The Effect of Nutrient-Allelochemicals Interaction on Food Consumption and Growth Performance of Alder Leaf Beetle, *Agelastica alni* L. (Coleoptera: Chrysomelidae)

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Abstract: In this study, the effects of secondary metabolites on the feeding preference and growth of generalist caterpillars, *Agelastica alni* L., were investigated. Feeding experiment has been applied with a total of 11 diet; 6 of which were prepared by adding different concentrations of gallic acid (1, 3, 5 %) and quinine (0.125, 0.25, 0.5 %) to the control diet, 3 diet of which prepared by adding different concentrations of gallic acid and quinine. According to the results, the amount of gallic acid consumed did not affect the food consumption and the amount of pupa lipids. However, the amount of gallic acid consumed positively affects the pupal mass and the pupal crude protein. In addition, the amount of quinine consumed negatively affected the developmental performance of larvae except for the food consumption. As the count of secondary metabolites in the diet increases, the pupal mass and the pupal crude protein decrease. Overall, during the co-evolution processs, *A. alni* larvae may be able to adapt to gallotannins. However, quinine, an alkaloid, is a feeding deterrence and growth suppressor for larvae.

1. INTRODUCTION

Herbivorous insects consume a wide variety of plant parts for their growth and reproduction that require a balanced nutrient intake that is a challenge for all animals [1] and nutritional requirements of animal include a variety of macro- and micronutrients [2]. Nitrogen is a macronutrient and nitrogen content of plant foods is a critical important feature for herbivores. These compounds play important roles in development of organisms as structural components of membranes, nucleotides and nucleic acids [2, 3, 4]. Availability of nitrogenous compounds of plant foods to herbivores has influential effects on animals feeding on plants. However, it is known that the presence of some secondary metabolites in herbivores' food plant, especially the polyphenols' affects the nitrogen availability to herbivores [4].

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Tannins and alkaloids are two important classes of secondary metabolites that are distributed widely in dicotyledonous plants. These compounds may influence the target insects in many ways [5-6]. Tannins by binding to the natural polymers such as proteins and carbohydrates may reduce the enzyme actions and the digestion of carbohydrates and proteins in the digestive tracts of herbivores [7]. Also, the esters of gallic acid and its polymers with sugars known as gallotanins are one of the major groups of tannins [8]. [9] reported that complexes could be formed between gallotannins and many proteins, polysaccharides and alkaloids. These complexes may cause enzyme deactivation and the shortage of some microelements in the body [9]. Alkaloids with their bitter taste to most animals play an important role as feeding deterrent to many herbivores [10-11]. Alkaloids can be affect different regions of the nervous system and they may block acetylcholine receptors. In addition, they can affect the development and reproduction of insects, as well as mortality [12].

The larvae and mature individuals of *Agelastica alni* L. are oligophagous herbivores feeding on the leaves of *Alnus* spp. (especially *A. glutinosa*) and *Salix* spp. Their population growth in some years may reach to a level of population explosion on these plant species. If there is a shortage of their normal foods, *A. alni* individuals may feed on hazel nut, birch and hornbeam [13]. So, they may cause differentiation in the landscape in the forests; this may lead to soil erosion in the forested areas and eventually to decrease in the forested area size. *A. alni* has a significant distribution range on the coastline of Black Sea Region of Turkey.

Plant produce the mixtures of structurally different secondary metabolites against an array of different herbivore attacks and microbial invaders. However, some herbivores may overcome the adverse effects of secondary compounds on their survival by evolving detoxification and sequestrations as handling mechanisms. Some herbivores may be adapted and become dependent on certain secondary metabolites of those plants in order to find and feed on new food plants and to lay eggs [14]. So, in this study, we aimed that the synergistic effects of the nutrient and the plant secondary metabolites constituents of artificial agar based diets are investigated in the performance of *A. alni* larvae.

