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The Influence of Dietary Cadmium on Changes in the Midgut Mass Related to the Mass of Gypsy Moth Larvae

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ABSTRACT

Cadmium pollution is becoming an increasing problem, especially in parts of the world that have developed industries. To consider the potentially harmful effects of cadmium, we need to examine changes at all different levels of biological organization. The main goal of this study was to detect a possible change in the percentage of midgut mass relative to larval mass (PMM) and determine the plasticity of this trait and the correlations between midgut enzymes and PMM under stress conditions. Fourth-instar larvae were exposed to acute and chronic effects of two cadmium concentrations, 10 and 30 µg Cd/g dry food, as well as a three-day recovery from chronic treatments. PMM is also an indirect indicator of food consumption and was found to be significantly reduced compared to control in both acute effects and chronic treatment at 30 µg and its three-day recovery. The PMM reduction during acute treatments is a consequence of cadmium action, while in chronic treatment, the genetic factor (egg mass) plays a crucial role in the change of PMM. According to the index of plasticity, distinct phenotypes were not produced. Significant correlations were shown between PMM and trypsin (Tryp) and leucine aminopeptidases (LAP) at acute and chronic treatment with higher cadmium concentrations, while significant correlations between proteases and PMM were detected at lower metal concentrations (Acute10 and Chronic10 and 30 µg Cd/g dry food). In contrast to chronic treatment, egg masses respond more uniformly by reducing PMM during the short-term effect of cadmium. Finally, we can conclude that, as an addition to biochemical and molecular research, PMM can be used for studying the cadmium effects to gain a better insight into the state of the organism under stress conditions.

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Authors' Contribution
MV designed, coordinated and participated in all phases of the project. DM, AG and AF collected insects, reared them in laboratory and treated larvae with cadmium. MM and JL processed data. LI and VPM collected literature data and participated in manuscript writing.

Key words
Cadmium treatments, Gypsy moth, Midgut mass, Correlations, Plasticity

INTRODUCTION

Cadmium is a metal of great public health significance. This carcinogen has the ability to accumulate in many organs as well as in food (vegetables, cereals, and starchy roots). Anthropogenic activities are the main source of cadmium in nature. Cadmium presence in drinking water is almost irrelevant compared to dietary exposure (WHO, 2019). Nowadays, thanks to anthropogenic activities, cadmium progressively affects ecosystems. It is, therefore, necessary to determine the toxic effect of xenobiotics at different levels of biological organization.

The gut is responsible for the secretion and synthesis of enzymes, digestion, absorption of nutrients, and detoxification. It can be a model organ in the analysis of the harmful effects of metals introduced through food. In addition, it is the major tissue for metal accumulation (Zhang *et al*., 2001; Vlahović *et al*., 2017). In insects exposed to cadmium, a decline in the length of the alimentary canal, histological modifications in the midgut (disruption of epithelial cells and presence of vacuoles) and disruption of the peritrophic membrane are detected (Wu *et al*., 2009; Osman *et al*., 2015). Many authors have shown ultrastructural changes in the midgut during cadmium exposure (Hopkin and Martin, 1983; Vandenbulcke *et al*., 1998; Rost-Roszkowska *et al*., 2020). Besides, cadmium presence in the gut can lead to mitochondrial swelling, which can affect energy metabolism (Hemelraad *et al*., 1990). Metals can cause variations in epithelial thickness in the invertebrate digestive system. Furthermore, apoptosis was detected in phytophagous insects exposed to heavy metals (Rodrigues *et al*., 2008). Rost-Roszkowska *et al*. (2019) showed that acute and chronic cadmium treatments trigger cell death processes in insects. In their study, non-

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specific autophagy was found to maintain homeostasis under conditions of short-term exposure to metal (Rost-Roszkowska *et al*., 2018, 2019).

