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The Effect of some secondary metabolites on food consumption and pupal parameters of male and female *Malacosoma neustria* L. (Lepidoptera: Lasiocampidae) larvae

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Abstract: Plants produce secondary metabolites for defense against herbivorous insects. In this study, the effects of different concentrations of quinine, nicotine, and tannic acid on food consumption and pupal parameters of the larva of a severe pest, *Malacosoma neustria* L. (Lepidoptera: Lasiocampidae), were investigated in 2007. Artificial diets containing 0.125%, 0.25%, and 0.5% concentrations of quinine or nicotine were prepared. In addition, diets containing 1.25%, 2.5%, and 5% tannin and a control diet without secondary metabolite were prepared. The feeding experiments revealed that the food consumption and pupal parameters of male and female larvae were different, and all parameters of female individuals were higher than those of male larvae. It has been determined that tannins cause more reductions in food consumption and pupal parameters than alkaloids in male and female individuals. We observed an increase in male larvae's pupal mass with increased concentration of nicotine to 0.5%. Nicotine did not cause a decrease in pupal parameters of larvae compared to quinine. In addition, all parameters of female individuals decreased with the addition of secondary metabolite. Although food consumption in alkaloid-containing diets was less than in the control group, there was a positive relationship between food consumption and pupal lipid and protein content. However, quinine had a more significant effect on triggering protein storage than nicotine. In diets containing tannins, more lipids were stored. A decrease was observed in the food consumption and pupal parameters of all individuals in the tannin-containing diets compared to the other diets.

1. INTRODUCTION

Most insects are herbivores and have co-evolved with plants for about 350 million years. During long-term coevolution, plants have also developed morphological, chemical, and biochemical defense mechanisms to prevent insect feeding (Belete, 2018; Yuan *et al.*, 2020). Compounds that play a role in chemical defense are secondary metabolites. Secondary metabolites are small molecular-weight organic compounds. They mainly contain phenols, terpenes, and nitrogenous organic compounds and play an essential role in plant resistance against phytophage insects

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(Jiang *et al.*, 2021). In addition to being a feeding deterrent, they may have inhibitory properties on insect development and growth (Yuan *et al.*, 2020). They are thus used as potential insecticides (Divekar *et al.*, 2022; Gajger & Dar, 2021).

Tannins, the second most abundant polyphenol, are found in all vascular plants and mainly function as defense compounds that protect plants against pests and other abiotic stresses. They are protease inhibitors; they can form complexes with digestive enzymes. They can cause the precipitation of proteins and reduce digestion by reducing the use of proteins by herbivorous insects (Pizzi *et al.*, 2009). In addition, they are oxidized in the insect gut in a highly acidic environment and bind to various enzymes in the gut. They cause significant nutrient loss as they bind to lipids and reduce their digestibility. They also inhibit insect growth by causing midgut lesions. Because of their bitter taste, they also act as nutritional deterrents (Price *et al.*, 2019).

Another group of secondary metabolites is nitrogen-containing alkaloids. Nicotine and quinine are derived from amino acids. They are highly reactive and have biological potential (Divekar *et al.*, 2022). Due to its bitter taste, alkaloids are nutritional deterrents after ingestion or consumption (Munoz *et al.*, 2020). Nicotine is a pyridine alkaloid that affects the central nervous system of insects, acting as an agonist of the post-synaptic nicotinic acetylcholine receptors. Thus, they are used as insecticides (Saremba *et al.*, 2018). Moreover, sublethal concentrations cause an increase in energy investment, antioxidant functions, and heat-shock responses in bees (Randt *et al.*, 2015). Quinine also reduces food preferences due to their bitter taste. Gustatory receptor neurons (GRNs) are activated by noxious compounds and mediate deterrent/aversive responses in many insect species (Munoz *et al.*, 2020).

Insects may tolerate or have acquired the ability to detoxify secondary plant metabolites during coevolution (Yuan *et al.*, 2020). Phytophage insects have developed several behavioral and physiological adaptive mechanisms as a defensive shield to protect themselves from the harmful effects of secondary metabolites and maintain their population development under selective pressures (Jiang *et al.*, 2021).

