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Basic Larval Structural Composition of *Thaumetopoea Pityocampa (Denis & Schiffermüller, 1775)* (Lepidoptera:Notodontidae) During Feeding Inhibition Due to Some Natural Chemicals

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Abstract

Thaumetopoea pityocampa (Denis & Schiffermüller, 1775) (Lepidoptera:Notodontidae) is the most important defoliating insect for several pine species and cedars. In this study, body nutrient composition of *T. pityocampa* larvae were analyzed under feeding inhibition caused by natural chemical agents. In no-choice assays, larvae were fed ponderosa pine needles treated with oleic acid and chlorogenic acid solutions, respectively, at each of four concentrations, 0%, 25%, 50% and 75%. The neetles were as given to separate test groups. At the end of feeding experiments, antifeedant index (AFI) was calculated for each solutions with different concentrations. Then, rates of protein, lipid, glycogen and water of larvae were calculated for control and test groups. It was determined that there had been a strong relation between concentrations of solution and AFI values regarding oleic acid ($r= 0.998$, $P < 0.05$). However, there was no significant relationship between concentrations of solution and AFI values regarding chlorogenic acid ($r= 0.663$, $P > 0.5$). The most remarkable finding was a sharp decline in the level of larval glycogen during starvation period in accordance with rising concentrations of both oleic and chlorogenic acid in its food. The glycogen level of the larvae was also affected by both chemical applications

Keywords: Antifeedant index, chlorogenic acid, feeding inhibition, oleic acid, pine processionary caterpillar

1. INTRODUCTION

Plant chemicals have antibacterial, antitumor, cytotoxic to human [1] and antifeedant effects for insects. The effects of plant chemicals used in pest control (or antifeedant) occurs in a variety of ways such as feeding inhibition [2-4] and developmental delay [5]. Antifeedant chemicals play a major role in the unsuitability of host plants as food for insects. Unsuitable plants are avoided by detection of other chemical cues; such [chemical substances may have repellent or toxic properties against insects [6]. These compounds have not any negative effect to humans and environment. Moreover, pests do not develop resistance against these compounds [7]. At this point, a question comes to mind: how chemical composition

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of the pest's body changes because of mentioned effects of these chemicals? Detecting the changes of this chemical composition is important in terms of nutritional value of pest for its natural enemies. Because, insects are significant protein resources for mammals and birds living in number of ecosystems [8]. Relationship between feeding inhibition and body components of pests such as the percentage of protein, glycogen, lipid and water, provide important clues about biological control. Chlorogenic acid as one of the plant chemical has antioxidant effect and insect repellent properties. This compound also causes delay in growth and development when consumed by insects [9]. In addition, it has been shown that chlorogenic acid has a reducing effect on the bioavailability of amino acids and reduces nutrient assimilation in Lepidopteran larvae [10], Coleopterans [11], Cicadellids [12] and small sap-sucking insects [13]. One of the most familiar fatty acids, oleic acid, released from dead members of species, has removal function to other members in American cockroaches [14-15] and two caterpillars [16]. Also, oleic acid exhibited potent feeding deterrent activity *Helicoverpa zea*, (Boddie,1850) (Lepidoptera:Noctuidae), *Lymantria dispar* (Linaaeus, 1758) (Lepidoptera:Erebidae), *Orgyia leucostigma* (J. E. Smith, 1797) (Lepidoptera:Erebidae), and *Malacosoma disstria* (Hübner, 1820) (Lepidoptera: Lasiocampidae) [17].

The pine processionary caterpillar, *Thaumetopoea pityocampa*, (Denis & Schiffermüller, 1775), is the most important defoliating insect for several pine species [18-20]. It is one of the most destructive species to pines and cedars in central Asia, North Africa, Mediterranean countries and southern Europe [21]). It is also deleterious in Anatolia due to attacks to mentioned pine species [22-23]. Pesticides used to control pests cause water and soil pollution [24]. This study focuses on the antifeedant activity, body nutritional composition of larvae against oleic acid and chlorogenic acid. Because, insecticidal activities of oleic acid and chlorogenic acid are known. It is not known whether it has antifeedant effects on *T. pityocampa*, which is such a harmful forest pest. Therefore, body nutritional composition of *T. pityocampa* has been analyzed during the last larval stage under feeding inhibition caused by natural chemical agents.

