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

Samir Moura Kadri[1], Paulo Eduardo Martins Ribolla[2], Diego Peres Alonso[2], David de Jong[3], Ricardo de Oliveira Orsi[1]*

[1]Universidade Estadual Paulista "Júlio de Mesquita Filho"/ FMVZ – Núcleo de Ensino, Ciência e Tecnologia em Apicultura Racional – Depto. de Produção Animal e Medicina Veterinária Preventiva, R. Prof. Dr. Valter Maurício Corrêa s/n – 18618-681 – Botucatu, SP – Brasil.

[2]Universidade Estadual Paulista "Júlio de Mesquita Filho"/ IBTEC – Alameda das Tecomarias s/n – 18607-440 – Botucatu, SP – Brasil.

[3]Universidade de São Paulo/FMRP – Depto. de Genética, Av. Bandeirantes, 3900 – 14049-900 – Ribeirão Preto, SP – Brasil.

*Corresponding author <ricardo.orsi@unesp.br>

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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 $\pm$ 1.64 °C, highest temperature 28.7 $\pm$ 3.06 °C, mean temperature 24.7 $\pm$ 2.60 °C, precipitation 12.3 $\pm$ 29.7 mm, relative humidity 53.3 $\pm$ 16.2 %, and mean daily wind speed 0.86 $\pm$ 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 oligodT 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

Table 1 – Oligonucleotide sequences and details.

Gene	Gene Bank Number Accession	Oligonucleotides sequences 5'-3'	Amplification	Ta
			pb	°Cᵃ
actin	AB023025	TGCCAACACTGTCCTTTCTG AGAATTGACCCACCAATCCA	155	61
catalase	XM_003699011.1	CCTGGTATTGGAGCAAGTCC GGCCATCACGTTGGTAGTTT	154	61
defensin	U15955.1	GTCGGCCTTCTCTTCATGG TGACCTCCAGCTTTACCCAAA	200	61
ERP60	XM_623279	ACTCTTGCTAAAGTTGATTGTACAG TAGATGCTGGACCAACTTGTG	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 cm², means $\pm$ 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 cm², means $\pm$ 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 mEq kg⁻¹, means $\pm$ 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 $\pm$ 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 $\pm$ 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 $\pm$ 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
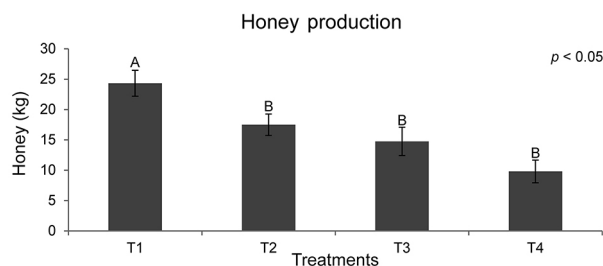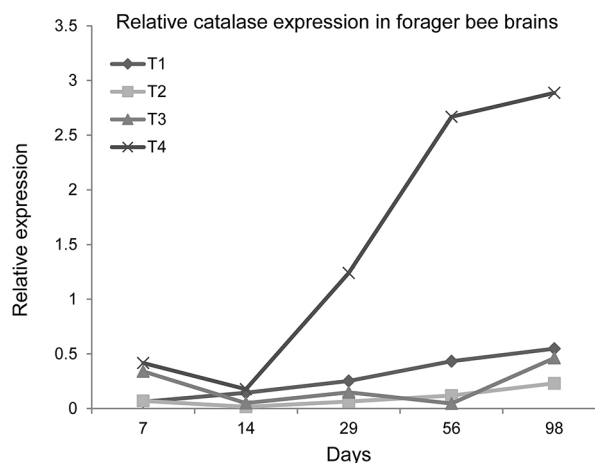

Figure 1 – Honey production (kg) by Africanized honey bee colonies managed for honey production with different supering regimes. The Y axis shows honey production (kg) and the x axis shows the treatments. T1 = one standard shallow super added at the beginning of the honey flow, with other supers added as needed. T2, T3, and T4 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; $p < 0.05$).