Malacosoma neustria is a widespread species inhabiting the whole territory of Europe and a large part of Asia (i.e., China, Iran, Japan, Mongolia, Russia, and Türkiye) and the countries of northern Africa (Zikic *et al.*, 2017). It is an essential defoliator of fruit trees, ornamental trees, and shrubs (Özbek & Çoruh, 2010). *M. neustria* is univoltine and overwinters in the form of egg masses on twigs of the host plants. Caterpillars are gregarious, eat together, and make a silken group tent as a shelter when resting. They feed on young shoots and leaves, often resulting in the defoliation of solitary trees or even entire forests (Zikic *et al.*, 2017). Secondary metabolites are one of the defense mechanisms of plants against herbivores. Tannins and alkaloids are also among plants' most common secondary metabolites (Pizzi *et al.*, 2009; Divekar *et al.*, 2022). During coevolution, herbivores can also adapt to secondary metabolites. *M. neustria* is a species with melanic dimorphism (Dhillon *et al.*, 2022). It is unknown whether males and females respond physiologically to secondary metabolites differently. The present study investigated the effects of tannins, nicotine, and quinine on the food consumption and pupal parameters of *M. neustria* larvae, a critical forest pest. Pupal mass, pupal total lipid amount, and pupal protein amount were investigated as pupal parameters.

2. MATERIAL and METHODS

2.1. Collection of Larval Samples

M. neustria larvae were collected from the Kızılırmak Delta (N 41° 30' E 36° 05') in Samsun directly on feeding plants (*Hippophae rhamnoides* L. *subsp. caucasica* Rousi) while they were feeding. They were placed into transparent labelled containers and brought to the laboratory, where they were fed artificial diets until the emergence of the adults. Adults laid eggs, and the

larvae hatched from these eggs were used in feeding experiments. Eggs and larvae were taken to the air-conditioning room at 24 °C, 70 ± 5% RH, at 16:8 h light/dark period.

2.2. Sex Separation in Larval Stage

The feeding experiment groups were repeated collectively. In the related feeding experiment, the sex determinations of the individuals emerging from the pupa were made. Larger pupae were considered females. These data were used to determine nutritional indices.

2.3. Artificial Diets

In order to reveal the food preference of *M. neustria* larvae, an artificial diet developed and modified by Yamamoto (1969) was used. The content of Yamamoto's artificial diet is wheat germ, casein as the protein, saccharose or arabinose as the carbohydrate, torula yeast, vitamin mixture, salt mixture, cholesterol, sorbic acid, methylparaben, linseed oil, agar, and water. Diets containing secondary metabolites were prepared by adding different concentrations of tannic acid, nicotine, and quinine to the Yamamoto's nutritional content. The concentration of alkaloids in plants is not as high as in tannins, so the concentrations of tannins and alkaloids in the artificial diet were also prepared differently. A diet containing tannic acid in 3 different concentrations was prepared by containing 1.25%, 2.5%, and 5% percent tannic acid. Nicotine and quinine were added at 0.125, 0.25%, and 0.5%. The control diet did not contain any secondary metabolite. A total of 10 different artificial diets were prepared.

2.4. Feeding Experiment

The larvae of the *M. neustria* were collectively fed on each food group tested. The larvae that reached the last larval stage were taken into plastic containers one by one, with ten larvae in each diet, and the daily feeding experiment was started. The food was given (2 cm²) by weighing on a balance with a precision of 0.001 grams. The food left unconsumed is packaged by giving new food every other day. The remaining food was dried in the oven until it reached constant weight. Thus, the amount of consumption was calculated. Thus, the food consumption amount of the larvae was determined. Weight changes in the larvae each day were also noted. This process continued until all larvae reached the pupal stage (Lee et al., 2002). A total of 100 larvae were used for feeding experiments.

2.5. Pupal Parameters

The pupa mass, lipid, and protein content of the pupa were determined for growth performance. Pupae from each experimental group were dried in an oven at 50 °C and weighed after they reached the constant weight. The pupal mass was determined by weighing the dry weight. The total lipid amount was calculated using the methods modified from Loveridge (1973) (Simpson & Raubenheimer, 2001). The total amount of lipids stored in each pupa was determined with chloroform extraction (Simpson & Raubenheimer, 2001). After this application, the pupae were dried again at 50 °C, and the lipid-free pupal weight was determined. The difference gives the pupal total lipid amount.

The nitrogen content of the pupae was measured by the semi-micro Kjeldahl method with a Kjeltec Auto 1030 analyzer (Tecator model, Sweden) (Bergvinson *et al.*, 1997). Lipid-free pupa samples were taken and wet burned in a mixture of concentrated sulfuric acid and potassium sulfate-copper sulfate (95-5), and then distilled by adding 40% NaOH. The resulting nitrogenous substances were kept in 4% boric acid. The boric acid solution was then back-titrated with 0.1 N HCl (Allen *et al.*, 1986). The % N (Nitrogen) amounts found at the end of this process were multiplied by the constant 6.25, and the % protein amounts were found (Oonincx *et al.*, 2015).

