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CADMIUM EFFECTS ON THE RATIO OF ACTIVITIES OF LYSOSOMAL AND TOTAL ACID PHOSPHATASES (ACP_{LYS}/ACP_{TOT}) IN *LYMANTRIA DISPAR* LARVAE

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Abstract - Searching for novel molecular biomarkers, we investigated cadmium effects on the ratio of specific activities of lysosomal and total acid phosphatases (ACP_{Lys}/ACP_{Tot}) in 4th instar gypsy moth larvae. After acute and chronic exposure to 10 and 30 μg Cd/g dry food, as well as after recovery from both concentrations, the trait values, plasticity, variability and genetic correlations were evaluated. The ACP_{Lys}/ACP_{Tot} ratio decreased during acute and chronic effects of both concentrations. Inhibition during long-term cadmium exposure was irreversible. Indices of phenotypic plasticity for ACP_{Lys}/ACP_{Tot} ratio were positive for all cadmium treatments. The variability of plasticity was higher after recovery from 10 μg Cd/g dry food, compared to recovery from 30 μg Cd/g dry food. A significant correlation coefficient was calculated between short-term cadmium treatments. Significant changes in the ACP_{Lys} activity fraction during all treatments indicate the examined trait (ACP_{Lys}/ACP_{Tot}) could be used as a pollution exposure biomarker.

Key words: Gypsy moth, cadmium, percent of lysosomal phosphatase activity, phenotypic plasticity

INTRODUCTION

Cadmium pollution has increased over the past decades as a result of anthropogenic activities. It is a non-degradable heavy metal that can be accumulated in animal tissues causing disruptions at all levels of organization. Cadmium was shown to reduce growth and longevity and to affect the development in various insect species, such as *Epirrita autumnata* (Van Oik et al. 2007), *Lymantria dispar* (Vlahović et al., 2009; Mirčić et al., 2010), *Folsomia candida* (Fountain and Hopkin, 2001), *Oncopeltus fasciatus* (Cervera et al., 2004). The effects of cadmium on detoxification and antioxidant systems in insects were noticed by Wang and Wang (2009) and Kafel et al. (2012). Planello et al. (2007, 2010) showed that cadmium exposure could alter the expression of ribosomal genes, as well as heat-shock and hormone receptor genes in *Chironomus riparius*. Ilijin et al. (2011) found the size of L2 type neurosecretory neurons was increased in cadmium-treated *Lymantria dispar* larvae. In addition, cadmium impedes cell cycle progression and cell proliferation (Templeton and Liu, 2010), DNA repair (Bertin and Averbeck, 2006) and cell-cell adhesion (Prozialeck et al., 2008).

After exposure, in *Orchesella cincta* approximately 90% of the cadmium is present in the gut (van Straalen and Roelofs, 2005). This study investigated cadmium effects on the midgut ratio of specific activities of lysosomal and total acid phosphatases (ACP_{Lys}/ACP_{Tot}). Phosphatases are metalloenzymes that catalyze the hydrolysis of various phosphomonoesters and take part in transphosphorylation. (Calvo-Marzal et al., 2001). Acid phosphatases (ACP) are located mainly in the cytosol of midgut cells of

Diptera and Lepidoptera, but can also be membrane-bound or present in the gut lumen (Terra and Ferreira, 1994). They participate in the final processes of digestion (Cheung and Low, 1975), excretion, water resorption and the mechanisms of active membrane transport, as well as in the cell replacement during apoptosis (Srivastava and Saxena, 1967). Lysosomal acid phosphatases take part in the hydrolysis of various macromolecules inside the lysosomal compartment, which is recognized as a center of heavy metal sequestration (Sterling et al., 2007). Cadmium is known to induce lysosomal damage (Fotakis et al., 2005), to increase lysosomal size and reduce its membrane stability (Lekube et al., 2000). Lysosomal enzyme release assay is generally considered as a biomarker of exposure to toxicants (Fotakis et al., 2005; Nazar et al., 2008).

The aims of this study were (i) to investigate the effects of cadmium exposure on the ratio of specific activities of lysosomal and total ACP (ACP_{Lys}/ACP_{Tot}) and to evaluate the possibility of using the trait as a biomarker of exposure; (ii) to study the variance and plasticity of the ACP_{Lys}/ACP_{Tot} ratio, and (iii) to evaluate the correlations of the ACP_{Lys}/ACP_{Tot} ratio across cadmium treatments.

MATERIALS AND METHODS

Experimental animals and rearing conditions

Female gypsy moths lay a single egg mass that is the product of a single mating. All larvae hatched from a single egg mass represent full sibs. Twenty egg masses were collected from the poplar forest at Opovo (45°03′49”N and 20°27′26”E). Egg masses were kept at +4°C until they were allowed to hatch. Larvae were reared individually in plastic cups on a high wheat germ diet (HWG) (Bell et al., 1981; Odell et al., 1984) at 23°C with a 12 h light/dark photoperiod. They were fed daily.