So far, we have found a small number of experiments that describe alterations in the midgut of phytophagous insects in the presence of cadmium. Changes in the morphological characteristics of organisms have long served as indicators of environmental quality (Lagisz, 2008). This type of analysis can complement biochemical and physiological biomarkers in environmental monitoring. Furthermore, beetles inhabit various biogeographical regions, have highly adaptive characteristics and are inexpensive, which is why there are numerous model systems available with well-described life cycles. Consequently, beetles can be a favorable bioindicator in studies of environmental protection.

We aimed to determine whether larvae of *Lymantria dispar* (Lepidoptera: Erebidae) exposed to chronic and acute dietary cadmium treatments have decreased midgut mass compared to the mass of the whole larvae. In the present paper, we focused on (1) midgut mass alteration in relation to the mass of the whole body (PMM); (2) plasticity of the examined trait during different cadmium treatments; (3) correlations between PMM and activities of midgut enzymes; (4) possible use of this parameter (PMM) as a biomarker of cadmium exposure.

MATERIALS AND METHODS

Experimental procedures

The egg masses of the gypsy moth analyzed in the experiment originate from a poplar forest. Twenty egg masses were collected at the Opovo site (45°03′07″ N and 20°25′49″ E). After collection, the 20 egg masses were held in the refrigerator at 4° C before placing in Petri dishes (volume 300 ml) for hatching. Larvae were reared at a density of 15 larvae per petri dish until the entry into the third larval stage when the density was reduced to five larvae/dish. Upon entering the fourth larval stage, the larvae were individually arranged in cups. The growing temperature was 23°C, with a 12h dark: 12h light photoperiod.

The larvae were reared on a high wheat germ diet (HWG), in which casein was replaced by yeast hydrolysate (Bell *et al*., 1981; O'Dell *et al*., 1984). Two cadmium concentrations were used: 10 g Cd/g dry food (further on referred to as 10) and 30 µg Cd/g dry food (designated as 30). Cadmium was added in the form of the nitrate salt: Cd $(NO_3)_2$ x $4H_2O$. Cadmium concentration in a diet was calculated with respect to its relative amount in the cadmium salt.

Experimental groups

Seven experimental groups were formed:

(i) Control a cadmium-free control group; (ii, iii) acute 10 and 30 acute treatment groups (larvae reared at 10 and 30 µg Cd/g dry food for three days of the fourth instar); (iv, v) chronic 10 and 30 chronic treatment groups (larvae reared at 10 and 30 µg Cd/g dry food from hatching until sacrificing); (vi, vii) recovery 10 and 30 recovery treatments (larvae reared at 10 and 30 µg Cd/g dry food until entering the fourth larval stage and then transferred to control for three days).

The analysis included 20 egg masses, 5 larvae per egg mass, and a total of 100 individuals within each experimental group (700 larvae in total). On the third day of the fourth larval instar, the larvae were measured and sacrificed. The abdominal cavity was opened, the midgut was separated from the foregut and hindgut and measured. All experimental procedures were in compliance with the Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković" National Institute of Republic of Serbia, University of Belgrade (Approval No. 54/10).

Calculation of the PMM

The percentage of the midgut mass (PMM) related to the mass of the larva on the third day of the fourth larval stage was calculated with the formula:

$PMM(\%) = (M_{midgut}/M_{larvae})$ x100.

This parameter gives us an insight into the degree of food consumption in different environments (Jindra and Sehnal, 1989).

Enzyme activities as well as detection methods are described in our previous papers (Vlahović *et al*., 2008, 2014, 2015, 2017).

Statistical calculations

Statistical processing of the results was done with the computer program STATISTICA version 4.5 and SAS (edition 9.1.3., Service Pack 4, SAS Institute Inc, Covy, NC, USA, 2002-2003).

Analysis of variance was used to examine the significance of the effects of cadmium treatment. It was performed on log-transformed values of the examined traits. Norms-of-reaction plots show the plasticity of the response of PMM from different egg masses to the presence of different cadmium treatments.

The coefficient of phenotypic plasticity was calculated according to Cheplik (1995) using the following formula:

$$PP_{Ch} = \frac{X_{Con} - X_{Treat.}}{X_{Con}} * 100$$

where PP_{Ch} is the index of phenotypic plasticity according to Cheplik and X_{con}, X_{Tr} are the PMM at the control and treatment, respectively.

