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Original article (Orijinal araştırma)

Diet-mediated modulation on the development and phenoloxidase activity in the Alder leaf beetle larvae, *Agelastica alni* (L., 1758) (Coleoptera: Chrysomelidae)[1]

Kızılağaç yaprak böceği, *Agelastica alni* (L., 1758) (Coleoptera: Chrysomelidae) larvalarının fenoloksidaz aktivitesi ve gelişiminde diyet etkenli değişiklikler

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Abstract

Food-induced changes in the phenoloxidase activity and development of *Agelastica alni* (L.,1758) (Coleoptera: Chrysomelidae) larvae reared on unbalanced artificial diets were examined. The study was conducted between 2015-2016. Food quality had an impact on the phenoloxidase activity and growth performance of alder leaf beetle. The maximum pupal mass was recorded in the 0.5:1 P:C (protein:carbohydrate) diet and the minimum pupal mass was recorded from the larvae fed on the 3:1 P:C diet. The amount of carbohydrate consumed affected the pupal mass positively, whereas the amount of protein consumed negatively affected the pupal mass. The highest amount of pupal lipid was found in the 0.5:1 P:C diet and the lowest pupal lipid amount in the 1:3 P:C diet. Also, imbalance diets affected phenoloxidase activity. There was a positive relationship between P ratio of the diet and phenoloxidase activity. Phenoloxidase activity decreases as the amount of carbohydrate consumed by larvae increases. As a result, unbalanced diet affects the immune system of larvae. Carbohydrate also has a significant effect on immune defenses as much as protein. In addition, larvae increase their body size with excessive consumption of carbohydrate.

Keywords: *Agelastica alni*, imbalance diet, insect immunity, nutritional ecology, phenoloxidase

Öz

Bu çalışmada gıda bakımından dengesiz diyetlerle beslenen *Agelastica alni* (L.,1758) (Coleoptera: Chrysomelidae) larvalarının fenoloksidaz aktivitesi ve gelişiminde meydana gelen besin kaynaklı değişiklikler araştırılmıştır. Çalışma 2015-2016 yılları arasında gerçekleştirilmiştir. Besin kalitesi kızılağaç yaprak böceğinin gelişim performansında ve fenoloksidaz aktivitesinde önemli bir etkiye sahiptir. En fazla pupa kütlesi 0.5:1 P:C (protein:karbonhidrat) besininde ve en az pupa kütlesi ise 3:1 P:C besininde beslenen larvalarda kaydedilmiştir. Tüketilen karbonhidrat miktarı pupa kütlesini pozitif olarak etkilerken, tüketilen protein miktarı pupa kütlesini negatif olarak etkilemektedir. Pupa lipid miktarı diyetler arasında farklılık göstermektedir. En fazla pupa lipid miktarı 0.5:1 P:C diyetinde kaydedilirken, en az lipid miktarı 1:3 P:C diyetinde kaydedilmiştir. Dengesiz diyetler fenoloksidaz aktivitesini de etkilemiştir. Besinin protein oranıyla fenoloksidaz aktivitesi arasında pozitif bir ilişki vardır. Fenoloksidaz aktivitesi larvaların tüketmiş olduğu karbonhidrat miktarının artışıyla azalmaktadır. Sonuç olarak, dengesiz diyetler larvaların fenoloksidaz aktivitesini etkilemektedir. Karbonhidrat savunma sistemi üzerinde protein kadar önemli bir etkiye sahiptir. Ek olarak, larvaların vücut büyüklüğü aşırı karbonhidrat tüketimiyle artmaktadır.

Anahtar Kelimeler: *Agelastica alni*, dengesiz diet, böcek bağışıklığı, gıda ekolojisi, fenoloksidaz

[1] This study was conducted as a master thesis of first author's at Recep Tayyip Erdoğan University and was presented as a poster presentation at the Ecology Symposium (11-13 May 2017, Kayseri, Turkey).

