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Effects of Selected Plant Secondary Metabolites in Mulberry, Apple, Plum, and Walnut on the Pupal Parameters of *Hyphantria cunea* Drury, 1773 (Lepidoptera: Arctiidae) Larvae Infected by *Bacillus thuringiensis* subsp*. kurstaki*

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ABSTRACT

In this study, the effects of various secondary metabolites in the economically important plants on the pupal weight, the pupal total protein and the pupal total lipid contents of *Hyphantria cunea* larvae infected by *Bacillus thuringiensis* subsp. *kurstaki* were investigated. In order to find out their effects on the larvae, the phenolic compounds present in *Morus alba* (mulberry), *Malus pumila* (apple), *Prunus dometica* (plum), and *Juglans regia* (walnut) leaves samples, which are the most preferred by *H. cunea* and have economic importance, were determined by phytochemical methods. The changes observed in the pupae's weights, total protein and lipid contents were related to the plant leaves' chemical contents. Among the non-infected groups, the mulberry-fed group had the highest both pupal weight and the pupal protein content. The minimum amount of gallotannin, as well as the catechin and rutin contents, was present in the mulberry leaves. The minimum pupal weight and the pupal protein contents were obtained in the larvae fed by the apple leaves with the highest rosmarinic acid and protocatechuic acid. The pupal weights and the pupal total protein contents were decreased by the bacterial infection, while the pupal total lipid contents increased by the bacterial infection. As a result of this study, the effects of both plant secondary metabolites experimentally used and *B. thuringiensis* infection on the pupal parameters of *H. cunea* were shown to be statistically significant.

Key words: *Bacillus thuringiensis*, gallotannin, *Hyphantria cunea*, phenolic compound, pupal weight.

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INTRODUCTION

Insects dominate more than half of the known living organisms in the world in terms of their number of species. Herbivore insects, which obtain their nutrients through various plant parts, and make up the greatest part of the total biomass in the world (Tek & Okyar, 2017). Among these, the herbivorous lepidopteran larvae consume large amounts of plant material throughout their development from the first instar to the last instar (Esperk & Tammaru, 2004; Gotthard, 2004). To prevent their attacks, plants defend themselves against herbivores through various physical and chemical defenses (Chen, Kim, Klinkhamer, & Escobar-Bravo, 2020). While physical defenses such as the leaf hardness and the trichomes adversely affect the performance and preference of herbivores (Chen et al, 2020); however, the chemical defenses involve various plant secondary metabolites (PSMs) biochemically generated in plants (Mazid, Khan, & Mohammad, 2011; Rosa, Woestmann, Biere, & Saastamoinen, 2018). PSMs are widely distributed in many plant taxa (e.g. phenolic acids, flavonoids). The phenolic compounds have a great importance to defend plants parts against herbivores and microbial attacks. PSMs function by interfering with the basic metabolic, biochemical, physiological functions of the cells, and even behavior of herbivorous insects (Kessler & Baldwin, 2002; Tan & Luo, 2011; Tangtrakulwanich & Reddy, 2014). In studies, chlorogenic acid (Kundu & Vadassery, 2019), rosmarinic acid (Khan et al, 2019), rutin (Silva et al, 2016), protocatechuic acid (Syafni, Putra, & Arbain, 2012), benzoic acid (Beran, Kollner, Gershenzon, & Tholl, 2019), and tannic acid (Ma et al., 2019) have been shown to biological activities against herbivores.

The autumn webworm, *Hyphantria cunea* Drury (Lepidoptera: Arctiidae), being a polyphagous species that feed on a vast number of plant species and is a serious invader in a wide variety of habitats. This insect larvae causes significant damage to both forests and orchard trees in urban areas throughout its range (Liao et al, 2010). As in many countries, this pest causes significant damage to the crop in Turkey too. Control measures are required especially to prevent economic damages to orchards and ornamental trees. In our study, *Bacillus thuringiensis* (*Bt*), the most commonly used, the most cost-effective and containing many spore-crystal toxins in its formulation, has been used in biological control of *H. cunea* larvae (Weinzierl, Henn, Koehler, & Tucker, 2005).

