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Investigation of the Effects of Essential Amino Acids on Overexpression of the *Vg* and *Sod* Gene in *Apis mellifera*

Research Article

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ABSTRACT

Honeybee immunity and health can be significantly affected by protein nutrition. Essential amino acids have significant effects on animal health, resistance to diseases and survival. The aim of this study was to evaluate the potential of a number of dietary amino acids (lysine, methionine and threonine) to increase honeybees' life span. To do so, 78 honeybee hives were studied divided into13 groups (6 hives each) with different concentrations of dietary amino acids. Parameters of honey and pollen production, winter survival, number of brood and mature bees in each group were evaluated. Moreover, expression of genes for immunity-related peptides (*Vg* and *Sod*) was compared among groups using real time polymerase chain reaction (PCR). The results revealed a significant effect of the different concentrations of amino acids on the parameters studied ($P<0.0001$). The honeybees fed with 1.51 g lysine, 0.3 g methionine and 0.572 g threonine at each hive showed the highest levels of *Vg* and *Sod* expression compared to other groups ($P<0.0002$). Therefore, our results strongly suggest that honey production could be increased by extending the life span of honeybees through the use of essential amino acids in their diet.

KEY WORDS *Apis mellifera*, epigenetics, essential amino acids, mitochondrial Mn superoxide dismutase, vitellogenin.

INTRODUCTION

In addition to genetic factors, environmental factors such as temperature, oxygen, food intake, and nutrition also highly affect the life span of metazoans. Among these, the honeybee *Apis mellifera* has been studied in great detail mostly due to its long affiliation with humans and contemporary ecological and economic importance (Rueppell *et al*. 2016). It is also known that a deficient or improper nutrition can have negative consequences on health and pathogens susceptibility (Wang *et al*. 2014). Moreover, the life span of worker honeybees, average daily brood production and individual productivity of worker bees are among the major factors that affect honey production. Nutrigenomics is a new scientific branch that integrates genomic tools with nutrition research due to the relationship between health and nutrition (Alaux *et al*. 2011).

Use of dietary supplements is common in some countries, as natural forage is not available in some seasons and honeybees must be manually fed. Moreover, reduction in floral abundance and diversity as a result of loss of natural habitats has been a global concern, leading to reduced natural nutrition (pollen and nectar) in many regions (Glavinic *et al*. 2017). Consequently, supplemental diets have been introduced (Brodschneider and Crailsheim, 2010; Stanimirovic *et al*. 2017) that can be used to fight colony losses by

alleviating protein stress (DeGrandi-Hoffman *et al*. 2010). Among the most common supplements for honeybees are amino acids and vitamins (Glavinic *et al*. 2017); however, scientific research about their influence on honeybees has not yet been well investigated.

Research showed that proteins from pollen had a beneficial role on physiological processes, brood rearing, adult population growth and production of royal jelly (Mattila and Otis, 2006; DeGrandi-Hoffman *et al*. 2008). Natural proteins of pollen are essential for maintaining colony healthy including immune responses, parasite tolerance and survival (DeGrandi-Hoffman *et al*. 2016; Basualdo *et al*. 2014), reproductive success (Czekońska *et al*. 2015), and worker longevity (Li *et al*. 2014). The efficacy of protein supplements on honey bees varied depending on formulation and composition.

Nevertheless, the proper amount of protein needed for honeybees has not yet been precisely determined. Herbert *et al*. (1977) reported that the mortality rate of honeybees living on a high protein diet (50%) was greater than those on a less protein diet (5 and 10%), which was likely due to defecation disabilities caused by the high levels of protein in their diet (Human *et al*. 2007). Different pollen-free commercial diets have been produced with various levels of success in enhancing honey bees growth and brood rearing (DeGrandi-Hoffman *et al*. 2008).

