Effects of Diet Quality on Survival of *Vanessa atalanta* (L., 1758) (Lepidoptera: Nymphalidae) Larvae Infected by *Bacillus thuringiensis* subsp. *kurstaki*

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Abstract: Effects of seven artificial diets with varying protein, carbohydrate and tannic acid contents on the survival of *Vanessa atalanta* larvae infected by *Bacillus thuringiensis* subsp. *kurstaki* (*Btk*) were studied. The highest survival rate among the infected larvae was observed from the group feeding on diet containing 10% of tannic acid. The highest mortality rate was observed among the larvae feeding on artificial diet with the lowest protein amount. Maximum mortality in each diet group was observed on the third day among the larvae infected by bacteria. This mortality was particularly higher among the larvae feeding on diet that contain less amount of protein. Among the infected larvae, the highest number of total haemocytes was found among those that fed on diet containing 2.5% of tannic acid. The lowest number of haemocytes was found in larvae that fed on diet with lower protein amount. The lowest pupal protein amount was found among the infected larvae feeding on artificial diet containing much lower amounts of protein.

Keywords: carbohydrate, protein, tannic acid.

Introduction

Bacillus thuringiensis (Berliner) is a gram-positive bacterium that produces crystal-shaped toxin with insecticidal characteristics against many members of the order Lepidoptera. The mechanism of its insecticidal effect on lepidopterans is associated with dissolution of prototoxin in the highly alkaline habitat in midgut (BRODERICK et al. 2006). It is known that *Bacillus thuringiensis* subsp. *kurstaki* (*Btk*) is more resistant than other conspecific subspecies (TABASHNIK et al. 1993).

Plant secondary compounds have various ecological roles such as allelopathy, deterrence of herbivores, attracting predators of herbivores as well as having antifungal properties (HARBORNE 1977, CHOU & KUO 1986, BAAS 1989, DICKE & SABELIS 1989). Tannins are very important for the management of *B. thuringiensis* resistance (GUAN et al. 2009). Some

studies have reported that tannins reduce the toxicity of *B. thuringiensis* (LUTHY et al. 1985, NAVON et al. 1993). However, others stated that the effect of toxin in *B. thuringiensis* is only achieved when tannins present (SCHULTZ 1983, GIBSON et al. 1995).

Experimental studies on diet choice of insect feeding have focused on the investigations of adjusting protein and carbohydrates (SIMPSON & RAUBENHEIMER 1995, LEE et al. 2003, 2004). Nutritional needs of an infected insect can be different from those needed for growth, reproduction and survival. Immune systems of insects are comprised of cellular and humoral components. Cellular means are immune reactions that include encapsulation, nodulation and phagocytosis through haemocytes showing quite differences from each other. Humoral responses include solvable proteins such as anti-mi-

crobial peptides and polypeptides. These molecules are produced in haemocytes. Depending on insect's immune system activity and maintaining its continuity, it is expected that when immune system faces an infection, its macronutrient intake needs will change (Povey et al. 2009). Infection has been shown to change the amount of protein and carbohydrate intake (Thompson & Redak 2005).

Vanessa atalanta (L., 1758) is a species found throughout Europe and Africa north of the Sahara (Emmet & Heath 1990, Tolman & Lewington 1997). It is a common species in Turkey. We have used it due to its accessibility in order to carry out pioneer studies prior to examination of other lepidopteran larvae.

Quality of diet is important for the survival of larvae and to fight against infection. Previous results of infection studies conducted in the presence of tannins were controversial. In the present study, we examine the effects of protein, carbohydrate and tannic acid in artificial diet on the survival of *V. atalanta* larvae infected by *Btk*.

Materials and Methods

Collecting larvae

Larvae of *V. atalanta* were collected from nettle plants (*Urtica dioica* L.) during our field survey conducted on the shoreline of Bafra District in the city of Samsun, Turkey, in 2014.

