Optimal Dietary Pantothenic Acid Level for Italian Honey Bee (Apis mellifera ligustica) Worker Larvae: A Postprint

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Abstract

This study aimed to investigate the effects of dietary pantothenic acid levels on the development, antioxidant capacity, and expression of Coenzyme A (CoA) synthesis-related enzyme genes in Italian honeybee (Apis mellifera ligustica) worker larvae, in order to determine the optimal dietary pantothenic acid level for the larval stage. A total of 1,800 1-day-old Italian honeybee worker larvae were selected and randomly divided into 5 groups with 3 replicates per group and 120 larvae per replicate. The five groups of worker larvae were fed experimental diets with measured pantothenic acid levels of 0.92 (control), 1.22, 1.52, 1.82, and 2.12 mg/g, respectively, until pupation. Body weight, body composition, hemolymph biochemical indices, antioxidant indices, and relative expression levels of CoA synthesis-related enzyme genes were measured in 5-day-old and 7-day-old larvae, and the pupation and eclosion rates of the larvae were calculated. The results showed that: 1) Dietary supplementation with pantothenic acid significantly increased fresh weight, dry weight, and crude fat content of the larvae ($P<0.05$); and when the pantothenic acid level was 2.12 mg/g, the eclosion rate of larvae was significantly higher than in other groups ($P<0.05$). 2) Dietary pantothenic acid level had significant effects on total protein (TP), total cholesterol (TCHO), high-density lipoprotein (HDL), and low-density lipoprotein (LDL) contents in the hemolymph of 5-day-old worker larvae ($P<0.05$), with the lowest values observed in the 1.82, 1.82, 1.22, and 1.52 mg/g groups, respectively. 3) Total antioxidant capacity (T-AOC) of larvae at both 5 and 7 days of age, as well as superoxide dismutase (SOD) activity in 5-day-old larvae, increased significantly with increasing dietary pantothenic acid levels ($P<0.05$). 4) Dietary pantothenic acid level significantly affected the relative expression levels of the pantothenate kinase 4 gene and phosphopantothenoylcysteine decarboxylase gene in 5-day-old larvae ($P<0.05$), with the highest relative expression observed at a pantothenic acid level of 1.82 mg/g. Using dry weight of 5-day-old larvae and eclosion rate for regression curve fitting,

the optimal dietary pantothenic acid level for Italian honeybee worker larvae was determined to be 1.85–2.01 mg/g.

Full Text

Appropriate Dietary Pantothenic Acid Level for *Apis mellifera ligustica* Worker Bee Larvae

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Abstract

This study investigated the effects of dietary pantothenic acid level on the development, antioxidant capacity, and expression of coenzyme A (CoA) synthesis-related enzyme genes in *Apis mellifera ligustica* worker bee larvae to determine the appropriate dietary pantothenic acid level for this developmental stage. A total of 1,800 one-day-old worker bee larvae were randomly assigned to five groups with three replicates per group and 120 larvae per replicate. The five groups were fed experimental diets containing measured pantothenic acid levels of 0.92 (control), 1.22, 1.52, 1.82, and 2.12 mg/g until pupation. Body weight, body composition, hemolymph biochemical indices, antioxidant indices, and relative expression levels of CoA synthesis-related enzyme genes were measured in 5- and 7-day-old larvae, and pupation and eclosion rates were calculated. The results showed: (1) Dietary pantothenic acid supplementation significantly increased larval fresh weight, dry weight, and crude fat content ($P<0.05$), and the eclosion rate at 2.12 mg/g was significantly higher than other groups ($P<0.05$). (2) Dietary pantothenic acid level significantly affected total protein (TP), total cholesterol (TCHO), high-density lipoprotein (HDL), and low-density lipoprotein (LDL) contents in hemolymph of 5-day-old larvae ($P<0.05$), with minimum values observed at 1.82, 1.82, 1.22, and 1.52 mg/g, respectively. (3) Total antioxidant capacity (T-AOC) in both 5- and 7-day-old larvae and superoxide dismutase (SOD) activity in 5-day-old larvae increased significantly with dietary pantothenic acid level ($P<0.05$). (4) Dietary pantothenic acid level significantly influenced the relative expression levels of pantothenate kinase 4 (PANK4) and phosphopantothenoylcysteine decarboxylase (PPCDC) genes in 5-day-old larvae ($P<0.05$), with maximum expression at 1.82 mg/g. Based on fitting curves for 5-day-old larval dry weight and eclosion rate, the appropriate dietary pantothenic acid level for *Apis mellifera ligustica* worker bee larvae is 1.85–2.01 mg/g.