2.6. Statistical Analyses

The food consumption and pupal performances of *M. neustria* female and male larvae in each food group were analyzed for normality. In order to determine the differences between the groups, One-Way ANOVA and TUKEY tests were performed on the data with normal distribution. Correlation analysis revealed the relationships between pupal mass, pupal protein, lipid content, and larvae's food consumption according to the quinine, nicotine, and tannic acid concentration. Regression analysis was performed after the relationship was determined. SPSS version 23.0 was used for statistical analysis.

3. RESULTS

The food consumption and development parameters of male and female individuals of *M. neustria* larvae differed from each other. The food consumption of females was higher than that of males (Figure 1, Figure 2). The highest food consumption in female individuals was determined in the control diet and the diet containing 0.125% quinine. It was determined that the food consumption of diets containing alkaloids at 0.25% and 0.5% concentrations was the same between male and female individuals. Minor food consumption is in diets containing tannins. However, there is no difference in food consumption at 2.5% and 5% concentrations in these diets (Figure 1). In male individuals, the highest food consumption occurred in the diet containing 0.125% nicotine. While food consumption decreased in the diet containing 0.25% quinine, it increased in the diet containing quinine at 0.5% concentration. However, with the increase in nicotine concentration, another alkaloid, food consumption decreased (Figure 2).

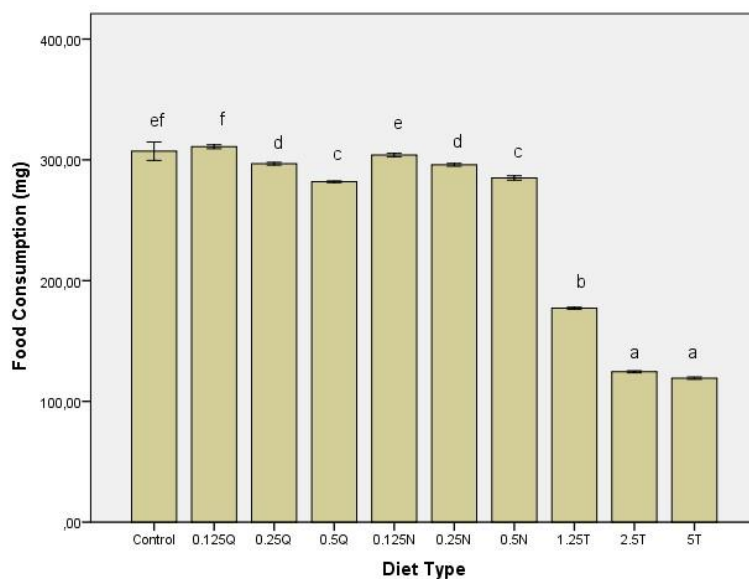


Figure 1. Amount of food consumption of female larvae in different diets (mg). Diets with the same letter are not significantly different.

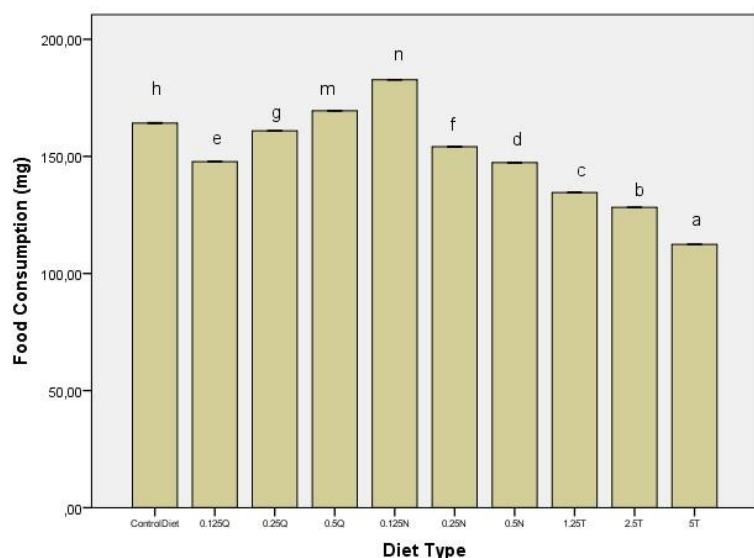


Figure 2. Amount of food consumption of male larvae in different diets (mg). Diets with the same letter are not significantly different.

