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Implications of long-term exposure of a *Lymantria dispar* L. population to pollution for the response of larval midgut proteases and acid phosphatases to chronic cadmium treatment

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ABSTRACT

Cadmium (Cd) presence in terrestrial ecosystems is a serious threat that requires continuous development of biomonitoring tools. Ideally, a suitable biomarker of exposure should respond to the toxicant consistently in different populations regardless of previous exposure to pollution. Here we considered the activities and isoform patterns of certain proteases and acid phosphatases (ACP) in the midgut of *Lymantria dispar* larvae as well as the integrated biomarker response (IBR) for application in Cd biomonitoring. We compared the responses of caterpillars originating from unpolluted and polluted localities after they had been chronically subjected to dietary Cd (50 and 100 μg Cd/g dry food). The population inhabiting the unpolluted forest was far more sensitive to Cd exposure as the activities of total proteases, trypsin (TRY) and leucine aminopeptidase (LAP) were mostly reduced while the activities of total and non-lysosomal ACP were increased. Non-lysosomal ACP activity was elevated in larvae from the contaminated site in response to the higher Cd concentration. Exposure to the metal resulted in numerous alterations in the pattern of enzyme isoforms, but the responses of the two populations were similar except that larvae from the polluted locality were more tolerant to the lower Cd concentration. Non-lysosomal ACP activity and the appearance of ACP isoforms 4 and 5 together with the IBR index are the most promising indicators of Cd presence, potentially applicable even in populations with a history of exposure to pollution. TRY and total ACP activities could be used to monitor populations at uncontaminated localities.

1. Introduction

Cadmium is a heavy metal that has become a threat to the living world as its concentration has increased in the environment over several decades due to processes of industrialization and urbanization (ATSDR, 2017; Genchi et al., 2020; IARC, 2012). Nowadays, a continuous effort is being made to develop and improve biomonitoring protocols and tools for the assessment of cadmium contamination levels in various ecosystems. More comprehensive information about toxic effects of pollution can be provided when the responses of different biomarkers are summarized into a single index such as the Integrated Biomarker Response (IBR), calculated by the procedure of Beliaeff and Burgeot (2002). The IBR is one of the most used methods in the multi-biomarker approach to environmental assessment (Marigómez et al., 2013; Serafim et al., 2012).

In terrestrial insects heavy metals most often enter the organism through contaminated food. After metals are ingested, the insects tend to accumulate them mainly in the gut (Chapman, 2013; Hensbergen et al., 2000). However, data about the effects of heavy metals, including cadmium, on gut proteases and phosphatases are very limited, although this topic deserves more attention. Namely, these enzymes directly affect insect fitness, being involved in digestion and metabolic processes related to energy production (Chitgar et al., 2013; Nathan et al., 2004). Due to such a close relation to fitness, they should be considered as potentially important biomarkers of cadmium exposure. Previously, we have shown considerable sensitivity of several midgut enzymes (proteases, acid phosphatases and esterases) in larvae of *Lymantria dispar* L. (Lepidoptera: Erebidae) exposed to dietary cadmium (Matić et al., 2020;

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Vlahović et al., 2013, 2014, 2015). Other authors have suggested the application of different digestive enzymes in biomonitoring (Hyne and Maher, 2003; Lagadic et al., 1994; Lai et al., 2011).

Various proteases have been identified in the midgut of Lepidoptera larvae, including trypsin, chymotrypsin, elastases, cathepsin B-like proteases, aminopeptidases and carboxypeptidases (Chougule et al., 2008; Terra and Ferreira, 2012). Serine protease activity (particularly trypsin and chymotrypsin) accounts for 95% of the total protease activity in the midgut of these caterpillars (Chougule et al., 2008; Srinivasan et al., 2006; Tabatabaei et al., 2011). Proteases are present in multiple isoforms whose expression can be altered to accomplish optimal protein digestion (Patankar et al., 2001; Srinivasan et al., 2006). Lepidopterans can switch to a different complement of isoforms during development or adapt to another plant-host (Gatehouse et al., 1997; Patankar et al., 2001). For example, the response to the presence of plant protease inhibitors may include overproduction of existing proteases, increased expression of inhibitor-resistant protease isoforms or activation of proteases that detoxify plant inhibitors (Zhu-Salzman and Zeng, 2015). Heavy metals have been shown to reduce proteolytic activity in *Musca domestica* (Diptera) (Blahovec et al., 2006) and *Rhynchophorus palmarum* (Coleoptera) (Egwim, 2011). Božić et al. (2003) found that cadmium, zinc and cobalt inhibited the activity of purified aminopeptidases in *Morimus funereus* (Coleoptera).

