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Synergistic Effects of Some Secondary Compounds Combined with Some Heavy Metals on *Hyphantria cunea* Drury (Lepidoptera: Arctiidae) Larvae

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ABSTRACT

In this study, the effects of phenolic compounds such as tannic acid and gallic acid, and heavy metals such as iron, zinc, copper, nickel and cobalt on the last larval stage of the first generation of *H. cunea* were investigated. The larvae were collected from mulberry and hazelnut trees in the district of Çarşamba, Samsun in 2016. 18 foods in total were prepared; two foods by adding 5% concentration of tannic acid and gallic acid to the artificial diet (dry weight), 5 foods by preparing nickel, zinc, copper, cobalt and iron solutions prepared at 8% concentrations, 10 foods by adding 8% concentrations of heavy metal solutions to 5% concentrations of tannic acid and gallic acid separately, and a control diet. Compared to those fed with control food, the consumption amount of the larvae whose diet contained iron, zinc and copper decreased, whereas it increased in the ones fed with nickel and cobalt containing foods. Pupae weight of the larvae fed with tannic acid and metals added(other than nickel) to it has decreased compared to the larvae in control group. While the amount of pupa protein was the lowest in larvae fed with the food in which both gallic acid and zinc used, it was highest in larvae fed with food containing gallic acid. The pupa lipid amount of the larvae fed with foods supplemented with iron, zinc, copper and cobalt were lower than the control group larvae. The longest development time was observed in the larvae in food groups where iron was added to food alone and tannic acid and iron were added together. The results in this study have shown that the addition of secondary compounds and heavy metals together to the artificial food content may increase toxicity of compounds (synergism) or reduce it (antagonism).

Key words: Hyphantria cunea, artificial diet, secondary compound, heavy metals, feeding, synergistic effect.

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INTRODUCTION

Plants produce proteins and secondary compounds that are toxic or repellent for herbivores or specialized morphological structures to protect themselves against herbivore attacks (Usha Rani & Jyothsna, 2010; War, Paulraj, War, & Ignacimuthu, 2011a; b). Through secondary compounds they produce, plants influence host plant preference of insects, thus their survival and reproduction success, which is direct defense against herbivores, or by attracting species that are natural enemies of pests, which is indirect defense mechanism against herbivores (Dudareva, Negre, Nagegowda, & Orlova, 2006; Howe & Jander, 2008; Arimura, Matsui, & Takabayashi, 2009).

Tannins, which are amassed in many plants especially in woody ones (Bernays, Driver, & Bilgener, 1989; Peters & Constabel, 2002) against herbivory, demonstrate their effects by binding many proteins. Their effects on majority of herbivores from insects to mammals have been reported (Bernays et al,1989). Tannins have a variety of biochemical activities ranging from beneficial antioxidants to harmful prooxidants and toxins (Barbehenn & Constabel, 2011). Gallic acid is a phenolic substance with low molecular weight. Gallic acid is synthesized by shikimic acid pathway directly, not by phenylalanine pathway. It is known that this phytochemical has characteristics of being antioxidant, antibacterial, anti-inflammatory, anti-mutagenic, and chemical preservative (Birošová, Mikulásová, & Vaverková, 2005; Kim et al, 2006; Kang, Oh, Kang, Hong, & Choi, 2008; Giftson, Jayanthi, & Nalini, 2009).

All metals, in spite of being naturally present on the earth's crust and many of them being necessary for cells (i.e., copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), zinc (Zn)), are toxic at high concentrations for living organisms (Yang, Feng, He, & Stoffella, 2005). Elemental defense hypothesis asserts that excessive metal accumulation at least affects some herbivores/pathogens in habitats negatively, and thus, acts as a defense mechanism evolved in hyperaccumulators against some natural enemies such as herbivores and pathogens. There are two different metabolic pathways which the defenses are successful. One of them is that the excessive toxicity of plant tissues containing metals (Boyd & Martens, 1994; Martens & Boyd, 1994), that is, consuming plant material causes death. Another one is the repellent way, which means the less consumption of plant tissue that has high levels of metals (Boyd & Martens, 1998). The first difference between elemental defense and organic plant defenses is that elemental defenses are acquired from soil, but not synthesized by plants. The second difference is elements cannot be degraded chemically; therefore, herbivore defense mechanism is blocked. The third one is that when metabolism considered, organic plant mechanisms are more costly than elemental defenses for plants (Boyd, 1998). It was shown in the previous studies that thrips were deterred by the cadmium and snails, locusts and caterpillars were deterred by the zinc in *Noccaea caerulescens* (formely *Thlaspi caerulescens*) (Pollard & Baker, 1997; Behmer et al, 2005; Jiang, Ma, Zhao, & McGrath, 2005), aphids by the selenium in *Brassica juncea* (Hanson, Lindblom, Loeffler, & Pilon-Smits, 2004), locusts by the arsenic in *Pteris vittata* (Rathinasabapathi et al, 2007), and snails by the nickel in *Streptanthus polygaloides* (Boyd & Jhee, 2005).