Pupal mass also differed between male and female larvae. The female individuals' pupal masses are higher than those of male individuals (Figure 3, Figure 4). In females, pupal mass decreased with increasing concentration in all diets containing secondary metabolite. While the highest pupal mass was detected in the control group, the minor pupal mass was detected in the diet containing a 5% concentration of tannin (Figure 3). When the alkaloids were compared, the increase in quinine concentration resulted in a more significant reduction in pupal mass than nicotine (Figure 3). There was a positive relationship between food consumption and pupal mass in all diets containing secondary metabolite (quinine; $r=0.98$, $p<0.01$; nicotine; $r=0.93$, $p<0.01$; tannin; $r=0.82$, $p<0.01$). In male individuals, the highest pupal mass was detected in the control diet and the diet containing 0.5% nicotine. The lowest pupal mass was determined in diets containing tannin at 2.5% and 5% concentrations (Figure 4). A decrease in pupal mass was detected in diets containing both quinine and nicotine. However, there was no difference in pupal mass with increasing quinine concentration. The pupal mass of larvae fed on diets containing 0.125% and 0.25% nicotine did not differ from those with quinine (Figure 4). In nicotine-containing diets, the pupal mass of the larvae fed on the diet containing 0.5% nicotine was not different from the control group. The pupal mass of the diets containing 0.125% and 0.25% nicotine was decreased compared to the control group (Figure 4). In males, there was a positive correlation between food consumption and pupal mass in diets containing quinine and tannin (respectively; $r=0.51$, $p<0.01$; $r=0.87$, $p<0.01$). A negative correlation was found between food consumption and pupal mass in diets containing nicotine ($r= -0.69$, $p<0.01$).

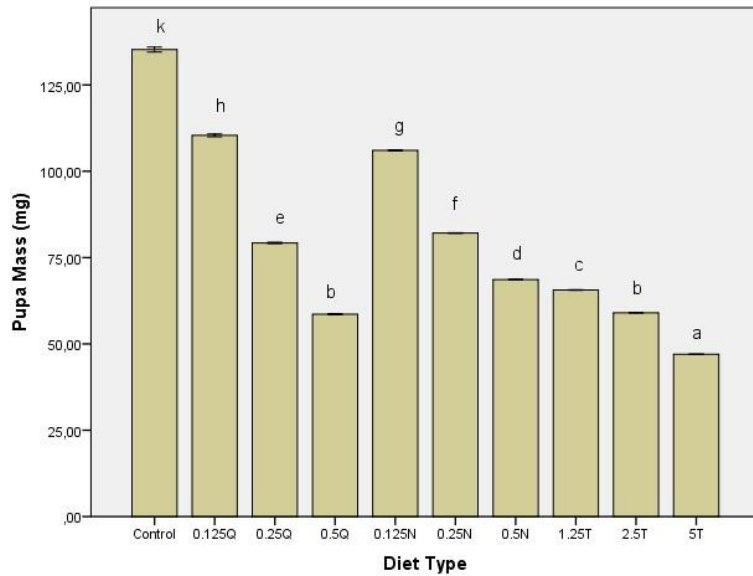


Figure 3. Pupal masses of females fed on different diets (mg). Diets with the same letter are not significantly different.

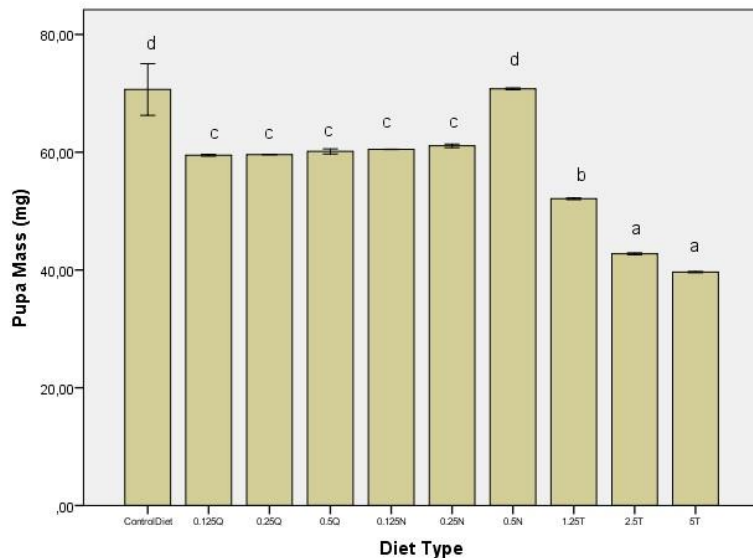


Figure 4. Pupal masses of males fed on different diets (mg). Diets with the same letter are not significantly different.