Phosphatases are involved in hydrolysis of different phosphomonoesters and transphosphorylation (Calvo-Marzal et al., 2001). Two groups, alkaline and acid phosphatases (ALP and ACP, respectively), can be distinguished based on the pH range of their maximal activity. ACP are ubiquitous enzymes in all living systems taking part in processes such as digestion (Cheung and Low, 1975), ion transport (Yi and Adams, 2001) and carbohydrate metabolism (Sreenivasan et al., 2011). Although ACP are generally considered to be a lysosomal marker, they are found predominantly in the cytosol of midgut cells of Lepidoptera larvae (Santos and Terra, 1984), but the roles of these so-called "non-lysosomal" enzyme forms remain unclear. Cadmium may change the number and the size of lysosomes, disrupt the integrity of lysosomal membranes or alter the activity of lysosomal enzymes (Lekube et al., 2000). If the lysosomal membrane is damaged, hydrolases from the lysosomal lumen may leak into the cytosol. ACP are considered to be a reliable lysosomal marker, frequently used as an indicator of environmental pollutants including cadmium (Chen et al., 2017; Izagirre et al., 2009). However, it is crucial to discriminate between these lysosomal ACP released into the cytosol and non-lysosomal ACP that are normally and abundantly present in the cytosol of Lepidoptera midgut cells, as mentioned above. In enzyme activity assay this can be achieved by adding NaF that specifically inhibits lysosomal ACP activity (Nemec and Ženka, 1996). Otherwise, if ACP were considered to be exclusively lysosomal marker, non-lysosomal ACP activity would be completely neglected, whereas cadmium effects on lysosomes or lysosomal ACP activity would be overestimated. Rare literature data about cadmium effects on arthropod ACP pointed to an increase in the enzyme activity, as in an *Aedes albopictus* C6/36 insect cell line (Braeckman et al., 1999), or in several tissues of the crab *Uca annulipes* (Suresh et al., 2016). However, in these studies only total ACP activity was measured, but it was implicitly attributed to the lysosomal ACP activity.

An exposure of arthropod populations to heavy metals for long periods of time may result in the phenomenon of metal tolerance (Kafel et al., 2012; Roelofs et al., 2009). The implication of metal tolerance in biomarker assessment is obvious. Namely, long-term exposure of a population to pollution might alter sensitivity of a potential biomarker. Our previous research confirmed decreased sensitivity to cadmium treatment at different levels of biological organization in *L. dispar* populations inhabiting polluted localities (Matić et al., 2016, 2020; Vlahović et al., 2017). According to the literature, it is unknown if such prolonged exposure of invertebrate populations affects protease and ACP activity or their isoform expression. The current study aimed to compare proteolytic activity (total proteases, trypsin (TRY) and leucine aminopeptidase (LAP)), ACP activity (total, non-lysosomal and lysosomal ACP) and enzyme isoform patterns between *L. dispar* larvae originating from uncontaminated and contaminated habitats after exposure to chronic dietary cadmium. Enzymatic parameters were evaluated individually and combined into the IBR as potential biomarkers of cadmium presence.

2. Materials and methods

2.1. Rearing of experimental insects and cadmium treatment

L. dispar inhabits areas across the northern hemisphere including Europe, Asia and North America. The caterpillars can devastate forests by feeding on the foliage of more than 300 species of trees, which may result in serious economic losses during outbreak years. *L. dispar* is considered to be a suitable model organism due to its large native range, short life cycle, and successful rearing on an artificial diet.

Egg-masses were collected in the autumn from two localities in Serbia – unpolluted Kosmaj mountain and a site near the Ibar highway in a suburban area of Belgrade city. Kosmaj mountain was declared a protected natural resource in 2005 (State Enterprise for Forest Management "Srbija Šume"). "The area has an unexpectedly high level of biodiversity, including a rich wildlife fund with numerous representatives classified as natural rarities, endangered, relict and endemic species" (Institute for Nature Conservation of Serbia). The Ibar highway is recognized as one of the busiest roads in Serbia. Traffic is a major contributor to metal pollution in urban soils in the state. The most common metals detected in excess of standard limits are Pb, Cr, Ni, Cu and Zn (Marjanović et al., 2009; Mihailović et al., 2015; Pivić et al., 2017). At the time when *L. dispar* egg-masses were collected for the current study, leaded gasoline was still permitted.