Figure 2 – Relative *catalase* gene expression in foraging honey 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. T1 = one standard shallow super added at the beginning of the honey flow, with other supers added as needed. T2, T3, and T4 indicate 2, 3, or 4 supers added at once at the beginning of the honey flow.

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 (mEq kg⁻¹)	17.6 ± 3.8	19.2 ± 4.1	16.8 ± 1.1	20.4 ± 0.9	0.2438
pH	3.7 ± 0.1	3.7 ± 0.1	3.7 ± 0.2	3.6 ± 0.2	0.8761
Moisture (%)	22.7 ± 1.1	22.3 ± 0.5	22.5 ± 0.6	23.1 ± 1.1	0.5219
Ash (%)	0.13 ± 0.04	0.16 ± 0.05	0.15 ± 0.01	0.13 ± 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.
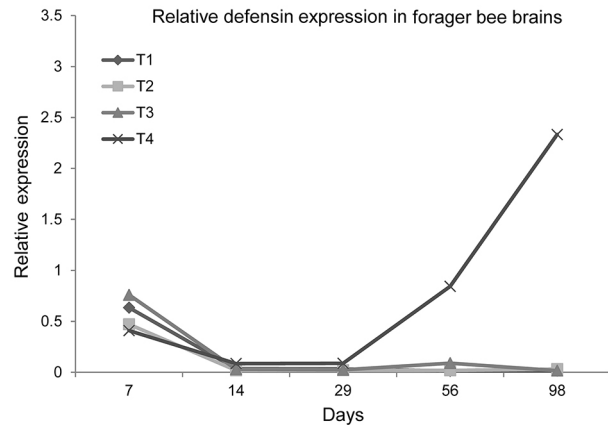
Figure 3 – Relative *defensin* gene expression in foraging honey bees in colonies managed for honey production under different supering regimes. The Y axis shows relative expression and the x axis shows the days of the experiment. T1 = one standard shallow super added at the beginning of the honey flow, with other supers added as needed. T2, T3, and T4 indicate 2, 3, or 4 supers added at once at the beginning of the honey flow.
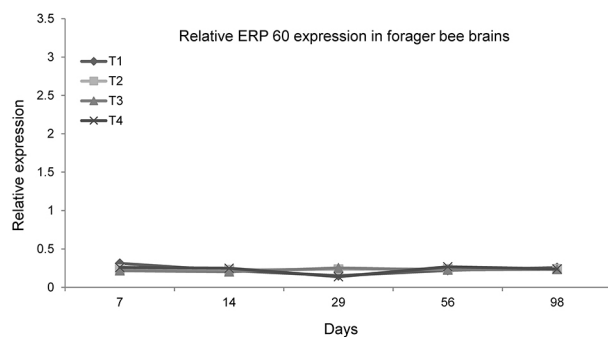

Figure 4 – Relative *ERP60* gene expression in foraging honey bees in colonies managed for honey production under different supering regimes. The Y axis shows relative expression and the x axis shows the days of the experiment. T1 = one standard shallow super added at the beginning of the honey flow, with other supers added as needed. T2, T3, and T4 indicate 2, 3, or 4 supers added at once 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). *ERP6*0 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 $\pm$ 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 beehives 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 T4 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 beehives 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 H_2O_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.

References

Bailie, M.L.; Ijichi, C.; O'Connell, N.E. 2018. Effects of stocking density and string provision on welfare-related measures in commercial broiler chickens in windowed houses. Poultry Science 97: 1503-1510.

Bertocchi, E.; Fusi, F.; Angelucci, A.; Pongolini, S.; Strano, R.M.; Riuzii, J.G.; Moroni, P.; Lorenzi, V. 2018. Characterization of hazards, welfare promoters and animal-based measures for the welfare assessment of dairy cows: elicitation of expert opinion. Preventive Veterinary Medicine 150: 8-18.