It was determined that male individuals' pupal lipid amount was less than those of female individuals (Figure 5, Figure 6). While 0.125% quinine concentration causes a decrease in the lipid amount, increasing the concentration to 0.25% increases the lipid amount. No difference was detected between 0.125% and 0.5% nicotine concentrations. The most effective concentration in tannin-containing diets was 0.25% (Figure 6). There was a negative correlation between pupal mass and pupal lipid amount in diets containing nicotine ($r = -0.47$, $p < 0.01$). A negative correlation was also found between the amount of lipid and the amount of pupal protein ($r = -0.36$, $p < 0.05$). A positive correlation was found between pupal mass and pupal lipid amount in tannin diets ($r = 0.92$, $p < 0.01$). Food consumption also positively affected pupal lipid amount ($r = 0.60$, $p < 0.01$). However, there was a negative correlation between pupal protein and lipid amount ($r = -0.77$, $p < 0.01$). In diets containing nicotine, no factor affecting pupal lipid content was detected ($p > 0.05$). While the highest pupal lipid amount in female individuals was in the control diet, the lowest pupal lipid amount was determined in individuals fed a diet containing 5% tannin (Figure 5). In all diets containing secondary metabolite, a decrease in

pupal lipid amount was detected with increasing concentration (Figure 5). There was a positive correlation between food consumption and pupal lipid content in all diets containing secondary metabolite (quinine; $r = 0.98$, $p < 0.01$; nicotine; $r = 0.91$, $p < 0.01$; tannin; $r = 0.98$, $p < 0.01$). Similarly, there was a positive correlation between pupal mass and pupal lipid content (quinine; $r = 0.99$, $p < 0.01$; nicotine; $r = 0.99$, $p < 0.01$; tannin; $r = 0.92$, $p < 0.01$). The relationship between pup protein amount and lipid amount was also positive (quinine; $r = 0.99$, $p < 0.01$; nicotine; $r = 0.99$, $p < 0.01$; tannin; $r = 0.79$, $p < 0.01$).

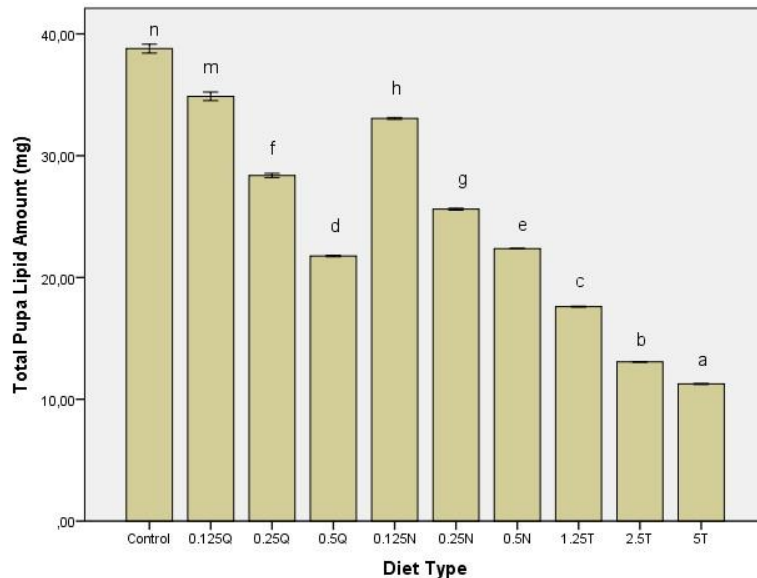


Figure 5. Total pupal lipid amount of female individuals fed on different diets (mg). Diets with the same letter are not significantly different.