2. MATERIAL AND METHODS

2.1. Collection of Test Insects

Larvae of *T. pityocampa* were collected from Ankara province, Beypazarı district, Turkey, from Ponderosa pine (*Pinus brutia*) (Lambert, Aylmer Bourke, 1761-1842) (Pinales:Pinaceae) trees in December 2016. The larvae were brought to laboratory and larvae to feed on Ponderosa pine needles. When the larvae reached 4th instar, they were separated and placed in a growth chambers for feeding experiment. Head capsules of the larvae were distinctively evaluated to determine the developmental stages [25]. 4th instar larvae were chosen for antifeeding activity because they were appropriate to achieve the bioassays.

2.2. Antifeedant Test

Antifeedant tests were done during the 4th larval stage. Experimental design was set up as a non-choice test with 30 larvae for each test and control groups. Each test and control group were set up with perforated 30 plastic boxes (10×15×5 cm) that include 1 larva (4th instar). Thin layer of wet sponge pieces were placed into the boxes before putting the larvae. Untreated group was formed with Ponderosa needles placed in the boxes. Test groups were separately set up with Ponderosa needles treated with oleic acid (Sigma- Aldrich, CAS Number:112-80-1) and chlorogenic acid

(Cayman, CAS Number: 327-97-9) solutions. The weight of each needle was measured before the experiment. Needles of control group were only immersed in the solvent (50% ethanol in H_2O). The solutions of test groups were prepared from chlorogenic acid powder and liquid oleic acid (Sigma- Aldrich) in different concentrations 25%, 50%, 75% using the same solvent (50% ethanol in H_2O). Then, all needles were incubated to let evaporate the solvent at 30 °C for 5 minutes before presenting the needles to larvae. Feeding experiments were conducted in a growth chambers (Caron 6015) (15±5 °C and L10: D14 Photophase). Each daily feeding experiment took 5 hour for all groups. At the end of the fifth hours, remnant of the leaves were collected and weighed. This test procedure was continued for 4 days. Amount of food consumed by larvae was calculated using initial fresh weight of needles and fresh weight of residual needles. The antifeedant index (AFI) was calculated to [26].

$$AFI = [(C-T) / (C+T)] \times 100$$

The meaning of the letters in the formula as follows, "C" is the consumption of control needles and "T" is the consumption of treated needles. Total and average consumptions were separately determined daily using thirty larvae for treated groups but the average consumption of thirty larvae of control group was also calculated. Finally, the AFI values were used in data analysis as average of 4 days for all treatments.

2.3. Determination of Protein, Glycogen, Lipid and Water Levels of Larvae

Extraction of glycogen was carried out using the method of [27] and quantification of glycogen was carried out using the method of [28]. Samples were homogenized with 10% TCA (Sigma- Aldrich, CAS Number:76-03-9) in ice for analysis of glycogen. After filtration of the obtained extracts, it was waited for precipitation of glycogen by adding ethyl alcohol (Sigma-Aldrich) into homogenates at 35-40 °C in water bath for an overnight. Then, the tubes containing the mixtures were centrifuged at 3500 rpm and the glycogen was allocated from supernatant. Alcohol was also removed at 35 °C. All samples were retained adding 10 ml. anthrone reagent (Sigma- Aldrich, CAS Number:90-44-8) for 30 min. at 80 °C water bath [29]. Blind and standard samples (0.1 mg/ ml glycogen) were prepared in appropriate procedures. Absorbance of the samples were determined at 620 nm as spectrofotometrically (Shimadzu UV-1700). Values of glycogen of the larvae were calculated as fresh weight (mg / 100 mg) with the aid of data obtained from samples using the formula that specified in terms of procedures referred above. Determination of protein levels of the larvae were achieved using the method of [30]. The regression equation was obtained thanks to the spectrophotometric readings at 750 nm in different concentrations of standard solutions prepared from 1% albumin (Biological Industries) stock solution. Protein content of samples was calculated by substituting the absorbance values in the equation. Lipid extraction and determination of total lipid of the larvae were ensured using the method of [31]. Firstly, it was ensured that lipids transfer from the samples to the organic solvent for the determination of lipids. For this transaction, samples were homogenized in chloroform (Sigma- Aldrich) / methanol (Sigma- Aldrich) mixture (2:1, v/v) using ultrasonic homogenizer (Bandelin-2450) rotating 24000 times for a minute. The homogenate was separated from the solvent using rotary evaporator (Bibby- RE 300) and the amount of lipid was determined. The amount of water was detected by calculating differences between fresh

weight and dry weight of the larvae. Homogenized larvae were dried in a sterilizer (Elektromag- M5040 P) at 50 °C for 4 hours during this process.