Keywords: pantothenic acid; *Apis mellifera ligustica*; worker bee; larvae; appropriate level

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Pollen and nectar, natural bee feeds, are rich in pantothenic acid [1-2]. Studies

have shown that pantothenic acid promotes growth [3], provides antioxidant effects [4-5], improves learning and memory [4], and enhances intestinal immunity [5]. Therefore, investigating the nutritional requirements of bees for pantothenic acid has important guiding significance for healthy beekeeping. In recent years, pantothenic acid has been extensively studied in poultry, ruminants, mice, and aquatic animals. Research has found that pantothenic acid can improve the metabolic rates of crude protein, crude fat, calcium, and phosphorus in broiler chickens [6]. Adult ruminants can synthesize pantothenic acid in the rumen to meet growth requirements, but young ruminants with incomplete rumen function require dietary supplementation to promote growth [3]. Dietary pantothenic acid supplementation can improve lipid antioxidant capacity and learning/memory abilities in mice [4] and enhance intestinal mucosal immunity and antioxidant capacity in grass carp [5]. Currently, research on pantothenic acid in insects is limited. Available literature indicates that pantothenic acid is an essential B vitamin for silkworms [7], and pantothenic acid supplementation can alleviate heat stress and prolong survival time in heat-exposed fruit flies [8]. Some studies suggest that pantothenic acid can promote the development of royal jelly glands in bees and serve as an indicator of royal jelly freshness [9].

Pantothenic acid primarily functions as CoA and acyl carrier protein (ACP) in organisms [10]. CoA is an important coenzyme in many acetylation reactions in carbohydrate, fat, and amino acid metabolism, while ACP plays a role equivalent to CoA in fatty acid carbon chain synthesis [11]. In bees, pantothenic acid forms 4' -phosphopantetheine through pantothenate kinase 4 (PANK4) or through phosphorylation to form 4' -phosphopantothenic acid, which then forms 4' -phosphopantetheine under the action of phosphopantothenoylcysteine synthetase (PPCS) and phosphopantothenoylcysteine decarboxylase (PPCDC), ultimately forming CoA through bifunctional CoA synthase (BCoAS) to participate in the metabolism of three major nutrients via acetylation [12]. Currently, there are few reports on the nutritional value and physiological functions of pantothenic acid in bees, and the nutritional requirements of bees for pantothenic acid have not been reported. Therefore, this study investigated the effects of dietary pantothenic acid level on the growth and development, antioxidant capacity, physiological functions, and expression of CoA synthesis-related enzyme genes in *Apis mellifera ligustica* larvae to determine the appropriate dietary pantothenic acid level.

1.1 Diet Composition

The basal diet was formulated according to Vandenberg et al. [13]. Five pantothenic acid supplementation gradients were designed based on the basal diet, with other nutrients remaining constant, forming five experimental diets. The composition and nutrient levels are shown in Table 1 . Pantothenic acid was supplemented as D-calcium pantothenate (98.30% calcium pantothenate content, with 91.62% active pantothenic acid, produced by Shandong Xinfa Pharmaceutical Co., Ltd., batch number: Lu [Feed][Add] 2014125001). High-performance

liquid chromatography measured the pantothenic acid levels in the five experimental diets as 0.92 (control), 1.22, 1.52, 1.82, and 2.12 mg/g. The prepared diets were stored at 4°C.

1.2 Experimental Design and Management

Sister queen colonies with similar colony strength were selected as experimental colonies. Using a grafting tool, 1,800 one-day-old honeybee larvae were transferred from sister queen colonies to 24-well culture plates (300 L diet per well, pre-warmed). According to a single-factor completely randomized design, larvae were divided into five groups with three replicates per group and 120 larvae per replicate, fed diets containing 0.92, 1.22, 1.52, 1.82, and 2.12 mg/g pantothenic acid, respectively. The culture plates were placed in an incubator with 15% glycerol humidification solution (V_{glycerol}:V_{water} = 3:17) [14] (temperature: 34.5°C; relative humidity: 95%). The diet was changed daily. On day 7, when larvae began defecating, they were transferred to 24-well plates lined with sterile filter paper for pupation (feeding stopped). The incubator temperature was maintained at 34.5°C and relative humidity at 75%.