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Introduction

As all organisms, insects need energy for growth and reproduction over their life span. Every organism meets the energy from their diets. The nutritional value of diet for animals varies according to the amount of nutrients in the diet and the amount of secondary metabolites of diet (Simpson & Raubenheimer, 2001). Food quality affects the growth (Joern & Behmer, 1997; Prasad & Mukhopadhyay, 2015, Rios et al., 2016), survival, reproductive success and immunity (Yang & Joern, 1994; Ponton et al., 2011) of animals. If the diet does not meet the needs of the organism, it is considered to be an unbalanced diet. Diets with unbalanced macronutrient affect the immune systems and development of larvae (Klemola et al., 2007; Lee et al., 2008; Cotter et al., 2011; Ponton et al., 2013). Immune defenses are important biological features that affect the fitness and development of organisms (Singer et al., 2014; Vogelweith et al., 2015). Invertebrates have a primitive immune system compared to vertebrates (Klemola et al., 2007). The innate system in insects contains specific and nonspecific responses to foreign agents. Phenoloxidase (PO) is one of the most important enzymes in these responses (González-Santoyo & Alex Córdoba-Aguilar, 2012). The PO enzyme is responsible for the activation of melanization in invertebrates. Melanization is responsible for the repair of tissues, defense against other pathogens such as bacteria, fungi and viral agents (Cerenius & Söderhall, 2004). PO is also an enzyme used against various pathogens (Santoyo & Aguilar, 2011). In insects, PO activity in the hemolymph is used to predict resistance to diseases (Adamo, 2004; Vogelweith et al., 2015; Srygley, 2017). That is, it is associated with resistance to some parasites/pathogens between species (Nigam et al., 1997). Dietary quality may also affect PO activity (Cotter et al., 2011; Kangassalo et al., 2018). Studies indicate that the food component that affects immunity is the amount of protein in diets (Lee et al., 2008, Singer et al., 2014). However, there may be differences between species. In addition to the individual effects of nutrients, the rate of macronutrient in the diet is one of the parameters affecting the development and immunity (Cotter et al., 2011). Every organism and growth stage of any organisms require a complex of the nutrient. In the literature, there is no report of a relationship between development parameters of larvae and PO activities.

Agelastica alni (L., 1758) (Coleoptera: Chrysomelidae) is an oligophagous leaf insect that usually occurs at a significant population density in alder (*Alnus* sp.) and willow (*Salix* sp.). Almost every year, a significant amount of leaf assimilation surface loss occurs in alder. In some years all the leaves of alder are consumed by *A. alni* (Firidin & Mutlu, 2009). In this study, the effect of unbalanced diets on the PO activity and the development of *A. alni*, which is an important forest pest, was investigated. Therefore, it will make a contribution to the literature and reveal the factors affecting the immunity and development of this species.

Materials and Methods

Study organism and diets

Agelastica alni larvae were collected from alder leaves in Maçka Çatak Village, Trabzon, Turkey in 2015. The larvae were brought to the laboratory and fed on artificial diets. The artificial diets used in feeding experiments were modified from the study of Yamamoto (1969). Artificial diets were wheat germ-base supplemented with casein. The contents of the base diet developed by Yamamoto (1969) are given in Table 1. In this study, additional total of eight artificial diets were used, with different protein and carbohydrate ratios. The protein and carbohydrate ratios of diets added to the based artificial diet are as follows: 1:1, 2:1, 1:0.5, 1:3, 1:5, 0.5:1, 3:1 and 5:1 P:C (protein:carbohydrate). P:C ratio in the diet was used to create nutrient imbalanced diets.

Table 1. Content of base diet (for 1 kg formula)

Content	Quantity (g/ml)
Wheat germ	80 g
Casein	30 g
Sucrose	30 g
Yeast Brewers	16 g
Vanderzant Vitamine Mixture	10 g
Wesson salt mixture	8 g
Cholesterol	0.2 g
Sorbic acid	2 g
Methyl parapen	1 g
Linseed oil	1 ml
Agar	20 g
Distilled water	800 ml

Larval growth performance

Trials were performed in two groups. In the first experimental group, the growth performance of larvae and the duration from the larval stage to the pupal stage were recorded. The pupal mass, pupa lipid and protein content of pupa were determined for growth performance. For this purpose, 10 larvae were used for each diet. Individuals were weighed on an electronic balance sensitive to 0.0001 mg; then each one was placed singly into a plastic cup with a cover. Each food block (about 1 g) prepared as described was pre-weighed before being presented to the larva for each treatment. Every second day, any uneaten food by the larvae remaining in the larval chamber was collected and replaced with fresh pre-weighed food block. The uneaten food left by the larva from each feeding chamber was collected separately and dried in an oven (50°C) and weighed after it reached a constant weight. Every other day, each larva was weighed. This procedure was repeated until all of the larvae entered the pupal stage (Lee et al., 2002).

