Effect of Dietary Protein Level on Homing Ability and Memory-Related Gene Expression in Italian Honey Bees (Postprint)

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Abstract

This experiment aimed to investigate the effects of protein level on the homing ability and expression of memory-related genes in Italian honeybees. Nine local Italian honeybee colonies of similar strength were selected and randomly divided into three groups, with three colonies per group. Groups I, II, and III were fed experimental diets with protein levels of 15%, 25%, and 35%, respectively. After 45 days of feeding, the return rates of forager bees at 1,000 m and 2,000 m and the expression of three memory-related genes [glutamate receptor gene (GluRA), N-methyl-D-aspartate receptor gene (Nmdar1), and tyramine receptor gene (Tyr1)] in worker bees of different ages (newly emerged, 10-day-old, 20-day-old) were measured. The results showed that the return rates of bees in groups II and III at both 1,000 m and 2,000 m were significantly higher than those in group I ($P<0.05$), but there was no significant difference between groups II and III ($P>0.05$). The relative expression level of GluRA in 10- and 20-day-old worker bees in group III was significantly higher than that in groups II and I ($P<0.05$), and the relative expression level of GluRA in group II was also significantly higher than that in group I ($P<0.05$). The relative expression level of Nmdar1 in 10- and 20-day-old worker bees in groups II and III was significantly higher than that in group I ($P<0.05$), but there was no significant difference between groups II and III ($P>0.05$). The relative expression level of Tyr1 in 10-day-old worker bees in groups II and III was significantly higher than that in group I ($P<0.05$), but there was no significant difference between groups II and III ($P>0.05$). There were no significant differences in the relative expression levels of GluRA, Nmdar1, and Tyr1 in newly emerged worker bees, nor in the relative expression level of Tyr1 in 20-day-old worker bees among all experimental groups ($P>0.05$). It can be concluded that excessively low dietary protein level affects the homing ability and expression of memory-related genes in Italian honeybees.

Full Text

Effects of Dietary Protein Level on Homing Capability and Memory-Related Gene Expression in *Apis mellifera ligustica*

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Abstract

This study investigated the effects of dietary protein level on the homing capability and memory-related gene expression in *Apis mellifera ligustica*. Nine honeybee colonies of comparable strength were randomly divided into three groups (n=3 per group). Groups I, II, and III were fed experimental diets containing 15%, 25%, and 35% protein, respectively. After 45 days of feeding, we measured the return rates of forager bees at distances of 1,000 m and 2,000 m from the hive, and examined the expression levels of three memory-related genes—glutamate receptor type A gene (*GluRA*), N-methyl-D-aspartate receptor type 1 gene (*Nmdar1*), and tyramine receptor type 1 gene (*Tyr1*)—in worker bees of different ages (newly emerged, 10-day-old, and 20-day-old).

The results showed that the homing rates at both distances were significantly higher in groups II and III compared to group I ($P<0.05$), with no significant difference between groups II and III ($P>0.05$). The relative expression of *GluRA* in 10-day-old and 20-day-old worker bees was significantly higher in group III than in groups II and I ($P<0.05$), and significantly higher in group II than in group I ($P<0.05$). The relative expression of *Nmdar1* in 10-day-old and 20-day-old worker bees was significantly higher in groups II and III compared to group I ($P<0.05$), but did not differ significantly between groups II and III ($P>0.05$). The relative expression of *Tyr1* in 10-day-old worker bees was significantly higher in groups II and III than in group I ($P<0.05$), with no significant difference between groups II and III ($P>0.05$). No significant differences were observed among groups in the expression of *GluRA*, *Nmdar1*, or *Tyr1* in newly emerged worker bees, nor in *Tyr1* expression in 20-day-old worker bees ($P>0.05$). These findings indicate that insufficient dietary protein levels impair both the homing ability and memory-related gene expression in *Apis mellifera ligustica*.

Keywords: *Apis mellifera ligustica*; protein level; homing rate; memory genes

Introduction

Honeybees are typical social and economically important insects belonging to the phylum Arthropoda, class Insecta, order Hymenoptera, superfamily Apoidea, family Apidae, and genus *Apis* [1]. As crucial components of ecosystems, they play a significant role in promoting biodiversity and maintaining ecological balance. However, since the winter of 2006, Colony Collapse Disorder (CCD) has devastated beekeeping operations across the United States, France, Sweden, Germany, and Australia, causing colony losses of 50–90%. CCD is characterized by the sudden disappearance of large numbers of adult worker bees from the colony within a short period, with no dead bodies found near the hive—only the queen, brood, some immature workers, and abundant honey and pollen remain. Potential contributing factors include disease, pesticides, climate, and nutrition [2-4], though no definitive cause has been identified. Nutrition may be a primary factor, as malnutrition can affect bee development and even impair memory and homing ability, leading to disoriented bees that fail to return to the colony. While reports on honeybee learning and memory genes are limited, researchers have identified the tyramine receptor type 1 gene (*Tyr1*), N-methyl-D-aspartate receptor type 1 gene (*Nmdar1*), and glutamate receptor type A gene (*GluRA*) as important genes involved in learning and memory in *Apis mellifera* [5-7]. Recent studies on honeybee protein nutrition have shown that protein levels affect egg hatchability, larval pupation rate, and antioxidant activity [8-12], but no studies have examined whether protein levels influence homing ability or memory gene expression. Therefore, this experiment was designed to investigate these effects and provide insights into the etiology of CCD.