1.3.1 Larval Body Weight Measurement

Three 5-day-old larvae and two 7-day-old larvae were randomly selected from each replicate, placed in EP tubes (pre-dried to constant weight and weighed), and weighed using an analytical balance. The difference between this weight and the tube weight was recorded as fresh weight. The larvae were then dried in an oven at (102$±$2)°C to constant weight, and the difference between this weight and the tube weight was recorded as dry weight.

1.3.2 Crude Protein Content Determination

Two 5-day-old and two 7-day-old worker bee larvae were randomly selected from each replicate and weighed, then dried at 65°C and weighed. The samples were transferred without loss to Kjeldahl digestion tubes, with 0.4 g copper sulfate pentahydrate and 6 g anhydrous sodium sulfate added, followed by 10 mL concentrated sulfuric acid. The tubes were digested on a digestion furnace in a fume hood for 5 hours. After the digestion solution became clear and cooled, a VELP automatic Kjeldahl nitrogen analyzer was used for titration, and the volume of hydrochloric acid used was recorded. The crude protein content was calculated as follows:

$$\omega(\mathrm{CP}) = \frac{(V_1 - V_2) \times c \times 0.014 \times 6.25}{m}$$

where ω(CP) is crude protein content (%), c is the concentration of standard hydrochloric acid titration solution (mol/L), m is sample mass (g), V_1 is the volume of standard hydrochloric acid titration solution required for sample titration (mL), and V_2 is the volume required for blank titration (mL).

1.3.3 Crude Fat Content Determination

Two 5-day-old and two 7-day-old worker bee larvae were randomly selected from each replicate and weighed, then dried at 65°C and weighed. The samples were placed in 5 mL centrifuge tubes and homogenized with a glass rod [pre-rinsed with 2 mL chloroform-methanol solution (2:1)], then 2 mL chloroform-methanol solution was added, mixed well, and extracted for 24 hours. After centrifugation at 3,000 r/min for 10 min, the supernatant was transferred to another centrifuge tube. The residue was mixed with 2 mL chloroform-methanol solution, centrifuged at 3,000 r/min for 10 min, and the supernatant was combined with the previous one. Then 1.2 mL of 1.6% calcium chloride solution was added to the tube containing the supernatant, mixed with a magnetic stirrer, left to stand for 1 hour, and centrifuged at 3,000 r/min for 10 min. The supernatant was removed. Next, 1 mL of the upper layer of 2% calcium chloride-chloroform-methanol (3:8:4) mixture was slowly added, centrifuged at 3,000 r/min for 10 min, and the upper layer was removed. The lower layer was transferred to a pre-weighed bottle (mass m_1), dried at 70°C, and weighed (m_2). The difference between m_2 and m_1 was the crude fat content of the larvae [15].

1.3.4 Hemolymph Biochemical Indices Determination

Using a 20 L capillary tube, hemolymph was collected from three 5-day-old and three 7-day-old worker bee larvae into 1.5 mL centrifuge tubes containing phenylthiourea and stored at -80°C. For measurement, samples were centrifuged at 3,000 r/min for 10 min at 4°C, and the supernatant was collected. A Hitachi 7020 automatic biochemical analyzer was used to determine total protein (TP), triglyceride (TG), total cholesterol (TCHO), high-density lipoprotein (HDL), and low-density lipoprotein (LDL) contents in larval hemolymph.

1.3.5 Antioxidant Indices Determination

One 5-day-old and one 7-day-old worker bee larva were randomly selected from each replicate and weighed. Physiological saline was added at a 1:9 mass-to-volume ratio for tissue homogenization in centrifuge tubes, then prepared into 10% and 1% homogenates with physiological saline. After centrifugation at 3,000 r/min for 10 min at 4°C, the supernatant was collected. The 10% homogenate was used to determine malondialdehyde (MDA) content and total antioxidant capacity (T-AOC), while the 1% homogenate was used to determine protein concentration and superoxide dismutase (SOD) activity. MDA content, SOD activity, and T-AOC were measured using MDA kit (A003-1), SOD kit (A001-1), and T-AOC kit (A015), respectively (all from Nanjing Jiancheng Bioengineering Institute). MDA content, SOD activity, and T-AOC were all corrected by larval protein concentration.