The total lipid amount was calculated using formula from Loveridge (1973) and Simpson & Raubenheimer (2001). The total lipid amount stored in each pupa was determined with three times chloroform extraction (Simpson & Raubenheimer, 2001). The pupae were dried to a constant weight at 50°C. The dry weights of the pupal are noted. Each of the dry pupae was placed in tubes and chloroform was added. The tubes were placed in an automatic shaker for 24 h, then chloroform in tubes evacuated and chloroform re-added to the tubes. This process was repeated three times. At the end of the third chloroform extraction, the pupae were redried and reweighed to calculate their per cent lipid contents.

The lipid free pupae obtained by the chloroform extraction were analyzed for their nitrogen content using Thermo Scientific Flash 2000 series-NCS analyzer instrument and Dumas method (Yi et al., 2013). The ground dry samples weighed approximately 2.5 mg were placed in a thin tin capsule and the capsule was sealed. The capsules were then placed in the autosampler portion of the device. When the sample enters the combustion reactor, it enters into a special furnace heated to 900-1000°C and a small amount of pure oxygen and helium gas is added to the system to allow the samples to burn. In this case, the samples turn into elemental (simple) gases. Element separation is determined without the need for a complex separation system by means of TCD detector and separation in the column. The gas generated by the TCD detector is transferred to the column and the N values are calculated by the peaks formed in the column. At the end of this process, the percentage of nitrogen was multiplied by the constant of 6.25 to convert to the crude protein quantities (Oonincx et al., 2015).

Phenoloxidase activity

The second experimental group was established to determine the specific PO activity. In this experimental group, larvae were fed collectively in each food group. Four days after ecdysis to the final larval stage, hemolymph was collected from individuals by piercing the final proleg with a sterile needle.

Hemolymph was collected in Eppendorf tubes and frozen at -20°C until needed. PO activity and the amount of protein were measured according to Lee et al. (2008). For the PO activity assay 100 µL of 10 mM L-Dopa (substrate) was added to 100 µL of buffered hemolymph (400 µL of ice-cold phosphate-buffered saline (PBS, pH 7.4) and the absorbance of the mixture was measured at 492 nm on a Versamax tunable microplate reader after 20 min (no full stop) of incubation at 25°C. PO activity is expressed as PO units, in which one unit represents the amount of enzyme required to increase the absorbance by 0·001/min. Hemolymph protein content was quantified by the method of Bradford (1976). Triplicate samples were used for examining PO activity and protein level.

Statistical analysis

The amount of food intake by each larva fed on each artificial diet, the pupal mass, the protein contents of the pupae, the lipid contents of the pupae, duration of growth and PO activity were analyzed statistically using SPSS Version 17. A normality test was performed to determine whether the variables were normally distributed. In order to determine the differences between the groups, ANOVA and Tukey test were performed in the data with normal distribution. Correlation testing was performed to determine whether there was a correlation between the amount of food consumed, the amount of pupae protein, the amount of pupae lipid, pupal mass and PO activity. Regression analysis was performed after the relationship was determined.

Results and Discussion

Growth performance

Diets affect the amount of food intake and growth performance of larvae. In the feeding experiments, 1:1 P:C group was accepted as the control group. The maximum amount of consumption was found for larvae fed on 2:1 P:C diet and the least amount of consumption was found for larvae fed on 1:5 P:C diet (Figure 1). There was a significant difference in point of food intake between diets (ANOVA, $F=545$, $p<0.00$). However, there was no significant difference between the 1:0.5 P:C diet and the 3:1 P:C diet according to the Tukey test. Dietary carbohydrate ratio ($R=-0.68$, $p<0.01$) has a negative effect on food intake. However, the 1:1 P:C ratio of the diet or protein ratio of the diet had no effect on the amount of food intake ($p>0.05$) (Figure 1).