Egg-masses were kept in a refrigerator at 4 °C until April to satisfy diapause requirements. After hatching, larvae were reared on an artificial diet at 23 °C under a 12 h light/12 h dark regime. The wheat germ diet cubes (Bell et al., 1981; Thompson et al., 2017) were replaced daily to ensure continuous availability of fresh food. The experimental groups from both localities were provided with food containing 50 or 100 μg Cd/g dry food starting from hatching until the larvae were killed. The lowest-observed-effect concentrations (LOEC) of cadmium at both biochemical and whole organism level that we determined previously in larvae from the unpolluted locality were 10 or 30 μg Cd/g dry food (Vlahović et al., 2013, 2014, 2015). However, preliminary experiments on the *L. dispar* population from the polluted site near Ibar highway showed insensitivity to these concentrations, which made us choose higher levels (50 and 100 μg Cd/g dry food) for the current study. Several authors who studied metal tolerance under laboratory conditions exposed insects to the similar range of cadmium concentrations (Kafel et al., 2012; Sterenborg and Roelofs, 2003). These high cadmium levels were registered in soils or plants at some heavily polluted areas (Roelofs et al., 2007; Wang et al., 2009).

On the third day of the fourth instar, larvae were anesthetized on ice for 1 min and decapitated. Immediately after it was dissected out, the midgut was frozen and kept at −20 °C.

2.2. Preparation of the samples for enzyme assays and electrophoretic analyses

Ten midguts per experimental group were randomly selected to prepare individual samples for the analysis of the proteolytic activity. Another ten individual midgut samples were used to investigate phosphatase activity. After the midgut from a single larva was weighed, a precooled (4 °C) 150 mM NaCl solution was added to a tube to obtain a final concentration of 100 mg tissue/ml in each sample. The tube was immediately immersed in an ice dish, and the content was homogenized using Ultra Turrax homogenizer (IKA-Werke, Staufen, Germany). After the homogenates were centrifuged at 10,000*g* for 10 min at 4 °C, the

supernatants were collected and kept at −20 °C for few days before the measurements. This protocol ensured that supernatants would contain cytosol and lysosomes. Protein concentrations in both individual and pooled samples were determined using BSA as the standard (Bradford, 1976).

The individual samples were used in enzyme assays, while the isoforms were analyzed in the two pooled samples. Each pooled sample, "protease" one and "phosphatase" one, was composed of equal volumes of three corresponding randomly selected individual samples that were used in protease or phosphatase activity assays. Pooling samples for electrophoresis contributed to simplicity and efficiency. Considering that the main goal of the current study was to analyze the applicability of parameters as biomarkers of an exposure to cadmium, simple and time-saving approaches, techniques and protocols are highly appreciated.

2.3. Spectrophotometric assays

2.3.1. Proteases

For determination of protease activity each group contained 9–10 samples derived from individual midguts. Total protease activity was measured in three technical replicates for each sample, while measurements of TRY and LAP activities involved two replicates per sample.

2.3.1.1. Total proteases. Total protease activity was measured by a proteolytic assay (Kunitz, 1947) using casein as the substrate. The sample was diluted 1:50 with 200 mM Gly/NaOH buffer (pH 10). 1% casein in the same buffer was incubated with the diluted sample in the ratio 1:1 for 60 min at 40 °C. The reaction was interrupted with 10% trichloroacetic acid. After precipitation of the remaining casein during 30 min at 4 °C, the content was centrifuged at 10,000*g* for 10 min. Absorbance in supernatants was measured at 280 nm. Total protease activity is proportional to the amount of free aminoacids and peptides.