Plants contain PSMs with the complex chemical composition (Guerriero et al, 2018). PSMs formed more than once from various structural classes, rather than a single compound, differ in both content and quantity. How these compounds affect the herbivores is important because it is essential to know the biology of the species in the combat against harmful species. Studies on insect feeding in the literature generally evaluate insect performance in terms of survival and development (Sousa et al, 2016; Rosa et al, 2018; Huang, Lv, Zhang, & Chang, 2020). In addition to these studies, studies addressing the parameters that affect the fitness and fecundity of insects are also crucial. Our aim in this study has been to determine how the phenolic compounds present in the leaves of *Juglans regia* (walnut), *Malus pumila* (apple), *Prunus dometica* (plum), and *Morus alba* (mulberry), which are the most preferred

food items by *H. cunea* and economically important for local people, affects the pupal weight, the pupal total protein contents, and pupal total lipid contents of the insect fed on them. Besides, we have aimed to determine how *B. thuringiensis* subsp. *kurstaki* (*Btk*) effects on these parameters.

MATERIAL AND METHODS

Collection of the larvae and the feeding experiments

The 1st generations of *H. cunea* larvae were collected from Çarşamba District of Samsun, Turkey, in June 2020 (N41°30'-E36°05'). The larvae collected were placed into the plastic containers (5×10×2 cm) one by one with 50 larvae in each group and were let to feed on the leaf samples of walnuts, mulberries, apples, and plums plants until they reached the pupal stage. In this study, the larvae of the 2nd generation obtained as the offspring of the 1st generations' adults were used. The larvae of 2nd generation were allowed to feed on the leaf samples of these four plant species until the pupal stage. The feeding experiments were carried out at 25±2°C, 70% humidity, 16 h light/8 h dark. The plant leaves used in the feeding experiments were collected daily; each leaf sample was sterilized with 50% ethyl alcohol, and then given to the larvae.

Preparing bacterial suspension

Bacillus thuringiensis subsp. *kurstaki* (*Btk*) was used in larval infection. The strain was obtained from culture collection of microbiology laboratory at Karadeniz Technical University. The *Btk* was grown overnight at 30°C in nutrient broth (AppliChem, Darmstadt, Germany). The optical density of the growing culture was measured at a wavelength of 600 nm and set to OD_{600} = 1.89 (Ben-Dov, Boussiba, & Zaritsky, 1995).

Experimental setup

Each group consisted of 50 *H. cunea* larvae. The larvae in the non-infected groups were fed with the non-contaminated leaves for five days. For infected groups, each plant leaf used for feeding the larvae was contaminated by 1 ml of the bacterial suspension. The larvae continued to feed until they became pupae. Since deaths also occurred due to infection, 30 pupae were used for analysis to keep the number constant in each group.

The phenolic and gallotannin contents of the plant leaf samples

The determination of PSMs was made with HPLC brand Thermo Finnigan Surveyor (Thermo Finnigan, San Jose, CA, USA). HPLC-UV analyses were performed on a reverse phase C18 column (150 mm × 4.6 mm id, 5 µm particle; Fortis, France) using a Thermo Finnigan Surveyor HPLC and UV detector which is simultaneously operating dual-UV wavelength. Gradient elution was used for HPLC analyses. The mobile phase was (A) 2% acetic acid in water and (B) 70:30 acetonitrile:water. The following gradient was used; 0-3 min 5% B; 3-8 min 5-15% B; 8-10 min 15-20% B; 10-12 min 20-25% B; 12-20 min 25-40% B; 20-30 min 40-80% B. The injection volume was 25 µl, the column temperature was 30°C and the flow rate was 1.2 ml/min.

The method used to determine gallotannin contents of the leaf samples was described by Bate-Smith (1977). For gallotannin analysis, a certain amount of leaf samples from each plant species were taken daily and dried in an oven until reaching constant weights. The dried leaves were ground. 4 tubes with 10 ml were used for each sample. A 5% KIO_3 solution was prepared for analysis. 0.5 mg of the leaves sample was taken on the 4 tubes prepared for each sample and put into the tubes. 1 ml of the previously prepared 5% KIO_3 solution was added to three of the samples placed in the tube. For the control, only 1 ml of distilled water was added to the 4th tube. The prepared samples were kept in an ice bath for 1 hour. The samples were then removed from the ice bath and their absorbance was measured in a spectrophotometer at a wavelength of 550 nm. A standard curve was prepared with tannic acid solutions (0.1-0.7 mg/ml) to calculate the gallotannin amounts of the samples.