2.4. Data Analysis

Glycogen, protein, lipid and water levels of larvae were evaluated using variance analysis followed by SNK test for determination of significant difference among the parameters. Pearson correlation test was preferred to define the relations between antifeedant index (AFI) and concentrations of treatments. All analysis were performed using the software SPSS version 17.0 for Windows (SPSS Inc., 2008).

3. RESULTS

3.1. Anti-feedant Index of Natural Chemical Agents

The relation between AFI and treatment concentrations of chemicals is very important. Because the determination of AFI values for different concentrations of chemicals give important clues about the relationship between chemicals and the pests. In this context, the relation between AFI values and the concentrations of oleic acid was found very strong ($r = 0.998^*$, $P < 0.05$; Fig. I). On the other hand, a strong relation was not found between AFI values and the concentration of chlorogenic acid ($r = 0.663\sim$, $P > 0.5$; Fig. II). At this point, a specific finding was remarkable, when the chlorogenic acid concentration rise to fifty percent from a quarter, the value of AFI was not affected from this increase. Even if this rising reached to 75 percent, the value of AFI showed unexpectedly a slight decrease ($P > 0.5$, 2-tailed, Fig. II). Briefly, these results showed that oleic acid had clearly caused a feeding inhibition but it was not possible to suppose same effect for chlorogenic acid in this experiment.

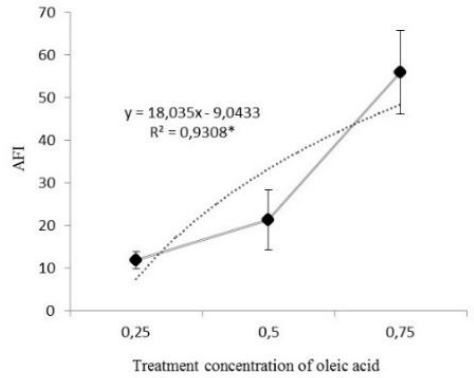

Fig 1 Relation between averages values of AFI of *Thaumetopoea pityocampa* and concentration of oleic acid. *Correlation is significant at the level of $P \leq 0.05$ (2-tailed)

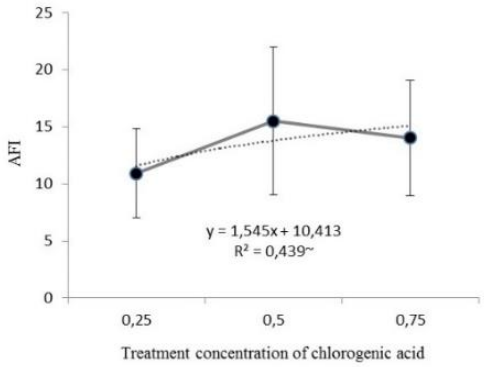

Fig. 2 Relation between averages values of AFI of *Thaumetopoea pityocampa* and concentration of chlorogenic acid. *Correlation is significant at the level of $P>0.5$ (2-tailed)

3.2. Body Nutritional Composition of Larvae

Any differences was not determined statistically in protein, lipid and water levels of the larvae for different oleic acid concentration ($P < 0.05$, Table 1). However, the larval glycogen levels decreased in accordance with the increase in the concentration of oleic acid. Especially, when the concentration of oleic acid raised to 50 percent, glycogen level of larvae decreased by more than half ($P < 0.05$, Table 2). Similar results was determined for the larvae in experimental group of chlorogenic acid. When the concentration of chlorogenic acid raised to twofold,

glycogen level of the larvae decreased by half ($P < 0.05$, Table 2). As a result of experimental studies, the glycogen level of the larvae was also affected by both chemical applications but the applications did not affect other analyzed body components

Table 1 Protein, lipid, glycogen and water ratio of *Thaumetopoea pityocampa* fed on fresh needle of pine added different concentrations of oleic acid. Protein, lipid, glycogen and water ratios are the average ± standard deviation of 3 replicates of measurements. Letters refers the difference between the averages as horizontal *(P < 0.05*)

Structural Substance (%)	**Concentrations (%)**		
	25	**50**	**75**
Protein	14,41 ± 1.53[a]	15.87 ± 3.68[a]	15.02 ± 2.43[a]
Lipid	8.02 ± 1.74[a]	8.65 ± 1.01[a]	8.14 ± 1.45[a]
Glycogen	0.86 ± 0.13[a]	0.30 ± 0.06[b]	0.22 ± 0.06[b]
Water	68.12 ± 8.33[a]	61.98 ± 5.04[a]	57.45 ± 6.27[a]