2.3.1.2. Trypsin. TRY activity was measured according to Erlanger et al. (1961) and Valaitis (1995). The method is based on the liberation of *p*-nitroaniline from the substrate Nα-benzoyl-DL-arginine 4-nitroanilide hydrochloride (DL-BAPNA). Enzyme activity is proportional to the amount of *p*-nitroaniline liberated from DL-BAPNA. The reaction mixture contained 50 mM Gly/NaOH buffer (pH 10), 20 mM substrate and 5% (v/v) sample. After incubation at 30 °C for 15 min, the reaction was stopped with 6% acetic acid. The absorbance was measured at 405 nm.

2.3.1.3. Leucine aminopeptidase. To evaluate LAP activity, the method of Erlanger et al. (1961) was applied. The reaction took place in 40 mM veronal/HCl buffer (pH 7.8–8), the substrate 20 mM L-leucine *p*-nitroanilide and 5% (v/v) sample at 30 °C during 10 min. Afterwards, it was interrupted with 6% acetic acid. LAP activity is proportional to the concentration of liberated *p*-nitroaniline. The absorbance was measured at 405 nm.

Specific activities of proteolytic enzymes are given in U per mg of total protein.

2.3.2. Acid phosphatases

ACP activities were measured in 8–10 midgut samples per group in two technical replicates.

Total ACP activity determined in the midgut samples came from lysosomal ACP that ended up in the cytosol, and non-lysosomal ACP which are normally localized in the cytosol. The term lysosomal ACP in this manuscript refers only to the lysosomal phosphatases found in the cytosol, whose activity was determined indirectly, as a fraction of total ACP activity after they were specifically inhibited with NaF (Nemec and Ženka, 1996; Holtzman, 1989). Determination of total ACP activity was based on the liberation of *p*-nitrophenol from the substrate *p*-nitrophenyl phosphate (pNPP) under acidic conditions (Nemec and Socha, 1988). The reaction took place in a mixture of 100 mM citrate buffer pH 5.6, 5 mM $MgCl_2$, 5 mM pNPP and 5% (v/v) sample. After incubation for 60 min at 30 °C, the reaction was stopped by adding 0.5 M NaOH, and the absorbance was measured at 405 nm. To determine the activities of lysosomal and non-lysosomal ACP, the reaction occurred under the same conditions in a mixture containing 50 mM NaF. The absorbance determined at 405 nm is proportional to the activity of the non-lysosomal fraction of total ACP. Specific activities of ACP are given in mU per mg of total protein.

2.4. Detection of enzyme activity after native polyacrylamide gel electrophoresis (PAGE)

2.4.1. Proteases

Isoforms of total proteases, TRY and LAP were detected in the midgut sample, pooled from three randomly selected biological samples that were used in protease enzyme assays. Three technical replicates were run for each enzyme or enzyme group.

2.4.1.1. Total proteases. The isoforms of total proteases were examined according to the modified method of Muhlia-Almazán and García-Carreño (2002). Aliquots containing 5 μg proteins were prepared using SDS sample buffer without reducing agent, and loaded on to 10% polyacrylamide gel. SDS-polyacrylamide gel electrophoresis was run at 100 V and 4 °C. The gel was washed twice for 10 min in deionized water, and equilibrated in 50 mM glycine buffer (pH 10) for 20 min. Then it was soaked for 30 min at 4 °C in 3% casein dissolved in the same glycine buffer and pre-chilled at 4 °C. The gel was incubated with the casein substrate for 1 h at 40 °C, washed with deionized water, and stained for 5 min with a solution containing 0.1% Coomassie brilliant blue R-250, 40% ethanol and 10% acetic acid. To remove the excess dye, the gel was discolored overnight with a solution composed of 40% ethanol and 10% acetic acid. Clear bands against a dark blue background indicated proteolytic activity.

2.4.1.2. Trypsin. TRY isoforms in a polyacrylamide gel were detected by a method modified from Erlanger et al. (1961). Each lane of 10% polyacrylamide gel was loaded with a sample that contained 5 μg protein. Electrophoresis was run under non-reducing and non-denaturing conditions at 100 V and 4 °C in 25 mM Tris and 192 mM glycine buffer, pH 8.3. Enzyme activity was detected by an overlay technique modified from Vinokurov et al. (2005). Nitrocellulose membrane (0.45 μm, Amersham) was presoaked for 50 min at room temperature in 2 mM DL-BAPNA diluted in 50 mM glycine buffer, pH 10.0 (DL-BAPNA was first dissolved to 20 mM in dimethylformamide). The gel was washed in deionized water twice for 10 min and then equilibrated in the same glycine buffer for 20 min. After removing the buffer, the gel was covered by the membrane and incubated in a moist chamber for 60 min at 37 °C (or until yellow bands coming from *p*-nitroaniline appeared on the membrane). Membrane was first soaked in 0.1% sodium nitrite in 1 M HCl for 2.5 min, then washed in 1% urea for no more than 30 s, and incubated in 0.05% 1-naphthylamine in 47.5% ethanol. When pink bands became visible, the membrane was soaked in deionized water to stop the reaction, and then was scanned.