The determination of the pupal total lipid contents

Hyphantria cunea pupae were kept in the incubator at 50°C for one month to achieve constant weights and thus calculate their dry weights. To calculate the lipid amounts of the pupae reaching constant weights, the pupae were placed in glass tubes and kept in pure chloroform for 24 hours on a rotary shaker. After this treatment, the supernatants of the tubes were discarded. This process was repeated three times so that the lipid contents of the pupae were removed. The pupae were put back into an oven and dried again to constant weights, and then the lipid-free weights of the pupae were determined by weighing out. The analyses were performed individually for each pupae. The total lipid contents of the pupae were calculated from the obtained data (Simpson, 1983).

The determination of the pupal total protein content

The nitrogen content determination of the lipid-free *H. cunea* pupae was made by semi-micro Kjeldahl method with Kjeltec Auto 1030 analyzer (Tecator, Sweden). The analyses were performed individually for each pupae. Total protein contents of the pupae were calculated by multiplying the nitrogen content of each pupal sample found as a result of the process by the constant 6.25 (Oonincx, van Broekhoven, van Huis, & van Loon, 2015).

Statistical analysis

In the study, whether the pupal weights, pupal lipid and protein contents of *H. cunea* were statistically different from each other were determined by ANOVA followed by post hoc Dunnet test. SPSS 21.0 software was used for statistical analysis.

RESULTS

The phenolic and gallotannin contents of the leaf samples

While the highest amount of chlorogenic acid contents was in the plum leaves, the lowest content was present in the mulberry leaves. Catechin and rutin were only present in the mulberry leaves. Benzoic acid was determined to be present only in the walnut

leaves. Protocatechuic acid and rosmarinic acid contents were the highest in the apple lowest content and the lowest content of them were found to be in the plum leaves (Fig. 1). Among all plant leaf samples, the highest gallotannin content was determined in the walnut leaves and the lowest content of it was in the mulberry leaves (Fig. 2).

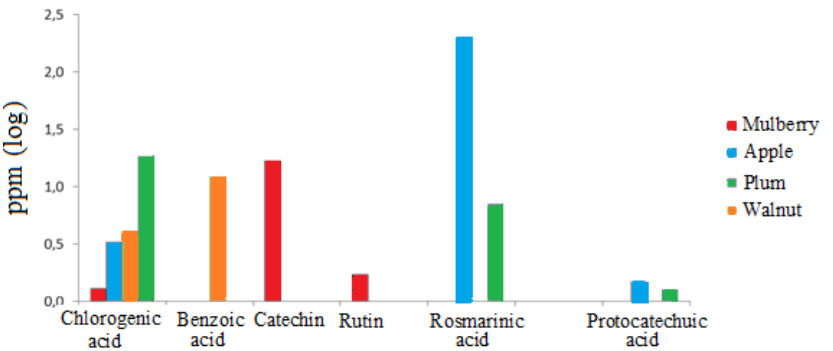

Fig. 1. The contents of phenolic compounds in leaves.

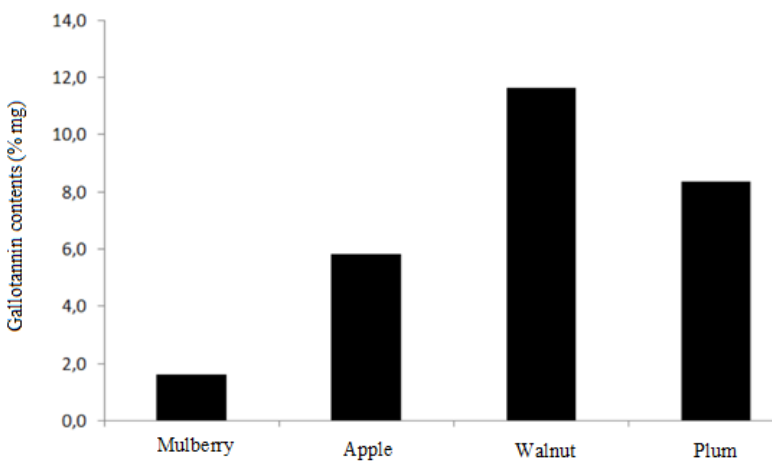

Fig. 2. The gallotannin contents in leaves.

The pupal weights

Among the non-infected groups, while the lowest pupal weight was obtained in the apple-fed group (21.7±1.4 mg, t=-0.7, P>0.05), and the highest pupal weight was found to be in the mulberry-fed group (25±1.7 mg, t=-40, P>0.05). The pupal weights of all groups infected with bacteria decreased compared to the non-infected ones. Among the infected groups, the lowest pupal weight was in the walnut-fed group (18.8±1.2 mg, t=-2.3, P<0.05) and the highest in the mulberry-fed group (23.2±0.8 mg, t=-40, P>0.05) (Fig. 3).

