Supering Apis mellifera (Hymenoptera, Apidae) beehives impairs honey production and biomarker genes

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Abstract: For honey production, beekeepers add one or more supers to the hives to allow honeybees to store their products. However, the increase in hive space can affect the social and health organization in the colony, promoting stress. This study assessed the management of honey production, physicochemical honey properties, population development, and forages immune system gene expression patterns to be used as biomarker for monitoring beekeeping welfare. The treatments comprised 40 beehives divided in four treatments. Treatment 1 - control, supers added according to storage necessity. Treatments 2, 3, and 4 presented two, three, and four supers at the beginning of the experiment, respectively. T1 presented greater honey production (39.4 % increased). No difference in open brood area in the colonies was observed and honey properties and only T2 showed closed brood area higher than the other treatments. Foragers from T4 showed higher catalase and defensin gene expression at the middle-end experiment. Thus, the increasing internal space at the beginning of honey season can affect honey production and immune system of foragers. Catalase and defensin can be used as biomarkers for monitoring honey production welfare.

Keywords: apiculture, gene expression, welfare

Introduction

Honey production in beekeeping requires the addition of one to several supers to the beehive, where bees can store and process nectar collected from flowers, which becomes honey (Delaplane, 1997). However, this increase in hive volume affects the social and health organization of the colony (Kumsa and Takele, 2014). In recent years, public concern about production practices in livestock has increase. Concerns with livestock welfare are a current topic for almost all animal production systems (Bertocchi et al., 2018; Rochais et al., 2018; Bailie et al., 2018; Hempstead et al., 2018). According to Fraser et al. (1997), animal welfare has three dimensions: animal functioning, animal feelings, and animal welfare.

Honey demand has been growing worldwide (FAO, 2016); therefore, beekeeping has focused on bee product production. Consumers appreciate relatively inexpensive and safe food supply; however, size and scale of modern operations may compromise the environment and animal welfare (Boogaard et al., 2011). Various physiological parameters have been used as welfare indicators in livestock production, such as cortisol levels (Carroll et al., 2018). However, there are no well-established biological indicators for measuring welfare in honey bees. Thus, finding ways to determine how beekeeping practices affect bee welfare is crucial. Whole-genome sequencing of Apis mellifera (Hymenoptera, Apidae) has enabled a wide range of studies on molecular genetics (The Honey Bee Genome Sequencing Consortium, 2006). The Real Time PCR has been widely used in gene expression studies of A. mellifera honey bees (Hagai et al., 2007; Mustard et al., 2010; Fang et al., 2012; Lichtenstein et al., 2018) and thus could be a useful tool to find biomarkers for monitoring bee welfare.

In this sense, we hypothesized that increasing internal space in hives by adding supers during the honey production season could affect colony health and consequently reduce honey production for A. mellifera honeybees. We posed the following questions: Does an increase in internal hive space during the honey production season affect [i] honey production and population development, [ii] honey physicochemical properties (total acidity, pH, moisture and ash), and [iii] candidate genes for stress measurement (catalase, defensin, and ERP60)?

Materials and Methods

Field experiment, population growth and honey production

The study comprised 40 Apis mellifera (Hymenoptera: Apidae) beehives, housed in standard single deep Langstroth hives, and distributed randomly into treatment groups, with 10 colonies for each treatment. Treatment T.1 control received one standard shallow super (13.5 cm height) at the beginning of the experiment and more supers gradually throughout the blossom period under the last super added, as combs in the supers became filled with honey. Treatments T.2, T.3 and T.4 received two, three, or four supers at the beginning of the experiment, respectively, over the brood chamber. All shallow super frames were prepared...
with new beeswax sheets for honey production at the beginning of the season.

Fifteen days before the beginning of the honey flow, the number of brood and food frames was equalized in all the beehives, totaling seven brood frames and three food frames. All colonies were kept in an experimental apiary [22°49’15” S, 48°23’24” W, altitude of 488.39 m] in the middle of a secondary forest, and the apiary produced wildflower honey during the main honey flow from December to March.

The climatic data for the period of the study were as follows: lowest temperature 18.9 ± 1.64 °C, highest temperature 28.7 ± 3.06 °C, mean temperature 24.7 ± 2.60 °C, precipitation 12.3 ± 29.7 mm, relative humidity 53.3 ± 16.2 %, and mean daily wind speed 0.86 ± 0.36 km.

Population growth was measured in the beehives monthly throughout the experimental period, including open brood area (O.B.A.) [larvae] and sealed brood area (S.B.A.) [cm²] in the central frame of the nest using the methods in Lomele et al. [2010].