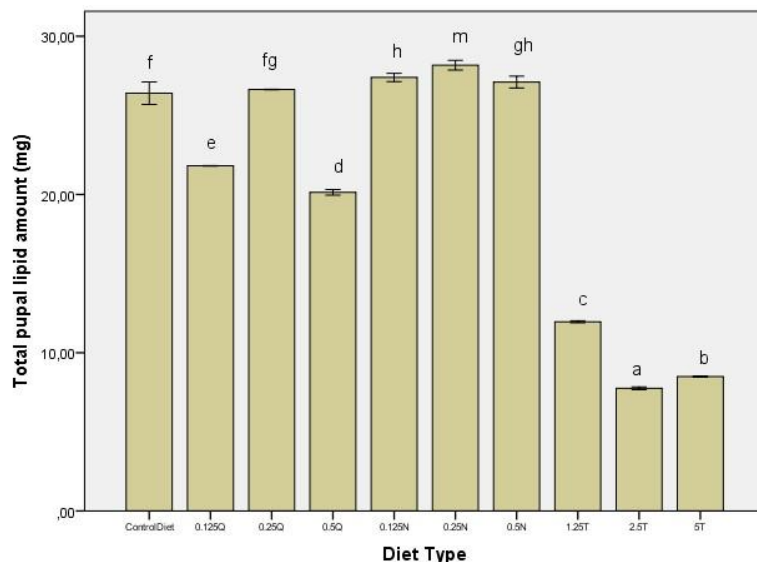


Figure 6. Total pupal lipid amount of male individuals fed on different diets (mg). Diets with the same letter are not significantly different

In females, the crude protein amount of pupae decreased with increasing concentration in diets containing secondary metabolite (Figure 7). Food consumption positively affected pupal protein amount in all diets (quinine; $r = 0.99$, $p < 0.01$; nicotine; $r = 0.95$, $p < 0.01$; tannin; $r = 0.66$, $p < 0.01$). Again, there was a positive correlation between pupal mass and pupal protein amount in all diets (quinine; $r = 0.99$, $p < 0.01$; nicotine; $r = 0.99$, $p < 0.01$; tannin; $r = 0.96$, $p < 0.01$). In males, pupal protein amounts increased in all diets containing secondary metabolite. (Figure 8).

In males, the increase in nicotine and quinine concentrations caused an increase in pupal protein content (Figure 8). The pupal protein amounts of larvae fed on tannic acid at 1.25% concentration were not different from those of the control group (Figure 8). Increasing the concentration to 2.5% increased the protein amount, while 5% tannin concentration caused a decrease in the pupal protein amount compared to the 2.5% concentration. However, the pupal protein amount in 5% tannin concentration was higher than in the control diet (Figure 8). In males, food consumption in diets containing quinine positively affected pupal protein amount ($r = 0.98$, $p < 0.01$). However, a negative correlation was found between food consumption and pupal protein in nicotine-containing diets ($r = -0.78$, $p < 0.01$). In diets containing tannin, food consumption did not affect the amount of protein ($p > 0.05$). A positive correlation existed between pupal mass and protein content in diets containing quinine and nicotine (respectively; $r = 0.51$, $p < 0.01$; $r = 0.99$, $p < 0.01$). A negative correlation was found between pupal mass and pupal protein amount in tannin diets ($r = -0.46$, $p < 0.05$).

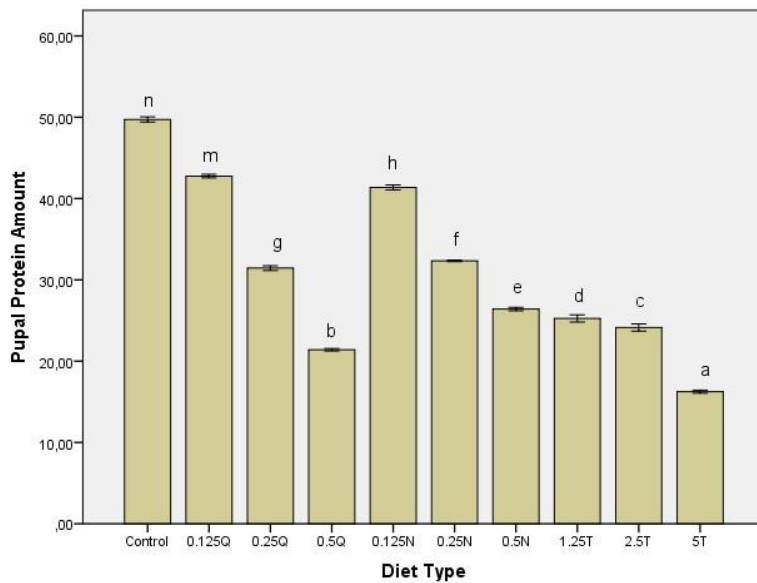


Figure 7. Pupal crude protein amount of female individuals fed on different diets (mg). Diets with the same letter are not significantly different.