1.3.6 Determination of Relative Expression Levels of CoA Synthesis-Related Enzyme Genes

Total RNA was extracted using the Trizol method, and reverse transcription kit (TaKaRa) was used to reverse transcribe the extracted total RNA samples into cDNA, which was stored at -20°C. Then 2 L of cDNA (4-fold dilution) was added to a 20 L fluorescence quantification system. According to the fluorescence quantitative PCR kit (TaKaRa) operation guidelines, an American ABI-7500 real-time fluorescence quantitative PCR instrument was used to detect the relative expression levels of target genes. Target gene primer design sequences were obtained from the NCBI database, and Primer 5.0 was used for primer design with β-actin as the internal reference gene. Primers were synthesized by Sangon Biotech Co., Ltd., and primer sequences are shown in Table 2 .

1.3.7 Pupation and Eclosion Rate Determination

Larval growth was observed daily, dead individuals were removed, and the numbers of successfully pupated and eclosed individuals were recorded. Pupation rate (%) = (total number of pupated larvae / total number of larvae) × 100; Eclosion rate (%) = (total number of eclosed individuals / total number of pupated larvae) × 100 [16].

1.4 Data Processing and Analysis

Data were analyzed using one-way ANOVA and Duncan' s multiple comparison in the General Linear Model (GLM) of SAS 9.1.3 statistical software. Results are expressed as mean ± standard error, with $P < 0.05$ as the criterion for significant difference.

2.1 Effects of Dietary Pantothenic Acid Level on Body Weight of *Apis mellifera ligustica* Worker Bee Larvae

Dietary pantothenic acid supplementation significantly increased the fresh weight of 5- and 7-day-old larvae ($P<0.05$), which first increased and then decreased with increasing dietary pantothenic acid levels (Figure 1 [Figure 1: see original paper]-A). Fresh weight was highest at 1.82 mg/g dietary pantothenic acid for both 5- and 7-day-old larvae, significantly higher than other groups ($P<0.05$), followed by the 2.12 mg/g group.

Similarly, dietary pantothenic acid supplementation significantly increased the dry weight of 5- and 7-day-old larvae ($P<0.05$). Dry weight of both 5- and 7-day-old larvae first increased and then decreased with increasing dietary pantothenic acid levels ($P<0.05$), reaching maximum values at 1.82 mg/g dietary pantothenic acid (Figure 1-B).

Based on the fitting regression curve between 5-day-old larval dry weight and dietary pantothenic acid level (Figure 2 [Figure 2: see original paper]), the

appropriate dietary pantothenic acid level for *Apis mellifera ligustica* worker bee larvae was determined to be 1.85 mg/g.

2.2 Effects of Dietary Pantothenic Acid Level on Body Composition of Worker Bee Larvae

As shown in Table 3 , dietary pantothenic acid supplementation significantly affected crude fat content in larvae ($P<0.05$). With increasing dietary pantothenic acid levels, crude fat content in both 5- and 7-day-old larvae continuously increased, reaching the highest value at 2.12 mg/g dietary pantothenic acid, but without significant difference from the 1.82 mg/g group ($P>0.05$). Dietary pantothenic acid supplementation had no significant effect on crude protein content in larvae ($P>0.05$).

2.3 Effects of Dietary Pantothenic Acid Level on Hemolymph Biochemical Indices of Worker Bee Larvae

As shown in Table 4 , dietary pantothenic acid level significantly affected TP, TCHO, HDL, and LDL contents in hemolymph of 5-day-old worker bee larvae ($P<0.05$). Compared with the control group, TP and HDL contents in hemolymph of 5-day-old larvae in all pantothenic acid-supplemented groups were significantly reduced ($P<0.05$). However, with increasing dietary pantothenic acid levels, TCHO and HDL contents in hemolymph first decreased and then increased, with TCHO reaching its minimum at 1.82 mg/g dietary pantothenic acid and HDL reaching its minimum at 1.22 mg/g.