Table 2 Protein, lipid, glycogen and water ratio of *Thaumetopoea pityocampa* fed on fresh needle of pine added different concentrations of chlorogenic acid. Protein, lipid, glycogen and water ratios are the average ± standard deviation of 3 replicates of measurements. Letters refer the difference between the averages as horizontal (*P<0.05*)

Structural Substance (%)	**Concentrations (%)**		
	25	**50**	**75**
Protein	12.44 ± 2.34[a]	14.36 ± 1.47[a]	14.67 ± 0.64[a]
Lipid	7.01 ± 1.51[a]	6.32 ± 0.57[a]	6.48 ± 0.70[a]
Glycogen	1.14 ± 0.16[a]	0.58± 0.09[b]	0.51 ± 0.12[b]
Water	70.56 ± 3.27[a]	62.93 ± 4.53[a]	66.10 11.4[a]

4. DISCUSSION

Relationships between the tested concentrations and AFI values were analyzed at the first stage of the present study. Quantification of antifeedant effect is important for insect pest management. From an ecological point of view, antifeedants are very important since they never kill the target insects directly and allow them to be available to their natural enemies and help in the maintenance of natural balance. Higher antifeedant index normally indicate decreased rate of feeding. Antifeedant is a chemical that inhibits the feeding without killing the insect pests directly, while it remains near the treated foliage and dies through starvation [6]. It is more important to focus on the reactions of pests to different concentrations of the same solution than comparing the AFI values of the different solutions with each other. These relations were important in order to determine the level of solution deterrence. In general, antifeedants have profound adverse effects on insect feeding behavior [32]. A familiar study was carried by [33] regarding the antifeedant effect of chlorogenic acid. These researchers found the important findings about the antifeedant properties of chlorogenic acid against various leaf beetles. They identified that chlorogenic acid had antifeedant property on three leaf beetles but excluding *Plagiodera versicolora* ssp. *distincta*. The results of the study are remarkable in terms of differences in the level of deterrence of chlorogenic acid among the same family of insects. For all that, chlorogenic acid had not an antifeedant effect against the larvae of *T. pityocampa* in this study. [34] transferred the second - fifth instar larvae of

cabbage butterfly *Pieris rapae* from cabbage to garden nasturtium, *Tropaeolum majus* (Tropaeolaceae). They observed that the larvae refused to feed and died after this transfer due to starvation. They also thought that this refusal can be stemmed from chlorogenic acid found in nasturtium leaves. However, we can easily express that chlorogenic acid has not any negative effect on the feeding of the larvae of *T. pityocampa*, because this determination was directly based on my observations on the larval feeding behavior. [35] stated that chlorogenic acid was mild stimulant at intermediate concentrations but deterrent at higher concentrations for japanese beetle *Popillia japonica* in artificial diets. In this study, when the concentration of cholorogenic acid was raised double, antifeedant index values changed from 10.95 to 15.52 (Fig 2). Even the concentration was increased tripled, AFI values changed from 10.95 to 14.04 (Fig 1). These results revealed that larvae of *T. pityocampa* were insensitive to higher concentration of chlorogenic acid. Similarly, [36] found that *Lochmaea capreae* was only affected from pure chlorogenic acid among four tested leaf beetle species. It is understood that the defensive effect of pure chlorogenic acid was very limited against willow leaf beetles compatible with the findings of my study on pine processionary caterpillar [37] showed that *P. rapae* caterpillars, reared on artificial diet, did not distinguish between leaf discs treated with chlorogenic acid and solvent, whereas cabbage-reared caterpillars avoided chlorogenic acid-treated leaf material. The results of mentioned study showed that the sensitivity of neonate larvae decreased over time against some secondary chemicals if the larvae were not encountered them in the natural foods or the development of sensitivity against deterrents may be directly related to larval stage as mentioned by [34]. In Lepidoptera larvae, taste neurons are located on the ventral surface of the labrum, maxillary palps, and galea. Insects that have been exposed to deterrent compounds in particular show reduced susceptibility. Sensitivity can change with habitutation [34]. Therefore, the reason why the chlorogenic acid AFI values of *T. pityocampa* larvae did not change depending on the dose may be due to habituation. If the feeding deterrent property is evaluated in terms of oleic acid, the results of present study are exactly different regarding the chlorogenic acid. Because, the relation between AFI and concentration of oleic acid was found to be very strong compatible with the study of [16] where used social caterpillars, *Hyphantria cunea* and *Malacosoma americanum*. The agreement between the results of two studies are very remarkable, because Pine processionary caterpillars also exhibits social life. The fact that oleic acid showed a strong feeding deterrent on the larvae of *T. pityocampa* was not obviously a surprise. Because there is a tight relationship between monofag-oligofag species and smell-taste of their host plants. Any external factors that disrupt this relationship can negatively affect to feeding behavior of the species [38]. In this context, it may be considered a repellent feature of oleic acid, but not such a feature of chlorogenic acid on feeding performance of *T. pityocampa*. [39] also demonstrated that oleic acid is produced by *Monosteria unicostata* and its predator *Piocoris luridus* as seen in most animals, although almond tissues provide very little oleic acid to the herbivore *P. luridus*. This finding supported the idea that oleic acid has some metabolic [40-41] and ecological [14-16] tasks in insects.

