

Rearing of *Bombyx mori* with a vitamin D enriched diet

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An experiment to find the most efficient diet resulting in silkworms with a high nutritional value

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Thesis

**Aeres Hogeschool
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Preface

In order to complete my fourth and last year of my study 'Biology, Food & Health' at the Aeres University of Applied Sciences in Almere, the Netherlands, I had to write a thesis. The whole document has been divided into 5 chapters, an introduction, the materials and methods, results, discussion and the conclusion and recommendations.

The feedback received from the preliminary study has been incorporated and included in this final report.

For this great opportunity, I would like to thank my supervisor and external coach for their excellent guidance and support during this process.

Mr. Ger van der Wal, who is the passionate owner of Insect Europe in the Netherlands and my supervisor during my internship, also he encouraged me to continue in this research area. Mrs. Elise Gieling, who is my external coach at the Aeres University of Applied Sciences in Almere and guided me during the process of writing this document.

As well I would like to thank all my colleagues at Insect Europe for the pleasant cooperation.

I hope you enjoy reading.

Laura de Wit
Almere, June 2017



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Abstract

Background

Insect farming has been suggested as a good alternative to conventional livestock farming for the growing demand of food and feed production. One of the most well-known edible insects is the silkworm, larva of the silk moth (*Bombyx mori*). This insect is reared because of their commercially valuable products, namely the silk filaments. As for food, sericulture provides larvae or pupae inside cocoons as sources of nutritious proteins.

Objective

The objective of this study was to investigate if silkworms can be successfully reared on a vitamin D enriched diet and can result in a healthy, high protein, and vitamin D enriched end product. The results may aid the current and worldwide protein transition and it may help in reducing the vitamin D deficiency problem.

Methods

One strain of silkworm larvae and two different diets were used in this study. One group was reared on a diet enriched with vitamin D and the other one, the control group, was reared on a non-enriched diet. After harvesting, protein, fat and vitamin D content were determined.

Results

Silkworm larvae with an enriched diet with vitamin D had a protein percentage of 61,23%, which was higher than the control group (58,77%). On the other hand, fat percentage of group with an enriched diet (37,26%) was lower than the control group (29,54%). From the data, it is evident that the concentration of vitamin D found in the larvae fed an enriched diet was approximately 60 times higher (1405 mcg/kg) than the group without vitamin D in their diet (23,15 mcg/kg). No silkworms died during the larval period in each experimental group.

Conclusion

Obtained results showed that it is possible to rear silkworms with a vitamin D enriched diet for larvae with a high protein content and high vitamin D concentrations without affecting the survival rate. However, further research is required to evaluate all the exact effects of adding vitamin D into the artificial diet.

Samenvatting

Achtergrond

Het kweken van insecten voor de groeiende vraag naar eiwitten voor humane consumptie en diervoeding is geïntroduceerd als een goed alternatief voor de veehouderij. Een van de meest bekende eetbare insecten zijn zijderupsen, de larven van de zijde mot (*Bombyx mori*). Dit insect voornamelijk bekend en gekweekt voor hun commercieel waardevolle producten, waaronder de zijdedraden. Wat voedsel betreft, levert zijdeteelt larven of poppen uit de cocons die bronnen zijn van hoogwaardige eiwitten.

Doelstelling

Het doel van dit onderzoek was onderzoeken of zijderupsen succesvol kunnen worden gekweekt op een vitamine D-verrijkt dieet en dit resulteert in een gezond eindproduct met een hoog eiwitgehalte én een hoge vitamine D concentratie. De resultaten kunnen de huidige wereldwijde eiwittransitie ondersteunen en het kan helpen bij het verminderen van het vitamine D-deficiëntie probleem.

Methode

In het experiment werd één soort zijderupsen gebruikt die gekweekt werd op twee verschillende diëten. Eén groep zijderupsen werd gekweekt op een dieet met toegevoegde vitamine D en de andere groep, de controlegroep, op een dieet zonder toegevoegde vitamine D. Na oogsten werden het eiwit-, vet- en vitamine D-gehalte gemeten.

Resultaten

De zijderupsen met een vitamine D verrijkt dieet hadden een eiwitpercentage van 61,23%, wat hoger is dan de controlegroep zonder toegevoegde vitamine D in het dieet (58,77%). Daarentegen, was het vetpercentage van de groep met een verrijkt dieet (37,26%) lager dan de controlegroep (29,54%). Uit de gegevens blijkt dat de concentratie van vitamine D in de rupsen die een verrijkt dieet werd gevoed ongeveer 60 keer hoger was (1405 mcg/kg) dan de groep zonder vitamine D in het dieet (23,15 mcg/kg). In de experimentele groepen zijn geen zijderupsen doodgegaan tijdens de kweekperiode.

Conclusie

De resultaten laten zien dat het mogelijk is om met een vitamine D verrijkt dieet zijderupsen te kweken met een hoog eiwitgehalte en hoge vitamine D concentraties, zonder dat de overlevingskans afneemt. Verder onderzoek is nodig om alle exacte effecten van het toevoegen van vitamine D in het samengestelde dieet te evalueren.

Chapter 1. Introduction

The world’s population has been growing exponentially over the last decades and global wealth has been increasing. This caused a rapid increase in the demand for animal protein (Boland et al., 2013). With the current population growth rate, livestock production is expected to be twice as high in 2050 compared to 1999 (Steinfeld et al., 2006, Robinson and Pozzi, 2011). Thereby, the current protein intake in the Western world by humans is already higher than necessary. The Recommended Dietary Allowance (RDA) of protein for average adults per day is 56 grams. Nowadays Dutch women already consume 60-75 grams and men 61-98 grams. In America about twice the RDA for protein is being consumed, which will most likely continue to increase (Van Dooren and Postma-Smeets, 2015, Pimentel and Pimentel, 2003).

