Effects of Vitamin B2 on Lifespan and Learning and Memory in Apis cerana Worker Bees (Post-print)

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Abstract

This experiment aimed to investigate the effects of vitamin B2 on the lifespan and learning-memory abilities of Apis cerana worker bees. The experiment consisted of two parts: worker bee rearing and learning-memory testing. Worker bee rearing experiment: One-day-old Apis cerana worker bees were divided into 3 groups, with 5 replicates per group and approximately 200 bees per replicate. The control group (Group I) was fed 1:1 sugar water, while the two experimental groups were supplemented with 100 (Group II) and 400 mg/kg (Group III) of vitamin B2 based on the 1:1 sugar water. The mortality of worker bees in each group was recorded daily until all bees died. Worker bee learning-memory experiment: The grouping method was the same as in the worker bee rearing experiment. After feeding one-day-old worker bees for 7 days according to the rearing method, the proboscis extension response method was used to determine the effects of different vitamin B2 concentrations on short-term and long-term learning-memory abilities in bees, and the relative expression levels of learning-memory-related genes in bees after successful learning were detected by fluorescence quantitative PCR. The results showed that the average lifespan of worker bees extended with increasing vitamin B2 supplementation, and the average lifespan of the experimental groups (Groups II and III) was significantly higher than that of the control group ($P < 0.05$), but the difference between Groups II and III was not significant ($P > 0.05$). The long-term and short-term learning-memory abilities of worker bees in Group III were significantly higher than those in the control group and Group II ($P < 0.05$), and the long-term learning-memory ability of worker bees in Group II was also significantly higher than that in the control group ($P < 0.05$). The relative expression levels of dopamine receptor gene 2 (Acdop3) and cAMP response element-binding protein (AcCREB) in worker bees of Group III were significantly higher than those in the control group and Group II ($P < 0.05$), and the relative expression of Acdop3 in worker bees of Group II was also significantly higher than that in the control

group ($P < 0.05$). It can be concluded that vitamin B2 affects the lifespan and learning-memory abilities of Apis cerana worker bees, and appropriate amounts of vitamin B2 should be provided when artificially feeding bee colonies.

Full Text

Effect of Vitamin B2 on Lifespan and Learning-Memory Ability of Worker Bees in *Apis cerana cerana*

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Abstract: This study investigated the effects of vitamin B2 on the lifespan and learning-memory capacity of *Apis cerana cerana* worker bees. The experiment comprised two components: worker bee feeding trials and learning-memory assays. For the feeding trials, one-day-old worker bees were divided into three groups with five replicates each (approximately 200 bees per replicate). The control group (Group) received a 1:1 sugar syrup, while the two experimental groups received the same syrup supplemented with 100 mg/kg (Group) and 400 mg/kg (Group) of vitamin B2, respectively. Mortality was recorded daily until all bees had died. For the learning-memory assays, bees were grouped and fed identically for 7 days, after which their short-term and long-term learning-memory abilities were evaluated using the proboscis extension response (PER) method. The relative expression levels of learning-memory-related genes were subsequently measured by quantitative real-time PCR in bees that successfully completed the learning task. The results demonstrated that worker bee lifespan increased with higher vitamin B2 supplementation, with both experimental groups showing significantly longer average lifespans than the control ($P < 0.05$), though no significant difference existed between Groups and ($P > 0.05$). Group exhibited significantly superior short-term and long-term learning-memory performance compared to both the control and Group ($P < 0.05$), while Group also showed significantly better long-term memory than the control ($P < 0.05$). Furthermore, the relative expression levels of dopamine receptor gene 3 (*Acdop3*) and cAMP response element-binding protein (*Ac-CREB*) were significantly higher in Group than in the control and Group ($P < 0.05$), and *Acdop3* expression in Group was significantly elevated compared to the control ($P < 0.05$). These findings indicate that dietary vitamin B2 influences both lifespan and learning-memory capacity in *A. cerana cerana* worker bees, highlighting the importance of adequate vitamin B2 supplementation in artificial diets for honeybee colonies.

Keywords: *Apis cerana cerana*; vitamin B2; lifespan; proboscis extension response

Honeybees, as crucial economic insects, have specific nutritional requirements analogous to livestock and poultry. Investigating these nutritional needs and establishing scientifically sound feeding standards not only promotes healthy bee development and enhances disease resistance but also maintains colonies at optimal production levels, thereby improving both the yield and quality of bee products [1]. Strong colony strength is fundamental for high honey yields, and robust colonies require two essential elements: rapid reproduction and extended longevity, which together ensure sufficient individual bee accumulation [2]. Therefore, improving nutritional conditions to extend bee lifespan can significantly increase economic returns for beekeepers. Recent research on honeybee nutrition has yielded substantial progress, establishing requirements for protein, vitamin A, vitamin C, amino acids, and minerals at different developmental stages, providing critical reference data for feed formulation [3-17]. Riboflavin (vitamin B2) converts to flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) within animal tissues, serving as a prosthetic group for various flavoenzymes that act as hydrogen carriers in biological oxidation processes. This vitamin is intimately involved in carbohydrate, lipid, and protein metabolism [18] and represents an essential nutrient for tissue metabolism and repair. This study examines how vitamin B2 affects the lifespan and learning-memory abilities of *A. cerana cerana* worker bees, providing theoretical data to guide rational diet formulation for honeybee colonies.