Boncristiani, H.; Underwood, R.N.; Schwarz, R.; Evans, J.D.; Pettis, J.; van Engelsdorp, D. 2012. Direct effect of acaricides on pathogen loads and gene expression levels in honey bees *Apis mellifera*. Journal of Insect Physiology 58: 613-620.

Bonnafé, E.; Drouard, F.; Hotier, L.; Carayon, J.L.; Marty, P.; Treilhou, M.; Armengaud, C. 2015. Effect of a thymol application on olfactory memory and gene expression levels in the brain of the honeybee *Apis mellifera*. Environmental Science Pollution Research 22: 8022-8030.

Boogaard, B.K.; Bock, B.B.; Oosting, S.J.; Wiskerke, J.S.C.; van der Zijpp, A.J. 2011. Social acceptance of dairy farming: the ambivalence between the two faces of modernity. Journal of Agricultural and Environmental Ethics 24: 259-282.

Brigida, D.J.; Antonelo, D.S.; Mazon, M.R.; Nubiato, K.E.Z.; Gómez, J.F.M.; Netto, A.S.; Silva, S.L. 2018. Effects of immunocastration and a β-adrenergic agonist on retail cuts of feedlot finished Nellore cattle. Animal 12: 1690-1695.

Carroll, G.A.; Boyle, L.A.; Hanlon, A.; Palmer, M.A.; Collins, L.; Griffin, K.; Armstrong, D.; O'Connell, N.E. 2018. Identifying physiological measures of lifetime welfare status in pigs: exploring the usefulness of haptoglobin, C-reactive protein and hair cortisol sampled at the time of slaughter. Irish Veterinary Journal 71: 1-10.

Corona, M.; Robinson, G.E. 2006. Genes of the antioxidant system of the honey bee: annotation and phylogeny. Insect Molecular Biology 15: 687-701.

Delaplane, K.S. 1997. Practical science-research helping beekeepers 2. Colony manipulations for honey production. Bee World 78: 5-11.

European Union. 2002. Council directive 2001/110/EC of 20 December 2001 relating to honey. Official Journal of the European Communities L10: 47-52.

Fang, Y.; Song, F.; Zhang, L.; Aleku, D.W.; Han, B.; Feng, M.; Li, J. 2012. Differential antennal proteome comparison of adult honeybee drone, worker and queen (*Apis mellifera* L.). Journal of. Proteomics 75: 756-773.

Food and Agriculture Organization [FAO]. 2016. FAOSTAT: food and agriculture data. Crop and livestock products. Available at: http://www.fao.org/faostat/en/ [Accessed Apr 4, 2019]

Fraser, D.; Weary, D.M.; Pajor, E.A.; Milligan, B.N. 1997. A scientific conception of animal welfare that reflects ethical concerns. Animal Welfare 6: 187-205.

Hagai, T.; Cohen, M.; Bloch, G. 2007. Genes encoding putative takeout/juvenile hormone binding proteins in the honeybee (*Apis mellifera*) and modulation by age and juvenile hormone of the takeout-like gene GB19811. Insect Biochemical Molecular 37: 689-701.

Haldar, S.; Ghosh, T.K.; Bedford, M.R. 2011. Effects of yeast (*Saccharomyces cerevisiae*) and yeast protein concentrate on production performance of broiler chickens exposed to heat stress and challenged with *Salmonella enteritidis*. Animal Feed Science Technology 168: 61-71.

Hempstead, M.N.; Wass, J.R.; Stewart, M.; Dowling, S.K.; Cave, V.M.; Lowe, G.L.; Sutherland, M.A. 2018. Effect of isoflurane alone or in combination with meloxicam on the behavior and physiology of goat kids following cautery disbudding. Journal of Dairy Science 101: 3193-3204.