2. MATERIALS and METHODS

2.1. Insects and Experimental Chambers

A. alni adults were collected from *Alnus glutinosa* leaves in Yildizli location of the town of Arakli in Trabzon Province in Turkey in the late May in 2012 and allowed to mate and lay eggs in the laboratory. The eggs laid by the females were collected and used to maintain a colony in the laboratory. The caterpillars from the laboratory colony were fed on an agar based artificial diet originally designed by [15] until the final instar. Immediately the individuals were weighed in 0.0001 mg sensitive scale; then each one was placed singly into a plastic cup with a cover. Both the culture and the experimental chambers were kept at the constant temperature at 25 ^{0}C with a 12h:12h light-dark regime.

2.2. Artificial Diets

The diet developed by Yamamoto has been modified. The diet developed by [15] has been modified. The protein and carbohydrate amounts of all diets are the same. Gallic acid, quinine or secondary metabolite mixture were added to the diets at different concentrations for feeding experiments. Totally eleven artificial diets were prepared. These artificial diets were labeled A, B, C, D, E, F, G, H, J, K and L. Diets and their contents are given in Table 1.

Table 1. The components of the artificial diets

Artificial Diets	Secondary Metabolite
A (control diet)	No secondary metabolites
B	1 % gallic acid (GA)
C	3 % gallic acid
D	5 % gallic acid
E	0.125 % quinine
F	0.25 % quinine
G	0.5 % quinine
H	1 % gallic acid + 0.5 % quinine
J	1 % gallic acid + 0.125 % quinine
K	5 % gallic acid + 0.125 % quinine
L	5 % gallic acid + 0.5 % quinine

2.3. Feeding Experiment

Each of the final instar larvae was weighed. Each food block prepared, as described above was pre-weighed before being presented to the larvae for each treatment. A total of 10 replicates were used for each diet treatment. Every other day, any food uneaten 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 ^{0}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 [16].

2.4. Pupal Lipid and Crude Protein Analysis

The total amount of lipids stored in each pupa was determined with chloroform extraction by three times [17]. After each chloroform treatment the pupae were dried to the constant weight at a drying oven at 50 ^{0}C. At the end of third chloroform extraction, the pupae were re-dried and re-weighed to calculate the lipid contents.

After the chloroform treatment, free lipid pupae were used for estimation of crude protein content. Crude protein content was determined by Dumas (Thermo Scientific Flash 2000 series-NCS analyzer) using a protein-to nitrogen conversion factor of 6.25 [18-19].

2.5. Statistical Analysis

The amount of food consumption by each larva fed on each artificial diet, the pupal dry weight, and the protein contents of the pupae, the lipid contents of the pupae were analyzed statistically using SPSS 17 version. The values were analysed with TUKEY test for identification of differences between groups. A Pearson correlation test was performed to determine whether there was an association between variables.

3. RESULTS

3.1. Food Intake

As expected, secondary metabolites at different concentrations affect the food consumption of larvae (Fig 1). The food consumption of larvae differs in different artificial diets (ANOVA, F=24355,96; p<0.001). The diet with the highest concentration of gallic asid was consumed by the larvae than other diets. The least consumed diet is that the diet containing the highest concentration quinine. (Fig 1). Interestingly, there is no difference in the food consumption between diet with the highest concentration of quinine and A diet. Diet A does not contain any secondary metabolite (Fig 1). The amount of quinine affected food consumption