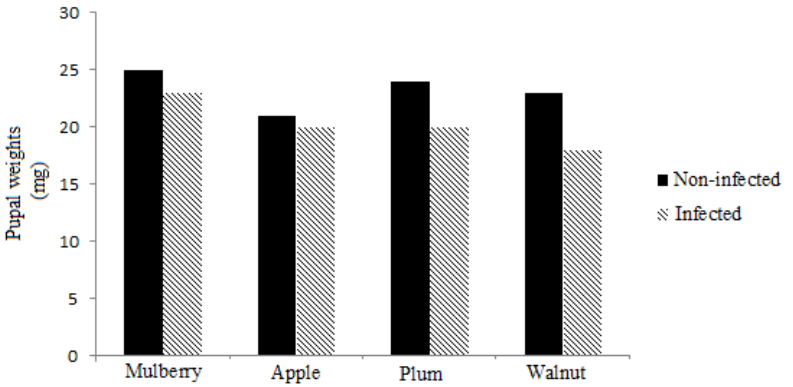

Fig. 3. The pupal weights of *Hyphantria cunea* according to different plant groups.

The pupal total protein contents

Among the non-infected groups, the pupal total protein contents were 13.3±1.1 mg, t=-2.1, P<0.05 in the mulberry, 11.2±0.7 mg, t =-2.5, P<0.001 in the apple, 13.1±0.7 mg, t=-5, P<0.001 in the plum, and 12.3±0.6 mg, t=-3.2, P<0.05 in the walnut-fed groups. Among the bacteria infected groups, while the lowest pupal total protein content was found in the plum-fed group (8.7±0.6 mg, t=-5, P<0.001) and the highest one in the mulberry-fed group (10.7±0.4 mg, t =-2.1, P<0.05). The pupal total protein contents of all groups were found to be decreased by the bacterial infection compared to non-infected groups (Fig. 4).

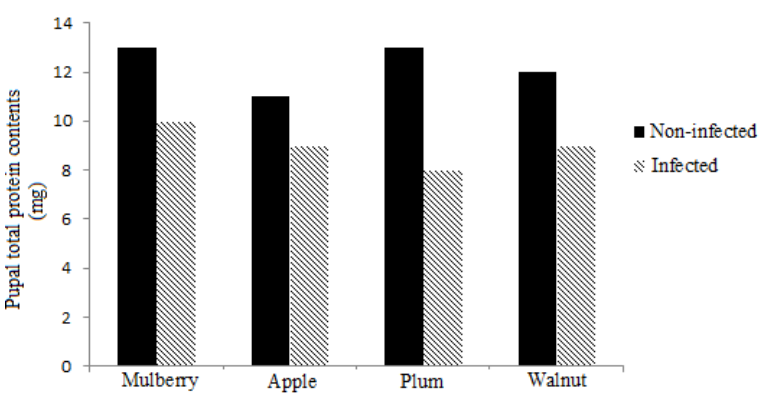

Fig. 4. The pupal protein contents of *Hyphantria cunea* according to different plant groups.

The pupal total lipid contents

The pupal total lipid contents in the non-infected groups were respectively in the plum (7.4±0.5, t=1.3 mg, P>0.05)> mulberry (7.3±0.5 mg, t=2.3, P<0.05)> apple (6.8±0.6 mg, t=1.6, P>0.05) > walnut (6.6±0.6 mg, t=-1, P>0.05). In the infected groups, the pupal total lipid contents were 9.0±0.6 mg, t=2.3, P<0.05 in mulberry, 8.1±0.5 mg, t=1.6, P>0.05 in

apple, 8.5±0.6 mg, t=1.3, P> 0.05 in plum, and 5.8±0.5 mg, t=-1, P>0.05 in walnut-fed group. It was determined that the pupal total lipid contents in all groups (except walnut) increased by the bacterial infection compared to the non-infected ones (Fig. 5).