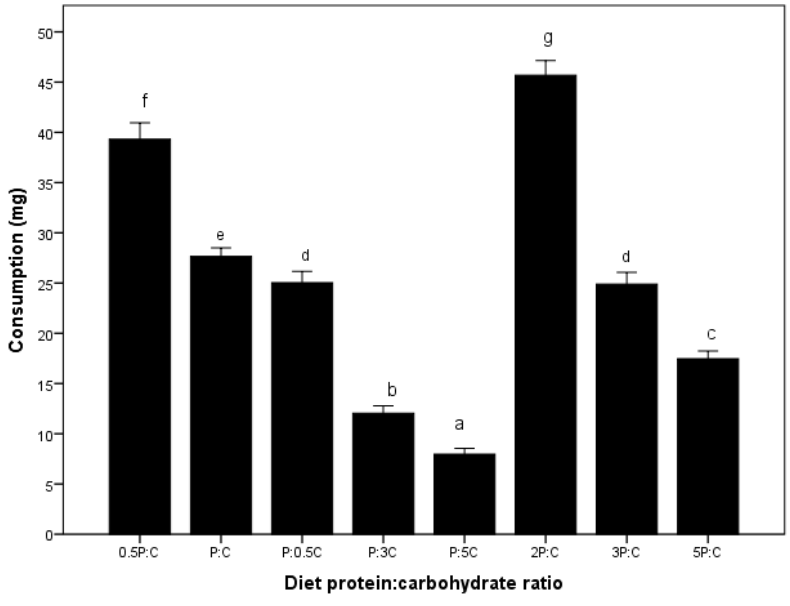

Figure 1. Food intake on different artificial diet. Diets with the same letter are not significantly different (p=0.05).

Diets affect pupal mass (ANOVA, F=11.74, p<0.00). The highest pupal mass was found for individuals fed on 0.5:1 P:C diet and the lowest pupal mass was found for individuals fed on 3:1 P:C diet (Figure 2). The P:C ratio of the diet did not affect pupal mass (p>0.05). However, the amount of carbohydrate consumed and the amount of protein consumed affected pupal mass. While the amount of carbohydrate consumed affects the pupal mass positively (R=0.28, p<0.05), the amount of protein consumed negatively affects pupal mass (R=-0.24, p<0.05), (Figure 2).

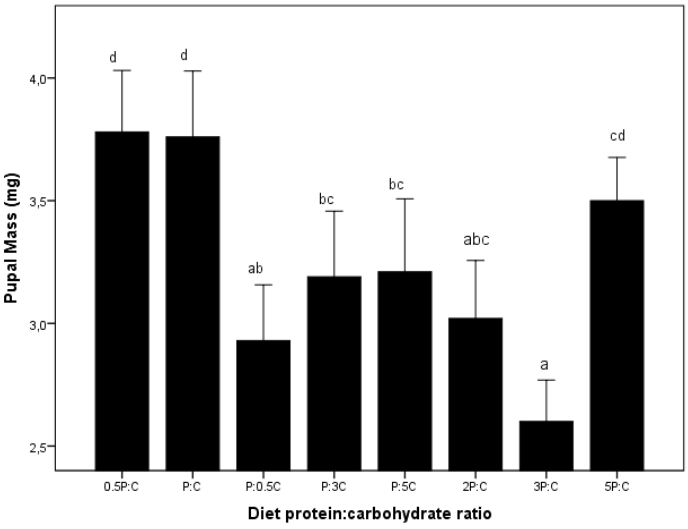

Figure 2. Pupal mass on different artificial diet (mg). Diets with the same letter are not significantly different (p=0.05).