Cadmium treatments

Ten larvae from each of the twenty egg masses were randomly selected and assigned to seven experimental groups. These are as follows: 1 – C or control larvae that were not exposed to cadmium; 2 and 3 – Ac1 and Ac2 or larvae acutely exposed to two cadmium concentrations (10 and 30 μg Cd/g dry food, respectively); after molting into the 4th instar they were fed the cadmium diet for three days; 4 and 5 – Chr1 and Chr2 or larvae chronically exposed to two cadmium concentrations (10 and 30 μg Cd/g dry food, respectively); these were provided a cadmium diet from hatching until sacrifice; 6 and 7 – Rec1 and Rec2 or larvae recovered after chronic exposure to two cadmium concentrations (10 and 30 μg Cd/g dry food, respectively); these were provided the cadmium diet from hatching until molting into the 4th instar and then fed a control diet for three days until sacrifice. The cadmium test concentrations were based on the active component in $Cd(NO_3)_2{\cdot}4H_2O$.

Preparation of midgut homogenates

Larvae were weighed and killed on ice by decapitation on the third day of the 4th instar. The midguts were removed and kept at -20°C until homogenized. The homogenization was done in ice-cold saline 0.15M NaCl, followed by 10 min centrifugation at 10,000 x g. Supernatants were used for enzyme assays. For each pooled homogenate, five midguts from a single egg mass were used.

Enzyme assays

The activity of total acid phosphatases was measured spectrophotometrically by the modified method of Nemec and Socha (1988) based on the dephosphorylation of p-nitrophenyl phosphate (pNPP) which is a general substrate of phosphatases. The reaction was performed at 30°C in a mixture containing 100 mM citrate buffer pH 5.6, 5 mM $MgCl_2$, homogenate and 5 mM pNPP. After 30 min, the reaction was stopped by the addition of 0.5 M NaOH, and the absorbance of the enzyme reaction product, p-nitrophenol, was measured at 405 nm. Lysosomal acid phosphatase activity was determined indirectly, by calculating the difference between the activities of total acid phosphatases and nonlysosomal phosphatases. The reaction mixture was the same as described above with

the addition of 50 mM NaF, which is the specific inhibitor of lysosomal acid phosphatases. Protein concentrations were estimated according to the method of Bradford (1976).

Statistical methods

Mean values (MV) and standard errors of mean values (±SE) were calculated for the ratio of specific activities of lysosomal and total ACP. To evaluate the cadmium influence on the ACP_{Lys}/ACP_{Tot} ratio, one-way ANCOVA was used with the larval mass as covariate. The analysis of variance was carried out on the *arc sin*-transformed values of the trait.

Phenotypic plasticity (PP) for each egg mass was expressed by an index of PP, which was calculated according to the formula of Cheplick (1995):

$$PP_{Ch} = \frac{Xc - Xt}{Xc} \times 100$$

where Xc is the ACP_{Lys}/ACP_{Tot} ratio in the pooled homogenate from a single egg mass during control treatment, while Xt refers to cadmium treatment. Wilcoxon's test was used for comparing the PP of the trait between different cadmium treatments, and significance in index variability was estimated by the F-test. Z-test was used for comparisons of correlation coefficients between different environments. Differences were considered significant when $p<0.05$ was achieved.

RESULTS

Fig. 1 shows the ratio of specific activities of lysosomal and total ACP (ACP_{Lys}/ACP_{Tot}). During acute and chronic effects of both cadmium concentrations there were significant differences in relation to the control. Inhibition of the lysosomal fraction during long-term exposure to the effect of cadmium was irreversible considering that the activities did not return to the control level during a three-day recovery period. One-way ANOVA showed that the ACP_{Lys}/ACP_{Tot} ratio significantly depended on the cadmium concentrations during all treatments (Table 1). The indices of phenotypic plasticity for the ACP_{Lys}/ACP_{Tot} ratio were positive for all cadmium concentration treatments (Table 2). The variability of plasticity was higher after recovery from 10 μg Cd/g dry food compared to recovery from 30 μg Cd/g dry food (Table 3). A statistically significant correlation coefficient was calculated between short-term cadmium treatments at 10 and 30 μg Cd/g dry food (Table 4).