The growing demand for animal protein is one of the important reasons to explore replacements. Insect farming has been suggested as a good alternative to conventional livestock farming for future food and feed production (Jansson and Berggren, 2015). Insects have a lower environmental impact in comparison to meat, due to less water use, less feed, a high fecundity and an almost complete consumption of the entire animal (Van Dooren and Postma-Smeets, 2015). The information in figure 1 clearly shows the differences between cattle and crickets in feed and water conversion efficiency and reproduction rate. Besides the remarkable benefits the crickets have shown below, they are also easy to farm in large quantities using very little space (Dossey, 2013).

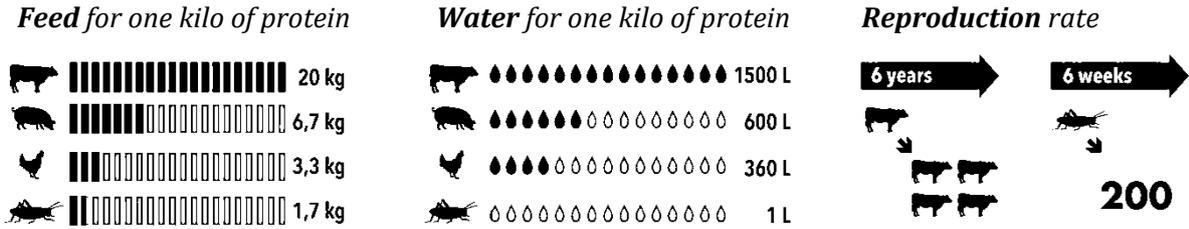


Figure 1. Crickets are an efficient protein source (EntoCube, 2017)

The practice of eating insects, also known as entomophagy, is a taboo in many westernised societies (Van Huis et al., 2013). At the same time, edible insects are incorporated in the diet of many societies in different countries around the world, and are known to be highly nutritious in polyunsaturated fatty acids, protein and generally rich in micronutrients and vitamins (Rumpold, 2013). Altogether, eating insects compared to other animals has a lower impact on the environment, has more health benefits and it can also improve livelihoods. For example, people in underdeveloped countries can easily become involved in the cultivation, processing and sale of insects, because rearing insects is more accessible than livestock farming. This can directly improve their own nutritional status and provide cash income (Van Huis et al., 2013).

One of the most well-known edible insects is the silkworm, larva of the silk moth (*Bombyx mori*). It is the second best-studied insect after the fruit fly (*Drosophila melanogaster*) (Goldsmith, 2013). Silkworms have been domesticated and can therefore be reared on a large scale. They can no longer survive independently in a natural environment. Due to domestication, a large number of strains with various sizes and colours exists. Nevertheless, this insect is mainly well-known and reared because of their commercially valuable products, namely the silk filaments (Van Huis et al., 2013). Sericulture, rearing silkworms for the production of raw silk for clothes and goods for houses, has been practiced for at least 7000 years (Rumpold, 2013). As for food, sericulture provides larvae or pupae inside cocoons as sources of nutritious proteins (Sumida and Suttikhum, 2015). However, the by-product of silk manufacturing, the pupae, could be promoted better as

food for human consumption. Silkworm pupae are known to be rich in protein, lipids, minerals and vitamins (Menzel and D’Aluisio, 1998). It is commonly said that the nutritional value of three silkworm pupae is equivalent to one hen’s egg (Mitsuhashi, 2010).

Like other insects, silkworms are consumed in various stages of their life cycle. They can be eaten as larvae (± 25 days) and pupae (± 31 days) (Siriamornpun and Thammapat, 2008). The life cycle of silkworms contains four distinctive main developmental stages, defined as embryo, larva, pupa and adult moth (Liu et al., 2009). The larval period consists of five different instar phases shown in figure 2. After the fifth instar phase (mature larva), the silkworm spins a cocoon and pupates inside that cocoon. During a period of about ten days, the pupa transforms into a moth. The moth emerges out of the cocoon by orally excreting the enzyme cocoonase. Female silk moths mate the same day and begin egg deposition (Takeda, 2009). The number of days of the stages in the life cycle may vary depending on the rearing temperature. Temperature plays a critical role on the growth of the cold-blooded animals. The average optimum temperature of rearing silkworms is about 25°C (Rahmathulla, 2012). The detailed requirements for each stage can be found in table 4 at page 10.

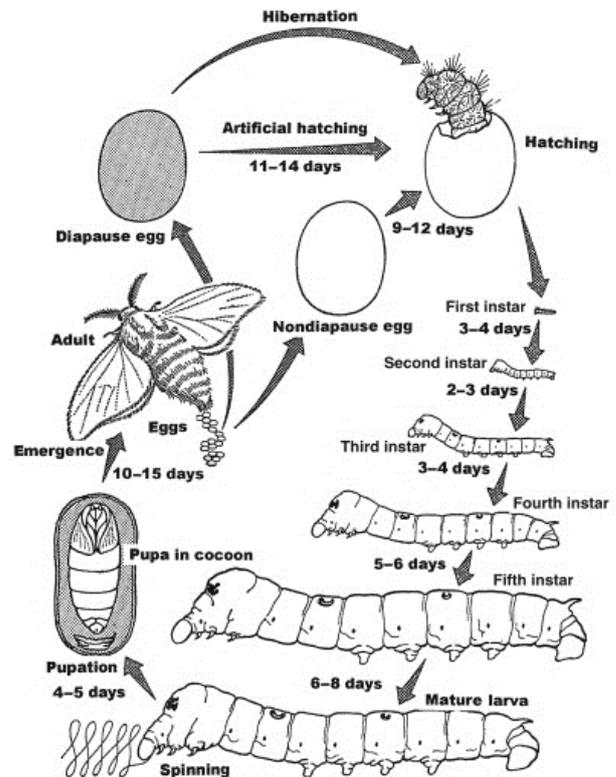


Figure 2. Life cycle of *Bombyx mori* (Mori, 1970)

