of the year (before, during, and after winter) were included as fixed effects, and "colony" was included as the random effect.

Generalized linear models of mixed-effects were used to evaluate the effects of the treatments on the abundance and prevalence of *Nosema* spp. in autumn (May 17), winter (August 5), and late winter (September 19), given the nature of the response variables and the structure of the sampling design (repeated measures of bees per colony) (Pinheiro & Bates, 2000; Gelman & Hill, 2006). Since the response variables for the analysis of the abundance of *Nosema* spp. follows a discrete nature (number of spores per bee), the model assumed a Poisson distribution. As the response variable for the prevalence analysis of *Nosema* spp. follows a distribution according to the Bernoulli test (infected versus uninfected bees), the model assumed a binomial distribution with logarithmic function. The diet treatment and its interaction with the time of the year (before, during, and after winter) were included as fixed

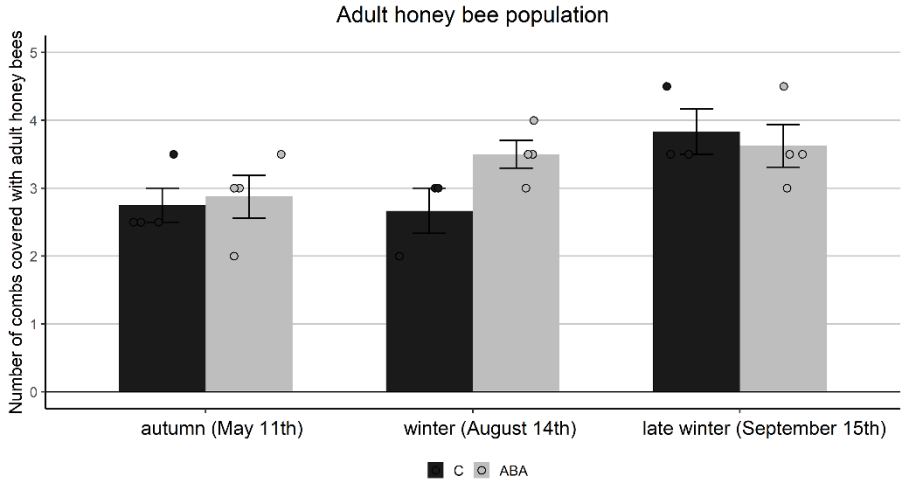

Figure 1. Adult honey bee population in nuclei with and without supplement of 50 µM ABA. The results indicate that no significant differences were found between the control group and the treatment with ABA over time (GLMM: F=0.976, p=0.336). Dots represent data points. Thin bars indicate +/- standard deviation (SD).

effects and "colony" as the random effect. Data analysis was carried out using the lmer function of the lme4 package (Bates *et al*., 2015). A post-hoc test was then anperformed using the Tukey test for multiple comparisons of means using the LSMean function of the package LSMeans (Lenth, 2017) of R software 4.0.0 (Team, 2016).

The effects of the treatments on gene expression levels were evaluated on the quantification time corresponding to the T2 sampling (August 4, winter period). T-test was applied to the data set to each gene that passed the normality test (Shapiro-Wilks test) and homogeneity of variances (Levene's test). R software 4.0.0 (Team, 2016) was used for the analyses.

RESULTS

Effects of the supplementary diet with ABA on the population dynamics of honey bee colonies

There were no significant differences between ABA and the control treatment in each time-point analysed (autumn, winter, and late winter) (GLMM: F=0.976, p=0.336) regarding the effects of phytochemical supplementation on the development of *A. mellifera* colonies during overwintering (Fig. 1). In autumn (May 11), at the beginning of the experiment, colonies of both treatments (C and ABA) had equivalent initial levels in the adult bee population (C=2.75; ABA=2.875, mean

