Dietary Vitamin D_3 Impact on Survival and Development of *Galleria mellonella* (Lepidoptera: Pyralidae) Reared on an Artificial Diet[1]

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Abstract *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae were reared on an artificial diet containing vitamin D_3, also known as cholecalciferol, at concentrations of 0.01, 0.10, or 1.00% (g/100 g of diet) to the adult stage under laboratory conditions. Survival rate and development through adult emergence were observed and recorded. Survival rate in postlarval stages was significantly reduced in response to increased vitamin D_3 concentrations. The tested concentrations of vitamin D_3 (0.01–1.00%) decreased adult emergence in comparison with the control; however, only the highest concentration (1.00%) prolonged the duration of the adult stage. The 1.00% concentration also significantly reduced pupal survival rate from mean (± standard error) of 85.00% ± 0.35% to 63.75% ± 1.14% for pupation, whereas adult emergence decreased to 42.50% ± 0.25%. However, it prolonged the duration of adults from 40.67 ± 1.80 d for the control to 46.07 ± 2.28 d. Although the highest concentration showed a prolongation in duration of the pupal and adult stages, we observed no statistical effects compared with the control group. Our results, however, indicate that vitamin D_3 may play a fortifying role in development as a nutritional additive for larval development, or as a toxin that may be used in small concentrations on postlarval developmental stages. Further research should assess if increased concentrations improve or deteriorate life table parameters in target insects.

Key Words developmental period, *Galleria mellonella*, survival rate, vitamin D_3

In recent years, chemical substances have been frequently used to control agricultural pests and reduce their populations. However, the use of environmentally friendly agents in combating agricultural pests is very important. In this context, new chemical substances are investigated on model organisms under laboratory conditions. The greater wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae), is widely used as a model organism in feeding physiology, biochemistry, molecular biology, and insecticidal effect studies (Kaczmarek and Boguś 2025; Kazek et al. 2024; Sugeçti et al. 2016, 2021). *Galleria mellonella* is also known as an agricultural pest as larval feeding causes serious economic losses in beehives (e.g., wax and honey) (Kwadha et al. 2017). Many studies have been conducted to rear this harmful insect using an artificial

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diet containing antifungals, antibiotics, and anthelmintics because of their potential to be used as a physiological model (Çelik et al. 2019, Kastamonuluoğlu et al. 2020, Keleş et al. 2021).

Dietary vitamin D is important in maintaining bone and blood calcium levels and regulating the immune system in humans and other animals (Aranow 2011, Cantorna et al. 2004). However, it is not yet fully known whether there is a direct relationship between the insect immune system and vitamin D (Oonincx et al. 2018). In addition to its important physiological roles, this vitamin also has toxic effects at high dietary concentrations (Jones 2010). There is no information on the use of vitamin D_3 in the control of agricultural pests. Thus, *Galleria mellonella* was used in this study to investigate the effect of vitamin D_3 on insects. The study aimed to test the hypothesis that dietary vitamin D_3 affects biological fitness in *G. mellonella*. Vitamin D_3 was registered as a pesticide (rodenticide) by the U.S. Environmental Protection Agency in 1984, in the form of a nutritional mixture of 0.075%, applied topically or manually against rodents (e.g., rats and mice) in and around closed areas and in transport vehicles (EPA 1984). Our objective was to assess the effect of vitamin D_3 incorporated in an artficial diet on the survival and development of *G. mellonella* larvae, pupae, and adults.

Materials and Methods

Insects. The *G. mellonella* colony from which the test insects were obtained had been established and maintained at our university. Neonates were placed on a semisynthetic artificial diet consisting of wheat bran (420 g), liquid honey (150 ml), glycerin (150 ml), ground dark honeycombs (20 g), and distilled water (30 ml) as per Bronskill (1961). Preparation of the diet and methods of handling developmental stages of the insect have been described in previous studies by Büyükgüzel et al. (2010) and Çelik et al. (2019). The colony was maintained in an environmentally controlled chamber (Nuve FN 400 and 500) at 28°C $\pm$ 2°C, 65% $\pm$ 5% relative humidity, and in continuous darkness. All experiments to study the effect of vitamin D_3 on the insects were conducted under these same conditions.

