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Research Article

Observations on the Life Stages of the Fruit Fly *Drosophila melanogaster* When Fed Vitamin D3 in Artificial Diet [1]

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Abstract

This study focused on the use of an important micronutrient, vitamin D3 for control of pest insects as environmentally sound alternative management. Vitamin D3 which is a lipophilic vitamin at nutritionally reguired doses not only have important physiological roles but also has a toxic effect at high dietary concentrations. The first instar larvae of *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae), which is an important pest model organism were reared on artificial diet containing vitamin D3 concentrations of 5.0, 20, 80, 320, 1280 and 2560 mg/L to adult stage in laboratory condition. The effects of this vitamin on developmental stages of the insects were microscopically observed. Morphometric changes depending on increased vitamin D3 concentrations in the developmental stages from larvae to adults were recorded by microscobic and personal observations. Vitamin D3 at increasing concentrations (especially 80-2560 mg/L) in larval diet increased melanization in abdominal region of the insect. This study refers vitamin D3 can play a fortifying role in development as a nutritional additive at low concentrations, or can be used as an insecticide at high concentrations due to its possible toxic effect.

Keywords: Developmental stages, *Drosophila melanogaster*, Microscopic observation, Vitamin D3

Introduction

Vitamin D is the general termination for a group of nutritional substance ensuring in balanced bone and blood calcium levels and immunmodulation function in human and animals. Vitamin D3 is the main dietary resource for vitamin D. A large amount of vitamin D (estimated around 80% but varies with sun exposure) is biosynthesized in the skin from 7-dehydrocholecalciferol as the function of ultraviolet B (UVB) rays. While 25-hydroxy cholecalciferol (25(OH)D) and 1, 25-dihydroxycholecalciferol (1,25(OH)2D) are source of vitamin D from diet, 1,25-dihydroxycholecalciferol (1,25(OH)2D), the most hormonally active form of vitamin D [1]. However, besides the innate immune system, it has autocrine and paracrine roles in cell propagation, differentiation, and programmed cell death [2-4].

There is no direct relationship between the immune system of insects and vitamin D, because it is still unknown whether insects can synthesize vitamin D3 de novo or activate it from precursor molecules with UV-B. Oonincx et al.[5], showed that when four insects which has different in ecology, migratory grasshopper, *Locusta migratoria* (L.), house cricket *Acheta domesticus* (L.), yellow mealworm, *Tenebrio molitor* and the black soldier fly (BSFL) *Hermetia illucens* (L.) were exposed to a low-irradiation UV-B source, vitamin D3 levels in house crickets, vitamin D2 levels in BSFL, and vitamin D2 and vitamin D3 in *T. molitor* were increased. Higher UV-B irradiation increased vitamin D3 concentrations in all insects except *H. illucens*. An increase in vitamin D2 was observed in both *H. illucens* and *L. migratoria*. Bah-Nelson et al.[6], found that the unirradiated Black field

locust *Gryllus bimaculatus* did not have detectable vitamin D3 content (below 0.50 IU/g) and irradiation with UV-B light did not enhance its synthesis, or at least the levels were still undetectable. These studies showed that some insects can synthesize vitamin D de novo and the amounts are dependent on UV-B irradiation and exposure time.

Although its exact physiological role is not known in various insect species where vitamin D has been detected, concentrations varied greatly between species. For example, vitamin D1 was low during larval to pupal transformation (Sláma's sterol reuse theory, 1998) and highest in the pupa ensuring advanced metamorphosis which is responsible for the development of digestive and muscular systems in *Galleria mellonella* adult. It has been revealed that vitamin D1 in *Manduca sexta* has a role in tissue regeneration after epidermis injury [7].