Feeding experiments

Feeding experiments were carried out in two different protocols. At the first one, within the purpose of determining the survival rates between infected and control group, total of 350 larvae were equally divided into 7 diet groups (50 larvae per diet group) and fed. At the second protocol, total of 350 larvae were also divided equally into 7 diet groups (50 larvae per diet group); however, 25 of larvae of each group were used to find the number of haemocytes, and the other 25 larvae were used to measure protein amount. This study was carried out with total of 700 larvae. For this experiment, last instar larvae were chosen. Except for the control group, *Btk* was added to diet used in all other diet groups and experiment continued at 28ºC; this temperature was chosen to be similar to the daytime temperature in the natural habitat of the larvae**.**

Artificial diet contents

Artificial diet developed by Yamamoto (1969) was used as control diet to feed control larvae. For the purposes of this study, artificial diets for the experimental larvae were prepared by using secondary substances such as tannic acid (TA) and adjusting the amount of protein (P) and carbohydrate (C). Thus, three diets were prepared by adding 2.5%, 5%, and 10% concentration of TA of dry weight to artificial diet, three diets by changing the amount of P and C in the artificial diet (15:15, 10:50, 50:10), and one as control diet. Seven diets were prepared in total (Table1).

Contents in Yamamoto (1969) artificial diet are wheat germ, casein as protein, saccharose as carbohydrate, torula yeast, vitamin mixture, salt mixture, cholesterol, sorbic acid, methyl paraben, linseed oil, agar and water.

Infection of bacteria in larvae

Bacterial suspension with the qualities of 600 nm (OD_{600}) wave length and 0.189 optical density was prepared in order to infect larvae with bacteria. After being fed with artificial diet for 10 days in laboratory, fifth instar larvae were treated by infecting the artificial diet with 1 ml of *Btk* bacteria from the prepared suspension for 3 days in climate chamber at 28ºC and under light setting.

Extraction of haemolymph

After the larvae fed on bacteria-infected diet for 3 days, their hemolymph was taken by cutting the third

Table 1. Diet types and contents used in the present study. Abbreviation: CD, control diet; TA, tannic acid; P : C, protein : carbohydrate ratio.

Diet Type	Diet Contents
A	CD + 2.5% TA
B	CD + 5% TA
C	CD + 10% TA
D	P : C = 50 : 10 g/kg
E	P : C = 10 : 50 g/kg
F	P : C = 15 : 15 g/kg
G	Control Diet (P : C = 30:30 g/kg)

Table 2. Total larvae number, number of larvae which have died and the survival rate of larvae infected by *Btk* and fed on the diet of different contents (Groups A-F) and the control (non-infected) group (G).

Diets	Larvae number	Dead larvae number	Survival rate (%)
A	50	35	30
B	50	20	60
C	50	10	80
D	50	25	50
E	50	45	10
F	50	45	10
G (Control)	50	0	100
Total	**350**	**180**	**48.6**

leg from the thorax, and 4 µl of haemolymph was obtained from each larva by using microcapillary tube in order to calculate the total number of the haemocytes. Haemolymph was transferred to eppendorf tubes including 36 µl anticoagulant (0.0098 M NaOH, 0.186 M NaCl, 0.017 M Na$_2$, EDTA and 0.041 M citric acid; pH 4.5). Haemolymph taken from eppendorf tubes was used to make Giemsa-stained smears and haemocytes number per cubic millimetre was calculated by using Jones' formula (Jones 1962).

Statistical analysis

In this study, Kaplan-Meier life-table analysis test was used to find the relation of survival rates of control-group larvae (not-infected) and the larvae infected by bacteria. ANOVA Dunnett test was used to determine the relation among development time, total haemocyte number and pupae protein amount. Cox-Regression analysis test was applied to compare the effects of protein, carbohydrate and tannic acid in artificial diet on survival.

Results

Survival analysis of larvae infected by bacteria depending on the type of artificial diets

Survival rates of *V. atalanta* larvae infected by *Btk* were compared with control group by Log-Rank test. It was found that there was significant statistical difference (df=1, *P*<0,001) between control and the infected larvae. The total number of larvae, number of the larvae which died and the survival rate of *V. atalanta* larvae, which were infected by *Btk* and fed with seven different diets, are given in Table 2. There were 350 larvae in total, equally divided in each diet group. Of them, 180 died and 170 became pupae. The highest mortality rate was seen in the larvae fed on E and F diets; the lowest mortality rate was in the ones fed on C diet. Mostly, the larvae fed on C diet became pupae, whereas larvae fed on E and F diets showed the lowest number of pupated larvae (Table 2).

The highest rates of mortality due to infection were observed two or three days after the infection in all diet groups (Table 3).