Feeding experiments. Vitamin D_3 (C9756; $\geq$98%; $C_{27}H_{44}O$; cholecalciferol, activated 7-dehydrocholesterol, calciol [Sigma-Aldrich, St. Louis, MO]) was added to the diet during its preparation. Preliminary experiments estimated dietary concentrations of 0.01, 0.10, and 1.00 g per 100 g of diet (% w/w), along with a control diet without vitamin D_3. The experimental vitamin D_3 concentrations were calculated depending on our previous studies (Aslan et al. 2019, 2025; Keleş et al. 2021; Özdoğan et al. 2024).

Survival rate and development. Twenty newly hatched larvae were placed on the respective diet treatments (0, 0.01, 0.10, 1.00%) for each replicate and allowed to feed for the duration of their larval development. Treatments were replicated four times. Survival and development time in the larval, pupal, and adult stages were recorded. The number of seventh-instar larvae was counted and recorded as a percentage; developmental time was recorded in days. Mature larvae (seventh instars) were transferred to separate jars lined with tissue paper to allow for pupation. After pupation and adult emergence, numbers of pupae and adults were recorded and their developmental times were calculated for each replicate.

Data analysis. The chi-square test (Snedecor and Cochran 1989) was used to compare treatment data using IBM SPSS statistics (version 28.0; 2021). Treatment

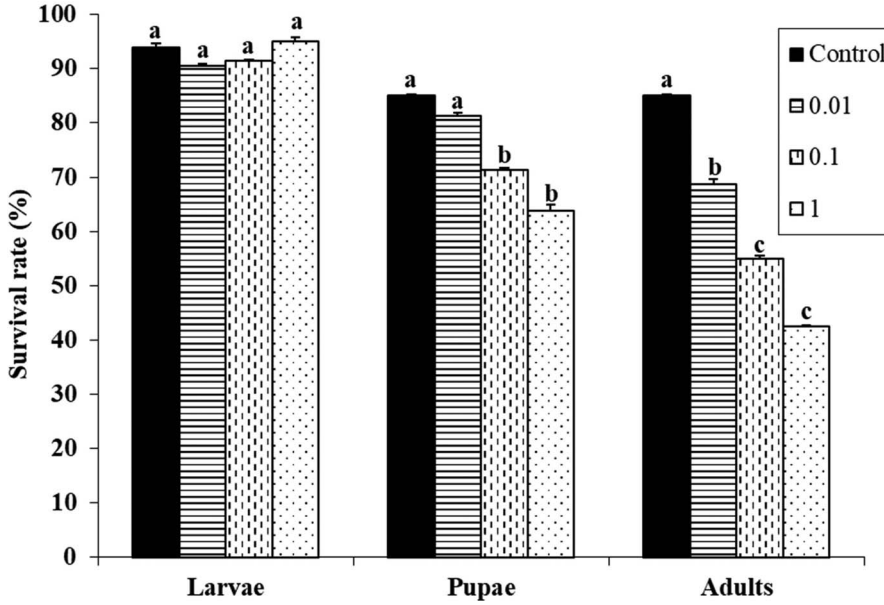

Fig. 1. Survival of *Galleria mellonella* larvae, pupae, and adults fed dietary vitamin D_3 in an artificial diet. Vertical lines above the means indicate ± standard error. Treatment means within a developmental stage with different lowercase letters are significantly different ($P < 0.05$, chi-square test).

data (e.g., life parameters) were compared using one-way analysis of variance. The least significant difference test was used to determine differences among significantly different means using a *P* value of 0.05.

Results

The chi-square analysis found no statistically significant ($P > 0.05$) differences between the control treatment and the three concentrations of vitamin D_3 in larval survival rate (Fig. 1). Mean (± standard error [SE]) survival was 94.00% ± 0.70% in the control, whereas survival in the 0.01, 0.10, and 1.00% concentrations were 90.45% ± 0.54%, 91.50% ± 0.25%, and 95.00% ± 0.75%, respectively. Larvae fed on dietary vitamin D_3, however, exhibited significantly lower survival in comparison with the control in the postlarval stages. Mean (±SE) pupal survival in the control was 85.00% ± 0.35%, whereas at the 0.01, 0.10, and 1.00% concentrations survival was significantly lower, with 81.25% ± 0.54% at 0.01% ($\chi^2 = 0.567$; df = 1; $P > 0.05$), 71.25% ± 0.41% at 0.10% ($\chi^2 = 5.711$; df = 1; $P < 0.05$), and 63.75% ± 1.14% at 1.00% ($\chi^2 = 11.607$; df = 1; $P < 0.05$). We recorded a similar pattern in the survival rates of the adults. At the highest concentration tested, adult emergence was significantly lower than that observed in the control ($\chi^2 = 38.281$; df = 1; $P < 0.05$). Likewise, adult emergence was significantly lower with the 0.01% concentration