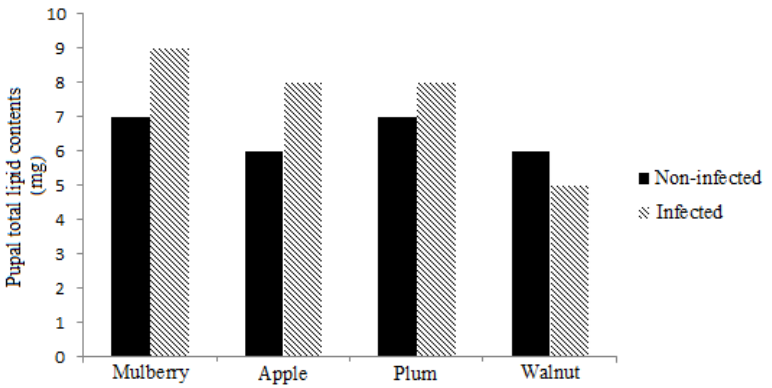

Fig. 5. The pupal lipid contents of *Hyphantria cunea* according to different plant groups.

DISCUSSION

Host preference by herbivorous animals is determined according to the characteristics of the plants (Stam et al, 2014), and the characteristics of the plants depend on the PSMs as well as the primary metabolites (Coyle, Clark, Raffa, & Johnson, 2011). Many phenolic compounds have antagonistic feeding effects on various taxa and other biological processes (Lindroth & St Clair, 2013). In the early stages of lepidopteran larvae, many phenolic compounds have growth inhibitory activities (Bhattacharya & Chenchaiah, 2007), which also affects subsequent biological parameters. The finding of the least pupal weight was in the apple-fed group among the non-infected groups in our study was supported by above mentioned studies. The apple leaves had maximum rosmarinic acid in our study, which adversely affected the pupal weight. In a study performed with *Anastrepha ludens*, Aluja et al (2014), found that the larvae fed by a cultivar containing the maximum amount of catechin and rutin had a minimum pupal weight. The result of this study contradicted with the result that we found in our study that the catechin and rutin only present in the mulberry leaves caused maximum pupal weight. Besides, in the mulberry leaves gallotannin content was minimal. Since tannins are astringent and bitter polyphenols (Ashok & Upadhyaya, 2012), the larvae may have reached maximum pupal weight by preferring more to be fed with the mulberry leaves containing minimum tannins. Tayal, Somavat, Rodriguez, Martinez, & Kariyat (2020) in a study with *Manduca sexta* larvae found out that the larvae fed on maize leaves with more tannin had lower pupal weights. Also, studies conducted with various species have shown that the tannin decreases pupal weight (Barbehenn et al, 2009; Topkara, 2019). Based on the results obtained from these studies, a possible reason may be that the emergence of semiquinone radicals in the presence of tannins increases the metabolic cost and decreases the efficiency of the conversion of consumed food into body mass (Barbehenn et al, 2009).

Exposure to sublethal concentrations of insecticides affects insect population dynamics by altering the biological parameters of individuals (Stark & Banks, 2003; Desneux, Decourtye, & Delpuech, 2007; Qu et al, 2015). Abedi, Saber, Vojoudi, Mahdavi, & Parsaeyan, (2014) showed that *Btk* infection decreased pupal weights of *Heliothis armigera*. Nouri-Ganbalani, Borzoui, Abdolmaleki, Abedi, & Kamita (2016) noted that *Bt* strains cause a low pupal weight in *Plodia interpunctella*. In our study, we found that the pupal weights of all groups infected with *Btk* decreased compared to the non-infected groups; these results may have likely to adversely affect the fitness of *H. cunea*. Besides this, the pupal weight and the fecundity are also related. Also in the current study, *Btk* toxicity may lead to decreased fecundity. In a study (Pineda et al, 2009), several Coleopteran pests have been reported to have decreased fecundity after exposure to pesticides. The fecundity of the adult females is greatly affected by bioinsecticides. Due to the strong correlation between the fecundity and the pupal mass in females (Bauerfeind & Fischer, 2009), it can be understood that low female pupal weight can directly affect reproductive abilities. Since oviposition is an important phenomenon, reduction in the fecundity may be a management strategy against the herbivore pests (Ketoh, Glitho, Koumaglo, & Garneau, 2000; Zhao, Yang, Wang-Pruski, & You, 2008).