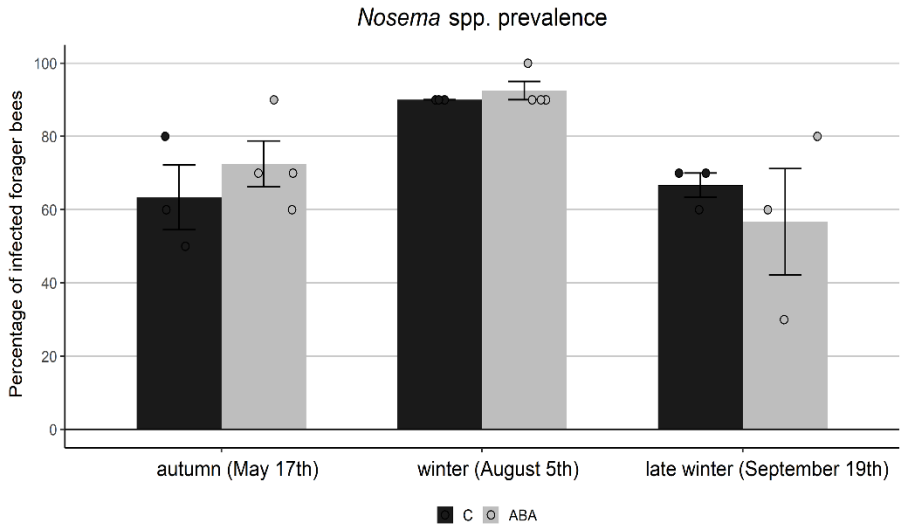

Figure 2. Changes in the prevalence of *Nosema* spp. during overwintering. The percentage of parasitized forager honey bees during overwinter did not vary significantly between the control and ABA treatments (GLMM: F=0.134, p=0.719). Dots represent data points. Thin bars indicate +/- standard deviation (SD).

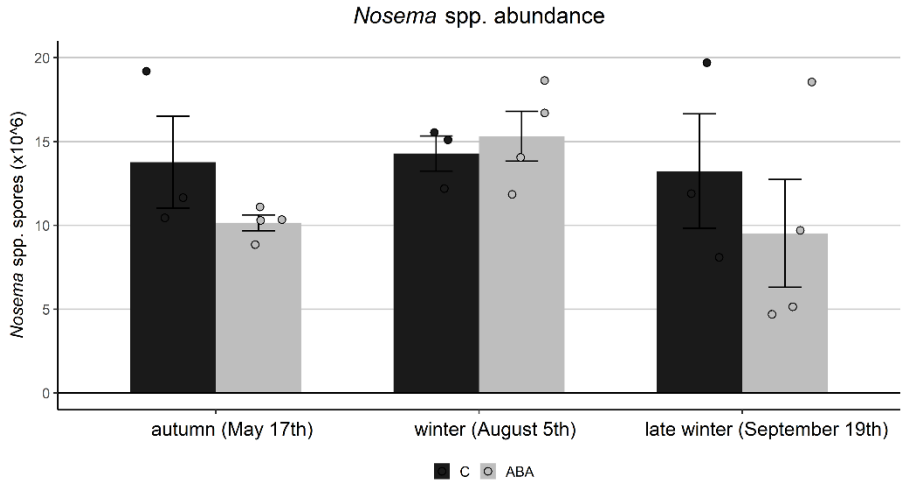

Figure 3. *Nosema* spp. spore abundance of forager worker bees during overwintering. The abundance of *Nosema* spp. during overwinter did not vary significantly between the control and ABA treatments (GLMM: F=0.97, p=0.36). Dots represent data points. Thin bars indicate +/- standard deviation (SD).

value of combs covered by adult bees or "ccab"). At the time-point corresponding to winter (August 14), the levels in adult bees for groups supplemented with ABA (3.5 ccab) increased their population compared to that of the control (2.67 ccab). However, these were not significant. At the last time-point corresponding to the late winter (September 15), the population levels of the control group were balanced with the colonies of the ABA group, resulting in 3.83 (C) and 3.63 (ABA) combs covered with adult bees, respectively.

The effects of ABA supplementation on *Nosema* spp. levels

The prevalence (percentage of bees parasitized) of *Nosema* spp. during overwinter did not vary significantly between ABA treatment and control group (C) (GLMM: F=0.134, p=0.719) (Fig. 2). The dynamics of *Nosema* spp. between autumn and winter behaved similarly in both treatments. We found an increase in the prevalence between autumn (May 17) and winter (August 5), and then towards late winter (September 19), decreasing again in both treatments.

The abundance of spores of *Nosema* spp. (n° spore/bee, from a pool of 60 individuals per colony) during the assay did not vary significantly between ABA treatment and the control group (C) (GLMM: F=0.97, p=0.36) (Fig. 3). For each diet treatment (C or ABA), spore loads per bee increased between autumn (May 17) and winter (August 5); then decreased towards late winter (September 19).