Drosophila melanogaster (Meigen) (Diptera: Drosophilidae) is a major pest for different fruit crops in agricultural area and has developed resistance to insecticide [8]. Therefore, the use of any nutritional micronutrient such as vitamin D or its metabolites can serve as an alternate strategy for *D. melanogaster* management. It has been shown that nutritional vitamin perception is an important determinant of nutritional behavior and plays an important role in development in *D. melanogaster* [9]. There are most important advantages for this insect as model in investigations such as ahving short life cycle, high reproduction capacity and low laboratory culturing costs. Since the fruit fly has similar biological, biochemical, physiological and neurological traits with mammals, this insect is used as an alternative model animal for vertebrates [10,11]. Using this insect as a model, the preventive effect of vitamin D3 on the mutagenicity and carcinogenicity of some chemicals in *D. melanogaster* was studied [12]. Vitamin D3 alone did not increase the overall tumor incidence, but significantly reduced the overall tumor incidence when co-administered with a tumor stimulator, doxorubicin (DXR), in its somatic cells in this insect. There are no studies on the other hand, most of the studies investigated the effects of vitamin A (retinol) and E (α-tocopherol), which are fat-soluble vitamins added to food in low amounts, and water-soluble vitamin C (ascorbic acid) on the longevity of *D. melanosgaster* in relation to its antioxidant properties under oxidative stress conditions [13]. In this study, it has been shown that the intensity of red color in the abdomen increased due to the increase in food intake as a result of feeding the adults with a special dye (0.2% sulforhodamine) added to the foods to which the vitamins were added [13]. For nutrional requirements, 0.00067 g vitamin D3 (500.000 IU/g) was added to the liter of the food to meet the vitamin D requirement for certain foods [14]. In some other pest insects, Amiri et al.[15], showed a decrease in the consumption rate of *Plutella xylostella* to destruxin, fungal toxin showing antifeedant effects in a dose related manner. Increased mortality of *P. xylostella* larvae with increasing concentrations of destruxin A was also reported [16]. In the light of these studies, some of vitamin D metabolites or other micronutrients especially at high concentrations have been demonstrated to have had some deteriorating effects on the insects including *D. melanogaster*. Nevertheless, appearently no researcher has measured or even observed effects of vitamin D3 or other vitamin D metabolites on size or morphological changes in any parts of insects. However, vitamin D3 was registered by the American Environmental Protection Agency in 1984 as a 0.075% nutrient mixture (rodenticide) in and around closed areas, in transport vehicles, against rodents such as rats and mice, by surface or hand application [17].

Melanization is part of the immune defense response after infection or injury in insects and some vertebrates. In this process, melanin pigment is produced and helps neutralize foreign matter or pathogens in the infected or injured area [18]. The prophenoloxidase enzyme in the hemolymph and other tissues is activated in response to the presence of foreign matter or pathogens, and melanin pigment is formed [19]. Melanization in mammals is not as in insects, but similar immune response mechanisms are used.

This study aimed to investigate the effect of dietary vitamin D3 on developmental morphology of *D. melanogaster.* This study is important because it can play a role as a nutritional additive at low concentrations, and it will give an idea whether it can be used as an insecticide at high concentrations due to its toxic effect.

Material and Methods

Ethical Statement

Insect studies do not require ethics committee approval.

Insect Culture

D. melanogaster (Diptera: Drosophilidae) (Oregon R strain) were aseptically reared in 250-mL glass container containing artificial diet in an ES 500 incubator (Nüve, Ankara, Türkiye) at 25±2°C and 60-70% relative humidity (RH) in a 12:12 hour light/dark photoperiod. First instar larvae were selected by removing from the main culture for use in the experiments described here.

The artificial diet used for rearing of the insects and for preparation of treatments was described by Roberts [20]. Briefly, per 1000 mL total volume; diet contained 8 g agar (Merck & Co., New York, USA), 20 g sucrose (Carlo Erba Reagents S.A.S, Sabadell, Barcelona, Spain), 11.78 g dry yeast (Dr. Oetker Food Industry and Trade, Inc., Torbalı-

İzmir, Türkiye), 36 g of potato puree (Knorr, Unilever Co., Ümraniye, İstanbul, Türkiye), 0.8 g L-ascorbic acid (Carlo Erba Reagents S.A.S), 7.72 mL of nipagin (p-hydroxybenzoic acid methyl ester, crystal (Sigma-Aldrich Co., St. Louis, MO, USA) prepared in 3.5% ethanol and 1000 mL of water. The preparation methods of the diet and pouring diets into bottles, and to obtain eggs and larvae and their placement onto diets were described previously by Aslan et al.[21].