Epigenetics studies show changes in gene activity that are inheritable but do not alter the DNA sequence. Many studies have proposed that morphological and behavioral differences between honeybee castes are ascribed to caste-specific protein isoforms, rather than changes in levels of gene expression (Vaiserman *et al*. 2018). Vitellogenin (*Vg*), an egg yolk protein, is present in growing oocytes and is used by queens for egg production. Moreover, it functions as an antioxidant to reduce oxidative stress and increase longevity in honeybees and hence, it is considered an indicator of longevity (Alaux *et al*. 2011). It is showed that bees with higher Vg expression have longer lifespan after paraquat injection, and knockdown of Vg using RNAi treatment significantly shortened lifespan. Thus, assessment of Vg expression in bees can be potentially an indicator of longevity (Seehuus *et al*. 2006).

In addition to *Vg*, the mitochondrial Mn superoxide dismutase (*Sod*) gene contributes to the degradation of H_2O_2 and superoxide radicals. In addition to vertebrates, researchers have investigated that *SOD1* gene express in insects, such as *Drosophila*, reporting that this enzyme also plays a significant role in eliminating ROS in insects (Gaál *et al*. 2006). For instance, Phillips *et al*. (1989) reported that insects with deactivated SOD1 had shortened lifespans because they are unable to remove reactive oxygen species (ROS).

Actually, the effects of ROS removal on life expectancy have examined in several insect species which ultimately highlighted the importance of SOD1 (Parkes *et al*. 1998; Sun and Tower, 1999; Koo *et al*. 2016). It is also assumed that the two genes are associated with pollen-induced molecular mechanisms in honeybees that increase life expectancy. Therefore, for the first time, the effect of 12 concentrations of essential amino acids on longevity and fecundity of honeybees was investigated in the present study. The results of this study can be used in diet management of honeybees worldwide.

MATERIALS AND METHODS

Honeybees and feeding

Honeybees (*A. mellifera*) were obtained from Khorasan Razavi Technical and Professional Center (Mashhad city, Honarestan) according to Wang *et al.* (2014). First, to stop egg production, a naturally mated queen was kept in a queen cage for 5 days; after which it was moved to a queen excluder box for approximately 24 h. An empty frame was also provided so the entire brood laid by the queen would roughly have the same ages. Then, three weeks after the egg laying, the frame was transferred from the colony to an incubator in a frame hive (dark, 32 ˚C and 70% RH). Finally, the emerging honeybees were partitioned into 78 hives. Therefore, we investigated the effect of 13 treatments of amino acid in 5 replications. The treatment of honeybees is presented in Table 1.

Measurement of productive traits

The experiment was carried out on 5 November to 22 December 2018 to provide sufficient space for growth of brood bees and avoid creating additional space and crowding the colony. The amount of honey production was measured weekly. The population of brood bees was determined using a specially wired frame (5×5 cm^2). The population of adult worker bees was measured by estimating the surface of the frame covered by mature bees. In this method, frames fully covered by worker bees were considered as a population frame, whereas partially covered frames were accordingly considered as a fraction of the frame. To measure the amount of pollen collected, pollen traps were used during the flowering period of each treatment and the production was estimated using a digital scale. Resistance and stability of colonies in winter is known to determine the durability and resistance of queens and their colonies as a winter survival parameter.

In order to determine winter mortality, starting from mid-December, colonies were weighed at the beginning of each day (before bees left). At the end of winter, the secondary weighing was carried out.

Table 1 The composition of diet provided to the honeybees

Diets	Lysine (g per hive)	Methionine (g per hive)	Threonine (g per hive)	Sugar
1	-	0.3	-	Available
2	-	0.6	-	Available
3	-	1.2	-	Available
4	1.51	-	-	Available
5	3	-	-	Available
6	6.05	-	-	Available
7	-	-	0.572	Available
8	-	-	1.145	Available
9	-	-	2.29	Available
10	1.51	0.3	0.572	Available
11	3	0.6	1.145	Available
12	6.05	1.2	2.29	Available
13	-	-	-	Available

The difference between the primary weighing and the secondary weighing (overnight=130 days) right after extracting the stored honey was estimated to represent winter survival (Mohebodini *et al*. 2013).