The accumulation of the storage proteins and the use of the proteins by insects in later stages are important events associated with the metamorphosis of holometabolous insects. The total protein contents of insect are essential for all stages of development of the insect. The assessment of changes in the total protein contents is important to determine whether the ingredients in the diets are used effectively by the insect and whether it effects on the development of the insects (Sak, Uçkan, & Ergin, 2006; 2011). Among the non-infected groups, we found that the pupal protein content was the lowest in the apple-fed group. The highest contents of rosmarinic acid and protocatechuic acid were present in the apple leaves adversely affected the pupal total protein content. These results in our study are supported by Dixit, Praveen, Tripathi, Yadav, & Verma (2017) research which was found that the increasing amount of PSMs caused a decrease in the total protein contents of *Helicoverpa armigera* and *Spodoptera litura*. Also, we found that the least of chlorogenic acid content in the mulberry leaves caused the highest pupal total protein content. Chlorogenic acid is known to be an antifeedant substance (Ikonen, Tahvanainen, & Roininen, 2001). Owing to its ability to bind proteins covalently, this substance can be especially harmful to the protein digestion in insects. Therefore, it is not surprising that a low chlorogenic acid content in the leaf samples causes the highest pupal protein content. Besides, the content of gallotannin in the mulberry leaves was the lowest among the leaf samples. Considering the interaction between tannins and proteins (Adamczyk, Simon, Kitunen, Adamczyk, & Smolander, 2017), the highest pupal total protein content in the mulberry-fed group with low tannins supports this result.

Considering that the pupal total protein content is an important parameter for the development of insects, it is a disadvantage for the insects that the pupal total protein contents of all groups obtained by the bacterial infection were decreased compared to the non-infected groups. The studies have demonstrated a relationship between the toxic properties of insecticides and the total protein amount (Ahmed, Wilkins, & Mantle,

2002; Guedes et al, 2006). It is known that *Bt* toxicity causes a decrease in the protein contents of insects (Abedi et al, 2014). Our study also is supported by the research of Elgizawy & Ashry (2019) showed that *Bt* infection reduced the total protein contents of insects compared to the control. Among the infected groups in our study, the lowest pupal protein content was determined in the plum-fed group. The plum leaves had the highest amount of chlorogenic acid among the plant leaves analyzed. Chlorogenic acid increases the toxicity of bacteria (Ludlum, Felton, & Duffey, 1991). Therefore, it is not surprising that the lowest total protein content was found in this group.

Lipids are the main component of all living organisms (Yi et al, 2013) and an important energy sources (Smit, Muskiet, & Boersma, 2004). Lipids are used by insects for various physiological functions such as development, flight, reproduction, in the structure of cell membranes, communication via pheromones, etc. (Beenakkers, Vanderhorst, & Vanmarrewijk, 1985; Lease & Wolf, 2011). The total lipid content of the insects is affected by their diets (Oonincx et al, 2015). In our study, it has been determined that the highest pupal total lipid content in the non-infected groups has found in the plum-fed group having the highest chlorogenic acid content. In the pupal stage, the lipid stores during the larval stages will be used to facilitate the metamorphosis and also to support the energy demands of reproduction and flight in adulthood (Ziegler, 1991), so that chlorogenic acid will be advantageous for the species. Furthermore, it was observed that the lowest pupal total lipid content was in the walnut-fed group among both the non-infected and infected groups. The highest amount of tannin content was found in the walnut leaves. Tannins can bind a wide variety of natural polymeric compounds, including lipids *in vitro* (Barbehenn & Constabel, 2011). In this case, the highest gallotannin content in the walnut leaves is a disadvantage for the insect feeding. Also, benzoic acid, present only in walnut leaves, may have had a detrimental effect on the total lipid contents of pupae.

Many insecticides have been shown to reduce biochemical components in the animal body (Khosravi, Sendi, & Ghadamyari, 2010; Zhao et al, 2016). In a study performed with *Agrotis ipsilon*, Xu et al (2016) observed that when insecticide was applied to the larvae, the lipid contents of them decreased compared to the control. Our study is also contradicted by the study of Elgizawy & Ashry (2019) in which *Bt* infection decreased the total lipid content of *Tribolium castaneum* compared to the control. It was determined that the pupal total lipid contents in all groups (except walnut) increased by the bacterial infection compared to the non-infected groups. It is surprising as the bacterial infection plays a triggering role on the lipid.

CONCLUSION

Holometabolous insects use the proteins and lipids they take and store in the larval stage for metamorphosis and for adult stage. In our study, it has been shown that the PSMs present in the plants consumed by the larvae affect the biological properties of *Hyphantria cunea* at the pupal stage. Our study has determined that PSMs affect the body weight of the insect by altering the total pupal lipid and total protein contents of

H. cunea. Furthermore, the effect of the bacteria used against the insect pests on the pupal parameters should not be ignored. It should be evaluated that the effects of both the PSMs and the infection can have a strong impact on the population dynamics of lepidopteran pests and contribute to their control.

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