Differences were also found between the pupal lipid for different diets (ANOVA, F=28.7, p<0.01). The highest amount of pupal lipid was determined in the 0.5:1 P:C diet and the lowest pupal lipid amount in the 1:3 P:C diet (Figure 3). The amount of carbohydrate consumed positively affected the amount of pupal lipid (R=0.41, p<0.01). Similarly, the dietary carbohydrate ratio positively affected the amount of pupal lipid (R=0.22, p<0.05). However, there was no effect of the amount of protein consumed to pupal lipid amount (p>0.05). The P:C ratio of the diet negatively affected the amount of pupal lipid (R=-0.26, p<0.05).

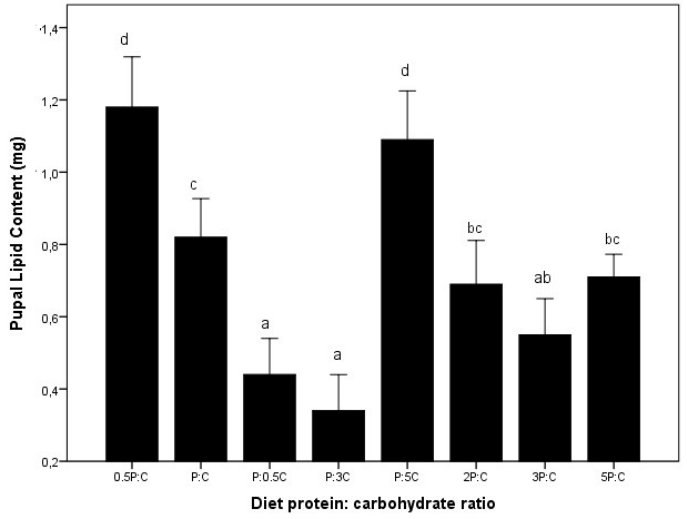

Figure 3. Pupal lipid content on different artificial diet (mg). Diets with the same letter are not significantly different (p=0.05).

The pupal crude protein also varies between diets (F=3.66, p<0.02). The highest pupa crude protein was found from individuals fed on 5:1 P:C diet, while the minimum pupa crude protein was determined from individuals fed on 3:1 P:C diet (Figure 4). However, the P:C ratio of the diet did not affect the pupa crude protein (p>0.05).

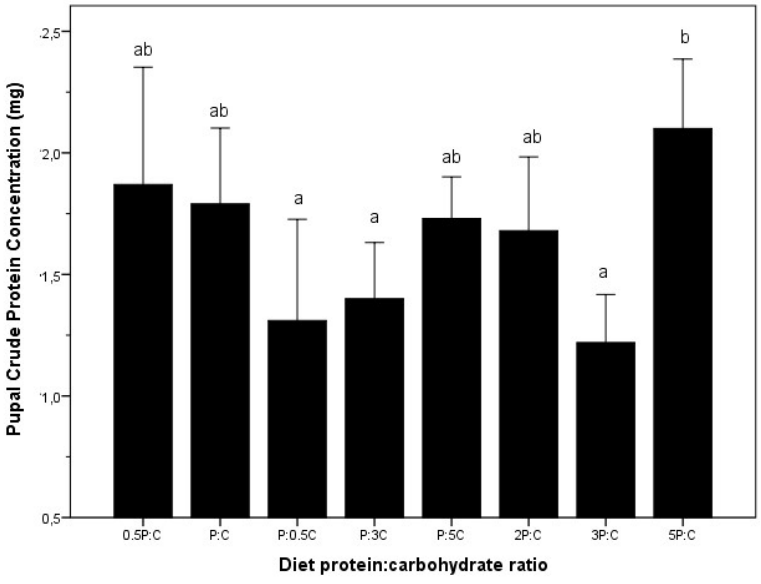

Figure 4. Pupal crude protein concentration on different artificial diet. Diets with the same letter are not significantly different (p=0.05).

Phenoloxidase Activity

PO activities of the experimental groups have been affected by diets used (ANOVA, F=21264.6 p<0.01). The highest specific PO activity (U/mg protein) was found for larvae fed on 1:0.5 P:C diet and the lowest PO activity was found for larvae fed on 2:1 P:C diet (Figure 5). The P:C ratio of the diet affected the PO activities of the larvae. There was a positive relationship between P ratio of diet and PO activity (R=0.31, p<0.01). The amount of protein consumed did not affect the PO activity (p>0.05). A negative correlation was found between the C ratio of the diet and PO activity (R=-0.39, p<0.01). Similarly, when the amount of carbohydrate consumed was increased, PO activity decreased (R=-0.68, p<0.01). A positive relationship was found between P:C ratio and PO activity of diet (R=0.52, p<0.01). The amount of pupal lipid also affects the PO activity. PO activity decreased with increasing of pupal lipid amount (R=-0.26, p<0.05).