RNA extraction and cDNA synthesis

According to Wang *et al*. (2014), we used liquid nitrogen to flash-freeze honeybees in each group. For real time PCR analysis, 12 samples per hive were collected and, for each sample, heads of 4 bees were pooled during RNA extraction. The heads of the four honeybees were put in a sterile 1.5-mL polypropylene microtube. Then, 200 µL of digestion buffer was added and the sample was homogenized using a sterile microtube pestle.

Therefore, we had 6 replicates and each treatment had 12 samples, so we had 156 samples for the entire study. Isolation of total RNA from the heads of honeybees was carried out with the RNeasy mini kit plus (QiaGene, USA). The next step was to prepare cDNA using RT Kit (Thermo, USA).

Real time PCR

The Maxima SYBR Green/ROX qPCR Master Mix Kit (Thermo, USA) was used for measurement of *Vg* and *Sod* genes. Primer sequences were investigated using the Primer Premier 5 program (Table 2) to amplify the target genes. Real time PCR analysis was carried out on an applied biosystem 7300 real time PCR system (ABI) 7300 (Applied Biosystems) system. As a means of quantifying the *Vg* and *Sod* mRNA relative expression, we implemented a method called the standard curve method, which used *b-Actin* gene as an internal control. The initial cycling conditions were 94 ˚C for 5 min, 40 cycles at 94 ˚C for 15 s, 62 ˚C for 30 s per cycle, and 72 ˚C for 30 s. Using the melting curve analysis, we confirmed that the PCR products of each sample was specified. In order to remove interpolate variation, we ran the control and target unigenes of each sample in the same plate. To calculate the Ct value of the replicates, we used the ABI software to estimate the arithmetic mean of two technical replicate values.

To measure gene expression levels, we used the $2^{-\Delta\Delta CT}$ analysis as a simple formula in order to calculate the relative fold gene expression of samples. To normalize each gene expression, we used β-actin as an internal control gene and median value of NI group as a calibrator.

Statistical analysis

We used one-way ANOVA tests to determine whether there were differences in the following measurements: honey production, pollen production, winter survival and adult and number of brood bees population. All tests were performed using the statistical software SAS Inc., Cary, NC (SAS, 2004). We set the level of statistical significance at $P < 0.05$ for all tests using Duncan test.

RESULTS AND DISCUSSION

Effect of treatment on production traits

Analysis of the data obtained from the traits showed that the effect of protein treatments on the amount of honey production was significant ($P<0.001$). The average production of honey was 4436.44 ± 516.08 g, among which diet 12 with an average production of 6066.07 g showed the highest performance (Table 3). Also, pollen production, winter survival and the number of brood and adult bees were significantly influenced in diets 10, 11 and 12 ($P<0.001$). Therefore, according to the results, it could be concluded that the combination of essential amino acids can effectively influence production traits.

Investigation of RNA extraction and cDNA synthesis

The quality of RNA extraction and cDNA synthesis was investigated by agarose gel electrophoresis and Nanodrop (Epoch, USA) (Figure 1). Clarity and intensity of bands represent high RNA concentration. The results showed that RNA concentration extracted from honeybees heads were greater than their bodies. Eyer *et al*. (2017) extracted RNA from the whole body of honeybees, but Moon *et al*. (2018) reported that bee brains are the best candidates for real time PCR analysis in different ages.