1 Materials and Methods

1.1 Experimental Animals

Worker bees of *Apis cerana cerana* were obtained from colonies maintained at the Honeybee Research Institute of Jiangxi Agricultural University.

1.2 Reagents and Equipment

Vitamin B2 (purity 98%, Supelco, USA), lemon and vanilla flavorings (food-grade, Queen Food, Australia), sucrose and sodium chloride (analytical grade, Xilong Chemical Co., Ltd.), Trizol reagent and RNase inhibitor (TransGen Biotech, Beijing), dNTP Mixture and Oligo(dT) (synthesized by Invitrogen, Shanghai), M-MLV reverse transcriptase and SYBR® Premix Ex Taq™ II (TaKaRa). Custom-made wooden boxes (11 cm × 13 cm × 15 cm), biochemical incubator (GZ-250-GSI, Guangzhi Technology, Shaoguan), quantitative PCR system (iQ™2, Bio-Rad, USA), and nucleic acid/protein analyzer (NanoPhotometer™ P300, Implen, Germany).

1.3 Experimental Procedures

1.3.1 Worker Bee Feeding Trial Four *A. cerana cerana* colonies of equal strength were selected, and queen egg-laying was restricted. Sealed brood near-

ing emergence was placed in an incubator (34 °C, 75% relative humidity) for eclosion. Newly emerged one-day-old worker bees were randomly divided into three groups with five replicates each (approximately 200 bees per replicate) and maintained in an incubator (34 °C, 75% relative humidity). The control group (Group) received a 1:1 sugar syrup, while experimental groups received the same syrup supplemented with 100 mg/kg (Group) and 400 mg/kg (Group) vitamin B2. All syrups were prepared fresh daily. Food was replaced every 24 hours to ensure adequate supply. Dead bees were removed after the first 24 hours, and mortality was recorded daily thereafter until all bees had perished [14].

1.3.2 Learning-Memory Assay Bees were grouped and fed as described in Section 1.3.1. After 7 days of feeding, they were immobilized with dry ice and secured in U-shaped tubes, then starved for 2 hours in an incubator (37 °C, 75% relative humidity). Bees in poor condition were subsequently removed. For conditioning, each bee received three training sessions at 6-minute intervals, pairing lemon odor with sucrose solution (positive stimulus) and vanilla odor with saline solution (negative stimulus). Short-term and long-term memory were tested at 6 and 24 hours post-training, respectively, by presenting the negative stimulus followed by the positive stimulus and recording proboscis extension responses. Each bee was tested three times, and data were analyzed statistically [19,20].

1.3.3 Measurement of Learning-Memory-Related Gene Expression
1.3.3.1 Sample Collection
Bees that correctly extended their proboscis during memory testing were collected (20 bees per group) in 1.5 mL RNase-free tubes and immediately snap-frozen in liquid nitrogen for subsequent analysis.

1.3.3.2 Total RNA Extraction and cDNA Synthesis
Four bees from each group were ground into powder in liquid nitrogen using a mortar and pestle. The powder was transferred to a 1.5 mL tube containing 1 mL Trizol reagent for total RNA extraction. RNA purity (OD /) and concentration were measured using a nucleic acid/protein analyzer, with three replicate measurements per group averaged. Reverse transcription was performed using a commercial kit in a 50 L reaction 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. Reaction conditions: 42 °C for 60 minutes, followed by 75 °C for 5 minutes to inactivate the enzyme. cDNA products were stored at -80 °C.

1.3.3.3 Quantitative Real-Time PCR
Primers were designed using Primer 5.0 software based on mRNA sequences for dopamine receptor genes 2 (*Acdop2*, GenBank KC814691) and 3 (*Acdop3*, KC814692), and cAMP response element-binding protein (*AcCREB*, KC814690) cloned from *Apis cerana* brain tissue. Glyceraldehyde-3-phosphate dehydroge-

nase (*GAPDH*) and -actin served as reference genes (Table 1).

The 10 L qPCR reaction 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. Cycling conditions: 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s and 60 °C for 1 min. A melting curve was generated by heating from 50 °C to 90 °C (1 °C increments every 6 s). Ct values were collected using Bio-Rad CFX 2.1 software [12], amplification efficiencies were calculated from technical replicates using the qPCR package, and relative gene expression was determined using the method described by Huang et al. [21].