Methods of rearing *Bombyx mori* are well established at a commercial scale. However, in areas with a colder climate, silkworms are reared only during the spring-summer season when fresh mulberry (*morus*) leaves are available. This host plant is of high nutritional quality and the only suitable (natural) food for optimal growth, development and reproduction of the larvae (Nayar and Fraenkel, 2014). To be able to rear this insect continuously throughout the year, an artificial diet of 13 different ingredients is created. This original diet was developed in 2004 and patented by CRA-API (Cappellozza et al., 2005). One of the ingredients is the natural food of the silkworms, mulberry leaves in dried and powdered form (Saviane et al., 2014). Nutrition, vitamins and mineral salts play an important role in the growth and development of the silkworm (Radjabi, 2010, Kanafi et al., 2007). The effect of vitamin supplementation on the growth of *Bombyx mori* has been investigated by many researchers (Kanafi et al., 2007). However, these researchers focus with their studies on increasing silk yield or improving the silk quality and not human consumption of the larvae or pupae (Goldsmith, 2013, Johnson, 2010). Additionally, enrichment of silkworms with fat-soluble vitamins (A, D, E and K) was not found to be studied in this species (Kanafi et al., 2007). This could be an interesting area of study aiming at rearing nutritious insects for human purposes.

Vitamin D is one of the vitamins that may possibly be a valuable enrichment of the diet of silkworms. If silkworms can be successfully reared on a vitamin D enriched diet and the end product results in healthy, safe, high protein, and vitamin D enriched ‘meat’, it can become very interesting for stakeholders. Vitamin D insufficiency is a public health concern (Hassannia, 2015). In the average European population, 13% has a vitamin D deficiency (Cashman et al., 2016). Also van der Meer (2008) found a large deficiency of vitamin D in non-Western people (37-51%). In

addition, many recent studies demonstrate a correlation between low vitamin D and prevalence of several chronic disorders, like cancer, heart disease and diabetes (Agmon-Levin et al., 2013, Judd and Tangpricha, 2010, Lips, 2007). Vitamin D is associated with a lot of health benefits, including good cognition, bone health and well-being, also it is an important key factor in the human innate immune system (Olliver, 2013). Moreover, it is one of the 24 micronutrients essential for human survival, so it is of high importance that the human diet contains sufficient vitamin D. The intake of vitamin D supplements seems to be associated with decreases in mortality rates (Autier and Gandini, 2007, Bjelakovic et al., 2011). Besides vitamin D in some foods and supplements, sunlight is also a key factor. The body produces vitamin D when the skin is exposed to sunlight (Voedingscentrum, 2012). This means that dietary and especially supplementary intake may be particularly significant during the winter months (Spiro and Buttriss, 2014). Sales of vitamin D supplements and vitamin D fortified foods have increased over the past years (Cashman et al., 2016). Also various National and European strategies already support a healthy lifestyle including a varied diet with vitamin D-containing foods (Spiro and Buttriss, 2014). Reducing vitamin D deficiency by eating food products cannot cause an overdose of vitamin D for humans. This is because the upper limit of intake will never be reached when following a normal diet, but can only occur as a result of prolonged use of too many supplements (Voedingscentrum, 2012). It is safe and legal to add vitamin D to foods. The Maximum Supplement Levels (MSL) for adding vitamin D directly to products is 35 µg per 100 gram/ml (European Commission, 2006). To achieve an optimal vitamin D status, daily intake of at least 1000 IU (International Unit) or more is required (Binkley et al., 2010).

Since 2008 three insect-rearing companies in The Netherlands started with producing grasshoppers (*Caelifera*) and mealworms (*Tenebrio molitor*) for human consumption (Huis, 2010). Another company, Insect Europe in Lelystad, started rearing crickets (*Acheta domestica*) in 2013. Besides rearing crickets they wondered whether it is possible and interesting for them to rear silkworms. This question arose from the owner of Insect Europe, G. van der Wal, who discovered a demand for silkworms for human consumption in Western societies (Van der Wal, G., personal communication, December 1, 2016). This research aims at discovering the possibilities in rearing high quality silkworms successfully on small scale without having to import silkworm larvae or pupae. Exploring if dietary vitamin D enrichment of silkworms leads to harvested animals with a high(er) vitamin D content is one aspect of this quest. Some other questions concerning successful rearing will be answered in a parallel study.

As said, the overall objective of this study is to investigate the effects of vitamin D enrichment of the diet of silkworms on the quality of the end product. The results may aid the current and worldwide protein transition and it may help in reducing the vitamin D deficiency problem in mainly countries further from the equator. This leads to the following main research question: *What is the effect of three artificial silkworm diets differing in vitamin D content on the nutritional value (protein, fat and vitamin D concentration), larval weight and survival of Bombyx mori (fifth instar larvae) compared to a control group on a standard artificial diet?*

Several sub questions will aid answering the main question:

- Which diet results in silkworms with the highest overall protein and fat content?
- What is the concentration of vitamin D in each silkworm group after harvesting?
- What is the effect of the different diets on the larval weight and survival of *Bombyx mori*?

To contribute to the protein transition by farming insects, the results of this study will be made accessible to interested parties. For example, this could support insect farmers and researchers to continue in the development in this innovative protein source.

Chapter 2. Materials and methods

2.1 Animals

Silkworm eggs were ordered from Sericulture and Agriculture Experiment Station in Vratsa, Bulgaria in February 2017. The chosen silkworm strain was especially selected for higher tolerance to unfavourable rearing conditions. The hybrid is obtained by crossing the single hybrid of Japanese type KK x Hesa 1 and the Chinese type Vesletz 2 x Gergana 2 and is characterized with medium values of the main productive characteristics. The larvae are plain and with normal marks, the egg hatchability is 98 – 99 %, the pupation rate is 96 – 98 %, and the larval duration is 25 – 27 days. Upon receiving, the eggs could be stored in the refrigerator if necessary until the start of the experiment. (Sericulture and Agriculture Experiment Station Vratsa, n.d.).