Analyses of pupae protein, larval haemocytes and development time

The highest amount of pupae protein was obtained from the larvae fed on G diet (control). On the other hand, the lowest amount of protein was found in the larvae fed on E diet. The total haemocytes number was the highest in the larvae given A diet, whereas the lowest haemocytes number was observed in the ones fed on F diet. An interesting finding about the

total haemocytes number was that two lowest values of survival were seen in the larvae fed on C diet, which had a high survival rate, and in the larvae that were given F diet, which had a low survival rate. The longest development time was found in the larvae fed on C, E and F diets. The shortest development time was observed in the larvae fed on G diet (Table 4).

Effects of total protein and secondary compounds on the survival of infected larvae

According to the results of Cox-regression analysis, protein, carbohydrate and tannic acid in the artificial diet had positive effects on the survival rate while infection increased the mortality risk 28 fold in the larvae (Table 5).

Discussion

The highest survival rate among infected larvae was found in the larvae fed on tannic acid added diet (C diet). This result may imply that tannic acid negatively affects *Btk* infection. Similar to our findings, increase in the condensed tannin concentration reduces the mortality rate in the larvae infected by bacteria (Hwang et al. 1995, Bauce et al. 2006). However, in contrast to our findings, it was found that the increased condensed tannin concentration did not reduce the mortality (Kleiner et al. 1998). In *Helicoverpa armigera*, it was determined that the synergistic effect of tannic acid and *Btk* Cry1Ac toxin increased the mortality (Guan et al. 2009). Another study demonstrated that the increased amount of tannic acid added to *Btk* medium resulted in a decreased bacterial growth (Çavdar 2013), which is in agreement with our results. Probably, the increased amount of tannic acid in the midgut of insects restricts bacterial growth. Although secondary compounds in plants are produced against herbivores' attacks, it can be supposed that they are used as resistance agents against pathogens in the intestinal tract of insects evolved as a result of the co-evolution.

The lowest survival rate was found in the larvae fed on E and F diets. It has been found that the diet quality has an effect on the immune function (Rolff & Siva-Jothy 2003, Schmid-Hempel 2005, Siva et al. 2005, Wilson 2005). In the larvae feeding on low quality diets, the power of pathogen resistance mechanism can be reduced as a result of the reduction of internal protein reserves (Lee et al. 2008). The infected larva prefers a protein-rich diet resulting into increase of the immune functions (e.g. antibacterial activity); therefore, this behaviour has a negative impact on bacterial infection and, as a result, the survival rate of larvae feeding on protein-

Table 3. The numbers of larvae, which have died or survived in accordance with the day of infection. The control group (G) is not presented due the the lack of mortality.

Day	A diet		B diet		C diet		D diet		E diet		F diet	
	Dead larvae	Sur-vived larvae	Dead larvae	Sur-vived larvae	Dead larvae	Sur-vived larvae	Dead larvae	Sur-vived larvae	Dead larvae	Sur-vived larvae	Dead larvae	Sur-vived larvae
1	-	50	-	50	-	50	-	50	-	50	-	50
2	10	40	5	45	1	49	8	42	10	40	15	35
3	15	25	7	38	4	45	10	32	20	20	20	15
4	6	19	1	37	4	41	4	28	10	10	5	10
5	4	15	1	36	1	40	3	25	-	-	-	-
6	-	15	3	33	-	-	-	-	-	-	-	-
7	-	15	3	30	-	-	-	-	-	-	-	-
8	-	15	-	-	-	-	-	-	-	-	-	-
9	-	15	-	-	-	-	-	-	-	-	-	-
10	-	15	-	-	-	-	-	-	-	-	-	-

Table 4. The pupae protein amount, total hemocyte number, and development time of *Vanessa atalanta* larvae in feeding experiments.

	Diet type	Pupal protein amount (mg)	Total haemocyte number	Development time (day)
Mean± standard error	A	11.0±0.03	160.4±0.5	6.0±0.1
	B	12.0±0.04	130.2±0.6	5.1±0.1
	C	12.7±0.04	24.0±0.5	6.9±0.1
	D	11.8±0.03	106.0±0.5	5.9±0.1
	E	10.0±0.04	56.3±0.5	6.9±0.1
	F	10.3±0.04	11.4±0.2	6.8±0.1
	G	13.0±0.03	75.2±0.4	4.1±0.1
ANOVA	d.f.*	349	349	349
	F	2247.2	16995.5	525.0
	P	< 0.001	< 0.001	< 0.001
	Dunnett test	A< 0.001 B < 0.001 C < 0.001 D < 0.001 E < 0.001 F < 0.001	A< 0.001 B <0.001 C < 0.001 D < 0.001 E < 0.001 F < 0.001	A< 0.001 B < 0.001 C < 0.001 D < 0.001 E < 0.001 F < 0.001