Table 2 Primer sequences and genes used for real time PCR assays

Gene	Primer name	Primer sequence	PCR target (length)
Vg	Forward	5′-AGTTCCGACCGACGACG-3′	63 bp
	Reverse	5′-TTCCCTCCCACGGAGTCC-3′	
Sod	Forward	5′-GTCGTTCCGTGTAGTCGAGAA-3′	73 bp
	Reverse	5′-TCCTTTGACTTCACCCTGAAGA-3′	
B-actin	Forward	5′-CGT GCC GAT AGT ATT CTT G-3′	100 bp
	Reverse	5′-CTT CGT CAC CAA CAT AGG-3′	

Table 3 Mean of production traits and investigation of significant effects

Diets	Honey production	Pollen production	Winter survival	No. of brood	No. of adults
1	3150^{ef}	91.667^{bd}	0.80^{a}	1^{c}	-1.3333^{h}
2	2783.3^{f}	65^{cd}	0.58333^{bc}	1^{c}	-0.3333^{f}
3	3350^{ef}	80^{d}	0.350^{de}	2.5^{ab}	1^{d}
4	3458.4^{e}	95^{c}	0.23333^{e}	2.6667^{ab}	1^{d}
5	4783.3^{cd}	88.333^{cd}	0.56667^{c}	2.1667^{b}	1^{d}
6	4375^{d}	103.333^{bc}	0.43333^{cde}	2.3333^{ab}	1.5^{cd}
7	5100^{c}	106.667^{bc}	0.33333^{ed}	2.3333^{ab}	0.3333^{e}
8	4766.7^{cd}	106.667^{bc}	0.25^{e}	1^{c}	0.3333^{e}
9	5283.3^{c}	110^{b}	0.76667^{ab}	1^{c}	-1.1667^{g}
10	5733.3^{b}	126.667^{a}	0.883333^{a}	2.3333^{ab}	2.3333^{c}
11	5883.3^{b}	131.667^{a}	0.83333^{a}	2.8333^{a}	3^{b}
12	6066.7^{a}	138.333^{a}	0.88333^{a}	2.8333^{a}	3.5^{a}
13	2983.3^{ef}	90^{cd}	051667^{cd}	0^{d}	-1.5^{h}
Df	17	17	17	17	17
F	23.0	6.86	9.86	18.9	19.68
P-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Df: degrees of freedom.
The means within the same column with at least one common letter, do not have significant difference (P>0.0001).

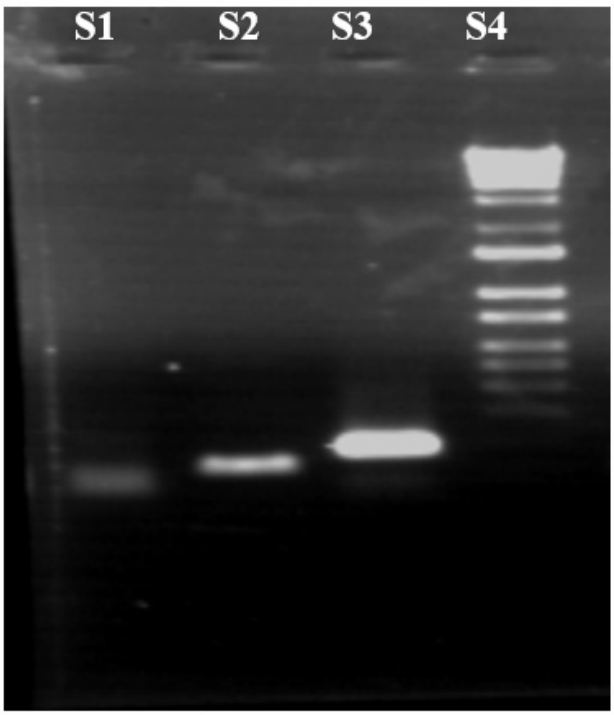

Figure 1 Specificity of the PCR assay for detection of target genes using electrophoresis on 1% agarose
S1: *Vg* PCR product; S2: *Sod* PCR product; S3: *b-Actin* product and S4: 1 Kb marker

The mean of RNA concentration and cDNA synthesis were 1500 ng and 3000 ng in this study, respectively.