The sizes of each group were of great importance and any unexpected drop out of silkworms should be taken into account. In order to perform proper laboratory work, there was a minimum of 100 grams of each group of silkworms required. For 100 grams, at least 60 fresh silkworms are needed of each group for one sample (Vos, P., personal communication, March 14, 2017). To make sure having enough silkworms in every experimental group, three groups of 90 silkworms in three boxes were used. Besides that, a sample size calculation would be desirable, both on ethical grounds (to reduce unnecessary animal use) and to gather reliable results when performing statistical analyses (Van Geloven et al. 2009). However, as estimation of the outcome of the study is not feasible because this would be the first study in this area, sample size calculation is unreliable. After this pilot study, a full study is probably required to validate the initial results. This means general guidelines were used for the setup of this pilot study (Sullivan and Feinn, 2012).

2.2 Experimental design

The experimental design in this study was 1 x 4 factorials: one strain of silkworm larvae (*Bombyx mori*) in three different groups (with three replications for each group) received a diet containing different amounts of vitamin D and one control group, where no vitamin D was added to the diet. During the experiment, observations and measurements made on the silkworm were performed by using the methods described in the following paragraphs.

Several parameters were measured to answer the main question defined in chapter 1. The diet was fed two times a day during the larval period, so the larvae had the chance to increase its size 10.000 times since birth (Mahesha, 2016). Among the four groups the composition of the diet was the same, but it differed in the added amount of vitamin D3 (table 3). Before harvesting the silkworms, the weight of each group (with the same group size) was determined, so the average weight of a larva was known and could be compared. The silkworms were stored in the freezer until the batch was sent to the laboratory for determining each group by chemical analyses. Besides these measurements, all unexpected findings during this experiment were collected.

2.3 Artificial diet

The artificial diet composition, developed by Cappellozza et al., is shown in table 2 and the detailed order list is shown in appendix 1. This diet was used as food for the silkworms. The powder of all ingredients, containing 25% dried and pulverized mulberry leaf out of the diet dry weight, was hydrated in the ratio 1 kg dry powder : 2.6 kg water. After the complete diet was well mixed, it was cooked in the Rational SelfCooking Center for 20 minutes at 91°C. This temperature was chosen by looking at the melting points of all the ingredients. The diet was cooled to room temperature and then kept in a refrigerator (5°C) until its utilization.

Table 1. Composition of the artificial diet

| Ingredients | Quantities/100 g (dry weight) |
|----------------------------|--|
| Dried mulberry leaf powder | 25.0 g |
| Defatted soybean meal | 36.0 g |
| Wheat meal | 15.0 g |
| Corn starch | 4.0 g |
| Soybean fiber | 5.0 g |
| Citric acid | 4.0 g |
| Ascorbic acid | 2.0 g |
| Salt mixture | 3.0 g |
| Agar | 4.2 g |
| Vitamin mixture | 0.399 g |
| Sorbic acid | 0.2 g |
| Propionic acid | 0.691 g |
| b-Sitosterol | 0.5 g |

Source: Cappellozza et al. (2005)

The three groups of larvae were fed under different diet regimens with regard to vitamin D3 (also known as cholecalciferol). D3 is chosen, because this form is more effective in the human body than vitamin D2 (Holick et al., 2008). The control group were fed the standard diet without vitamin D. To investigate the optimal amount of vitamin D in the diet, three different amounts were added to the artificial diet. The amounts of vitamin D in the diet were quite high in comparison to other studies where they used between the 500 and 1,500 UI/kg (Marques et al., 2011). The relative high amounts were chosen because personal experiences has known that a large part of the given food dried out or were left over, so the silkworms do not eat all given food. Also a logarithmic scale was used to define the different dosages of vitamin D in the diet. Before adding vitamin D to the diet, the vitamin D3 powder first had to be dissolved in a solution with 70% ethanol, this aids spreading the powder equally through the diet.

Table 2. Groups and their amount of vitamin D on the diet (dry weight)

| Group (box) | Amount silkworms | Food per group (5 x 30 = 150 g) | Vitamin D per group per 100 g in IU | Vitamin D per group in g per 100 g |
|------------------------|-----------------------------|--|--|---|
| 1a | 30 | 150 g | 0 | 0 |
| 1b | 30 | 150 g | 0 | 0 |
| 1c | 30 | 150 g | 0 | 0 |
| 1 | 90 | 450 g | 0 | 0 |
| 2a | 30 | 150 g | 4.000 | 0,0001 g |
| 2b | 30 | 150 g | 4.000 | 0,0001 g |
| 2c | 30 | 150 g | 4.000 | 0,0001 g |
| 2 | 90 | 450 g | 12.000 IU | 0,0003 g |
| 3a | 30 | 150 g | 40.000 | 0,001 g |
| 3b | 30 | 150 g | 40.000 | 0,001 g |
| 3c | 30 | 150 g | 40.000 | 0,001 g |
| 3 | 90 | 450 g | 120.000 IU | 0,003 g |
| 4a | 30 | 150 g | 400.000 | 0,01 g |
| 4b | 30 | 150 g | 400.000 | 0,01 g |
| 4c | 30 | 150 g | 400.000 | 0,01 g |
| 4 | 90 | 450 g | 1.200.000 IU | 0,03 g |
| 12 | 360 | 1800 g | 1.332.000 IU | 0,0333 g |

The three groups of silkworms fed with different amounts of vitamin D were compared after harvesting. During the 1st and 2nd instar phase all animals were given the same diet, because this were the most critical and important stages of the life cycle from the silkworms. The different diets were provided when the animals reached their 3rd instar phase, after ±8 days, and when each larva started to eat a greater amount of food. The food recipe per silkworm, 5 gram per larva per cycles was based on professional advice (Tzenov, P., personal communication, December 7, 2016).