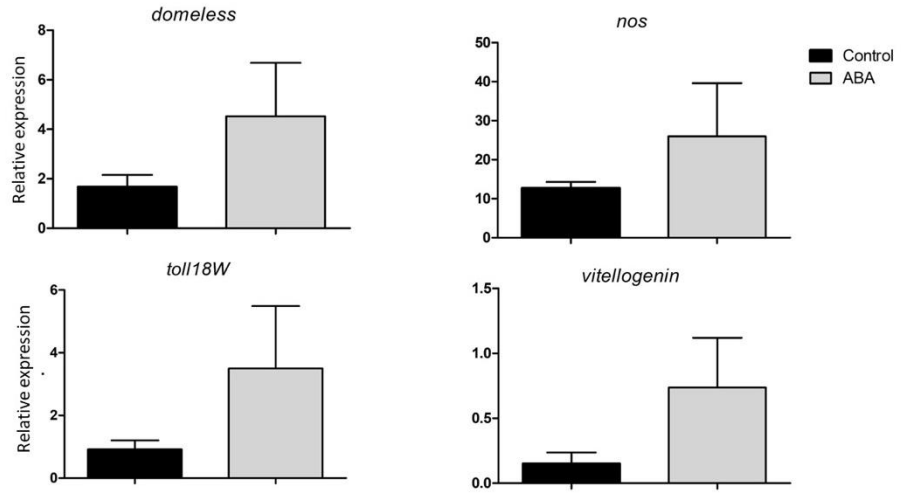

Figure 4. Nutrition- and immune-related gene expression of adult honey bees responding to supplementation (with and without 50 µM ABA) during winter. The relative expression (RE) of the genes was studied in the sampling associated with winter (August 4) in nurse honey bees (n=10). The RE analysis for all genes showed no significant effect when comparing ABA treatment with control (domeless T-test, p=0.269; nos T-test, p=0.45; toll18W T-test, p=0.325 and vitellogenin T-test, p=0.212). Thin bars indicate + standard deviation (SD).

Effects of ABA dietary supplementation on the expression of nutrition- and immune-related genes in adult honey bees during winter

Gene expression analyses in nurse bees, calculated as relative expression (RE), involved three immune system genes (*domeless*, *nos*, *toll18W*) and a nutritional response gene (*Vitellogenin)*. All showed increased levels compared to the control when the nucleus of honey bees was fed with ABA in winter (T2=August 4). However, this increase in ABA treatment was not significant to the control group (*domeless* T-test, t=-1.283, p=0.269; *nos* T-test, t=-0.818, p=0.45*; toll18W* T-test, t=-1.092, p=0.325 and *vitellogenin* (Vg) T-test, t=-1.483, p=0.212) (Fig. 4).

DISCUSSION

In this study, we evaluated the effect of ABA supplementation on the overwintering of *A. mellifera* nuclei. ABA application (50 µM) matched with the range of concentrations previously reported in nectar, honey, and honey bees (Tuberoso *et al*., 2010; Negri *et al.*, 2015; Ramirez *et al*., 2017). In addition, this concentration has been shown to stimulate the immune response of bee larvae reared in the laboratory against different stress conditions (Ramirez *et al*., 2017; Negri *et al.*, 2017; 2020).

ABA supplementation did not impact the population level of the treated colonies during overwintering. However, positive tendencies could be seen in the time-point corresponding to winter (August 14) for colonies supplemented with ABA. This treatment maintained higher adult bee levels in treated colonies than in the control group, where the number of combs covered with adult bees (ccab) was 3.5 and 2.67, respectively. According to Delaplane *et al*. (2013), these values represent 8,500 and 6,480 adult bees for ABA and control groups, respectively. By the end of the winter (September 15), both treatments reached a similar adult bee population size (Fig. 1). This final equality between the groups could be attributed to sporadic fluctuations in climate and blooming of some plant species in the area of study, impacting the population dynamics of honey bee colonies (Döke *et al*., 2015). Although blooming was not measured in this study, it could be hypothesized that a high availability of floral resources at the end of winter promoted colony growth levelling between groups.

In a previous study by our research group (Szawarski *et al*., 2019) carried out at Miramar, another area of the province of Buenos Aires, results showed a positive effect of ABA on both nutritional managements evaluated (with syrup or honey), observing higher population levels of adult bees at the end of winter than those in their respective controls. This dynamic is different from that seen in this experiment, where the population levels of the control group were balanced with the colonies of the ABA group during a similar season (September 15). In addition, this could be due to differences in the seasonal dynamics of the floral resources present between the environments of both experiments. Although flowering curves were not taken into account in either of the two assays, the apiary located in Miramar had specimens of the plant species *Myoporum laetum* in its surroundings. This plant species is characterized by being nectariferous and its flowering begins at the end of winter. Perhaps the contribution of its nectar stimulates the development of the colonies associated with this experimental apiary. However, to adequately answer these questions, we should study and define in future experiments the corresponding flowering curves of each of these environments. In addition, another factor that could have influenced may be linked to the differences between the population size of the honey bee colonies of both experiments. As previously mentioned, the colonies in this study were assembled into a nucleus with smaller bee colonies, than the honey bee colonies in the study of Szawarski *et al*. (2019) (average of 3 vs 7 combs covered by adult bees, respectively). In this context, a nucleus with low population levels, given the supply of floral resources from the environment, could fastly reach similar population levels between both treatments. In view of the results reported by Negri *et al*. (2015), we also showed in this experiment that colonies supplemented with ABA maintain the population levels of adult bees after winter.