Wilcoxon's test is used to compare the phenotypic plasticity indexes of different traits between different treatments. The significance of differences in index variation was determined by the F-test. The z-test was employed to compare correlation coefficients between different environments.

RESULTS

Figure 1 presents the mean values of the percentage of midgut weight at different treatments, while the significance of the changes was assessed by the LSD test. The percentage of gut mass decreases significantly during acute treatment at both cadmium concentrations. However, during the chronic effect of cadmium, the percentage of gut mass compared to the control decreases significantly only on 30 µg Cd/g dry food. In contrast to the recovery 10 group, where the percentage of gut mass did not differ significantly from the control, recovery from higher cadmium concentrations showed a significant decrease in the examined trait compared to control – no recovery occurred. A decrease in the PMM is an indirect indicator of reduced intake of contaminated food. The norm of reaction (lines-of-reaction norms) is a term that places phenotypic plasticity in the context of genotype-specific response. It shows an array of phenotypes that are developed by a genotype in different environments. The norms of reaction in Figure 2 show great variability in response to the same type of stress. In relation to control, in 13 egg masses, PMM decreased at Acute10, while at Acute 30, PMM decreased in 15 egg masses. During the recovery from 30 µgCd/g dry food, in as many as 15 egg masses, the PMM remained reduced to the level detected during chronic treatment (irreversible reduction).

Two-way ANOVA showed that cadmium significantly affects the change in the mean value of the PMM, $P<0.01$ (Table I) only in acute treatment. However, during the recovery from 30 µg Cd/g dry food, the genetic factor significantly contributes to the variation of the examined parameter, $P<0.001$.

The mean value of the index of phenotypic plasticity has only one negative value, namely, in chronic treatment at a lower concentration of cadmium (Table II). Comparison of the mean values of the phenotypic plasticity index and standard deviations of the PMM revealed there were no significant differences between the experimental groups, which means that the plasticity and its standard deviations cannot be taken as sensitive parameters to the presence of cadmium in food (Table II).

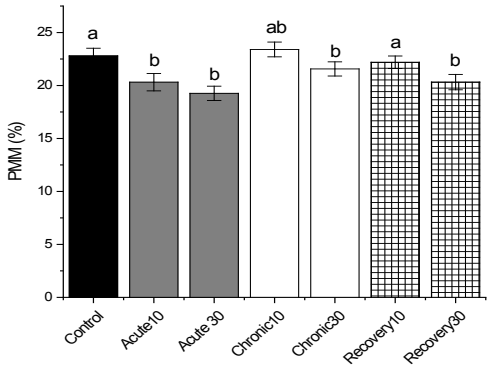

Fig. 1. Changes in PMM expressed as MV±SD (mean value, standard deviation) during different cadmium treatments. The groups were compared by LSD multiple range test followed by ANOVA. Different letters signify statistically different groups. Control cadmium is not added to food; Acute 10 (30) three-day treatment with 10 and 30 µg Cd/g dry food; Chronic 10 (30) cadmium treatment with 10 and 30 µg Cd/g dry food from hatching until sacrificing; Recovery 10 (30) three-day recovery after chronic treatments at 10 and 30 µg Cd/g dry food.