Our main finding was that nutrient imbalance has an impact on pupal lipid amount and PO activity of alder leaf beetle. Unbalanced diets are known to affect the growth of larvae. According to nutritional ecology, animals develop mechanisms to regulate nutritional imbalances in their diet (Cotter et al., 2011; Prasad & Mukhopadhyay, 2015; Rho & Lee, 2015). Even herbivores can change the amount of consumption to compensate for nutritional imbalance (Waldbauer & Friedman, 1991; Bernays, 1998; Cotter et al., 2011; Ravenscraft & Boggs, 2016). Also, for *A. alni* larvae, food intake varies between artificial diets. However, as the carbohydrate content of the diet increased, it was shown that the amount of food intake decreased. Other factors related to diet had no direct effect on consumption. *A. alni* larvae may have regulated the intake of nutrient by reducing the food intake to ingest amount of carbohydrates required for the development. In addition, carbohydrates are nutritional stimulants for many species (Bernays et al., 2004; Juma et al., 2013). However, for this species sucrose may have a role as a feeding deterrent over a certain concentration.

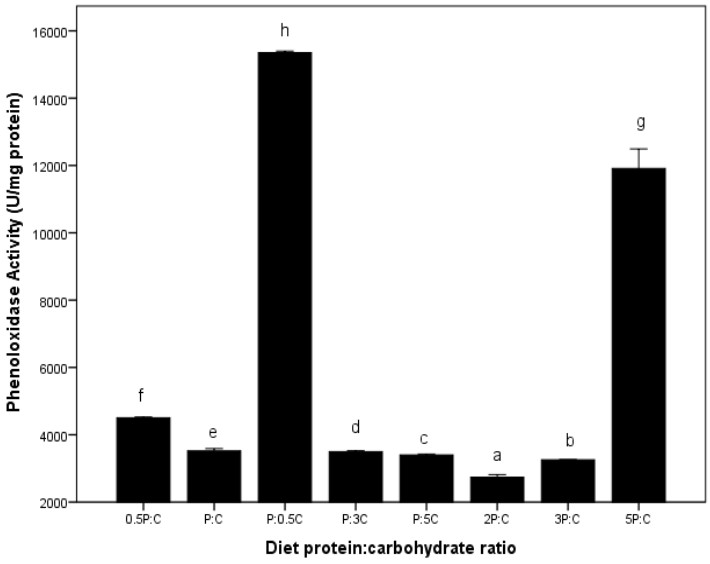

Figure 5. Phenoloxidase activity of larvae on different artificial diet. Diets with the same letter are not significantly different (p=0.05).

As the amount of protein consumed by *A. alni* larvae increases, the pupal mass decreased. This result is consistent with Honek (1993) and Lee et al. (2002). Excess protein ingested along with diet may have increased metabolic activity (Schroeder, 1986). The catabolizing of the excess protein and the removal of the feces from the body can lead to decreased pupal mass. However, insects maintain the balance of pupal crude protein while they fed on diets restricted to protein content. Given the amount of pupal crude protein is an important parameter for the development of insects, larvae may have managed to convert and use nutrition even though the diet is unbalanced (Joern & Behmer, 1997). The amount of pupal lipid increases with the amount of carbohydrates consumed and the carbohydrate rate of the diet. This result is consistent with the literature by Raubenheimer & Jones (2003). Increasing the protein: carbohydrate ratio of the diet resulted in a decrease in the amount of lipid. One way to avoid overly ingesting food is to increase metabolic rate through dietary thermogenesis as seen in some herbivores and omnivores (Trier & Mattson, 2003). This way, lipids can be used as energy reserves in a long-term starvation (Warbrick-Smith et al., 2006).