Regarding the effect of ABA on the levels of *Nosema* spp., we selected two parameters to study the parasite dynamics: prevalence and abundance. In agreement with previous studies (Higes *et al*., 2008; Botías *et al*., 2013; Retschnig *et al*., 2017), our results showed that, during winter, there was an increase in parasitized bee levels and spore load of *Nosema* spp. This increment could be seen in colonies from both treatments (with and without ABA). Therefore, this field experiment would show no direct effect of ABA on the percentage of parasitized bees and on the development of *Nosema* spp. spores. Unlike our previous research (Szawarski *et al*., 2019) where we had only studied the dynamics of nosemosis in two time-points, autumn and late winter, in the current experiment an extra intermediate time-point was included, over the winter,

in which the highest levels of the prevalence and abundance of *Nosema* spp. were obtained in both treatments. However, as reported in Bernklau *et al*. (2019), where some phytochemicals are shown to promote worker bee longevity and *Nosema* tolerance, future laboratory assays should be required to possibly determine similar effects of ABA on *Nosema* parasitized honey bees. In addition, during the whole assay period, high values of prevalence of nosemosis were found, which could be a variable acting on the results obtained. In future assays we should consider, before conducting the experiment, standardizing all bee colonies according to prevalence levels of parasitosis.

The studied genes were associated with the immune system of *A. mellifera*. Some are markers of the immune pathways that respond to a diversity of risk factors: *toll18W* gene is associated with Toll pathways, and *domeless* gene is associated with the JAK / STAT pathway (Evans *et al*., 2006); *nos* gene (Gregorc *et al*., 2012) regulates the synthesis of the enzyme nitric oxide synthase, associated with the production of nitric oxide (NO), a molecule that acts in signalling pathways and in cytotoxic mechanisms against parasites and pathogens (Rivero, 2006); and *vitellogenin* gene (Vg) (Corona *et al*., 2007) regulates the synthesis of the lipoprotein vitellogenin, usually used as a biomarker of the nutritional status of individuals (Negri *et al*., 2019). Concerning the expression of nutrition- and immune-related genes of *A. mellifera* (Evans *et al*., 2006; Negri *et al*., 2019, 2020), none of the studied genes (*domeless*, *nos*, *toll18W*, and *vitellogenin*) showed significant differences between the control group and the ABA treatment group in the time-point associated with winter (August 4). The lack of statistical differences between ABA and control group was probably related to the variability of the data and the low number of replications for each treatment (N = 4 of ABA, N = 3 of control, where N = honey bee nucleus). In addition, another factor that could have masked the effects of the genes studied is the age of the nurse bees sampled, since the individuals were not of the same age, which could have influenced on the variability of the data.

However, the four genes studied tended to increase gene expression levels in the ABA treatment compared to the control group. The tendency in the results of the *toll18W* gene, which encodes the Toll immune pathway receptor (Evans *et al*., 2006), correlates with that reported by Negri *et al.* (2020), where they demonstrated that bee larvae reared *in vitro* and supplied with ABA show higher expression of the *toll18W* receptor. This suggests that the ABA could have a central role as an inducer molecule of the Toll immune pathway in honey bee larvae. In addition, Negri *et al.* (2020) also demonstrated that the expression of the gene encoding for nitric oxide synthase (*nos*), the enzyme responsible for NO synthesis, is induced by the ingestion of ABA in *A. mellifera* larvae. This is supported by previous reports where it has been demonstrated that both ABA and NO were involved in the immune response of honey bees (Negri *et al*., 2015, 2017). Regarding gene *domeless*, a key component of the JAK/STAT pathway (Evans *et al*., 2006), the results of Negri *et al*. (2020) demonstrated that larvae only fed with ABA tended to increase the level of expression of this gene. Yet, when larvae were exposed to cold stress, both groups, with and without ABA, showed higher significant inductions of the *domeless* gene.

Another gene that tended to increase its expression level between the ABA treatment and the control was *vitellogenin* (*Vg*), related to stress responses (Münch & Amdam, 2010; Ramirez *et al*., 2017) and nutritionally regulated functions (Negri *et al*., 2019). The lipoprotein Vitellogenin is also thought to play a central role in the extended lifespan of the winter honey bees (Münch & Amdam, 2010). In Ramirez *et al*. (2017), supplementation with ABA in honey bee larvae reared *in vitro* and exposed to cold stress led to a more significant induction of *Vg* expression, which could be correlated with the tendencies seen in our study.

CONCLUSIONS

The results showed no significant differences after administering ABA on the population levels of adult bees, *Nosema* spp. levels and on the expression levels of the genes studied in a key period of the survival of the honey bee colonies. However, taking into account the positive tendencies observed in the variables measured, more studies should be conducted on this topic, with more colonies per group and measuring the blooming period and plant species involved.

DISCLOSURE STATEMENT

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