Dietary pantothenic acid supplementation had no significant effects on TP, TG, HDL, and LDL contents in hemolymph of 7-day-old worker bee larvae ($P>0.05$), but significantly affected TCHO content ($P<0.05$). With increasing dietary pantothenic acid levels, TCHO content in hemolymph first decreased and then increased, reaching its minimum at 1.82 mg/g dietary pantothenic acid, but without significant difference from the 1.52 and 2.12 mg/g groups ($P>0.05$).

2.4 Effects of Dietary Pantothenic Acid Level on Antioxidant Indices of Worker Bee Larvae

As shown in Table 5 , at 5 days of age, dietary pantothenic acid level significantly affected larval T-AOC ($P<0.05$), which continuously increased with increasing dietary pantothenic acid levels, with significant differences between groups ($P<0.05$), reaching the highest value at 2.12 mg/g dietary pantothenic acid. MDA content in larvae was higher at 1.22 and 1.52 mg/g dietary pantothenic acid, significantly higher than at 0.92, 1.82, and 2.12 mg/g ($P<0.05$). Additionally, dietary pantothenic acid supplementation significantly increased larval SOD activity ($P<0.05$), which continuously increased with increasing dietary pantothenic acid levels, with significant differences between groups ($P<0.05$).

At 7 days of age, dietary pantothenic acid supplementation significantly increased larval T-AOC ($P<0.05$), which continuously increased with increasing dietary pantothenic acid levels, reaching the highest value at 1.82 mg/g dietary pantothenic acid. Dietary pantothenic acid level had no significant effects on larval MDA content or SOD activity ($P>0.05$).

2.5 Effects of Dietary Pantothenic Acid Level on Expression of CoA Synthesis-Related Enzyme Genes in Worker Bee Larvae

As shown in Figure 3 [Figure 3: see original paper]-A, compared with the control group, the relative expression levels of PANK4 in 5-day-old larvae were significantly increased in the 1.52 and 1.82 mg/g pantothenic acid groups ($P<0.05$), and the relative expression levels of PPCDC were significantly increased in the 1.22 and 1.82 mg/g groups ($P<0.05$). As shown in Figure 3-B, dietary pantothenic acid supplementation increased the relative expression levels of CoA synthesis-related enzyme genes in 7-day-old larvae, but the increases were not significant ($P>0.05$).

2.6 Effects of Dietary Pantothenic Acid Level on Pupation and Eclosion Rates of Worker Bee Larvae

As shown in Table 6 , dietary pantothenic acid level had no significant effect on pupation rate ($P>0.05$), but significantly affected eclosion rate ($P<0.05$). With increasing dietary pantothenic acid levels, eclosion rate showed an upward trend, with the 1.82 and 2.12 mg/g groups being significantly higher than the control group ($P<0.05$), but without significant difference between these two groups ($P>0.05$).

Based on the fitting curve equation between larval eclosion rate and dietary pantothenic acid level (Figure 4 [Figure 4: see original paper]), the optimal dietary pantothenic acid level for *Apis mellifera ligustica* worker bee larvae was determined to be 2.01 mg/g.

3.1 Dietary Pantothenic Acid Level Affects Development of *Apis mellifera ligustica* Worker Bee Larvae

Honeybees undergo metamorphic development, experiencing four stages: egg, larva, pupa, and adult. Normal growth at each stage depends on nutritional status and resistance to external environmental stress. Pantothenic acid is a precursor of CoA, which participates in protein synthesis, transformation, and lipid metabolism in organisms. Pantothenic acid also promotes fatty acid synthesis through its acyl carrier protein form [5,17], and the balance between lipid and protein metabolism is crucial for bee development. Pupation rate reflects the success rate of transition from larval to pupal stage, while eclosion rate reflects the success rate of transition from pupal to adult stage. Increased pupation and

eclosion rates indicate that pantothenic acid promotes bee development. In this study, dietary pantothenic acid level had no significant effect on pupation rate of *Apis mellifera ligustica* worker bee larvae, but pupation rates in pantothenic acid-supplemented groups were generally higher than in the unsupplemented control group. Pantothenic acid supplementation significantly increased larval eclosion rate, reaching the highest value at 2.12 mg/g dietary pantothenic acid. Liu et al. [18] reported that appropriate pantothenic acid supplementation increased crude protein and crude fat contents in grass carp. Huang et al. [19] found that appropriate pantothenic acid supplementation increased crude fat content in the body but decreased crude fat content in the liver of GIFT tilapia. Bees lack liver tissue, but their fat body performs equivalent functions [16]. In this study, appropriate dietary pantothenic acid increased crude fat deposition and body weight of 5- and 7-day-old *Apis mellifera ligustica* worker bee larvae, consistent with findings in fish (grass carp [5,18], blue tilapia [20], cobia [21], GIFT tilapia [19]) and poultry (breeding chickens [22], broiler chickens [6], geese [23]). The mechanism by which pantothenic acid affects bee pupation and eclosion rates may involve promoting fat synthesis through its acyl carrier protein form [6] to store energy for molting.