Vitamin D3 (C9756; ≥98%; $C_{27}H_{44}O$; Cholecalciferol, Activated 7-dehydrocholesterol, Calciol, Sigma-Aldrich (St. Louis, MO, USA) was added to diets by dissolving in Tween 80 (1% tween 80 in 3% etanol) establish concentrations of 5, 20, 80, 320, 1280 and 2560 mg/L. The control was the diet without vitamin D3 added. Tween 80 control (1% tween 80 in 3% etanol) was also used as solvent control. These vitamin D3 concentrations were calculated depending on to the research with preliminary experiments on *D. melanogaster* or the effect of other nutritional additives on *D. melanogaster* [21,22].

Feeding Experiments

D. melanogaster larvae to be used in the study were obtained from eggs laid by adults placed in glass bottles under condition for maintaining stock culture. The 1st instar larvae hatched in the food from the stock colonies were selected under a stereo microscope. 20 first instar larvae were transferred into 5 mL of diet poured into small 15-mL glass bottles and bottles were covered with hydrophilic cotton. Twenty larvae were transfered on control diet and diets containin vitamin D3 by using a soft-tipped brush (No: 0, Goya Toray). Bottles were kept in incubators maintained at condition used for stock insect cultivation (25±2°C and 60-70% RH on a 12:12 h light/dark photoperiod). We noted that 3rd-instar larvae migrated from the diet to the inside surface of the glass container where they pupated. Newly-formed pupae were marked on the inside surface of the glass and recorded. Survivors to the third instar, pupal stage, and adult stage were photographed (Olympus SZ61, SZ2-LGB) in each rearing bottle to determine the effects of vitamin D3 added into diet at tested concentrations on each developmental stage of the insects. Photographs scale bars were added with the LCmicro (version 5.1) software which is developed by Olympus Soft Imaging Solutions GmbH. The experiments were done as 4 replicates, and 20 larvae were used in each replication.

Statistical Analysis

Average intensity value in pupal stages in each concentrations of vitamin D3 were calculated by applying the photos to Adobe Photoshop [23]. Intensity data were analyzed by one-way "analysis of variance" (ANOVA) [24]. To determine significant differences between means least significant difference (LSD) test was used [24]. Significance was considered at 0.05 level.

Results

Our study showed that increased intensity of melanization occurs towards the last abdominal segments especially in the pupal stage of *D. melanogaster*. The results of the study also show the normal size and appereance of each developmental stage of *D. melanogaster* on the control artificial diet and the small size and abnormal melanization through the last abdominal segments of larval, pupal and adult stages of the insects reared on given dietary concentrations of vitamin D3 as dietary additives *(Fig. 1)*.

Histogram analysis shows average intensity values in pupal stages in different concentrations in comparison to control group *(Table 1)*. High intensive value (92.61±2.39), especially in pupae reared on 20 mg/L was recorded. However low intensity values in pupal stages at 320 and 2560 mg/L of vitamin D3 was recorded in relative to other tested concentrations of vitamin D3.