Honey was harvested at the end of the blooming period, weighed for each beehive, and processed in stainless steel equipment. Honey samples (500 g) were taken for the following physicochemical analyses: total acidity (mEq kg⁻¹), pH, moisture percentage, and ash percentage [Sodré et al., 2007].

Gene expression

Candidate biomarkers were investigated by harvesting adult worker bees at days 0, 7, 14, 29, 56, and 98. Foragers are bees directly involved in honey production once they collect nectar for honey production and are most exposed to the environment; thus, we collected five returning foragers from the entrance of each hive for the analyses. The bees were immediately stored at −80 °C for later RNA extraction.

For RNA extraction, the head of each worker bee was separated from the body with a disposable scalpel [Scharlaken et al., 2008]. Their brains were dissected at 4 °C under a stereomicroscope and processed immediately for RNA extraction (Bonnafé et al., 2015). Each sample consisted of a pool of five brains. RNA extraction was performed by using the TRizol method, with 500 µL of TRizol (GIBCO BRL) for each sample to disrupt the cells and release their contents following the manufacturer’s instructions. The product extracted was visualized on a 1 % agarose gel and quantified using a NanoDrop instrument [ND-1000 Spectrophotometer]. Thereafter, all samples were stored at −80 °C until further analyses.

We used 1000 ng of each RNA extracted treated with DNase for the cDNA synthesis reaction: 0.75 mM of a mix of oligoT solution [nucleotides = 18]; random oligonucleotides (n = 8) 0.15 mM; 0.75 mM dNTP and 11 µL of RNA treated with DNase in the previous step and then was prepared and incubated at 65 °C for 5 min and then placed on ice for 1 min. To prepare this solution, we added 0.50 mM DTT, 40 U of RNase, and 100 U of Super Script III. The reaction was then incubated at 50 °C for 1 h and then at 70 °C for 15 min.

As candidate biomarkers, we analyzed changes in the patterns of catalase, defensin, and ERP60 gene expression [Scharlaken et al., 2008], all involved in oxidative stress. The actin gene was used as an internal control for the quantitative PCR reactions [Scharlaken et al., 2008].

Gene expression was determined by the Real Time PCR in triplicate, on a Real Time ABI 7300 instrument [Applied Biosystems] using the SYBR Green PCR Master Mix kit under the following conditions: one cycle at 50 °C for 2 min, another cycle at 94 °C for 10 min, followed by 40 cycles at 94 °C for 15 sec, and 60 °C for 1 min. The melting curve was obtained as follows: 95 °C for 15 sec, 60 °C for 30 sec, and 95 °C for 15 sec.

The oligonucleotides sequences used and details are shown in Table 1.

Relative quantification (R) was determined according to the Pfaffl [2001] formula. The relative quantification of stress genes data was calculated using the first day as a control for the 7, 14, 29, 56, and 98 crop days of the experiment.

Statistical analysis

The results of honey production, gene expression, population growth and physicochemical honey

| Gene | Gene Bank Number Accession | Oligonucleotides sequences 5’-3’ | Amplification | Ta °C |
|------|---------------------------|---------------------------------|---------------|------|
| actin| AB023025| TGCCAACACTGTCCTTTCTGAGAATTGACCCACCAATCCA| 155 | 61 |
| catalase| XM_003699011.1| CCTGGTATTGGAGCAAGTCCGGCCATCACGTTGGTAGTTT| 154 | 61 |
| defensin| U15955.1| GTCGGCCTTCTCTTCATGGTGACCTCCAGCTTTACCCAAA| 200 | 61 |
| ERP60| XM_623279| ACTCTTGCTAAAGTTGATTTGATTTCTGTAGATGCTGGACCAAACCTGTG| 178 | 61 |

ªoptimal annealing temperature specific to each oligonucleotide.
properties were tested first for normality (Anderson-Darling test) and homogeneous variance (Levene’s test). If significant deviations were detected ($p < 0.05$), the data were compared by nonparametric Mann-Whitney tests and presented as the median and interquartile intervals ($Q1$, $Q3$). If no significant deviations in normality and homoscedasticity were detected, the data was analyzed with the Student’s $t$ test. A $p$ value lower than 0.05 was considered significant. Data analyses were performed using Minitab statistical software (v. 17).