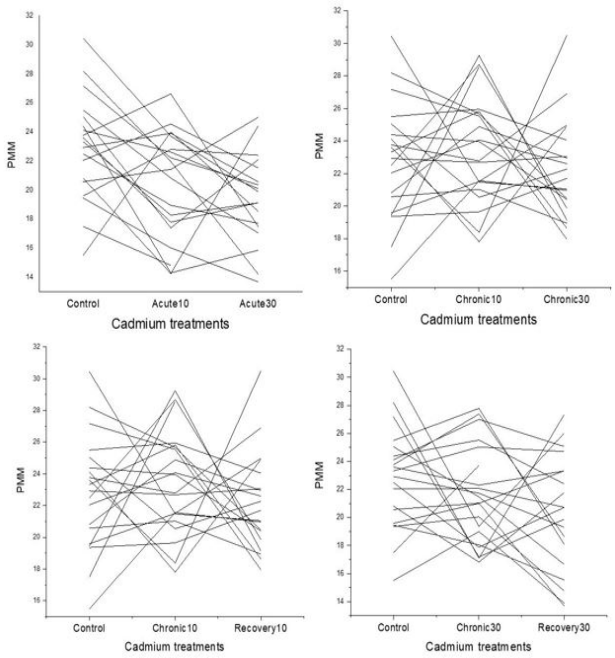

Fig. 2. Norms-of reaction plots for PMM during different cadmium treatments (Control; Acute 10(30) three-day treatment with 10 and 30 µg Cd/g dry food; Chronic 10(30) cadmium treatment with 10 and 30 µg Cd/g dry food from hatching until sacrificing; Recovery 10(30) three-day recovery after chronic treatments at 10 and 30 µg Cd/g dry food. The response of a single egg mass is represented by one line.

Table I. Mean squares (x10^3) from the two-way ANOVA of PMM in the gypsy moth larvae exposed to cadmium treatments; df, degrees of freedom, P < 0.05.

Treatments	Variation	df	MS	F	P
Acute	Egg mass (EM)	17	8.408	1.122	0.3351
	Cadmium(Cd)	2	53.657	6.430	0.0043
	EMxCd	34	8.345	1.113	0.3172
	Error	204	7.497		
Chronic	Egg mass (EM)	19	7.765	1.181	0.2749
	Cadmium(Cd)	2	13.437	1.417	0.2550
	EMxCd	38	9.485	1.442	0.0545
	Error	235	6.576		
Recovery10	Egg mass (EM)	18	5.404	0.877	0.6072
	Cadmium(Cd)	2	3.342	0.381	0.6858
	EMxCd	36	8.769	1.423	0.0661
	Error	224	6.163		
Recovery30	Egg mass (EM)	18	17.217	2.706	0.0003
	Cad mium(Cd)	2	23.321	3.158	0.0545
	EMxCd	36	7.385	1.161	0.2557
	Error	224	6.363		

Statistically significant correlations were detected between PMM and (1) total proteases at Acute10 and both chronic cadmium treatments, (2) trypsin at chronic treatment with 30 µg Cd/g dry food as well as LAP at Acute 30 (Table III). The only negative correlation was found between PMM and total protease during short-term effect at 10 µg Cd/g dry food.

DISCUSSION

It is clear that a three-day regimen of cadmium primarily leads to a reduction in PMM, which is also proof that it is the organ first hit by xenobiotics in food. In addition, these changes were also detected after treatment with 30 µg Cd/g dry food. The decline of PMM is irreversible, as this parameter did not return to the control level even after the three-day recovery. Anthropogenic factors such as heavy metal contamination can lead to the selective survival of individuals of certain genotypes. In our experiment, statistically significant changes of PMM in acute treatments originate directly from cadmium, and the irreversible decline at chronic treatment is due to the influence of egg mass, i.e., genotype (ANOVA). The lines of reaction norms, representing individual variability per egg mass, show that egg masses respond much more uniformly by decreasing PMM during acute than during long-term treatment. In chronic cadmium treatment, there is greater variability. Genetic variability among different genotypes (20 egg masses) may give a diverse response to longer-lasting and stronger stress. The variability of population response to changes in the environment depends on genetic variability (Guttman, 1994; Templeton, 1995). However, it should be stressed that PMM is an indirect indicator of food consumption because the gut includes ingested food and not just tissue. We can conclude that cadmium in the fourth larval instar may lead to reduced food intake as well. It is likely that, for this trait, the concentration of 30 µg Cd/g dry food is too high to increase consumption, or a three-days regime is insufficient for recovery after chronictreatment. These findings are consistent with the

Table II. The mean values (MV) and standard deviations (±SD) calculated according to Cheplik (PP_{ch}) and the significance of differences between the mean values (Z-Wilcoxon's test) and standard deviations (F-test) of the index of phenotypic plasticity.