2.4 Silkworm rearing and harvesting

Rearing was carried out in an acclimatized room where temperature, relative humidity and photoperiod were controlled. The temperature was set between 25°C and 26°C and the relative humidity (R.H.) between 70-80%. The photoperiod was 8 hours light and 16 hours of darkness. The animals were reared in 12 numbered and open plastic boxes (with plastic mesh in it for ventilation). Bed cleaning was performed once during the first, second and third instar stages and daily during the fourth and fifth instar phase, to prevent unwanted moulds. Caretakers wore gloves to prevent bacterial infection (Cappellozza et al., 2005). The detailed list of needed materials to make rearing possible, is shown in appendix 2.

Table 3. Optimum temperature and humidity requirements of silkworm during various stages

| Environmental factors | Incubation | I instar | II instar | III instar | IV instar | V instar | Spinning | Cocoon preservation |
|-----------------------|------------|----------|-----------|------------|-----------|----------|----------|---------------------|
| Temperature | 25°C | 28°C | 27°C | 26°C | 25°C | 24°C | 25°C | 25°C |
| Relative humidity | 75-80% | 85-90% | 85% | 80% | 70-75% | 65-70% | 70% | 80% |

Source: Rahmathulla (2012)

Prior to harvesting, the silkworms were fasted for one day to clean their intestinal system. After harvesting, the animals were killed by decreasing their body temperature (-20°C) and stored in the same freezer in closed plastic containers. All samples were send to the specialised laboratory Nutrilab (Giessen, Netherlands) who analysed protein and fat content, moisture and vitamin D. Detailed analysis protocols used by Nutrilab to determine protein, fat and moisture content are not public, but the basic methods Nutrilab used are visible in table 4 below.

Table 4. Methods used by the laboratory Nutrilab

| Material/product | Operation/research method | Internal reference number |
|------------------|---|------------------------------------|
| Food products | Determining moisture content: Gravimetrie | A002, A010 |
| Food products | Determining protein content: Dumas | A100, A110, A120 |
| Food products | Determining fat content: Petroleumether extractie | A100, A101, A110, A111, A120, A121 |

Source: Raad voor Accreditatie (2015)

2.5 Analysis

The received data of the protein, fat and moisture contents were expressed in percentages of the total percentages of the sample. Protein and fat analyses were done on the fresh ground larvae (called wet product). These percentages were converted to dry matter after knowing the moisture content.

The data of the vitamin D concentrations received from the laboratory were expressed in IU (International Unit) per kilogram, but was translated to microgram (mcg) with the formula: 1 IU is the biologic equivalent of 0,025 mcg/ μ g cholecalciferol (vitamin D3) (Cannell and Hollis, 2008). The original analysis reports from the laboratory with the results of the vitamin D concentrations, can be found in appendix 4.

After knowing the data of the experimental groups, it was analysed by descriptive statistics. Descriptive statistics were applied because the size and measurements of this pilot study do not allow any further statistical analyses.

Chapter 3. Results

The effects of supplementing the artificial diet for silkworms with vitamin D were tested in the present study. Three experimental silkworms groups received a vitamin D enriched diet from day 8 till day 38 after hatching. The fourth experimental group, the control group, was fed the artificial diet without additives.

Although four groups were studied, during the study because of financial limitations it became impossible to determine the set parameters of all the experimental groups in the laboratory. Due to these circumstances two out of four groups were picked. The control group with no added vitamin D to the diet (group 1 in table 2) and the group with the highest added vitamin D percentage (group 4 in table 2) were chosen for further analyses, as the largest differences were expected to be found between these two groups. Also the concentration of vitamin D in silkworm larvae without added vitamin D to the diet (basal levels), could be determined. The results of the analyses of the two groups of silkworms are shown and compared in the following paragraphs.

3.1 Protein and fat analyses

After knowing the percentages of the moisture content of the samples (enriched diet group: 83,0% control group: 84,6%), the data were converted to protein and fat content percentages on dry matter.

Figure 3 shows that the highest protein percentage was observed in the group of silkworm larvae fed the vitamin D enriched diet (61,23%). A lower protein percentage was observed in the larvae group fed a non-enriched diet (58,77%). The difference between the two groups is 2,46%.

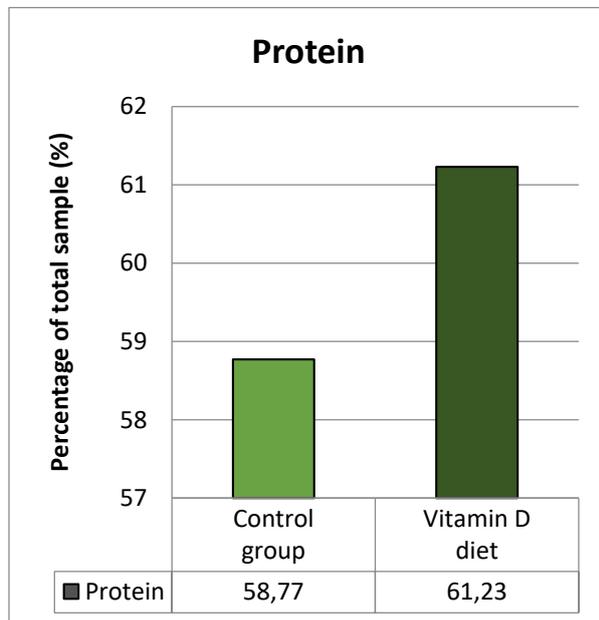


Figure 3. Protein percentage in the control group (silkworms fed a non-enriched diet) and an experimental group (silkworms fed a vitamin D enriched diet)

The difference in fat percentage between the two groups is 2,28%. The larvae reared on the vitamin D enriched diet have a lower fat percentage (37,26%) in comparison to the control group (39,54%) (fig. 4.).