**Results**

Colonies in the control treatment showed greater honey production ($p = 0.012$) than the other treatments (Figure 1). No significant difference ($p = 0.309$) in open brood area in the colony was observed in the different treatments during the experimental period ($199.0 \pm 26.3, 193.0 \pm 27.3, 173.5 \pm 16.8, 176.5 \pm 28.0 \text{ cm}^2$, means ± standard deviation) for treatments T.1, T.2, T.3, and T.4, respectively. However, when closed brood area was analyzed, T.2 was different ($p = 0.021$) from the other treatments ($596.0 \pm 196.6, 1176.0 \pm 371.6, 553.5 \pm 258.2, 695.0 \pm 188.7 \text{ cm}^2$, means ± standard deviation) to treatments T.1, T.2, T.3 and T.4, respectively).

Physicochemical honey properties were shown in Table 2. Total acidity did not differ significantly among the treatments ($p = 0.2438$) ($17.6 \pm 3.8, 19.2 \pm 4.1, 16.8 \pm 1.1$ and $20.4 \pm 0.9 \text{ mEq kg}^{-1}$, means ± standard deviation to treatments T.1, T.2, T.3, and T.4, respectively). The pH did not differ significantly among the treatments ($p = 0.8761$) ($3.7 \pm 0.1, 3.7 \pm 0.1, 3.7 \pm 0.2$, and $3.6 \pm 0.2$, means ± standard deviation to treatments T.1, T.2, T.3, and T.4, respectively). The moisture did not differ significantly among the treatments ($p = 0.5219$) ($22.7 \pm 1.1, 22.3 \pm 0.5, 22.5 \pm 0.6$, and $23.1 \pm 1.1 \%$, means ± standard deviation to treatments T.1, T.2, T.3 and T.4, respectively). Ash did not differ significantly among the treatments ($p = 0.5244$) ($0.13 \pm 0.04, 0.16 \pm 0.05, 0.15 \pm 0.01$, and $0.13 \pm 0.04 \%$, means ± standard deviation to treatments T.1, T.2, T.3 and T.4, respectively).

The results of relative quantification ($R$) for catalase, defensin, and ERP60 stress genes, using actin as the endogenous gene in T.1, T.2, T.3, and T.4 are presented in Figures 2, 3, and 4. Catalase gene in forager bees in colonies managed for honey production under different supering regimes. The Y axis shows relative expression and the x axis shows the days of the experiment. T.1 = one standard shallow super added at the beginning of the honey flow, with other supers added as needed. T.2, T.3, and T.4 indicate 2, 3, or 4 supers added at once at the beginning of the honey flow. Different letters above the bars indicate significant differences between treatments (Student’s $t$ test; $p < 0.05$).

**Table 2** – Mean and standard deviation of physicochemical analysis of honey parameters in Africanized honeybees managed under different methods.

| Physicochemical analysis | T1          | T2          | T3          | T4          | $p$ value |
|-------------------------|-------------|-------------|-------------|-------------|-----------|
| Total Acidity ($\text{mEq kg}^{-1}$) | $17.6 \pm 3.8$ | $19.2 \pm 4.1$ | $16.8 \pm 1.1$ | $20.4 \pm 0.9$ | 0.2438    |
| pH                      | $3.7 \pm 0.1$ | $3.7 \pm 0.1$ | $3.7 \pm 0.2$ | $3.6 \pm 0.2$ | 0.8761    |
| Moisture (%)            | $22.7 \pm 1.1$ | $22.3 \pm 0.5$ | $22.5 \pm 0.6$ | $23.1 \pm 1.1$ | 0.5219    |
| Ash (%)                 | $0.13 \pm 0.04$ | $0.16 \pm 0.05$ | $0.15 \pm 0.01$ | $0.13 \pm 0.04$ | 0.5244    |

$T1$ = control, one super at the beginning of the experiment and more supers added gradually over the flowering period; $T2$, $T3$, and $T4$ = two, three, and four supers on the brood chamber at the beginning of the honey flow.
bees over expressed significantly in T.4 at experimental days 29, 56, and 98 [Figure 2]. **Defensin** gene expression over expressed in T.4 at experimental days 56 and 98 [Figure 3]. **ERP60** gene expression did not differ significantly among the treatment groups [Figure 4].

**Discussion**

Honey production management usually consists of adding supers for honey production and storage. The number of supers added vary according to the amount of equipment available and the management scheme adopted by the beekeeper [Kumsa and Takele, 2014]. Compared to adding a single super initially and then subsequent addition of others, we found that adding two or more supers at the beginning of the honey flow negatively affected honey production. In T.1, supers were added according to the storage necessity of the beehive, which yielded the highest honey production. We added 2.8 ± 0.42 supers during the honey flow, providing 10.5 kg to super honey storage. We found that honey production was increased 39.4 % (comparing T.1 and T.3 honey production) when supers were added according to need. Neupane et al. [2012] show that strong colonies produce more honey during the blossom period, which explains our results. The addition of fewer supers initially probably did not affect the colony strength.