The fall webworm (*Hyphantria cunea* Drury), a native North American moth species, has become an invasive pest in Europe and Asia (Tadić, 1963; Yang, Wang, Wei, Qu, & Qiao, 2008).The fall webworm (*Hyphantria cunea* Drury) is a moth species native to North America but an invasive pest in Europe and Asia (Tadić, 1963; Yang et al, 2008). *H. cunea* causes serious damage to agricultural and forest lands. The plants that cause damage include fruit trees with economic importance such as hazelnuts, plums and apples.

In this study, the purpose was to show the effect of some heavy metals combined with secondary compounds on the last instar of first generation of *H. cunea*, which is a polyphagous species, exotic to Turkey, and causes severe damages to agriculture and forest areas in the north of Turkey.

MATERIALS AND METHODS

Obtaining the larvae

H. cunea larvae were collected during the field studies conducted at different times in the borders of Çarşamba district of the City of Samsun in Turkey in 2016.

Contents of artificial diet

To feed the larvae, the artificial diet developed by Yamamoto (1969) was used as the control diet. The content of Yamamoto's artificial diet is wheat germ, casein as the protein, saccharose as the carbohydrate, torula yeast, vitamin mixture, salt mixture, cholesterol, sorbic acid, methyl paraben, linseed oil, agar, and water. Diets were prepared by adding the secondary compounds such as tannic acid (TA) and gallic acid (GA), and heavy metals such as nickel (Ni), zinc (Zn), copper (Cu), cobalt (Co), and iron (Fe) to the diet at specific concentration suitable for the purpose of this research. Tannic acid and gallic acid were added into artificial diet at 5% concentration of dry weight (1.036 g). We decided on these concentrations of secondary compounds by reference to our previous work (Yanar, Topkara, & Gömeç, 2016). 5 diets were provided by preparing nickel, zinc, copper, cobalt, and iron solutions. Stock solutions were obtained by weighing 1.576 mg of heavy metals. 10 diets were prepared by adding heavy metals solutions into tannic acid and gallic acid's 5% concentrations. In total 18 diets, one of which is control diet, were prepared (Table 1).

Feeding experiments

The last instar larvae were put in plastic cups one by one, 30 larvae in each cup, then feeding experiment in which the larvae were fed every other day was started. In these feeding experiments, new diet was weighed on 0.001 precision scale and given to the larvae, and the remaining diets were dried in incubator, their dry weights were measured. In addition, weight changes of the larvae were noted every other day, and this process was continued until the larvae became pupae.

Table 1. Diet types and diet contents.

Diet Types	Diet Contents		Diet Types	Diet Contents
A	Control Diet (CD)		K	CD+ 5% TA+ Zn solution
B	CD+ 5% TA		L	CD+ 5% TA+ Cu solution
C	CD+ 5% GA		M	CD+ 5% TA+ Ni solution
D	CD+ Fe solution		N	CD+ 5% TA+ Co solution
E	CD+ Zn solution		O	CD+ 5% GA+ Fe solution
F	CD+ Cu solution		P	CD+ 5% GA+ Zn solution
G	CD+ Ni solution		R	CD+ 5% GA+ Cu solution
H	CD+ Co solution		S	CD+ 5% GA+ Ni solution
J	CD+ 5% TA+ Fe solution		T	CD+ 5% GA+ Co solution

Pupal lipid and protein analysis

The pupae at the end of the feeding experiments were placed in incubator at 45°C to dry. Then, in order to determine the lipid amount, they were kept in chloroform for 24 hours and this process was repeated three times. They were put into the incubator and redried. When this process was finished, the weights of the pupae without lipid were calculated (Simpson, 1983). The determination of nitrogen in pupae was carried out with semi-micro Kjeldahl method and Kjeltec Auto 1030 analyzer (Tecator, Sweden) (Kacar & İnal, 2008). The nitrogen amounts calculated at the end of this process were multiplied by 6.25 constant and the percentage of the protein amounts was found (Monk, 1987). Each treatment was repeated three times.