2.4.1.3. Leucine aminopeptidase. The activity of LAP isoforms was determined in the same way as for trypsin (Erlanger et al., 1961; Vinokurov et al., 2005) described in detail above. The substrate in which the nitrocellulose membrane was presoaked was 20 mM L-leucine *p*-nitroanilide dissolved in dimethylformamide and diluted to 2 mM in veronal/HCl buffer (pH 7.8–8). The same veronal/HCl buffer was used to equilibrate the polyacrylamide gel after native electrophoresis.

2.4.2. Acid phosphatases

A pool made up of three randomly selected biological samples that were used for phosphatase enzyme assays was subjected to electrophoresis in three technical replicates. ACP activity after native PAGE was detected by the modified method of Allen et al. (1963). Using 12% polyacrylamide gel, 10 μg protein aliquots per well were separated at 100 V and 4 °C. Then the gel was washed with deionized water for 1 min and equilibrated for 10 min in 100 mM acetate buffer (pH 5.2) at 30 °C. Nitrocellulose membrane was pre-soaked in 0.13% α-naphthyl phosphate dissolved in the same acetate buffer for 50 min at room temperature. The gel was covered with the membrane, and incubated in a moist chamber for 60 min at 30 °C. Afterwards, the membrane was soaked in 0.3% Fast Blue B stain dissolved in acetate buffer until bands became visible, and immediately washed with deionized water to stop the reaction.

The intensities of enzyme bands in all experiments were measured using ImageJ software (U. S. National Institutes of Health, Bethesda, Maryland, USA).

2.5. Integrated biomarker response (IBR)

The current study identified specific activities of total proteases, TRY and non-lysosomal ACP as the most promising biomarkers that demonstrated notable sensitivity and consistent responses to cadmium exposure. These three biomarkers were combined into an IBR according to the method developed by Beliaeff and Burgeot (2002). A standardized value S was calculated for each biomarker and each data set (control and cadmium-treated groups of both populations). The results were displayed as vectors in the star plot for each data set. The IBR value was then calculated as an area of the triangle formed by connecting endpoints of the vectors:

$$IBR = \sum_{i=1}^{n} A_i$$

where the area A_i is obtained by connecting the ith and the $(i + 1)$th endpoints,

$$A_i = S_i/2sin\beta\ (S_i cos\beta + S_{i+1} sin\beta),$$

$$\beta = tan^{-1}\left(\frac{S_{i+1} sin\alpha}{S_i - S_{i+1} cos\alpha}\right),$$

$\alpha = 2\pi/n$ radians, $S_{n+1} = S_1$, and $n = 3$ (the number of the biomarkers).

Excel software (Microsoft, USA) was used to calculate IBR values and to generate star plots.

2.6. Statistical methods

Statistical analyses were conducted in GraphPad Prism 7 (GraphPad Software, Inc., USA) and using QI Macros (version 2021) (KnowWare International Inc., USA). After the Kolmogorov-Smirnov test was applied to check if the data followed normal distribution, an assumption of homogeneity of variances was verified by parametric Levene's test. All enzyme activities were analyzed by one-way ANOVA followed by Tukey's post-hoc test. The level of statistical significance was $p < 0.05$ for all statistical tests.

3. Results

3.1. Effects of cadmium on specific activities and isoforms of proteases

3.1.1. Total proteases

According to one-way ANOVA, cadmium exposure significantly affected total protease activity in larval midgut, $F(5,53) = 5.3$, $p = 0.0005$ (Fig. 1A). Enzyme activity was reduced in larvae from the unpolluted locality after treatment with 100 μg Cd/g dry food ($p < 0.05$).