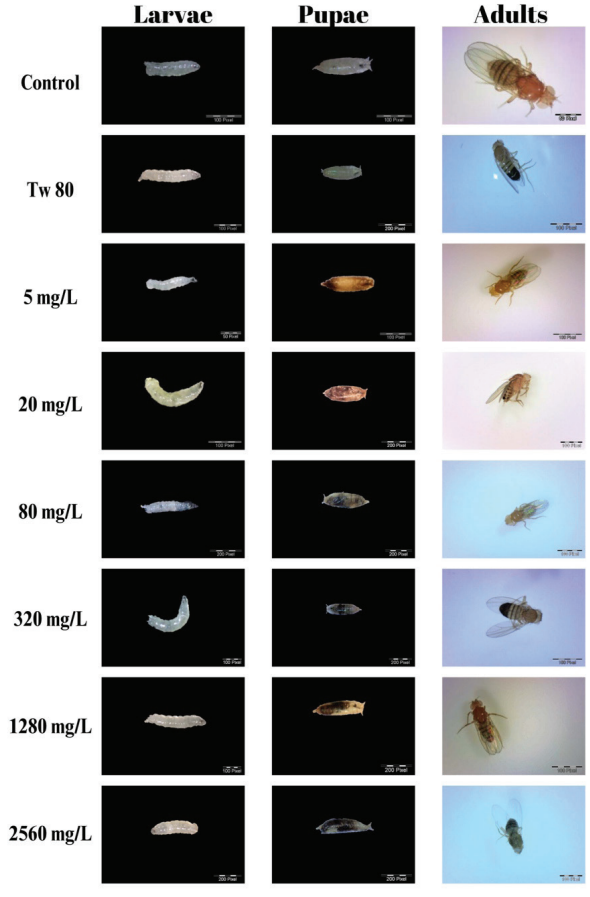

Fig 1. The effects of vitamin D3 on morphology of *D. melanogaster* reared on artificial diet. The magnification was ×20 and the scale bars represent pixels (1 Pixel = 0.14000 µm)

Table 1. *Intensity value of melanization in pupae reraed on artificial diet with vitamin D3*

Vitamin D3 Concentrations (mg/L)	Intensity Value in Pupal Stages (Mean*±S.E)†
0.000§	72.12±1.43[a]
Tw 80	68.74±1.65[a]
5	71.96±2.16[a]
20	92.61±2.39[bc]
80	69.47±3.78[a]
320	59.08±2.87[b]
1280	73.63±3.93[a]
2560	51.19±1.16[b]

**Four replicates with 5 pupae per replicate were used. †Means with the different letter are significantly different (P<0.05). §Control (without Vitamin D3). Tween 80 (Tw 80 solvent control)*

Discussion

The metabolic system of the insect may be affected biochemically by toxic proporties of these fat soluble vitamins at high concentrations as in vertebrates including human and animals. The effects of vitamin D alone have already been known in the some metabolic process of the insects [6]. The feeding experiments might be supported by the imaging techniques to determine their effects on wellfare of insects. Because, determination of only survivorship and developmental period sometimes may be insufficient to ascertain the effects of nutritional impairment of any dietary nutrient [25,26]. This study focused on the use of an important micronutrient, vitamin D3 for control of pest insects as environmentally sound alternative management.

In coincidence with our results with abdominal melanization appearence, abnormalty in vertebrates in relation to melanization is Peutz-Jegher syndrome showing formation of multiple polyps in different regions of the gastrointestinal canals, especially the large intestine [27]. Animals produce melanin pigments for the coloration of their skin and protection from harmful solar radiation. Insects also synthesize melanins even more ingeniously than mammals and use them for exoskeletal pigmentation, cuticular hardening, wound healing and innate immune responses [19]. Firstly, melanin was massively deposited at the wound region in insects for two major reasons with other biochemical processes to prevent further blood loss. Secondly, formed quinonoid products during melanogenic process are well known cytotoxic metabolites to kill any microbial agents entered the insect body through the wound site.

Melanization is an crucial and indispensable phase of the innate immune defence to biotic and abiotic invader [19,28]. The melanization in abdomen region of insects reared vitamin D3 might be attributed to deterioration in guts of larvae which are active feeding stage in response to toxic effects of high concentrations of vitamin D3. For example most insects consumpt the green leaves of plants but excrete black feces in an as yet unknown mechanism similar to our observation after vitamin D3 consumption by larvae. Here we show that the melanization of midgut and most probably hindgut content induced by high concentration of vitamin D3, with its possible toxicity that triggers the melanin synthesis around exces accumulation of vitamin D3 or toxic metabolites or at wound sites formed by toxic effects of vitamin D. Shao et al.[29], found that prophenoloxidase production in hindgut cells and secretion into the hindgut contents causing blackening in a model insect, silkworm *Bombyx mori*. Some researches showed the blackening of the insect feces was due to activated phenoloxidase enzim cascade, which decrease and finally death of bacteria in the hindgut. Our observation of melanization in abdomen region discloses why the fruit fly abdominal region is black showing melanization and provides understanding into innate immunity of hindgut, which is still not known in detailed in insects as stated by Shao et al.[29], for mammals.