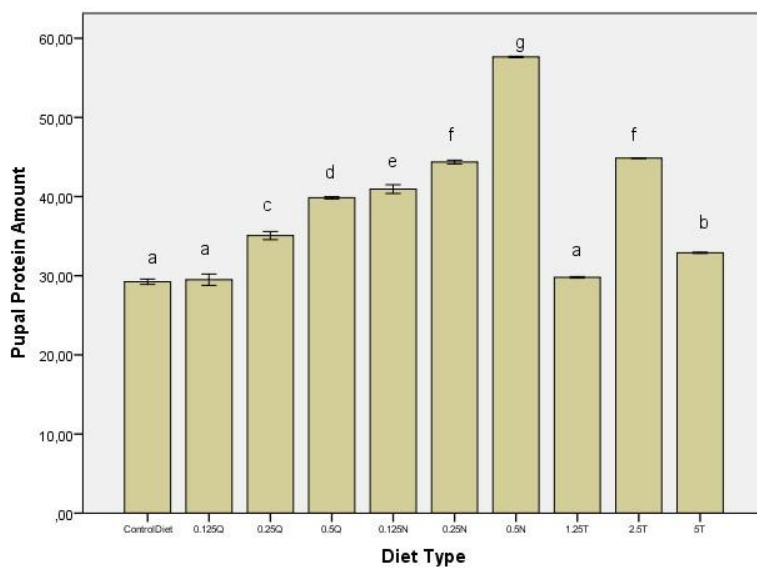


Figure 8. Pupal crude protein amount of male individuals fed on different diets (mg). Diets with the same letter are not significantly different.

4. DISCUSSION and CONCLUSION

Male and female insects may have sex-specific dietary preferences and differences due to differences in nutritional requirements (Carrel & Tanner, 2002). In this study, the food preferences of *M. neustria* female and male individuals were different. Food consumption of female individuals is higher than that of male individuals. Yoder and Grojean (1997) stated that female individuals of the *Gromphadorhina portentosa* (Blattodea:Blaberidae) consume more food than male individuals. Consequently, sexual differences in size tended to increase with increasing body size in these species (Teder & Tammaru, 2005). Therefore, *M. neustria* female individuals may have consumed more nutrients than males. In addition, it was determined that food consumption in *M. neustria* male individuals was less than the control group, except for diets containing nicotine. In female individuals, it was determined that the food consumption in all food types containing secondary metabolites was less than in the control group.

Similarly, Manosalva et al. (2019) stated that alkaloid-containing extracts of *Berberis microphylla* are a nutritional deterrent for *Plutella xylostella* (Lepidoptera: Plutellidae) individuals. The first step in protecting against herbivores of secondary plant metabolites is to be a nutritional deterrent. Insects avoid feeding on alkaloids in the pre-ingestive or post-ingestive stage, primarily because of the bitter taste of the alkaloids. However, it was determined that the food consumption in diets containing 0.5% nicotine in males was higher than in diets containing 0.25% nicotine. Noxious compounds stimulate olfactory (Thompson & Redak, 2007) and gustatory receptor neurons (GRNs) and induce aversive responses in many insects (Munoz et al., 2020). Those adapted to alkaloids also cause the gustatory code to change (Munoz et al., 2020). The increase in food consumption with an increase in concentration from 0.25% to 0.5% in the quinine-containing diet in male individuals may be related to the change in the gustatory code. Alkaloids are insecticides at low concentrations. They affect the acetylcholine receptor in the nervous system and sodium channels of nerve cell membranes. Since they are not volatile, they are directly toxic to insects (Rattan, 2010). Senthil-Nathan (2013) stated that secondary metabolites affect the nutritional indices of insect populations.

Low food consumption should not be considered alone for food to be classified as a deterrent. Therefore, when pupa weights are examined, in *M. neustria* males, pupal mass and protein content increased, although food consumption at 0.5% concentration was decreased in nicotine-containing diets. Diets containing nicotine are not effective as they cause an increase in pupal mass, although they are a food deterrent. In the literature, it has been stated that secondary metabolites, which are the defense metabolites of plants, cause a decrease in the pupa mass of insects. According to Yuan et al. (2022), it was determined that pupal weights of *Spodoptera litura* (Lepidoptera: Noctuidae) individuals fed with different pepper plants decreased with the amount of flavonoids. Similarly, Harvey et al. (2007) reported that the pupal mass of *Manduca sexta* (Lepidoptera: Sphingidae) was negatively correlated with nicotine concentration. Pupal mass is indicative of fecundity (Myers et al., 2000). Pupal masses of male individuals fed a diet containing 0.5% nicotine did not differ from the control group. Therefore, even if the increase in nicotine concentration reduces food consumption, it is ineffective for males because it may not reduce fecundity. However, other diets containing secondary metabolite may reduce fecundity as they cause a reduction in pupal mass of both female and male individuals.