($r=0.309$, p<001). However, the amount of gallic acid consumed did not affect food consumption. It was determined that the concentration of gallic acid in the diets and the concentration of quinine affect the amount of consumption. While there was a positive correlation between gallic acid concentration and food consumption ($r = 0.767$, p <0.001), there was a negative correlation with quinine concentration and food consumption ($r = -0.598$, p> 0.001). Any relation was not found between the count of secondary metabolites in the diet and the amount of consumption (p>0.05).
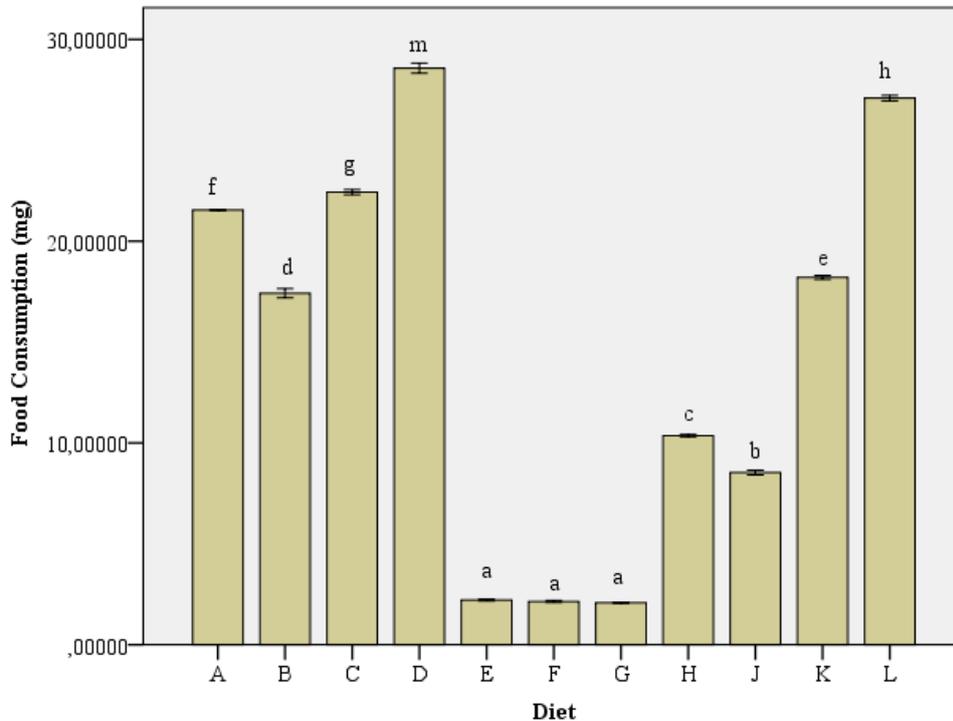
Fig 1. Food consumption on different artificial diet. *Diets with the same letter are not significantly different