Real time PCR analysis

The optimization of *Vg*, *Sod* and *b-Actin* PCR products electrophoresed on1% agarose gel is shown in Figure 2. As demonstrated in Figure1, the length of the amplified fragments of *Vg* primer, *Sod*, and *b-Actin* were 63, 73, and 100 bp, respectively, which correspond to the expected range. This indicates that the primers were designed and selected properly and also, PCR was performed correctly. Furthermore, it would suggest that the selected primers only had one binding site on target cDNA and no sequences have been identified for them elsewhere. Moreover, no non-specific bands, smear or dimer were observed and the bands were all clear. The melting curve of real time PCR confirmed that primers were entirely specific (data not shown).

Gene expression analysis under different diets

The real time PCR analyses showed that the transcript abundance of *Vg* and *Sod* significantly differed between bees fed with different diet contexts. In all groups, *Vg* expression increased and *Sod* expression was influenced in groups 10, 11 and 12. Also, bees fed with lysin, methionine and threonine amino acid (groups 10-12) had a higher expression level of *Vg* compared to bees exposed to diets with exclusively one amino acid (groups 1-9). Bees fed on diet 10 showed the highest *Vg* and *Sod* expression compared to other groups (Figure 2).

For years, beekeepers from all over the world have used commercial amino acids as dietary supplements for honeybees (DeGrandi-Hoffman *et al*. 2016). However, there are no available scientific reports about the effect of essential amino acids on honeybees, hence we aimed to examine their potential to reduce induced immunosuppression in honeybees.

In a study a potential role of amino acids on the regulatory feeding strategy of ants has been suggested and has showed that amino acids are the main elements eliciting the decrease of lifespan under high-protein diets. Moreover, it is identified that four amino acids which are harmful when present at high concentrations relative to other amino acids and whose toxicity had been independently reported in organisms from yeasts to mammals (Arganda *et al*. 2017).

The important result of the present study was that bees exposed to diets with amino acids showed the highest survival rate when essential amino acids were used together. According to Schmidt *et al*. (1987), the total amount of protein consumed by honeybees is the main factor that determines their life span. High protein pollen of *Camellia sinensis* (>26% protein) has 10 essential amino acids that are necessary for efficient growth including methionine, leucine, lysine, arginine, histidine, threonine, tryptophan, valine, phenylalanine, and isoleucine (Su *et al*. 2000; Li, 2003). The results of this article recommend that a diet with essential amino acids (lysine, methionine, threonine) be applied during early winter to enhance colony health in winter.