			10 µg Cd/g dry food			30 µg Cd/g dry food	
	N	**MV**	**±SD**	**N**	**MV**	**±SD**
Acute	19	9.600	22.381	19	14.339	20.374
Chronic	20	-5.871	24.429	20	3.245	19.858
Recovery	19	1.726	19.908	19	11.666	14.617
			Z	**P**	**F**	**P**
Comparisons	Acute10	-Acute30	1.2847	0.1989	1.2067	0.6944
	Chronic10	-Chronic30	1.4933	0.1354	1.5133	0.3745
	Recovery10	-Recovery30	1.8511	0.0642	1.8550	0.1996
	Chronic10	-Acute10	1.7707	0.0766	1.1914	0.7139
		-Recovery10	0.9256	0.3547	1.5057	0.3902
	Chronic30	-Acute30	1.8511	0.0642	1.0526	0.9101
		-Recovery30	1.2475	0.2122	1.8457	0.1997

Table III. Correlations between PMM and midgut enzymes during different cadmium treatments. Bold numbers represent correlations that are statistically significant P<0.05.

PMM	α-glucosidase	β- glucosidase	Esterase	Leucine aminopeptidase	Trypsin	Total protease	Glutathione S-transferase
Control	0.0323	0.2026	-0.1769	-0.1876	-0.0334	-0.1373	-0.3718
Acute10	0.0376	0.4196	-0.5029^{+}	-0.2698	-0.4163	-0.6288*	0.1821
Acute30	0.0433	0.2715	0.0818	0.5430*	0.3241	0.1405	-0.1642
Chronic10	0.2743	-0.2975	-0.1197	-0.0715	0.4042	0.4943*	-0.2249
Chronic30	0.323	0.0311	0.1770	0.3244	0.5823*	0.5970*	0.0425
Recovery10	-0.0168	0.4366^{+}	0.1126	0.1017	-0.1260	-0.0879	0.0315
Recovery30	-0.1609	-0.1273	0.0584	0.1118	0.2666	0.2330	-0.2034

results obtained by Fred and Brommer (2005), who found that larvae of two populations of *Parnassius apollo* reduced the intake of cadmium-contaminated food. It is possible that during long-term cadmium exposure, the chief priorities of individuals are survival and reaching adulthood. Therefore, all resources are directed towards proper development and thus adequate assimilation of food.

Restricted expression of enzymes may be associated with cadmium treatments as well as gut morphology. The decrease in midgut mass is accompanied by increased synthesis of enzymes that are crucial for survival in such conditions. There is probably a trade-off in the synthesis of digestive enzymes depending on which nutrients are most needed and/or available at the same time. In addition, under stress conditions, energy is redirected to the detoxification mechanisms that occur in the gut (sequestration, synthesis of metallothionein and oxidative enzymes, and the formation of granules containing metals).

All the enzymes that we claimed in our earlier researches (Vlahović *et al*., 2008, 2014, 2015, 2017) to be indicators of cadmium contamination show statistically significant correlations with PMM. The only negative correlation indicates different regulation of enzyme synthesis and/or activity in such conditions. Possibly, inhibition of protease isoforms leads to redistribution and preservation of homeostasis, while LAP and trypsin become the carriers of proteolytic function. In addition, during stronger cadmium stress (chronic treatment), a decrease in the activity of proteases and trypsin reduces PMM, the utilization of ingested food, and the survival rate. As previously stated (Vlahović *et al*., 2014, 2015), we have already detected the decline in digestive enzymes (LAP, TRYP and total PROT) during acute and chronic cadmium treatments. Several studies have suggested there are correlations between enzymatic activities in response to environmental stress or changes in food (Geer *et al*., 1985; Clark, 1989), and that correlated activities may result from simultaneous selection of the activity of several enzymes (Lande and Arnold, 1983). It can be concluded that, during cadmium presence, digestive enzymes can be mutually correlated and depend on environmental stress, the genetic basis, and most likely the structure of the enzyme itself.