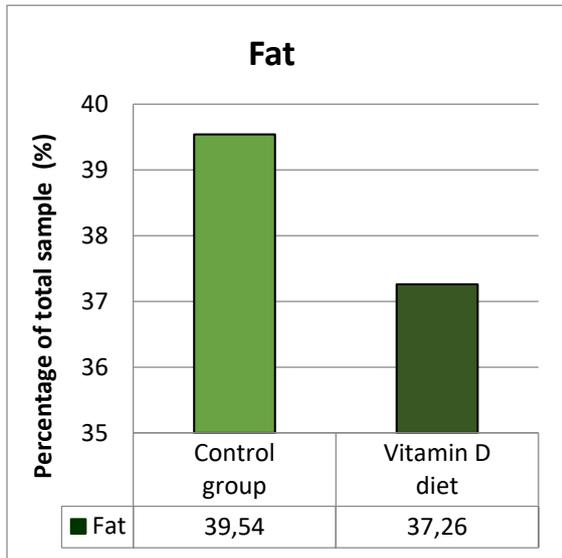


Figure 4. Fat percentage in the control group (silkworms fed a non-enriched diet) and an experimental group (silkworms fed a vitamin D enriched diet)

3.2 Vitamin D analyses

Figure 5 shows an enormous difference between the control group and the group reared on a vitamin D enriched diet. The larvae from the control group contain 23,15 mcg/kg without vitamin D added to their diet. The larvae on a vitamin D enriched diet contain approximately 60 times more vitamin D than the control group (1405 mcg/kg).

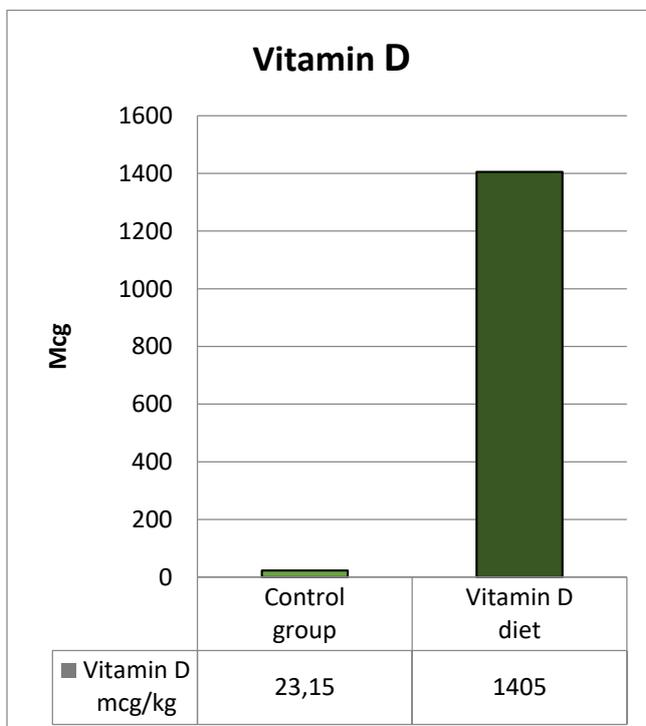


Figure 5. Average vitamin D concentration in the control group (silkworms fed a non-enriched diet) and in the experimental group (silkworms fed a vitamin D enriched diet)

3.3 Weight and survival

The experimental groups were checked regularly for larval weight and mortality. The average weight of the four groups is higher in the vitamin D enriched groups compared to the non-enriched group. The average extra weight per silkworm larvae is 0,25 gram between the group with the most added vitamin D (group 3) and the control group (group 4). The larval survival rate from hatching till harvesting is found to be 100% in all groups.

Table 5. Overview of the larval weight and survival rate of the silkworm groups

| Group | 1 | 2 | 3 | 4 |
|----------------------------------|----------|----------|----------|----------|
| Vitamin D (g) | 0,0003 | 0,003 | 0,03 | 0 |
| Animals (N) | 60 | 60 | 60 | 60 |
| Group weight (g) | 124 | 130 | 138 | 123 |
| Average weight/animal (g) | 2,07 | 2,17 | 2,30 | 2,05 |
| Survival rate (%) | 100 | 100 | 100 | 100 |

Chapter 4. Discussion

The objective of this study was to investigate enrichment of an artificial silkworm diet with vitamin D3 and its effect on the nutritional value of the animals after harvesting. Two silkworm groups, one reared on a vitamin D enriched diet and a control group reared on a non-enriched diet, were compared looking at the protein and fat percentages, concentration of vitamin D and survival rate.

Silkworm larvae reared on an enriched diet showed a protein percentage of 61,23%, which was higher than the control group without an enriched diet (58,77%). On the other hand, fat content of the group with an enriched diet (37,26%) was lower than the control group (29,54%). These percentages are close to prior studies of Finke (2002) who found comparable nutritional percentages of protein and fat content in *Bombyx mori*. It does not seem that vitamin D in the diet caused these differences in protein and fat percentages. Further research with larger numbers of animals must show this.

From the data, it became evident that the concentration of vitamin D found in the larvae fed an enriched diet was approximately 60 times higher (1405 mcg/kg) compared to the control group. In the control group, the basal level of vitamin D was measured (23,15 mcg/kg). This amount is comparable to vitamin D levels found in real butter (20 mcg/kg) (Dochy, 2014). The vitamin D content in the enriched diet group showed to be approximately 10 times higher in *Bombyx mori* than in fresh oily fish such as salmon and mackerel (Katan and Dusseldorf, 1987).