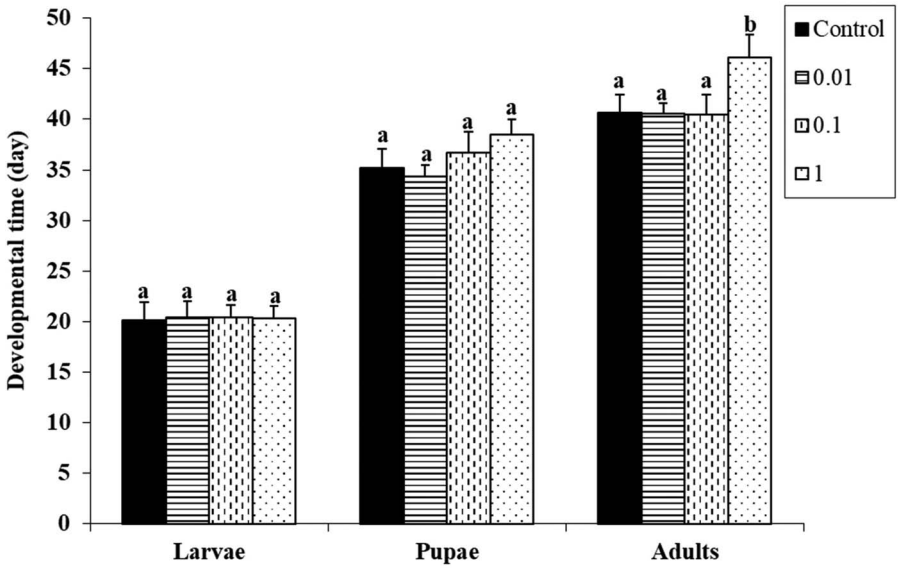

Fig. 2. Comparison of duration of developmental stages of *Galleria mellonella* fed different concentrations of vitamin D_3 in an artificial diet. Vertical lines above the means indicate ±standard error. Treatment means within a developmental stage with different lowercase letters are significantly different ($P < 0.05$, least significant difference).

($\chi^2 = 7.228$; df = 1; $P < 0.05$) and the 0.10% concentration ($\chi^2 = 21.429$; df = 1; $P < 0.05$) (Fig. 1).

Larval feeding on dietary vitamin D_3 resulted in a longer larval and pupal developmental period than the control; however, none of these differences was statistically different compared with the control group (Fig. 2). Consumption of the diet containing 1.00% vitamin D_3 increased the duration of the pupal stage from mean (±SE) of 35.21 ± 1.90 d for the control to 38.48 ± 0.50 d for the vitamin D_3 treatment; however, the difference was not statistically significant ($F = 0.114$; df = 3; $P > 0.05$). This high concentration also extended the duration of the adult stage from 40.67 ± 1.80 d for the control to 46.07 ± 2.28 d, which was statistically significant ($F = 1.551$; df = 3; $P < 0.05$) (Fig. 2).

Discussion

Although the importance of vitamin D in vertebrate physiology is well documented, little is known for invertebrates except for determination of adequate dietary vitamin D concentration required for growth and development (Shiau and Hwang 1994). Furthermore, its metabolism has not been studied in insects. Although their physiological roles are unknown, vitamin D and its metabolites have been identified in several insect species. This study explored the biological effects of fat-soluble vitamin D_3, one of the micronutrients required for growth and development, on *G. mellonella*, which is

frequently used as a model insect. In particular, the possible toxic effects of high doses of vitamin D_3 on biological parameters were examined.