Statistical analysis

In the study, whether total diet consumption amounts of *H. cunea* larvae, the pupal weights, lipid and protein amounts of the pupae and their development times are statistically different from each other or not were determined by ANOVA with Dunnett's post-hoc test. SPSS Statistics 21 was used for these tests.

RESULTS AND DISCUSSION

Total consumption amount

It was found that, when compared with the control group, adding tannic acid into the diet reduced the total diet consumption amount, whereas gallic acid increased this amount. Although tannic acid and gallic acid are phenolic compounds, their effects are different. This result is similar to the affirmation that the ecological activities of phenolic compounds depend on their chemical structures (Barbehenn & Constabel, 2011). Tannins are astringent bitter polyphenols and therefore act as feeding repellents against many pests (War et al, 2012). The reason why total diet consumption amount declines might be due to direct repellent feature of tannic acid (Simpson & Raubenheimer,

2001) (Table 2). In a study (Sorvari, Rantala, Rantala, Hakkarainen, & Eeva, 2007), conducted between the local and translocated colonies of *Formica aquilonia* living in a soil contaminated with heavy metal, the former showed higher heavy metal levels than the latter, but the encapsulation response was similar between the two groups. A study with *Epirrita autumnata* (van Ooik, Pausio, & Rantala, 2008) indicated that moderate amount of Ni and Cu in the diet of moth larvae increased their encapsulation rate, but a large amount of Cu caused the immune function to decrease. Dubovskiy, Grizanova, Ershova, Rantala, & Glupov (2011) showed *Galleria mellonella* larvae fed a low dose of nickel had significantly higher GST, phenoloxidase activity and encapsulation responses than controls fed on a nickel-free diet. When iron was added to the diets of *Orchesella cincta*, it caused a significant reduction in consumption, especially at high concentrations (Nottrot, Joosse, & van Straalen, 1987). This shows that heavy metals have different effects. In this study, iron, zinc, and copper added into diet have negative effects on consumption.

Many insects can usually tolerate the great amounts of tannins (Barbehenn & Constabel, 2011). The consumption amount of the larvae fed on the diet containing tannic acid (B diet) was lower than that of control group. In the groups where tannic acid and metals were put together, the consumption amount was seen to increase compared to the groups in which only metals used. As a result, while tannic acid caused a decrease in the consumption amount of the larvae (Hemming & Lindroth, 1995; Becker & Makkar, 1999; Hemming & Lindroth, 2000; Simpson & Raubenheimer, 2001), it increased the consumption amount when used together with the metals. Gallic acid scavenges free radicals due to its anti-inflammatory effect (Kim et al, 2006). Thus, it reduces the negative effect of some metals. The consumption amount of the larvae fed on C diet containing gallic acid was higher than that of control group. When this secondary compound used together with metals, it caused an increase in the consumption amount of all the diet groups (except S). As a result of this experiment it was determined that consumption amounts of all the diet groups were statistically different than that of control group.

Pupal weight

While adding tannic acid into the diet decreased the weights of the pupae compared to the control group, gallic acid addition increased the weights. In a study of Simpson & Raubenheimer (2001) on *Locusta migratoria*, they found that in the pupae whose grasshoppers had been fed on diet containing tannic acid, increase in the amount of tannic acid caused a decrease in the pupal weight. Yanar et al (2016) found that the pupal weights of *H. cunea* larvae fed on diet containing tannic acid were less than the larvae which became pupae by feeding on other diet groups. Reduction in pupal weight can affect the fecundity of their imagoes (Honek, 1993). Being heavier due to feeding with the diet containing gallic acid can positively affect the fecundity.