It is not known if the vitamin mix (van der Zant), part of the composition of the given diet, contained vitamin D. Also it is unknown if the larvae ate all the vitamin D in the diet, although all ingredients were mixed securely. Finally, it was not measured if the larvae used or excreted vitamin D ingested by both diets.

Vitamin D in experimental settings is mostly applied in very small amounts. Weighing, dissolving and spreading evenly in the diet, must be done very accurately to achieve reliable results. The vitamin D in this experiment came as a powder, so it had to be dissolved. Also vitamin D is a fat-soluble vitamin, so it first had to dissolve in alcohol before mixing it with water. Even with the help of a test tube mixer it was difficult to dissolve the lumps of the powder in the alcohol. Possibly small lumps were not completely dissolved in the solution and were unequally divided among the diet eaten by the larvae. It is therefore advisable to use liquid forms or order already weighted and dissolved vitamins. This would enhance acquiring more reliable results.

To ensure silkworms had enough food during the experiment, a little bit more food was given than expected. This was because the given food dried out or was left over. This means also a little bit more vitamin D was given to the experimental group with an vitamin D enriched diet. This may cause higher concentrations of vitamin D in the larvae.

The survival rate of both the experimental and the control groups during the rearing period was 100%. An average mortality rate of silkworms during the larval period, is so far unknown. However, in the literature is found that sericulture farmers suffer significant losses due to various viral infections during the larval stages. Studies are currently working to develop disease resistant silkworm breeds (Gupta et al., 2015).

However, it was unknown, but quite possible, that the (relatively high) addition of vitamin D to the diet would affect the silkworms health or survival negatively. Apparently the chosen addition of vitamin D was not that high it was toxic for the larvae what subsequently would cause a lower survival rate. Due to insufficient experimental groups and because there was no individual larval weighting, averages were used and therefore it can not be concluded that vitamin D influenced

the survival rate of 100%. For this reason, more animals in more groups should be investigated. Beside more animals, higher amounts of vitamin D can be added to the diet to examine if the concentration of vitamin D in the larvae after harvesting could be increased.

The rearing conditions chosen were apparently optimal as no ill or decreased silkworms were found. However, the experiment only lasted one life cycle of the silkworm, so more repetitions of rearing silkworms under the same conditions in the same experimental rooms may possibly cause survival rates to decrease. This because pathogens may occur after a longer time for development. In general conditions during rearing, like the (humidity of the) food, cleaning of the bedding and the survival of pathogens can possibly influence the survival rate in an 'insect farm'. Additionally the conditions of the previous generation influence the egg quality and hatching rate significantly (Wang, 2009).

A factor to take into account is that examining the survival rate of silkworms shortly after hatching can be difficult, because of the very small size of the silkworms during the first weeks of their life cycle. Devices, such as a microscope can make this easier.

Chapter 5. Conclusion and recommendations

Two different groups, the control group (silkworms fed a non-enriched diet) and the experimental group (silkworms fed a vitamin D enriched diet), were compared. Enrichment of the diet with vitamin D, caused in a higher protein content, but a lower fat content. These findings cannot be explained.

Silkworm larvae already contain vitamin D, but the concentration of vitamin D can be increased by adding vitamin D to the diet and the concentration can be 60 times (or possibly more) higher in the end product.

Adding (relatively high amounts) vitamin D to the diet of the silkworms can influence the vitamin D concentration inside the larvae fed that enriched diet without decreasing the survival rate.

Because of a small budget for this study, it was not feasible to meet all the needed tests. For all data obtained in the laboratory, repetitions are required to make the results more reliable. Also testing other parts, like the feces, the given diet or larvae in other stages will provide more information about the process of the vitamin D uptake, storage and accumulation.

The results of the study suggests that *Bombyx mori* larvae can be fed with a diet enriched with vitamin D for increased weight, protein content and vitamin D concentrations. However, further studies are required to support and elaborate this experiment to find out if it is economic efficient to rear enriched vitamin D silkworm larvae for human consumption. For analysing results in the next study, it is advisable to observe silkworms individually and weigh them and the given food with or without added vitamin D, before and after feeding each day or otherwise regularly to determine the exact food and vitamin D uptake of the larva. Also measurements on individual animals yields statistical analyses. There are still a lot of opportunities to optimise rearing methods and to continue research on vitamin D enriched diets with silkworms.

As a part of the ongoing and necessary protein transition, interest for edible insects in Europe is growing, so the results of this study are of great importance to aid insect farmers in the right direction. Especially the results of survival and vitamin D content in the enriched silkworm group are very promising. As this was only a pilot study, further research would be highly recommended.

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Appendices

Appendix 1. Order list

Table 2 shows the composition of the 13 ingredients for the artificial diet. All the details of the ordered products are shown in the order list below. These quantities are enough for in total 10 kilogram of dry feed which is approximately sufficient for 2500 silkworms.

| Product | Price | BTW | Send costs | Quantity | Supplier |
|-----------------------------------|---------|--------|------------|------------|--|
| Dried mulberry leaf powder | €150,- | - | €65,- | 15 kg | Sericulture and Agriculture Experiment Station, Vratsa, Bulgaria |
| Defatted soybean meal | €7,86 | €0,89 | €6,95 | 5 kg | Verse bollen |
| Wheat meal | €1,72 | - | - | 2 kg | Albert Heijn |
| Corn starch | €1,10 | - | - | 1 kg | Duryea Maizena |
| Soybean fiber (cellulose) | €37,29 | €7,83 | €7,95 | 500 g | Hinmeijer Chemie |
| Citric acid (≥99,5%) | €7,15 | €1,50 | - | 1 kg | Hinmeijer Chemie |
| Ascorbic acid (vitamin C, ≥99,5%) | - | - | - | 900 g | Hinmeijer Chemie |
| Salt mixture (Wessons) | €92,10 | - | - | 2 kg | Sigma Aldrich |
| Phyto agar | - | - | - | 500 g | Duchefa Biochemie |
| Vitamin mixture (van der Zant) | €110,- | - | - | 100 g | Sigma Aldrich |
| Sorbic acid (≥99,5%) | €38,52 | €8,09 | - | 250 g | Hinmeijer Chemie |
| Propionic acid (99%, pure) | €23,51 | €4,94 | - | 1 l | Hinmeijer Chemie |
| Beta-Sitosterol | €26,56 | - | - | 100 g | Pure Bulk |
| Vitamin D3 (cholecalciferol) | €44,39 | €9,31 | - | 1 g | Hinmeijer Chemie |
| Product | Price | BTW | Send costs | Quantity | Supplier |
| Silkworm eggs | €15,- | - | €5,- | 12500 eggs | Sericulture and Agriculture Experiment Station, Vratsa, Bulgaria |
| | Price | BTW | Send costs | | Total costs |
| Total | €555,20 | €32,56 | €84,90 | = | €672,66 |