3.2. Growth Performance

Pupal mass, amount of pupal crude protein and amount of pupa lipid were analysed to investigate the effect of diets on development performance. Differences were found between the pupal mass for different artificial diets (ANOVA F=169.662, p<0.001). The highest pupal mass was determined for A diet (no secondary metabolite). The minimum pupae mass was determined in the diet containing 0.25% quinine (Fig 2). The amount of gallic acid consumed and the amount of quinine consumed affects the pupal mass. The increase in the amount of gallic acid consumed causes the increase in pupa mass ($r=0.22$, p<0.05). The increase in the amount of quinine consumed resulted in a decrease in the pupa mass ($r= -0.501$, p<0.001). Gallic acid consumption affects the pupa mass, whereas gallic acid concentration of the diet does not have any effect on pupa mass (p>0.05) However, quinine concentration has a negative effect on the mass of the pupa mass just like the amount of quinine consumption ($r= -0.695$, p <0.001). Secondly, the number of secondary metabolite in the diet also negatively affected the pupal mass ($r= -0.436$, p<0.001).
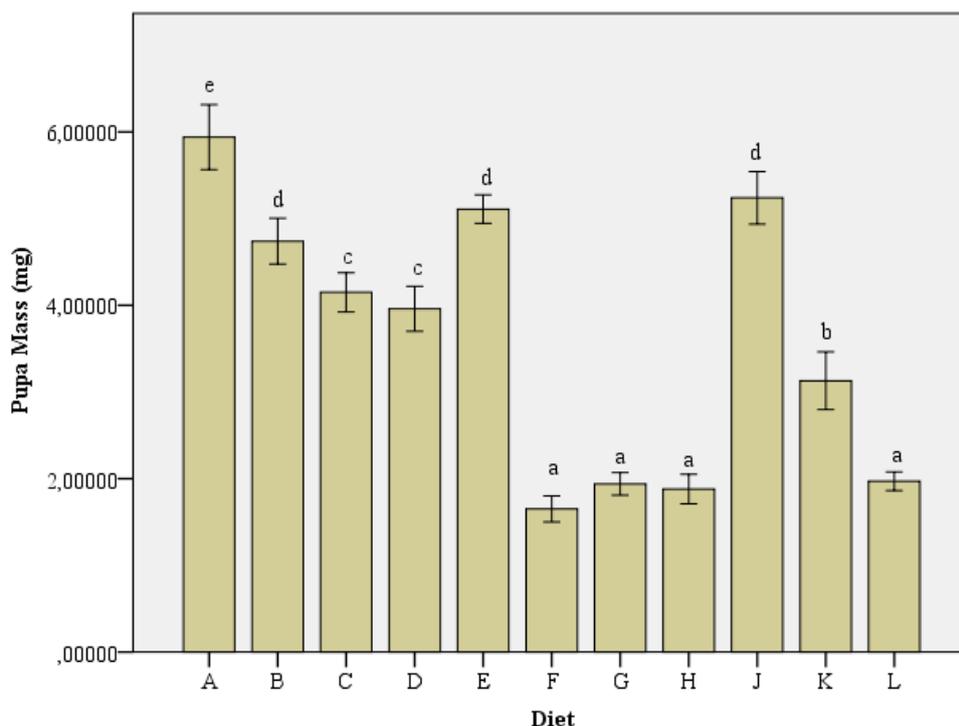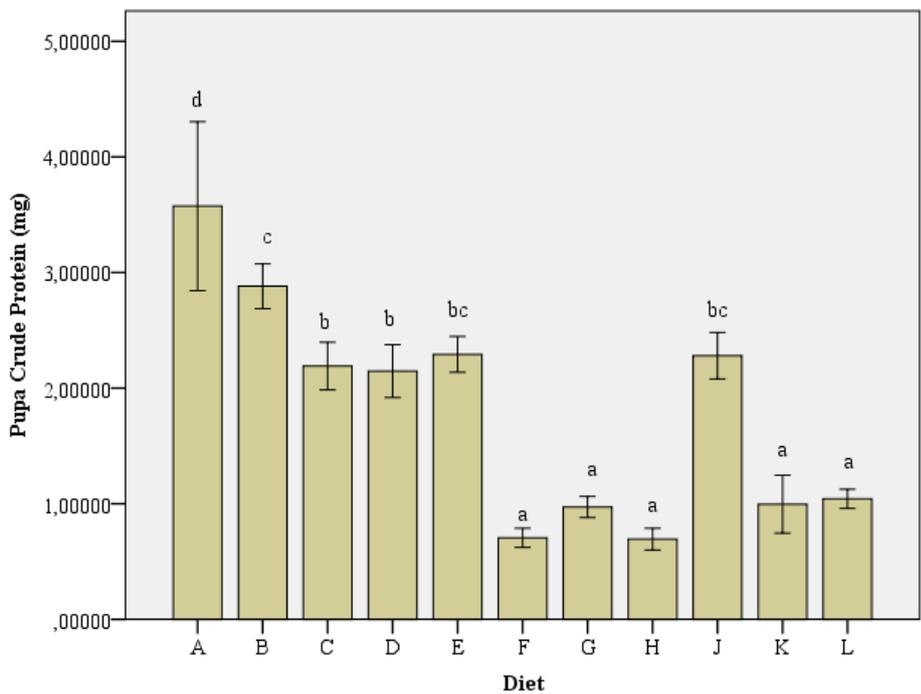
Fig 2. Pupa mass on different artificial diet (mg). *Diets with the same letter are not significantly different.