Jongman, E.C.; Rice, M.; Campbell, A.J.; Butler, K.L.; Hemsworth, P.H. 2017. The effect of trough space and floor space on feeding and welfare of lambs in an intensive finishing system. Applied Animal Behavior Science 186: 16-21.

Koivunen, P.; Helaakoski, T.; Annunen, P.; Veijola, J.; Räisaänen, S.; Pihlajaniemi, T.; Kivirikko, K.I. 1996. ERP60 does not substitute for protein disulphide isomerase as the b-subunit of prolyl 4-hydroxylase. Biochemical Journal 316: 599-605.

Kumsa, T.; Takele, D. 2014. Assessment of the effect of seasonal honeybee management on honey production of Ethiopian honeybee (*Apis mellifera*) in modern beekeeping in Jimma Zone. Greener Journal of Plant Breeding and Crop Science 2: 67-75.

Lichtenstein, L.; Grübel, K.; Spaethe, J. 2018. Opsin expression patterns coincide with photoreceptor development during pupal development in the honey bee, *Apis mellifera*. BMC Development Biology 18: 1-11.

Lomele, R.L.; Evangelista, A.; Ito, M.M.; Ito, E.H.; Gomes, S.M.A.; Orsi, R.O. 2010. Natural products in the defensive behavior of *Apis mellifera* L. Acta Scientarium Animal Science 32: 285-291 (in Portuguese, with abstract in English).

Mustard, A.J.; Pham, P.M.; Smith, B.H. 2010. Modulation of motor behavior by dopamine and the D1-like dopamine receptor AmDOP2 in the honey bee. Journal of Insect Physiology 56: 422-430.

Nardone, A.; Ronchi, B.; Lacetera, N.; Ranieri, M.S.; Bernabucci, U. 2010. Effects of climate changes on animal production and sustainability of livestock system. Livestock Production Science 130: 57-69.

Neupane, K.R.; Woyke, J.; Wilde, J. 2012. Effect of initial strength of honey bee colonies (*Apis mellifera*) supered in different ways on maximizing honey production in Nepal. Journal of Apicultural Science 56: 71-81.

Pfaffl, M. 2001. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Research 45: 2001-2007.

Raj, P.A.; Dentino, A.R. 2002. Current status of defensins and their role in innate and adaptive immunity. FEMS Microbiology Letters 206: 9-18.

Rochais, C.; Henry, S.; Hausberger, M. 2018. "Hay-bags" and "Slow feeders": testing their impact on horse behaviour and welfare. Applied Animal Behavior Science 198: 52-59.

Scharlaken, B.; De Graaf, D.C.; Goossens, K.; Peelman, L.J.; Jacobs, F.J. 2008. Differential gene expression in the honeybee head after a bacterial challenge. Development and Comparative Immunology 32: 883-889.

Sodré, G.S.; Marchini, L.C.; Moreti, A.C.C.C.; Otsuk, I.P.; Carvalho, C.A.L. 2007. Physical-chemical characterization of honey samples of *Apis mellifera* L. (Hymenoptera: Apidae) from Ceará state. Ciência Rural 37: 1623-1624 (in Portuguese, with abstract in English).

Taylor, P.S.; Hemsworth, P.H.; Groves, P.J.; Gebhardt-Henrich, S.G.; Rault, J.L. 2018. Ranging behavior relates to welfare indicators pre-and post-range access in commercial free-range broilers. Poultry Science 97: 1861-1871.

The Honey Bee Genome Sequencing Consortium. 2006. Insights into social insects from the genome of the honey bee *Apis mellifera*. Nature 443: 931-949.

Yang, X.L.; Cox-Foster, D.L. 2005. Impact of an ectoparasite on the immunity and pathology of an invertebrate: evidence for host immunosuppression and viral amplification. Proceedings of the National Academy of Science 102: 7470-7475.