Table 1 Primers used in real-time quantitative PCR

Gene Name	Forward Primer (5' -3')	Reverse Primer (5' -3')
AcCREB (cAMP response element-binding protein)	TGAAAATCCAGTTTGATCATTTCCAA	TTCCAATATAATCAGCAAATCATGCAC
Acdop2 (Dopamine receptor gene 2)	TTGGTTCTCCCTCTCTCCGA	ACTGTGCGTGTTATTGCGTTC
Acdop3 (Dopamine receptor gene 3)	AGAAGGACAAGAAAAATGCCG	CGAAGAGGTCACTATGAATGCG
-actin	GGC TCC CGA AGA ACA TCC T	GCA AAC ACC GTC ACC CG
GAPDH	CTGGTTTCATCGATGGTTT	ACGATTTCGACCACCGTAAC

Statistical analysis was performed using SPSS 20.0 software, with $P < 0.05$ considered statistically significant.

2 Results

2.1 Effect of Vitamin B2 on Worker Bee Lifespan

As shown in Table 2 , the average lifespan of worker bees increased with vitamin B2 supplementation level. Both experimental groups (and) exhibited significantly longer average lifespans than the control group ($P < 0.05$), though no significant difference was observed between the two experimental groups ($P > 0.05$). The median lifespan also increased with higher vitamin B2 concentrations.

Table 2 Effects of vitamin B2 on lifespan of worker bees for *Apis cerana cerana*

Group	Average Lifespan (days)	Median Lifespan (days)
(Control)	13.995 ± 0.221	14
(100 mg/kg)	18.566 ± 0.341	19
(400 mg/kg)	21.381 ± 0.315	21

Values in the same column with different letter superscripts differ significantly ($P < 0.05$).

2.2 Effect of Vitamin B2 on Learning-Memory Ability

As illustrated in Figure 1 [Figure 1: see original paper], Group demonstrated significantly superior short-term and long-term learning-memory performance compared to both the control and Group ($P < 0.05$). Additionally, Group exhibited significantly better long-term memory than the control group ($P < 0.05$).

Figure 1 Effects of vitamin B2 on short-term (A) and long-term (B) learning-memory abilities of worker bees for *Apis cerana cerana*. Data columns with different letters differ significantly ($P < 0.05$). The same notation applies below.

2.3 Effect of Vitamin B2 on Relative Expression of Learning-Memory-Related Genes

As depicted in Figure 2 [Figure 2: see original paper], the relative expression levels of *Acdop3* and *AcCREB* were significantly higher in Group than in the control and Group ($P < 0.05$). Moreover, *Acdop3* expression in Group was significantly elevated compared to the control ($P < 0.05$). No significant differences were observed in *Acdop2* expression among any groups ($P > 0.05$).

Figure 2 Effects of vitamin B2 on relative expression levels of learning-memory-related genes in worker bees for *Apis cerana cerana*.

Honeybee nutrition plays a vital role in normal growth and reproduction and remains a primary concern for beekeepers. Various vitamins, including vitamin B2, are routinely supplemented in artificial diets. Vitamin B2 is widely distributed in nature and participates in the formation of flavoenzyme prosthetic groups, serving as a hydrogen carrier in biological oxidation and playing a crucial role in carbohydrate, lipid, and protein metabolism. Vitamin B2 deficiency during the honeybee life cycle inevitably impairs biological oxidation and causes metabolic dysfunction. Our results demonstrate that worker bee lifespan in *A. cerana cerana* increases with dietary vitamin B2 concentration, likely because vitamin B2 enhances metabolic capacity for sugars, fats, and proteins, thereby promoting individual development and extending longevity. This strengthens colony populations and improves productivity.

Learning-memory capacity is closely linked to orientation flights and homing ability after foraging. Superior memory enables more efficient return to the hive, enhancing resource acquisition and increasing hive products. Our findings show that Group exhibited significantly enhanced short-term and long-term memory compared to the control and Group , while Group showed improved long-term memory relative to the control. This indicates that vitamin B2 supplementation improves learning-memory ability, with higher concentrations producing more pronounced effects.

Learning and memory are behavioral processes underpinned by molecular mechanisms. *AcCREB* regulates transcription of specific genes, strengthens intercellular connections, influences neuronal development and regeneration, and participates in memory formation [22]. Dopamine is a critical neurotransmitter in the central nervous system that regulates various insect behaviors and physiological processes, including learning-memory. Specifically, *Acdop3* activation induces cAMP changes in the brain that trigger memory formation [23]. Our study revealed that *Acdop3* and *AcCREB* expression levels were highest in Group , intermediate in Group , and lowest in the control, correlating directly with the observed learning-memory performance. This confirms that dietary vitamin B2 concentration modulates expression of learning-memory-related genes and corresponding cognitive abilities. The lack of significant difference in *Acdop2* expression among groups suggests this gene may be more associated with locomotor behavior, though this requires further investigation.

In conclusion, vitamin B2 significantly affects both lifespan and learning-memory capacity in *Apis cerana cerana* worker bees, underscoring the necessity of providing adequate vitamin B2 in artificial feeding regimes for honeybee colonies.

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