DISCUSSION

The decrease in the ACP_{Lys}/ACP_{Tot} ratio during short- and long-term stress at both cadmium concentrations, as well as the failure of the lysosomal phosphatase fraction to recover from inhibition during chronic treatment, show that this enzyme is susceptible to cadmium toxicity. This finding is expected considering that lysosomes are known to respond intensely to heavy-metal stress. According to Fotakis et al. (2005), cadmium-induced lysosomal damage is one of the earliest events in the cell stress response, preceding mitochondria and DNA damage. It includes the permeabilization and disintegration of lysosomes (Messnera et al., 2012), leading to content leakage and pH changes. Consequently, the ACP_{Lys} activity fraction might be reduced in such suboptimal conditions. Severe disruption of the lysosomal system could explain the irreversible nature of ACP_{Lys} inhibition that we have shown in this study.

Being accumulated in lysosomes, cadmium might inhibit ACP_{Lys} directly, due to its high affinity to thiol groups and the forming of cadmium-thiol complexes with proteins (van Straalen and Donker, 1994). Since it is able to replace cations in the metalloproteins (Moulis and Thevenod, 2010), cadmium could target the ACP_{Lys} active site, disabling enzyme function. In addition, it might prevent de novo synthesis of the enzyme. According to Planello and coworkers (2007), cadmium can inhibit the expression of ribosomal genes, consequently leading to the depletion of ribosomes. Stress may cause an energy redirection towards detoxification processes, which can also account for alterations in expression profiles in favor of proteins involved in stress adaptation response. Previously, we found that larval mass did not change during acute cadmium treatment, but de-

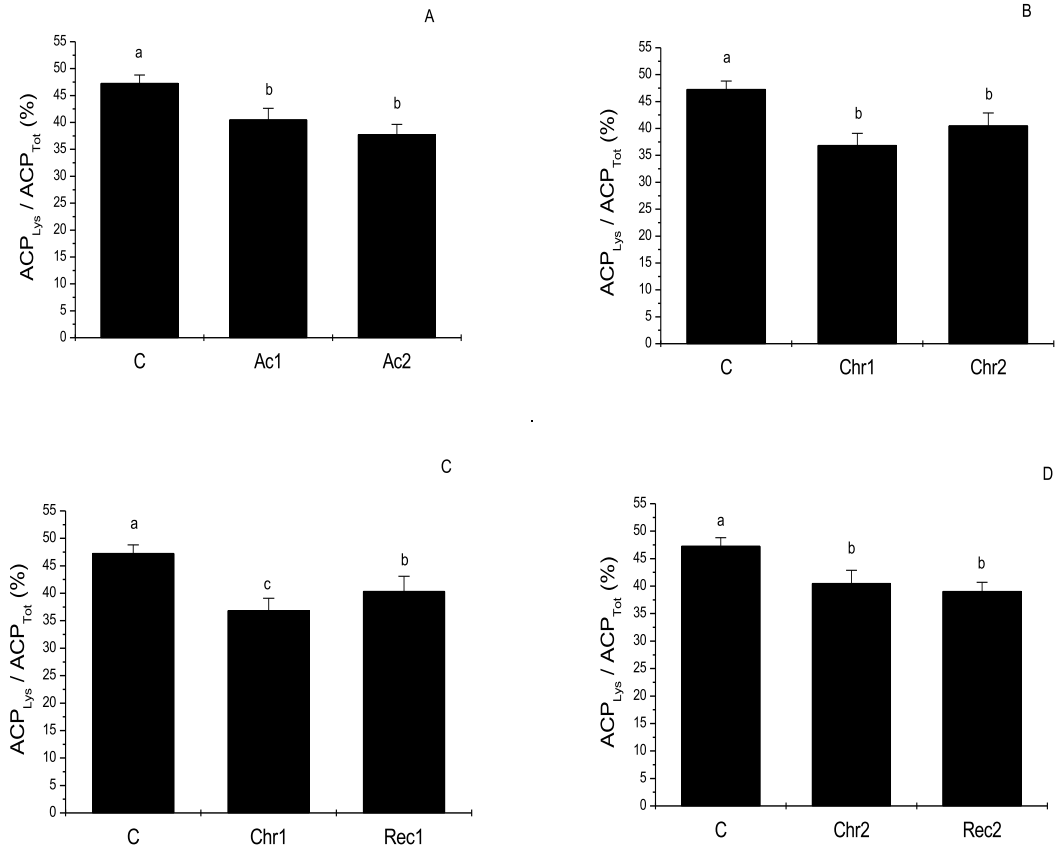

Figure 1. Changes in ratio of specific activities of lysosomal and total ACP (ACP_{Lys}/ACP_{Tot}) (MV±SE) after acute (A) and chronic treatments (B) and after recoveries (C, D). Experimental groups were compared by one-way ANCOVA followed by LSD multiple range test. Bars marked with different letters differ significantly. C-control; Ac1 and Ac2 – acute exposure to 10 and 30 μg Cd/g dry food; Chr1 and Chr2 – chronic exposure to 10 and 30 μg Cd/g dry food; Rec1 and Rec2 – recovery from exposure to 10 and 30 μg Cd/g dry food

creased during long-term exposure (Vlahović, 2009). These findings support the assumption of energetic shift during cadmium stress, as life history traits take longer to manifest changes compared to those at the molecular level.