In a study conducted by van Ooik, Rantala, & Saloniemi (2007), when the moths of *E. autumnata* were fed on metal (sulphur, copper, nickel, cadmium, lead, iron and zinc) contaminated leaves, their pupal weights went down compared with the ones that fed on control leaves. In an study with *Apis mellifera* (Di, Hladun, Zhang, Liu,

& Trumble, 2016), it was reported that pupal weights of larvae fed in artificial diets containing different concentrations of copper decreased compared to the control group. In our study, when iron, zinc, and copper added to the diet, pupal weights were recorded to be decreased. Pupal weight of the larvae fed on B diet containing tannic acid was lower than that of control group. Pupal weights of the groups whose diet contained tannic acid and iron, copper, and nickel together were higher than the groups where only these metals were used. It can be said that tannic acid increased the effects of these metals by dominating them. On the other hand, when tannic acid was used together with zinc and cobalt, pupal weights were lower than the groups where only these metals were used. When gallic was used together with iron and copper, it caused an increase in the pupal weight, whereas combining it with zinc and nickel caused a decrease in the weight. It did not change the effect which cobalt would cause itself. In accordance with these results, we can see three effects (positive, negative, neutral) of gallic acid on pupal weight. The group containing cobalt (H group) and the group in which gallic acid and cobalt used together (T group) were statistically same as control group.

Pupal protein amount

It was found that when tannic acid was added to the diet, this reduced the pupal protein amount compared to control group, whereas adding gallic acid into the diet increased this amount. Simpson & Raubenheimer (2001) found that *L. migratoria* feeding on diet containing tannic acid had higher pupal protein amount when compared with the ones that were fed with the diet which did not contain this compound, thus this finding is opposite of our data. Stored proteins that are transferred from larval to imago stage can have important function in herbivore insects especially in the imagoes due to limited nitrogen consumption (Hahn, 2005). Therefore, gallic acid in diets can be advantageous for *H. cunea* to store protein.

It was found that when the heavy metals, except cobalt, were added into diet, pupal protein amounts lessened compared to the ones in the control group. Even though Ni, K, Ca, Zn, and Fe are important cofactors of functional proteins (Dadd, 1985), in this study, pupal protein amounts of groups containing iron, zinc, and nickel (D, E, and G) were less than that of control group.

Pupal protein amounts in all the groups whose diet contained tannic acid and metals, except M group, were lower than the control. Negative effects of tannins in insects may depend on high tannin concentration (Aerts, Barry, & McNabb, 1999). Protein amounts of the groups whose diet contained both gallic acid and the metals were lower than that of control group, except for T diet group. All the groups, except G, H, and T diet groups, were statistically different than control group.

Pupal lipid amount

Storage function of insect's fat body is essential for the survival of holometabolous insects. Throughout the larval feeding stages, energy reserves are accumulated to be used along metamorphosis as well as for new imagoes. Insects need to accumulate

Table 2. Total diet consumption, pupal weight, pupal protein and lipid amount and development time of *H. cunea* in the feeding experiment.

	Diet types	Total diet consumption (mg)	Pupal weight (mg)	Pupal protein amount (mg)	Pupal lipid amount (mg)	Development time (day)
Average ± standard error	A	365.6±0.4	58.0±1.0	27.1±0.4	17.3±0.2	3.0±0.0
	B	310.8±0.4	40.9±0.6	21.4±0.3	12.4±0.2	3.4±0.1
	C	420.5±0.5	63.7±1.1	34.8±0.6	18.1±0.3	3.0±0.0
	D	322.7±0.4	31.8±0.2	17.2±0.1	7.9±0.5	3.8±0.0
	E	333.4±0.4	43.7±0.9	22.5±0.4	11.7±0.2	3.0±0.0
	F	278.3±0.3	29.3±0.2	16.9±0.1	7.7±0.1	3.2±0.1
	G	375.7±0.5	63.2±1.0	25.8±0.4	17.5±0.2	2.8±0.1
	H	369.0±0.5	60.9±1.0	27.6±0.4	15.7±0.2	3.3±0.1
	J	343.8±0.4	37.5±0.9	19.7±0.4	8.3±0.2	3.8±0.0
	K	346.9±0.4	40.9±0.5	17.9±0.2	17.1±0.2	3.2±0.1
	L	340.3±0.4	44.2±0.9	23.1±0.4	11.6±0.2	3.1±0.0
	M	381.0±0.5	65.0±1.0	30.0±0.4	20.3±0.2	3.2±0.1
	N	388.1±0.5	42.5±0.9	24.1±0.5	9.4±0.2	3.3±0.1
	O	373.8±0.5	39.5±0.9	18.3±0.4	17.9±0.2	3.2±0.1
	P	383.4±0.4	34.0±0.9	15.5±0.4	13.7±0.2	3.2±0.1
	R	356.5±0.5	42.6±0.9	20.0±0.4	16.6±0.2	2.8±0.0
	S	346.7±0.4	41.2±0.9	18.9±0.4	16.4±0.2	3.0±0.0
	T	389.6±0.5	60.2±1.0	27.6±0.4	21.7±0.2	2.9±0.0
ANOVA	df	269	269	269	269	269
	F	4365.5	169.6	142.5	279.4	8.5
	P	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
	Dunnet test	B < 0.001 C < 0.001 D < 0.001 E < 0.001 F < 0.001 G < 0.001 H < 0.001 J < 0.001 K < 0.001 L < 0.001 M < 0.001 N < 0.001 O < 0.001 P < 0.001 R < 0.001 S < 0.001 T < 0.001	B < 0.001 C < 0.001 D < 0.001 E < 0.001 F < 0.001 G < 0.001 J < 0.001 K < 0.001 L < 0.001 M < 0.001 N < 0.001 O < 0.001 P < 0.001 R < 0.001 S < 0.001	B < 0.001 C < 0.001 D < 0.001 E < 0.001 F < 0.001 J < 0.001 K < 0.001 L < 0.001 M < 0.001 N < 0.001 O < 0.001 P < 0.001 R < 0.001 S < 0.001	B < 0.001 D < 0.001 E < 0.001 F < 0.001 H < 0.001 J < 0.001 L < 0.001 M < 0.001 N < 0.001 P < 0.001 T < 0.001	D < 0.001 J < 0.001