The micronutrient requirements of organisms are important according to major factors such as age and gender. Micronutrients are substances that are necessary in trace amounts for optimum metabolism, homeostasis, and a healthy life and that function as coenzymes in various biochemical reactions. Although vitamins are small molecules, their physiological functions are of critical importance. Vitamin D and its metabolites play an important role in calcification in living things and in maintaining the calcium and phosphorus balance in body fluids. Excessive intake of vitamins A and D can result in vital physiological disorders and even death. Conditions that occur as a result of excessive intake of these vitamins or excessive storage in the body are known as hypervitaminoses. Hypervitaminosis D manifests itself in vertebrates with clinical symptoms such as morphological anomalies and crippled immune systems, leading to deteriorated survival (Marcinowska-Suchowierska et al. 2018). These results in vertebrates led us to investigate the biological and biochemical effects of vitamin D on a model invertebrate organism, *G. mellonella*, and to determine whether it would have similar toxic effects at high concentrations. Therefore, this study will also provide preliminary information for the use of vitamin D as an insecticide in the control of agricultural pests.

Fat-soluble vitamins in vertebrates can be toxic at high concentrations, and this can affect the metabolic system in insects as well, both physiologically and biochemically. The effects of vitamin D_3 on calcium accumulation or depletion, depending on changes in concentrations, have been studied in some insects without ultraviolet (UV) B exposure (Bah-Nelson et al. 2021). *Drosophila melanogaster* (Meigen) larvae fed with low doses of vitamin D_3 exhibited normal development with no morphological changes. At high concentrations (especially 80–2,560 mg/L), however, increased melanization was observed in the abdominal region of the insects (Özdoğan et al. 2024). These findings suggest that vitamin D_3 may play a development-supporting role as a food additive at low concentrations but may have a lethal effect on insects at high concentrations.

Similarly, as seen in previous studies (House and Barlow 1965, Thompson 1986), determining only the changes in survival rate and developmental duration may sometimes be insufficient to understand the adverse effects caused by a dietary nutritional or nonnutritional additive and the mechanisms of these effects. This preliminary study is very important as it is an attempt to determine the physiological effects of vitamin D_3, which is an important micronutrient for all living things and has recently been determined to have hormonelike effects on the developmental biology of *G. mellonella*. Thus, it was thought that vitamin D_3 could be used as an environmentally friendly alternative method for the chemical control of pest insects at high concentrations. The effects of vitamin D_3 on the survival and development of insects at low and high concentrations were investigated in detail to understand their biological functions and to evaluate their potential importance as an environmentally friendly insecticide. The results of this study showed that survival rate to adult stage and development period are important parameters in determining the effect of vitamin D_3 on *G. mellonella* and possibly for other pest insects.

The negative effect of vitamin D_3 on the survival rate in the pupal and adult stages of *G. mellonella* increased with increasing concentrations, indicating the most toxic effect at high dietary concentrations. Careful regulation of vitamin D_3 doses in diets is

important for maintaining chemical pest control without environmental impairment. Oh et al. (2021) conducted a 2-yr study on Gwanankong soybean enriched with vitamin A, revealing significant differences in insect populations grown in different regions and conditions. Therefore, this study highlights the multifaceted effects of vitamin supplementation and pest-control strategies.

The study conducted by Oonincx et al. (2010) on vitamin D_3 supplementation in the vertebrate *Pogona vitticeps* Ahl, commonly known as the bearded dragon, showed that oral supplementation did not have a positive effect on survival in increasing plasma $25(OH)D_3$ and $1,25(OH)_2D_3$ to normal levels, and activation from precursor molecules by exposure to UVB was more effective. It is not known if UVB activates the precursors of vitamin D_3 to active form or not; thus, dietary concentrations of vitamin D_3 should be well adjusted according to the purpose of use on insects. Inconsistent with our suggestion, Jin et al. (2024) reported that the effects of dietary vitamin D_3 in combination with different yeast levels on the lifespan of *D. melanogaster* is diet and concentration dependent.

Our study is the first to show biological effects of dietary vitamin D_3 as a nutritive component on *G. mellonella*, leading to decreased survivorship and delayed developmental period at high concentrations. We infer from these results that vitamin D_3 can also be used as an alternative environmentally sound insecticide in the control of pest insects at concentrations above the nutritional needs of the insect. Investigations are in progress to determine the molecular mode of action of this vitamin on survival and development of *G. mellonella*.

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