The amount of pupae protein and pupae lipid amount are very important for development. Feeding on different artificial diets influenced the pupae lipid amount (ANOVA, F=78.393, p<0.001). The maximum amount of pupa lipids was determined in the diet containing at least quinine. The lowest pupa lipid amount was determined for L diet (Fig 3). Concentrations of two secondary metabolites are the highest in L diet. Neither the amount of gallic acid consumed nor the gallic acid concentration influenced the amount of pupa lipid (p>0.05). In contrast, both the amount of quinine consumed and the concentration of quinine in the diet affected the amount of pupal lipid. Increased quinine consumption of the larvae as well as the increase in quinine concentration of the diet resulted in a decrease in the amount of pupal lipid (respectively, r= -0.495, p<0.01; r= -0.534, p<0.01). The count of secondary metabolite in the diet did not affect the amount of lipid (p>0.05).

Artificial diets affect the pupae crude protein (F=50.086, p<0.01). The maximum pupal crude protein was obtained with the control food A. A diet contains no secondary metabolite. The minimum pupal crude protein was obtained with the food H (Fig 4). The H diet is the diet with the minimum concentration of gallic acid and quinine. Both the concentration of secondary metabolite, the consumption amount of secondary metabolite and the count of secondary metabolite negatively affected the amount of pupae protein. Just ingested gallic acid amount affected the pupal crude protein positively (r=0.327, p<0.01) There is a negative relationship between gallic acid concentration of diet and pupa crude protein (r= -0.191, p<0.01). Also, there is a negative relationship between quinine concentration of diet, secondary metabolite counts and pupal crude protein (respectively, r= -0.667, p<0.01; r= -0.577, p<0.01). Ingested quinine negatively affected the pupal crude protein (r= -0.429, p <0.001).
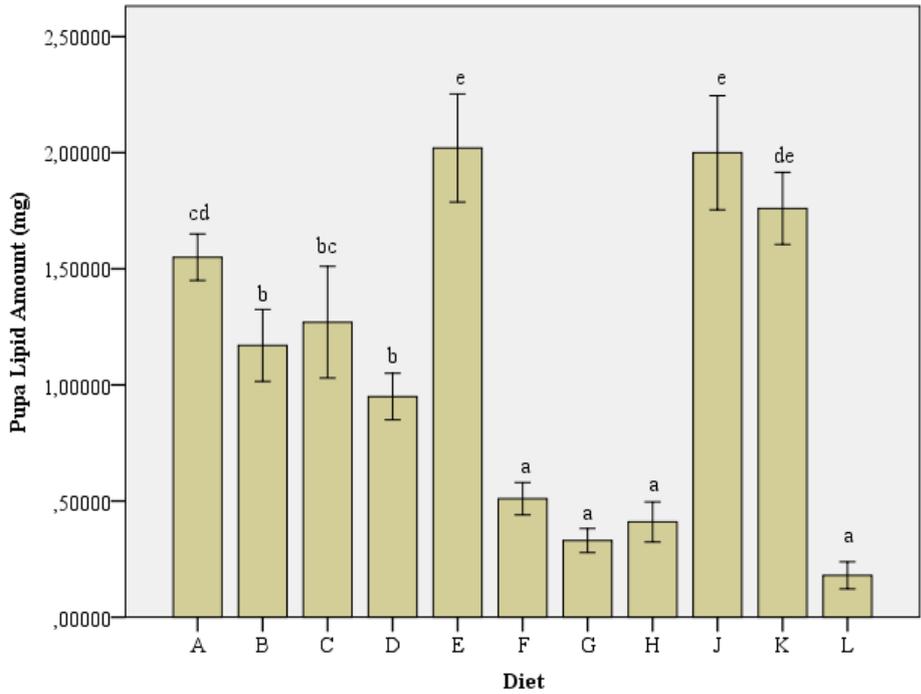
Fig 3. Pupa lipid amount on different artificial diet (mg). *Diets with the same letter are not significantly different.