Table 5. Comparison of bacterial infection and the effects of protein, secondary compounds on survival through Cox-regression analysis. Abbreviations: B: Coefficient of regression, SE: Standard error, Wald: Significance of the regression coefficients, df: Degree of freedom, *P*: Significant, Exp (B): Hazard proportion.

	B	SE	Wald	df	P	Exp(B)
Infection by bacteria	3.323	.843	15.528	1	.000	27.753
Protein amount	-.039	.007	34.587	1	.000	.962
Carbohydrate amount	-.024	.006	14.038	1	.000	.976
Tannic acid amount	-.615	.171	12.945	1	.000	.541

rich diets increases (Povey et al. 2009).

Our study has shown that the survival rate of infected larvae fed on D (protein-rich) diet is lower than of larvae fed on B and C diets. Richness in the amount of protein in diet activates the immune system or sustains it (Lee et al. 2006). In *V. atalanta*, higher survival rate has been found in larvae fed on the diet containing tannic acid. This result may mean that not only protein but also secondary compounds contribute to the activation and sustenance of the immune system.

Amount of the protein in all infected larvae is lower compared to the control group. The highest amount of protein in the infected larvae has been observed in larvae feeding on C diet. It has been shown that individuals of *Locusta migratoria* Author, Year consuming diet with tannic acid have higher protein content in comparison to specimens of the same species supplied with diet without tannic acid (SIMPSON & RAUBENHEIMER 2001). This result may imply that there is no change in insect physiology as a result of the infection. In our study, the lowest protein amount was found in the pupae of larvae feeding on the unbalanced diets (E and F diets). Tannin in insects' diet reduces the blocking effect of the CryIA(c) toxin on feeding (NAVON et al. 1993). Similarly, the highest protein amount observed in our study may depend on not decreasing of feeding.

The longest development time in this study was observed in the larvae which were given C, E and F diets. Ten % of tannic acid in C diet could have prolonged the development time. In their study with *L. migratoria*, SIMPSON & RAUBENHEIMER (2001) showed that the larval stage was longer in the larvae supplied with diet containing tannic acid. The other two diets (E and F diets) are unbalanced. When the protein amount in the diet in *Spodoptera exempta* larvae infected by *Bacillus subtilis* got lower, it was seen that the development time prolonged (POVEY et al. 2009).

E and F diets had lower amounts of protein. Similarly to the results we found with E and F diets, it was observed that the number of haemocytes decreased when the protein amount consumed by *Spodoptera littoralis* larvae infected by nucleopolyhedrovirus has been reduced (LEE et al. 2006). The highest number of haemocytes was found in the two diets (A and B) with added tannic acid and there were more haemocytes than in the larvae fed with the control diet (G diet). In addition, unlike A and B diets, the number of haemocytes is lower in the infected larvae feeding on C diet. This is one-third of the number of haemocytes of the larvae in the control group. This may mean that tannic acid in midgut suppresses the reproduction of *Btk* significantly.

Most of the mortality within each diet group of larvae infected by bacteria was seen on the 3rd day. It was observed that the mortality was high, especially in the larvae supplied with the artificial diets containing low protein levels. Results of this study may indicate that the pathogen resistance, which is shown above, is reduced by the decrease in the amount of intake protein. Moreover, as the amount of tannic acid intake increased, reduction in the effect of pathogen was seen. Phytochemicals taken by feeding can get attached to occlusion body in the midgut of larvae and can reduce the various infections to hosting insect (FELTON & DUFFEY 1990). This interaction can be adjusted by digestion practices in the intestine system of the host insect (GLARE et al. 2003).

This study shows the importance of protein for the immune system of infected insects. Also, it emphasizes the importance of balanced diet of insects in fighting against pathogens. It underlines that tannic acid in connection with tannins creates an important resistance against pathogen in the midgut of insects where infection takes place, thus protect the insects, and negative effects of secondary compounds can be beneficial. Future studies can focus on different researches about synergistic effects of secondary compounds.

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