High intensive value (92.61±2.39), especially in pupae reared on 20 mg/L may be attributed to melanization reaction as humoral immune adaptation in response to toxicity of this concentration vitamin D3. As in registered rodenticide effects of vitamin D3 [17], compared to other concentrations of vitamin D3, low intensity value of dietary 2560 mg/L vitamin D3 for *D. melanogaster* may be a result of vitamin D3 chronic toxicity because of rapid degradation of melanins. Similar results were obtained by a study with higher animals which reported a correlation between melanotic melanoma and lower survivorship than pigmented melanomas [30]. In the other hand, in consistent with our results showing low intensity value at the highest dose of vitamin D3, melanin might be degraded by a redox mechanism or lysosomally to polycyclic hydrocarbon and may form some reactive oxidant molecules a short after the melanization as suggested by Clancy and Simon [31].

Melanization can also be further induced around wounds to prevent additional mechanical injury in midgut or hindgut of *D. melanogaster* larvae caused by exces consumption of higher dietary vitamin D3 as a possible insecticide. The results of a study with a burrowing bug Cydnidae insects [32], in consistent with our results that this insect synthesize a chemicals such as hydrocarbonate, odorous substance. This causes red-brown volar melanotic pigmented discolored area in diffrent size and appearance at the site of contact with this chemicals on these insects. There is an evidence for our suggestion for vitamin D3 cause pigmentation that when silkworm *Helicoverpa armigera* larvae were orally fed with *Bacillus bombysepticus*, black chest septicaemia

and finally larval death were reported. A mucocutaneous pigmentation (peutz) on the thoracoabdominal region or the first three abdominal segments and then covering the whole body [33].

Consequently, a fat-soluble vitamin D3, as dietary ingredient in quantities above the normal requirement might be crucial nutritional alternatives for pest control. The midgut of insect is the first defensive barrier developing resistance and induces the immune response to fight microbial agents or chemicals exposed such as exsessive accumulation of vitamin D3 in midgut or hindgut of *D. melanogaster* in our study. Absorption rate of other nutrients may be changed as a result of overaccumulation of the vitamin D3 in the gut of the insect as in mammals. It has been already known that bioavailability of vitamin D is important factors including absorption, transportation and metabolism [34].

As Singh and House [35], stated for morphological changes in size of another dipteran pest insect *Agria affinis* with some dietary nonnutritional additive antibiotics; similar detrimental effect on larval, pupal and adult appearance in *D. melanogaster* varied with the level of vitamin D3 in a synthetic diet; for example, in proportion to the level of vitamin D3 was observed. This suggests some caution in use of dietary concentrations of such nutritional additives where normal insect species or damaged insects for pest management are desired. We infer from or results that a model for the process by which vitamin D3 damages the fruit fly midgut and hindgut decreasing survivorship. Although these results may shed a light for the registration of vitamin D3 as an insect control agent like rodenticide [17], further work on biological parameters, mass rearing, formulation, and field application should be undertaken.

Declarations

Availability of Data and Materials: The data sets during and/or analyzed during the current study are available from the corresponding author (E. Ö. Özdoğan) on reasonable request.

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Author Contributions: EÖÖ, GÜ and CÇ: Formal analysis, investigation, resources, visualization, writing-review and editing. KB: Conceptualization, project administration, formal analysis, investigation, methodology, resources, supervision, validation, visualization, writing-review and editing. All authors read and approved the final version of the manuscript.

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