An increase in pupal protein was observed in male individuals with all secondary metabolites. Dixit et al. (2017) stated that the increase in secondary metabolite in plants caused a decrease in the pupal protein in *Helicoverpa armigera* (Lepidoptera: Noctuidae) and *S. litura* individuals. In this respect, the decrease in pupal protein content of male *M. neustria* individuals differs from the literature. Also, in *M. neustria*, male pupal lipid content increased with nicotine concentration. However, as the pupal mass increases, the amount of lipids decreases. Larva stores more protein than lipids in increasing mass. This situation may be due to the use of

protein for development. The increase in storage proteins is significant for holometabolous insects in terms of their use in metamorphosis (Sak *et al.* (2006; 2011). However, insects use lipids for development, flight, reproduction, participation in the structure of cell membranes, and communication through pheromones (Beenackers *et al.*, 1985; Lease & Wolf, 2011).

Interestingly, nicotine poisoning symptoms follow the general sequence of excitation, convulsions, paralysis, death, and mimics as a part of the action of acetylcholine by interacting with the nicotine acetylcholine receptor as the receptor fails to distinguish between nicotine and acetylcholine (Rattan, 2010). However, male larvae differ from the result of Rattan (2010). In diets containing quinine, high food consumption does not affect the pupal mass. This result may be due to the inability of the larvae to use the food. Detoxification of secondary metabolites is a process with an energy cost. Therefore, it caused an increase in energy requirement and decreased food availability in *Spodoptera eridania* (Lepidoptera: Noctuidae) larvae (Cresswell *et al.*, 1992). Secondary metabolites may have reduced the availability of food for *M. neustria* larvae. However, pupal lipid content did not differ from the control group at 0.25% quinine concentration. Bate-Smith (1973) states that secondary metabolites cause enzyme inhibition after a particular concentration or the threshold level required for the sensory deterrent effect is exceeded. Wink (2000) stated that secondary metabolite analogs also interfere with cell signal system components, vital enzymes, and nervous system signals such as neurotransmitter synthesis and receptor activation and block metabolic pathways. Therefore, the nutrients taken after a particular concentration may not be used for development. Although the consumption amount at 0.5% quinine concentration was higher than the control group, the low lipid amount also supports this.

Secondary metabolite caused a decrease in pupal protein amount in female *M. neustria* individuals. However, nicotine has a more significant effect on triggering protein storage than nicotine. In diets containing tannins, more lipids are stored. Proteins are essential for female individuals, especially in egg production (Telang *et al.*, 2001). Therefore, the decrease in pupal protein amount with the addition of secondary metabolite may cause a decrease in egg production. Tannins show pharmacologically toxic effects as a nutritional deterrent, binding to digestive enzymes in the intestines of foods and animals, forming lesions in the intestinal epithelium, causing oxidative stress, destroying microbial symbionts, and pharmacologically toxic (Mole & Waterman, 1987; Bernays *et al.*, 1989; Schultz & Lechowicz, 1989). They also reduce the availability of proteins. Therefore, it may have triggered lipid storage compared to protein.

Food consumption and pupal parameters decreased in all tannin diets in male and female larvae. Tannins are absorbed in herbivores' midgut and cause high ROS production levels due to oxidative stress (Barbehenn & Kochmanski, 2013). In this case, food availability decreased, causing a decrease in pupal parameters.

As a result, some allelochemicals, as chemical defense compounds, affect organisms molecularly and physiologically (Rattan, 2010). Thus, secondary metabolites are used as insecticides. Many insecticides have been shown to reduce biochemical components (Roya *et al.*, 2010; Zhao *et al.*, 2016) and lipid parameters (Xu *et al.*, 2016) in the animal body. Therefore, since the type of alkaloids affects male and female larvae differently, determining their effects and fighting against them will be beneficial.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

Authorship contribution statement

Mahmut Bilgener: Investigation, Resources, Methodology, Analysis, and Writing - original draft. **Nurver Altun:** Methodology, Statistical Analysis, and Writing. Authors may edit this part based on their case.

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