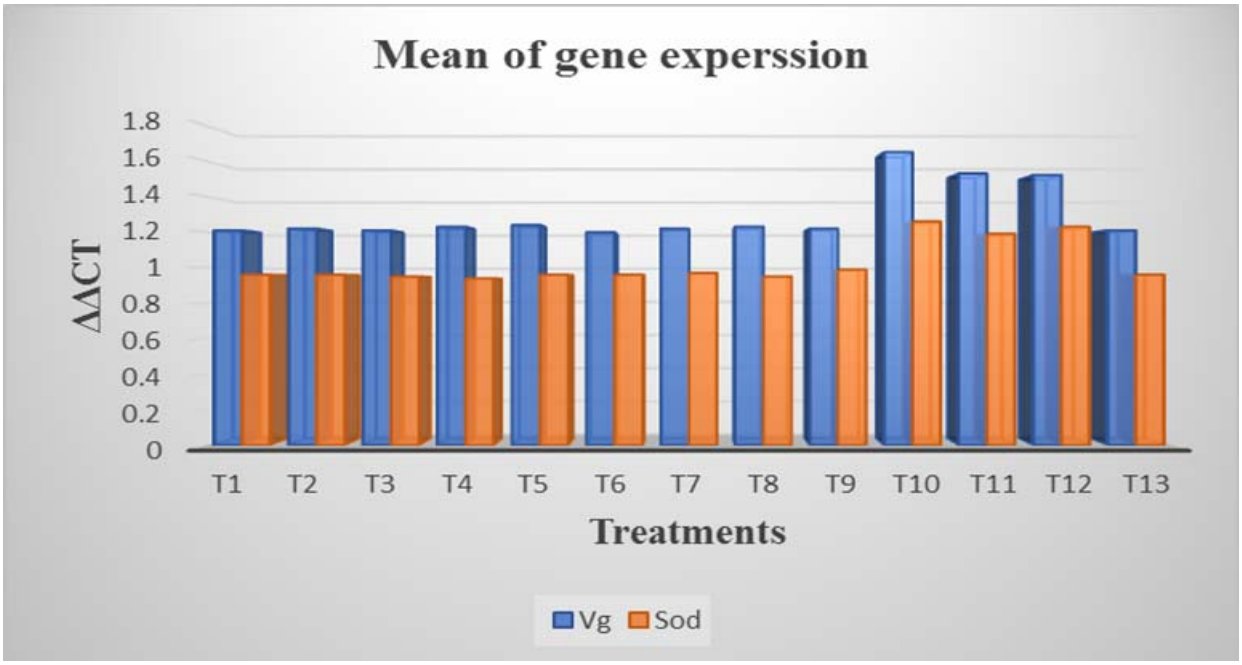

Figure 2 The mean of *Vg* and *Sod* gene expression in ΔΔCT stages for 13 treatments

Several authors have highlighted the significant relationship between honeybees' nutrition, particularly protein-rich diets, and their survival rates, immunity as well as their ability to fight diseases (Alaux *et al*. 2010; Alaux *et al*. 2011; Basualdo *et al*. 2014; Antunez *et al*. 2015).

In a study, the nutritional resilience of honey bee colonies was examined. The results support the preference of honey bees for dietary diversity hypothesis, and that they do not just take in novel sources but specifically target nutritionally complementary ones. Honey bees' ability to counter lacking nutrition contributes to the mechanisms which social insects use to sustain homeostasis at the colony level (Hendriksma and Shafir, 2016).

Examination of the essential amino acids (EAA), or a control treatment of nonessential amino acids (NAA) for supplementing bees demonstrated that caged bees fed EAA developed significantly greater head weights than controls, weights that were similar to nurse bees. Furthermore, caged bees fed EAA developed significantly greater thorax weights than controls, weights that were similar to foragers. Moreover, colonies fed by EAA may increase individual bee growth and brood rearing (Hendriksma *et al*. 2019).

Our findings reveal that the amino acids of methionine, lysine and threonine have the same beneficial effects on bee health, which conform with the previous studies suggesting a significant association between honeybees' protein nutrition and their longevity, immunity and resistance to diseases (Alaux *et al*. 2010; Di Pasquale *et al*. 2013; Basualdo *et al*. 2014; Antunez *et al*. 2015). Using chemical analyses (ICP-OES) to investigate seasonal variation of micronutrients in honey bee workers and floral resources in the field, it is found that honey bees use mineralized water to supplement their floral diet probably and may be limited by availability of potassium and calcium. These results suggested that honey bees may search for specific micronutrients seasonally, perhaps in preparation for overwintering as well (Bonoan *et al*. 2018). The optimization of suitable markers that can report hive health are very important for farmers. Therefore, Alaux *et al*. (2011) was testing which of 8000 genes can be effective in nutrigenomics of honeybee with use of digital gene expression (DGE) analysis. Among these 8000 genes, the best candidate for nutrigenomics were *Vg* and *Sod*. However, other researchers confirmed the same results by gene expression analysis (Eyer *et al*. 2017; Moon *et al*. 2018). In this study, we could optimize effective methods for detection of hive health by using real-time PCR. Results showed that *Vg* and *Sod* can be used as markers for status of hive health.

CONCLUSION

In this study, we showed for the first time that manually supplying essential amino acids can be very effective on life span of honey bees. Also, the treatment of 1.51 g lysine, 0.3 g methionine and 0.572 g threonine were suitable candidates for supplements of essential amino acid requirements of honey bees. Results showed that the life span genes (*Vg* and *Sod)* can be used as excellent markers for estimation of economic justification of the hive. Hives with over expression of *Vg* and *Sod* genes had greater economic traits.

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