Midgut structure influences enzymatic activity. The reason for this enzyme inhibition may be the altered structure and functioning of intestinal cells as a result of apoptosis and necrosis, which are direct consequences of cadmium action. Some ingested xenobiotics are detoxified via midgut epithelium (Johnson *et al*., 2009; Higes *et al*., 2013). Rost-Roszkowska *et al*. (2019) showed that acute and chronic cadmium treatments trigger the cell death process in *Lithobius forficatus* (Miriapoda). Non-specific autophagy of damaged organelles was mainly detected (Rost-Roszkowska *et al*., 2018, 2019). Reserve materials that occur in autophagosomes are utilized and removed from the gut as a way to diminish intracellular stress and reduce food intake (Wlodarczyk *et al*., 2019; Rost-Roszkowska *et al*., 2019). Short-term cadmium stress increases the metal concentration in the body. At the same time, ATP concentration is decreased regardless of the length of exposure to the metal. This can lead to reduced growth and changes in the structure and function of some organs (Rost-Roszkowska *et al*., 2020). Reduced amount of energy under stress conditions may affect the altered structure and function of the midgut in gypsy moth larvae. Degenerative necrotic changes in the midgut of the ground beetle are visible already after 1-2 days of cadmium exposure (Bednarska *et al*., 2016). With such substantial damage, cells are eliminated by necrosis, passive cell death. After metal exposure, apoptosis may be disturbed, and necrosis favored. Most likely, in our experiment, degeneration of the midgut epithelium resulted in reduced function and size of the midgut. Depending on the duration and severity of the treatment, the tissue damages may be

irreversible (there is no recovery after the cadmium effect).

The degenerated gut epithelium is most probably unable to properly synthesize digestive enzymes, as seen in our previous work. Buchon *et al*. (2013) proved that in *Drosophila melanogaster*, most digestive gene families are organized in genomic clusters. Proteases, trypsins, α-esterases, mannosidases, and lipases form clusters, and their activation depends on where each of its members is located along the gut. A possible explanation for the selective enzyme inactivation may be the arrangement of enzymes into clusters in *L. dispar* larvae.

Due to all of the above, reduction of the alimentary canal can occur. The insects' avoidance of contaminated food and inhibition of enzyme activities can be a consequence of the alterations in morphology and the changed functioning of the digestive system. In addition, under stress conditions, energy is redirected to the mechanisms of metal detoxification: The synthesis of metallothionein (Matić *et al*., 2020), synthesis of oxidative enzymes (Perić-Mataruga *et al*., 2019) as well as the formation of different granules (Hopkin, 1989). All these processes affect digestive enzymes and the utilization of nutrients in gypsy moth larvae. Thus, irreversible changes in fitness, metamorphosis and development arise, leading to reduced survival.

It is very important to note that the irreversible decline in larval mass begins only after the effect of a higher metal concentration upon chronic treatment at 30 µgCd/g dry food (Vlahović *et al*., 2014). During acute cadmium treatment, larval mass is constant, unlike reduced PMM. This phenomenon indicates that the gut is the first organ struck by the harmful effect of food toxicants and that its alteration does not have to be correlated with the size of the larvae.

Unlike midgut enzymes, statistically significant changes in the index of phenotypic plasticity or its standard deviations of the PMM between the treatments were not detected.

We demonstrated that metabolic and functional losses even at a low cadmium concentration, such as 10 µgCd/g dry food, which represents the NOEC for larval growth (Vlahović *et al*., 2001), can damage insects' midgut. The digestive system is the first to react to the presence of cadmium in food. We can conclude that the synthesis of digestive enzymes is correlated with the size of the intestine and vice versa, and a comprehensive study of the effect of dietary cadmium should thus analyze both biochemical parameters and structural changes in the midgut. This rapid screening method can be used to demonstrate the harmful effects of xenobiotics in food in a fast and inexpensive way or may complement biochemical tests in environmental studies.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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