*Statistically significant means according to Dunnet's Multiple Range Test (P<0.05) at least a small portion of stored nutrients in order to survive during metamorphosis (Mirth & Riddiford, 2007). Lipids that are used at imago stage are met from the lipids stored along growth phases before reaching imago (Giron & Casas, 2003). While having lower lipid amount in pupae of the larvae due to feeding on the diet (B) containing tannic acid can be disadvantageous for species, having higher lipid amount as a result of feeding on diet containing gallic acid (C) can be advantageous.

Adding iron, zinc, copper, and cobalt into diet caused a decline in pupal lipid amount. The level of nutrient reserves accumulated in insect fat body regulates some important points in the life of insects such as insect growth rate, timing of metamorphosis, and egg growth (Mirth & Riddiford, 2007). In the diet groups where metals and secondary compounds are used together, except N and S, increase in the lipid amount due to secondary compounds is important with respect to this matter. Lipid amounts of the groups, except C, G, K, O, R, and S diets, were found to be statistically different than that of control group.

Development time

Behavior, growth, and development of some insects are affected by plant flavonoids (Simmonds, 2001, 2003; Simmonds & Stevenson, 2001). It was found that in the group fed with tannic acid containing diet (B), development time was longer than that of control group, whereas this period did not change in the group whose diet contained gallic acid (C). Sublethal doses of metals cause the growth rate of herbivores to decrease (Williams, 1999), thus prolonging the development time. It was seen that when iron, copper, and cobalt were added into the diet, it took longer for the larvae to develop compared to the ones in the control group. Boyd & Moar (1999) found that nickel concentrations in the leaves up to 93 μg/g prolonged reaching pupae stage in *Spodoptera exigua* larvae. In our study, development time of the group whose diet contained nickel was shorter than that of control group.

A study carried out by Jhee, Boyd, & Eubanks (2006) showed that when the amount of tannic acid and nickel added to artificial diet increased, the survival percentage and the percentage of becoming pupae in *Plutella xylostella* larvae went down. In our study, when tannic acid and nickel used together, development time was found to be prolonger compared to the group in whose diet only nickel is used. This finding shows that the synergistic effect is different. In the groups where iron and zinc are used together with gallic acid development time was slower than that of control group. Extension in development time causes an increase in the possibility of encountering with natural enemies while feeding or looking for food (Bernays, 1997), or with the extension in growth and feeding the risk of preying/parasitism increases (Moran & Hamilton, 1980; Loader & Damman, 1991; Benrey & Denno, 1997), and these are true for this species, as for many others. Of all the diet groups, D and J groups were found to be statistically different from the control group.

Since plants provide the basis of many terrestrial food chains, secondary compounds and metals in plants play important roles for the livings at higher levels of food chain. These compounds can be repellent or toxic against the herbivores.

When secondary compounds and heavy metals are used together, they show different effects on larvae. When these compounds are combined, these combinations can cause a more complex influence on an herbivore. Since compounds can interact and affect each other, their combined effect becomes different than their individual effect. Findings in our study prove that the interaction can increase the toxicity of such combined compounds (synergism), or decrease it (antagonism).

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