Lipid has an ecological and evolutionary effect on developmental performance which also relates to immunity of the species (Klemola et al., 2007; Cotter et al., 2011; Ponton et al., 2011; Singer et al., 2014). Studies have shown that diet quality is more effective than the amount of food intake in immune defenses of insects (Siva-Jothy & Thompson, 2002; Klemola et al., 2007; Singer et al., 2014). In our study, there was no significant effect of food intake on PO activity of *A. alni* larvae. However, the P:C ratio of the diet have been found to be important for the PO activities of the larvae. The invertebrate immune system includes the PO-profenoloxidase (PO-PPO) system. PO is a defense component of oxidative and melanism used against eukaryotic parasites (Cerenius & Soderhall, 2004; Lee et al., 2008; Santoyo & Aguilar, 2011; Vogelweith et al., 2015). This activity is an important component of the innate immune system (Lee et al., 2008). PO activity in hemolymph can be used to measure disease resistance (Adamo, 2004). Therefore, high PO activity can be interpreted as high resistance to pathogens. The increase in P:C ratio of the diet also causes an increase in PO activity. Therefore, nutritional imbalance may be perceived as a stress condition by larvae and may cause an increase in PO activity. PO may also increase due to population density or response to parasites (Klemola et al., 2007). Low-quality diets cause to undergo stress on autumn moths, *Epirrita autumnata* (Borkhausen, 1794) (Lepidoptera: Geometridae) and thus show high PO activity (Klemola et al., 2007). For *A. alni* larvae, the increase in the P:C ratio of the diet increases the PO

activity. Protein deficiency in the diet can affect the immune system of insects (Lee et al., 2008; Srygley et al., 2009). Therefore, an increase in the rate of PO activity with an increase in protein ratio is expected. However, one of the interesting results in study is that the increase in the C ratio of the diet and the amount of carbohydrates consumed causes the PO activity of the larvae to decrease. Srygley et al. (2009) suggested that protein is more important than carbohydrate in immune defenses. According to our results, carbohydrate might play n important roles in immune defense as well as protein. The amount of carbohydrates consumed and the carbohydrate rate of the diet has a positive effect on the level of lipids in the pupae. Pupal lipid content also suppresses the PO activity. Adamo et al. (2008) reported that the high lipid level in the hemolymph caused a decrease in the concentration of apolipophorin III protein. Apolipophorin protein also has a role in the activation of pro-PO cascade (Adamo et al., 2008; Zdybicka-Barabas & Cytrynska, 2010). Therefore, consumption of large amounts of carbohydrates increases the amount of pupal lipids, and the increase in the amount of pupal lipids reduces the concentration of apolipophorin III protein and suppresses the PO activity. However, the effect of the amount of lipid in hemolymph is reported (Adamo et al., 2008; Zdybicka-Barabas & Cytrynska, 2010). According to the results of our study, it must have concluded that the increase in the amount of storage lipid causes the concentration of apolipophorin III protein to decrease. This has another advantage for larvae. With the increase in the amount of carbohydrates consumed, the larvae used nutrition for growth rather than immunity. Syrgley (2017) stated that protein consumption has a more important role in the determinant of fitness than carbohydrate. On the contrary, the amount of protein consumed decreases the pupal mass. The amount of protein consumed has no effect on the amount of pupal lipid. The excess C ratio in the diet may have encouraged the use of nutrient for the development of larvae. In the evolutionary process, larvae must have developed a strategy, that by increasing its body size, they have developed fecundity in order to increase fitness rather than strengthen immune defense to increase fitness, because for many insects, body size is associated with fecundity (Garrad et al., 2016; Srygley, 2017; Togashi & Yamashita, 2017).

As a result, unbalanced diets may impair immune function and affect defenses against pathogens (Amar et al., 2007; Vogelweith et al., 2015). In addition, limiting nutrition can stress insects. This situation leads to the suppression or increase of immune defenses. When considered the results obtained from different experimental diets in the present study, carbohydrates also might be an important compound in insect immunity. For example, the diet 3:1 and 2:1 P:C did not show high PO activity despite the increased protein content. All of these data suggest that the insect immunity might be a result of complex feeding containing enough of the essential macronutrients.

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