Materials and Methods

1.1 Experimental Animals

The experimental subjects were *Apis mellifera ligustica* colonies maintained at the Honeybee Research Institute of Jiangxi Agricultural University.

1.2 Reagents and Equipment

The study utilized artificial pollen substitute diets with varying protein levels (provided by Shandong Agricultural University), rapeseed honey, a radio frequency identification (RFID) system for bee tracking (Guangzhou Invengo Information Technology Co., Ltd.), a GPS global positioning device (eTrex Vista HCX), Trizol total RNA extraction kit and RNase inhibitor (Beijing TransGen Biotech), dNTP Mixture and oligo(dT) synthesized by Shanghai Invitrogen, M-MLV reverse transcriptase and SYBR® Premix Ex Taq™ II (TaKaRa), custom-made wooden boxes (25 cm × 25 cm × 20 cm), a quantitative PCR instrument (iQ™2, Bio-Rad), and a nucleic acid/protein analyzer (NanoPhotometer™ P300, IMPLEN).

1.3 Experimental Procedures

1.3.1 Colony Selection and Management Nine local *Apis mellifera ligustica* colonies of comparable strength (without pollen combs) were randomly divided into three groups with three replicates each. Colony strength was standardized to five full frames before the experiment. Each group was fed diets containing 15%, 25%, or 35% protein, with diet formulations based on reference [10]. Since natural pollen sources were available during the experiment, pollen traps were installed on all colonies, and sugar syrup was provided as supplementary feeding. The three protein diets were mixed with sugar and honey at a ratio of 2:3:1, kneaded with appropriate water until a dough-like consistency was achieved, then formed into patties. Each colony received 400 g of diet placed on the top bars, replaced every 3 days. After 45 days of continuous feeding, subsequent tests were conducted.

1.3.2 Effect of Protein Level on Forager Homing Ability Pollen-carrying foragers were collected at the hive entrance using a bee vacuum and electronically tagged following the method of He Xujiang [13]. Using GPS, precise release points were established at 1,000 m and 2,000 m from the hive. Tagged bees that had consumed sufficient sugar syrup were transported to the release sites and allowed to fly back, with their return recorded by the RFID system. This procedure was repeated six times.

The effective number of released bees was defined as those that initiated flight toward the hive within 5 minutes; bees that failed to fly normally were excluded. The homing rate was calculated as: (number of bees returning to the hive) / (effective number of bees released).

1.3.3 Effect of Protein Level on Memory-Related Gene Expression in Worker Bees

1.3.3.1 Sample Collection Twenty worker bees each of newly emerged, 10-day-old, and 20-day-old ages were collected from each colony and placed in 1.5 mL RNase-free EP tubes, then immediately immersed in liquid nitrogen for subsequent analysis.

1.3.3.2 RNA Extraction and cDNA Synthesis Three bees from the same colony and age group were pooled as one sample. Heads were ground to a powder in a liquid nitrogen-cooled mortar and transferred to 1.5 mL EP tubes containing 1 mL Trizol. Total RNA was extracted following the method of Qin Qiuhong [14]. RNA integrity was assessed by agarose gel electrophoresis, and purity was determined using a nucleic acid/protein analyzer to measure the OD260/280 ratio (acceptable range: 1.9–2.1) and concentration. Each sample was measured three times and averaged.

Total RNA was reverse-transcribed in a 50 L reaction mixture containing 8 L total RNA, 10 L buffer, 8 L dNTP Mixture, 1.5 L M-MLV reverse transcriptase, 3 L oligo(dT), 1 L RNase inhibitor, and 18.5 L DEPC-treated water. The reaction conditions were: 42°C for 60 minutes, followed by inactivation at 75°C for 5 minutes. cDNA products were stored at -80°C.

1.3.3.3 Primer Design and Quantitative Real-Time PCR Primers were designed using Primer 5.0 software based on *Apis mellifera* genomic sequences from the National Center for Biotechnology Information (NCBI) database . The glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) was used as the reference gene due to its stable expression during honeybee development [15].

The qPCR reaction mixture (10 L) contained 1 L cDNA, 5 L SYBR® Premix Ex Taq™ II, 0.4 L each of forward and reverse primers, and 3.2 L ultrapure water. The reaction conditions were: initial denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 10 s and 60°C for 1 min. After amplification, a melting curve was generated by slowly heating from 55°C to 95°C (0.5°C increments every 10 s). Each cDNA sample was run in triplicate, and relative expression levels were calculated using the method described by Huang et al. [15].

1.4 Data Analysis

Statistical analysis was performed using the "ANOVA and t-test" function in StatView software, specifically the "ANOVA or ANCOVA" procedure.