Appendix 2. List of materials

| Material | Brand |
|---|--------------|
| Clean room with light controller | |
| Digital temperature and relative humidity (H.R.) controller | DisQounts |
| Plastic box (12) | |
| Markers for each box (12) | |
| Lab Analytical Balance Digital Electronic Precision Scale | KERN |
| SelfCooking Center | Rational |
| Plastic Ziploc bags (16) | |
| Plastic mesh | |
| Nitrile gloves | CMT |

Appendix 3. Analysis reports Nutrilab

The data obtained from the laboratory Nutrilab is shown in the following reports.

1. Experimental group reared on a diet without added vitamin D3.

| Bepaling | | Resultaat | |
|-----------|--|-----------|--------|
| Vitamines | | | |
| E 592 | Vitamine D3 (cholecalciferol) (verw. gehalte < 1000 IE/kg) | 926 | IE./kg |

Startdatum analyse: 16.05.2017, einddatum: 26.05.2017.

De analyseresultaten hebben alleen betrekking op het monster. Nadere informatie over toegepaste methoden is te verkrijgen bij de operationeel manager.

De interpretaties van analyseresultaten vermeld op dit rapport vallen buiten de scope van de accreditatie.

Met de ozonheid % wordt m/en% bedoeld, tenzij anders vermeld.

Dit certificaat mag zonder schriftelijk schriftelijke toestemming van Nutrilab BV niet anders dan in zijn geheel worden gereproduceerd.


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BTW NL002007654B01

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De betekenis van de gebruikte tekens is:
Q Analyse met IFA accreditatie (ISO/IEC 17025)
I Analyse door Nutrilab BV uitgevoerd
E Analyse door Nutrilab BV uitbesteld

2. Experimental group reared on a diet with added vitamin D3.

| | | | |
|---|--|--|--------------------------------|
|  | | <h1 style="margin: 0;">nutrilab</h1> | |
| <h2 style="margin: 0;">Analyserapport</h2> | | Order-nr : 544687 | |
| Insect Europe B.V. T.a.v. Dhr. G. van der Wal Runderweg 6 8219PK LELYSTAD | | | |
| <hr/> | | | |
| Omschrijving | : 10j. EXP LAURA | Monsternamedatum | : |
| Ontvangstdatum | : 16-05-2017 | Monstertransport | : Klant |
| Rapportdatum | : 26-05-2017 | Monstertemperatuur | : Bevroren |
| Monsteremmer | : | Monsterconditie | : Monster en verpakking intact |
| Verpakking | : Plastic bakje | | |
| Verzegeld | : N | | |
| <hr/> | | | |
| Bepaling | | Resultaat | |
| <hr/> | | | |
| Vitamines | | | |
| E 592 | Vitamine D3 (cholecalciferol) (verw. gehalte < 1000 IE/kg) | 56.200 | IE./kg |
| <hr/> | | | |
| Startdatum analyse: 16-05-2017, einddatum: 26-05-2017. | | | |
| De analysesresultaten hebben alleen betrekking op het monster. Nadere informatie over toegepaste methoden is te verkrijgen bij de operationeel manager. | | | |
| De interpretaties van analysesresultaten vermeld op dit rapport vallen buiten de scope van de accreditatie. | | | |
| Met de eenheid % wordt m/m% bedoeld, tenzij anders vermeld. | | | |
| De certificaat mag zonder uitdrukkelijk schriftelijke toestemming van Nutrilab BV niet anders dan in zijn geheel worden gereproduceerd. | | | |
|  | | | |
| Operationeel manager : ing. T. Boogaard | | | |
|  | | | |
| Nutrilab bv Postbus 7, 4284 ZG Rijswijk Burgstraat 12, 4283 GG Giessen t. (0)183) 44 63 05 f. (0)183) 44 25 97 | | info@nutrilab.nl www.nutrilab.nl KvK 18114291 BTW NL002007654801 | |
| | | Pag. 1 / 1 De betekenis van de gebruikte tekens is: Q Analyse met IFA accreditatie (ISO/IEC 17025) I Analyse door Nutrilab BV uitgevoerd E Analyse door Nutrilab BV uitbesteld | |

Appendix 4. Photo logbook

1. Date: 22-03-2017

Most eggs are hatched



2. Date: 27-03-2017

The larvae (1st instar) are placed on a plastic mesh with food



3. Date: 05-04-2017

A number of 30 larvae (3rd instar) are selected and placed on new food



4. Date: 12-04-2017

4th instar larvae on (old and new) food



5. Date: 19-04-2017

5th instar larvae on food with mesh and some silkworm feces underneath



6. Date: 22-04-2017

One group silkworms (N = 60) ready for weighting



7. Date: 22-04-2017

Cleaned and frozen silkworms



8. Date: 23-04-2017

Silkworms from another experimental group with spinning larvae and cocoons