Phenotypic plasticity represents a change of the phenotype of a specific genotype in response to environmental factors. The capacity of plasticity depends on genotype, hormones and environment, and does not have to be an adaptive trait (Scheiner, 1993). Cheplick's index of PP is used to explore plasticity when some covariates are expected to influence the target variable or trait showing the direction of full-sib plastic response (Valladares et al., 2006). The variability of plasticity was higher after recovery from 10 μg Cd/g dry food compared to recovery from 30 μg Cd/g dry food.

The significant positive correlation of the ACP_{Lys}/ACP_{Tot} ratio between short-term treatments at 10 and 30 μg Cd/g dry food points to an overlapping in the genes that influence the ACP_{Lys}/ACP_{Tot} ratio at two different concentrations during acute treatment. Inter-environmental genetic correlations can considerably affect the rate and direction of evolution in traits related to the use of environmental resources (Via, 1984). Being significantly different from "one", the significant correlation we obtained is not a constraint for the evolution of plasticity. On the other

Table 1. Mean square ($x10^3$) from the one-way ANCOVA of ratio of specific activities of lysosomal and total ACP (ACP_{Lys}/ACP_{Tot}) in gypsy moth larvae exposed to different cadmium treatments. Larval mass was used as the covariate. Bold values indicate P<0.05; df = degrees of freedom

Effect	Source of variation	df	MS	F	P
Acute	Covariance	1	0.960	0.131	0.7193
	[Cd]	2	49.621	6.745	**0.0024**
	Error	54	7.357		
Chronic	Covariance	1	17.470	1.891	0.1747
	[Cd]	2	51.856	5.613	**0.0061**
	Error	55	9.239		
Recovery1	Covariance	1	12.747	1.253	0.2679
	[Cd]	2	53.015	5.212	**0.0085**
	Error	54	10.172		
Recovery2	Covariance	1	0.098	0.012	0.9118
	[Cd]	2	35.066	4.424	**0.0165**
	Error	55	7.926		

Table 2. Mean values (MV) and standard deviations (±SD) of index of phenotypic plasticity according to Cheplick (PP_{Ch}) for the ACP_{Lys}/ACP_{Tot} ratio depending on cadmium treatments. C1 = 10 μg Cd/g dry food; C2 = 30 μg Cd/g dry food

	C1			C2		
Effect	N	MV	±SD	N	MV	±SD
Acute	19	18.095	23.589	19	17.524	20.160
Chronic	19	20.108	20.762	20	13.939	19.889
Recovery	19	12.387	25.020	19	15.348	14.027

Table 3. Significance of differences between means (Z, Wilcoxon's test) and standard deviations (F-test) of index of phenotypic plasticity for (ACP_{Lys} / ACP_{Tot}) ratio. Bold value indicates P<0.05

Comparison		Z	P	F	P
Ac1	Ac2	1.677	0.0936	1.369	0.5118
Chr1	Chr2	0.523	0.6009	1.090	0.8520
Rec1	Rec2	0.523	0.6009	3.182	**0.0183**
Chr1	Ac1	1.502	0.1330	1.291	0.5937
	Rec1	0.762	0.4460	1.452	0.4364
Chr2	Ac2	0.604	0.5461	1.027	0.9508
	Rec2	0.443	0.6580	2.010	0.1448

Table 4. Significance of correlations between cadmium treatments for percent of lysosomal acid phosphatase (ACP_{Lys} / ACP_{Tot}). ✠ P<0.1 * P<0.05

	C	Chr1	Chr2	Ac1	Ac2	Rec1	Rec2
C		0.223	0.120	0.113	0.039	0.058	0.412✠
Chr1			0.170	0.204	0.361	0.173	-0.338
Chr2				0.462✠	-0.054	0.269	0.133
Ac1					0.512*	-0.072	-0.032
Ac2						-0.316	-0.111
Rec1							0.194
Rec2							

hand, the marked absence of correlations between the treatment groups suggests that independent genetic mechanisms mediate the responses in different stress environments. Javakumar et al. (2008) found that in the mussel *Lamellidens marginalis* cadmium affected the activity of lysosomal acid phosphatase (ACP_L) in an exposure duration-dependent manner, and Stubberud et al. (2000) evaluated ACP enzymes as a sensitive biomarker.

The significant influence of all cadmium treatments on the ACP_{Lys}/ACP_{Tot} ratio points to its great sensitivity, suggesting that ACP_{Lys} could be considered as a bioindicator of dietary cadmium exposure.

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