However, this interference was not observed for population growth, except for T.2 in S.B.A (sealed brood area). Similarly, supering the bees did not affect honey physicochemical quality. All the physicochemical analyses, except for moisture percentage, are in accordance with European Regulation of Quality [European Union, 2002]. The honey harvest management of the experiment caused the highest moisture. We harvested all honey in the supers, capped and uncapped, for honey production data. Hive supering did not affect brood area; nevertheless, honey production was affected by internal space allocations.

We analyzed gene expression related to the immune system as a factor to measure the stress impact caused by honey production methods. The gene **catalase** was overexpressed after the middle part of the honey flow (days 29, 56, and 98) in T.4 than the other treatments [comparing T.1 to T.4 results, 0.2525, 0.433, 0.5474, and 1.239, 2.668, 2.886, respectively] [Figure 2]. Thus, **catalase** may be used as an indicator of increase of β-oxidation of fatty acids that produces hydrogen peroxide involved in oxidative stress [Boncristiani et al., 2012]. Supering of bees tripled hive space by adding supers at the beginning of the honey flow and increased **catalase** gene expression in foraging bees, which are more stressed, spending more energy from β-oxidation of fatty acids. **Catalase** is directly involved in the degradation of superoxide radicals and \( \text{H}_2\text{O}_2 \). Reactive Oxygen Species (ROS) are constantly generated as by-products of aerobic metabolism. Oxidative damage to cellular components induced by ROS is a major cause of degenerative diseases and ageing [Corona and Robinson, 2006] and can affect directly honey production [Figure 1].

Figure 1 shows that supering the beehive can significantly affect honey production. Thus, the need for studies on the increase of productivity, coupled with animal welfare, is evident and has been the subject of recent research in various livestock production systems [Nardone et al., 2010; Haldar et al., 2011], but not for beekeeping production. **Defensin** expression was examined because its expression changes whenever honey bees experience stress that affects their immune
system. Defensin is a cysteine-rich cationic antimicrobial peptide that acts against a variety of microorganisms and constitutes the primary defense system of most organisms (Raj and Dentino, 2002). Defensin can be produced, upon infection or injury, in body fat or hemocytes and secreted subsequently into the hemolymph (Yang and Cox-Foster, 2005).

Foraging bees from T.4 presented higher ($p < 0.05$) expression of defensin compared to other treatments at the end of the honey flow (days 56 and 98). The fact that foragers from T.4 increased expression of defensin may be related to lowered honey production, compared with the other treatments.

The relation between honey production and defensin and catalase genes high patterns of expression in T.4 at the end of the honey flow can be related to the end of floral source and the exhaustive nectar collection work during the season by foragers to fill all supers with honey. This extreme biological situation could affect directly the immune system and β-oxidation of fatty acids of foragers, altering the expression patterns of these genes. Extreme managements in animal production that affect natural biological patterns in livestock production animals have been widely reported (Jongman et al., 2017; Taylor et al., 2018; Brigida et al., 2018).

We also studied expression of ERP60 gene, involved in general processes in the A. mellifera for stress response. This gene encodes proteins that show similarity to proteins of the disulfuram isomerase family. In Drosophila, the ERP60 gene is similar to that found in humans. In other species, it is involved in stress response and encodes a protein that takes part in oxidative protein folding (Koivunen et al., 1996). In our study, the ERP60 gene expression showed no difference ($p = 0.7959$) among the four treatments evaluated during the experimental period.

**Conclusion**

We conclude that honey production increases when supers are added according to storage necessity, compared to the addition of two, three, or four supers at the beginning of the honey flow period. Greater internal space in the beehives increased expression patterns of defensin and catalase genes in foragers at the end of the honey production season, without interfering in colony population development or physicochemical honey parameters. Thus, we recommend these genes as useful biomarkers to monitor bee welfare in beekeeping.

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**Author’s Contributions**

Conceptualization: Kadri, S.M.; Orsi, R.O. Data analysis: Kadri, S.M.; Orsi, R.O.; Alonso, D.; Ribolla, P.E.M.; De Jong, D. Design methodology: Kadri, S.M.; Orsi, R.O. Writing and editing: Kadri, S.M.; Orsi, R.O.; Alonso, D.; Ribolla, P.E.M.; De Jong, D.

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