Fig 4. Pupa crude protein on different artificial diet. *Diets with the same letter are not significantly different

4. DISCUSSION

It is known that gallotanins with other secondary compounds and primary metabolites are effective protective agents against herbivores [20]. The results presented in the study show that the digestion, the post digestion regulation and the development of the larvae were affected by their consumed nutrients by the larvae and the nutrient-secondary metabolites interaction. [21] reported that morphology and plant nutritional content affect growth and development of

herbivores. Secondary metabolites and nutritional quality of food are main factors to regulate between plant and its environment [4, 22-23].

Tannins are divided into 2 groups as condensation tannins and hydrolysable tannins (such as gallotanens). The effects of tannins on herbivores are as feeding deterrents or to reduce the availability of nutrients [8, 24, 25]. The hydrolysable tannins may not affect the digestion of proteins. These tannins are reduced to smaller phenolic substances in the gut. These phenolics do not bind proteins. However, they have positive or negative effects in the digestive system according to hydrolysis products [8]. In our study, the amount of gallic acid consumed does not affect the consumption of larvae. Therefore, it supports the view in the literature that the hydrolysable tannins are not feeding deterrent. With increasing concentration of gallic acid, the pupae mass and pupa protein amount of *A. alni* individuals increased. According to the co-evolution theory, herbivores can adapt to secondary metabolites of plants and they can use nutrients [26]. *A. glutinosa* contains gallotannin as a secondary metabolite [27]. *A. alni* larvae have been adapted to the secondary metabolite of these plant for feeding and they have managed to be protected from the negative effects of gallotannins. Also, *A. alni* larvae by increasing the food consumption were able to obtain enough nutrients that were otherwise hardly obtainable because of the tannic acid in their foods [23].

Alkaloids are toxic compounds to herbivores. They have bitter, nitrogenous compounds. Alkaloids that have an impact on nervous system [28-29] and cell division inhibit to larval development and growth [23, 30-31]. According to the results of the study, quinine has a negative effect on the development of *A. alni*. Also, alkaloids have a negative effect on food consumption because of the bitter taste. The binding to chromosomes (especially to the chromosome Y) is well known characteristics of quinine [32]. The absorption of quinine through animals' guts into the blood stream may distribute it through out the body. When it reaches to the growth zones where mitotic division takes place, quinine may bind to the chromosomes and may cause the interruption of mitotic division; therefore, animals' bodies may not grow as their actual sizes [32]. The larvae were negatively affected by the toxic effects of quinine because they were not adapted to the quinine of their nutrients. Quinine may affect the DNA structure of *A. alni* larvae. Also this may cause possible decrease in the body size of the larvae.

Plants can provide an advantage to protect the herbivores by increasing the number of secondary substances. As the count of secondary metabolites in the diet increased, the mass of *A. alni* pupae and the amount of pupa protein decreased. Fecundity of individuals will be adversely affected as the count of secondary metabolite in the diet. Because the increase in the count of secondary metabolite caused a decrease for pupa mass. The development of larval stage is important for insects rather than the development of the adult stage [26]. Pupae proteins and pupa mass are also important parameters for reproduction.

Herbivores can adapt to secondary metabolites in the evolutionary process, so plants can provide protection against herbivores with more than one secondary metabolite [33]. In addition, this result indicates that the *A. alni* larvae were not able to adapt to alkaloids. As a result, *A. alni* larvae have been adapted to the gallotannins during the process of co-evolution. However, quinine is a deterrent for larvae and adversely affects the development of larvae. [34] submitted that new defense substances arose on the plants parts approximately distribution and speciation of insects at co-evolution process and new defenses evolved against insect herbivores.

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