Results

2.1 Effect of Dietary Protein Level on Forager Homing Ability

As shown in , the homing rates of foragers at both 1,000 m and 2,000 m were significantly higher in groups II and III compared to group I ($P<0.05$), with no significant difference between groups II and III ($P>0.05$).

2.2 Effect of Dietary Protein Level on *GluRA* Relative Expression

The results are presented in [Figure 1: see original paper]. No significant differences were observed in *GluRA* expression among groups in newly emerged worker bees ($P>0.05$). However, the relative expression of *GluRA* in 10-day-old and 20-day-old worker bees was significantly higher in group III than in groups II and I ($P<0.05$), and significantly higher in group II than in group I ($P<0.05$).

[Figure 1: see original paper]

2.3 Effect of Dietary Protein Level on *Nmdar1* Relative Expression

The results are shown in [Figure 2: see original paper]. No significant differences in *Nmdar1* expression were found among groups in newly emerged worker bees ($P>0.05$). The relative expression of *Nmdar1* in 10-day-old and 20-day-old worker bees was significantly higher in groups II and III compared to group I ($P<0.05$), but did not differ significantly between groups II and III ($P>0.05$).

[Figure 2: see original paper]

2.4 Effect of Dietary Protein Level on *Tyr1* Relative Expression

The results are depicted in [Figure 3: see original paper]. No significant differences in *Tyr1* expression were observed among groups in newly emerged or 20-day-old worker bees (P>0.05). The relative expression of *Tyr1* in 10-day-old worker bees was significantly higher in groups II and III than in group I (P<0.05), with no significant difference between groups II and III (P>0.05).

[Figure 3: see original paper]

Discussion

Honeybees undergo complete metamorphosis through four developmental stages: egg, larva, pupa, and adult. Both larval and adult stages require nutritional intake, digestion, and absorption. Adult worker bees primarily consume natural foods of pollen and honey, while early-stage larvae are fed royal jelly secreted by nurse bees (the composition of which depends on the nurses' diet), and later-stage larvae are fed bee bread—a fermented mixture of pollen and honey. During early spring when natural pollen and nectar sources are scarce, beekeepers often use pollen substitutes (protein diets) and sugar syrup. Previous studies have demonstrated that different protein levels in pollen substitutes significantly affect individual bee weight, hypopharyngeal gland development, and antioxidant capacity [8–12], consequently influencing colony strength and royal jelly production. Our findings show that groups II and III exhibited significantly higher homing rates at both distances compared to group I, with no significant difference between groups II and III. This indicates that low dietary protein levels impair forager homing ability, likely because newly emerged workers require substantial protein for continued development and maturation. Insufficient protein compromises their physiological function, resulting in reduced homing capacity.

GluRA is widely distributed in the central nervous system and is considered a metabotropic glutamate receptor gene in honeybees that influences long-term learning and memory, serving as a key neurotransmitter in the memory formation process [7]. Our results demonstrate that *GluRA* expression in 10-day-old and 20-day-old worker bees was significantly elevated in group III compared to groups II and I, and significantly higher in group II than in group I. This suggests that dietary protein level significantly affects *GluRA* expression in older worker bees. After emergence, workers must consume high-protein diets to support further development of internal organs and optimize physiological functions, particularly the long-term memory capacity essential for successful foraging and homing.

Nmdar1 is associated with honeybee learning and memory and is broadly expressed in mushroom body Kenyon cells and other neural tissues [6]. We found that *Nmdar1* expression in 10-day-old and 20-day-old worker bees was significantly higher in groups II and III than in group I, with no significant difference between groups II and III. This indicates that low dietary protein levels affect the development of neural structures in the brain, reducing memory-related gene expression and consequently impairing homing ability—consistent with our homing assay results.

Tyr1 is an important neurotransmitter in insects that regulates flight, learning, and memory [16,17] and participates in honeybee learning and memory processes [5]. Our data show that *Tyr1* expression in 10-day-old worker bees was significantly higher in groups II and III compared to group I, with no significant difference between groups II and III. This suggests that dietary protein level influences *Tyr1* expression and that low protein levels impair memory function. However, protein level did not significantly affect *Tyr1* expression in 20-day-old worker bees, possibly because *Tyr1* is primarily involved in short-term memory processes such as orientation flights rather than long-term memory associated with foraging. He Xujiang [13] reported that honeybees' first orientation flights occur mainly at 7–11 days of age, coinciding with the period when higher *Tyr1* expression is required. The specific mechanisms warrant further investigation.

Additionally, we observed no significant differences in *GluRA*, *Nmdar1*, or *Tyr1* expression among groups in newly emerged worker bees. This may be because the royal jelly secreted by nurse bees fed different protein diets did not differ significantly in key active components such as 10-hydroxy-2-decenoic acid (10-HAD, also known as royal jelly acid) [18], resulting in minimal impact on larval development. The underlying reasons require further study.

Dietary protein level significantly affects memory-related gene expression and consequently influences homing ability in *Apis mellifera ligustica*. For routine colony management, dietary protein levels should not be lower than 25%.

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Note: Figure translations are in progress. See original paper for figures.