Another important point was the body nutrient composition of the larvae during feeding experiment period on this study. The most statistically remarkable findings about this issue were a sharp decline (from

0.86% to 0,22%) in the level of larval glycogen of *T. pityocampa* with increasing concentrations of oleic acid and relatively soft decline (from 1.14 to 0.51%) with increasing concentrations of chlorogenic acid in their food. For all that, other parameters (protein, lipid, water) were not affected from rising concentrations of these chemical. These results differ from those of [42]. When *H. cunea* larvae were fed on different foods, the highest amount of pupal protein was determined in the pupae of larvae fed with mulberry leaves containing the least chlorogenic acid. Another difference is that the highest amount of lipid was detected in pupae of individuals fed with plum leaves containing the highest chlorogenic acid [42]. In addition, increasing the dose of oleic acid does not affect the amount of lipid in our study (Table 2). Administration of lufenuron to *Xanthogaleruca luteola* individuals showed no significant change in storage macromolecules at LC_{30} concentration [43]. This result is similar to our study. Hovewer, [43]. an increase in the amount of storage macromolecules was observed at the LC_{50} dose. It is known that important energy sources are also glycogen and fat tissue in insects such as other organisms. At the same time, the first energy sources is glycogen in the execution of metabolic and physical activity in insects [44-46]. Probably, glycogen was primarily used by the larvae during emerged starvation after the deterrent effect due to oleic acid. Especially, at the end of the fourth day of the experiment with the solution of oleic acid (75%), We clearly observed that a disease state occur in the larvae. Nutrient composition of insects's body is definitely important for their predators. It brings to mind the idea that pine processionary caterpillars developed several adaptations against predation risks with respect to life characteristics such as to be nocturnal and social. These life features of caterpillars may be associated with being a nourishing prey. How body nutrient composition of the larvae will change while the state of starvation. As a result of this study, It was found that there is not any significant change in the body nutritional composition of the larvae, except glycogen.

As a conclusion, *T. pityocampa* is an important forest pest. Although many studies have been carried out on the species, an effective control method has not been determined. Antifeedant index value is important for insect management. In this study, chlorogenic acid had not an antifeedant effect against the larvae of *T. pityocampa*. When the concentration of chlorogenic acid was raised double, AFI values raised. Even the concentration was increased triple, the increase in value was less. It was determined that there had been a strong relation between concentrations of solution and AFI values regarding oleic acid. The most remarkable finding was a sharp decline in the level of larval glycogen during starvation period in accordance with rising concentrations of both oleic and chlorogenic acid in its food.

In future studies, more comprehensive and analytical assessment can be made about conversion of organic and inorganic matters of the caterpillars by incorporation specific feces analysis during starvation periods in this concept. Besides deterrence effects of the solutions, it should not be overlooked that reverse effect of the solutions on chemical digestion as stated that [47]. This is important in terms of nutritional composition of the insect's body.

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No conflict of interest and common interest has been declared by the authors.

Authors' Contribution
"The first author contributed 60%, the second author 40%." expressions such as should be included.

The Declaration of Ethics Committee Approval
This study doesn't require ethics committee approval and any special permission

The Declaration of Research and Publication Ethics
In the writing process of this study, international scientific, ethical and citation rules were followed, and no falsification was made on the collected data. Sakarya University Journal of Science and its editorial board have no responsibility for all ethical violations. All responsibility belongs to the responsible author and this study has not been evaluated in any academic publication environment other than Sakarya University Journal of Science.

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