Insect hemolymph functions as both blood and lymph. Blood nutrients come from metabolic products of digestive organs and decomposition products of tissue cells, and stable blood biochemical properties reflect organism health [24]. The amount of TP storage directly affects insect metamorphic development and serves as an important source for tissue construction [25]. Blood cholesterol content reflects free fatty acid deposition in the body. The results of this study showed that TP content in hemolymph of 7-day-old larvae in pantothenic acid-supplemented groups was higher than in the unsupplemented control group, indicating that pantothenic acid supplementation benefits TP synthesis in worker bee larvae. Conversely, pantothenic acid affected TG, TCHO, HDL, and LDL contents in hemolymph, suggesting that pantothenic acid supplementation enhances nutrient metabolism. Excessive accumulation of TG, TCHO, and LDL is considered detrimental to organisms and may cause cardiovascular diseases [26]. However, no reports have been published on the effects of pantothenic acid supplementation on TP, TG, TCHO, HDL, and LDL contents in bee hemolymph.

3.2 Dietary Pantothenic Acid Level Affects Antioxidant Capacity of *Apis mellifera ligustica* Worker Bee Larvae

Numerous studies have investigated how nutrients can improve bee antioxidant capacity, but whether pantothenic acid can enhance bee antioxidant capacity has not been reported. An in vitro study on tumor cells reported that pantothenic acid and its derivatives could resist lipid antioxidant damage to cell membranes [27], indicating that pantothenic acid has potential antioxidant capacity. MDA is one of the main end products of lipid peroxidation, and its production can exacerbate cell membrane damage [28-29]. MDA content is an important marker for evaluating oxidative damage. T-AOC and SOD are impor-

tant components of the antioxidant system, and their activities are proportional to free radical scavenging capacity, thus indirectly reflecting the degree of tissue cell peroxidation and free radical production. Therefore, lower MDA content and higher T-AOC and SOD activity indicate better antioxidant status. The comprehensive results of this study indicate that the best antioxidant status in *Apis mellifera ligustica* worker bee larvae occurred at dietary pantothenic acid levels of 1.22–1.52 mg/g.

3.3 Dietary Pantothenic Acid Level Affects Expression of CoA Synthesis-Related Enzyme Genes

Branched-chain amino acid aminotransferase (BCAT) can promote pantothenic acid formation by catalyzing amino group transfer from branched-chain amino acids. PANK4 plays a role in the initial step of pantothenic acid metabolism and is the rate-limiting enzyme in the CoA synthesis pathway [30]. Pantothenic acid ingested by bees from the diet is degraded to CoA through the actions of PANK4, PPCS, PPCDC, and BCoAS, which then participates in nutrient metabolism. The results of this study showed that the relative expression levels of CoA synthesis-related enzyme genes in pantothenic acid-supplemented groups were generally higher than in the unsupplemented control group, suggesting that dietary pantothenic acid supplementation may promote CoA formation by increasing the expression of CoA synthesis-related enzymes. This study demonstrated that pantothenic acid supplementation significantly affected the expression of PANK4 and PPCDC, though no reports have been published on the effects of dietary pantothenic acid level on its metabolism-related enzymes. In this study, the relative expression levels of BCAT in pantothenic acid-supplemented groups were higher than in the control group. Zhang et al. [31] reported that BCAT expression is positively correlated with the malignancy degree of gliomas, but current research on pantothenic acid and BCAT has mostly focused on nutrition, with no reports on the effects of pantothenic acid on BCAT.

Based on fitting curves for 5-day-old larval dry weight and eclosion rate, the appropriate dietary pantothenic acid level for *Apis mellifera ligustica* worker bee larvae is 1.85–2.01 mg/g.

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