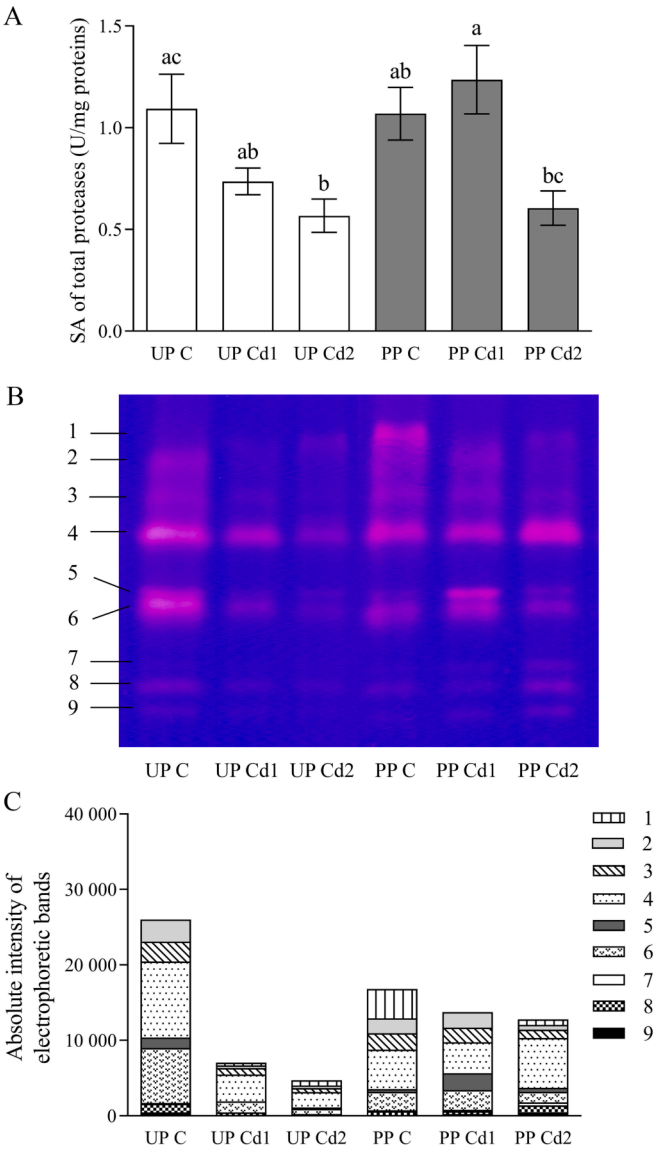


Fig. 1. Specific activity of total proteases (A), SDS PAGE gel stained for protease activity (B), and densitometric analysis of the bands (C) in the midgut of *L. dispar* larvae after chronic exposure to cadmium. UP and PP – populations from the unpolluted and polluted sites, respectively; C - control, Cd1 and Cd2 - 50 and 100 μg Cd/g dry food, respectively. The bars show means±SE (n = 9–10 larvae per group). Different letters denote significant differences among the experimental groups (Tukey's post-hoc test, $p < 0.05$). The results shown in panels B and C were obtained from a pooled sample, whereas panel C represents the mean of three technical replicates.

In the larval population from the locality near the Ibar highway it was lower after feeding with 100 μg Cd/g dry food than with 50 μg Cd/g dry food ($p < 0.01$).

Nine bands revealing total protease activity among the larval groups were detected on the gel (Fig. 1B). The activities of most isoforms in larvae from the unpolluted site were notably diminished after exposure to cadmium (Fig. 1C). While the intensities of bands with the highest electrophoretic mobility (7, 8 and 9) were reduced in the population from Kosmaj after receiving a cadmium diet, they tended to increase in the Ibar larvae.

3.1.2. Trypsin

Chronic cadmium treatment significantly affected TRY specific activity in larval midgut, $F(5,52) = 4.171$, $p = 0.0029$. The post-hoc test

showed that exposure to both 50 and 100 μg Cd/g dry food reduced TRY activity in the population inhabiting the unpolluted forest on Kosmaj mountain ($p < 0.01$, $p < 0.05$, respectively) (Fig. 2A). However, cadmium effects were not registered in larvae from the polluted area near the Ibar highway. Differences between equivalent groups of the two populations were not detected ($p > 0.05$).

Two TRY isoforms (marked 2 and 3) were detected in the midgut of control larvae from both populations and at 50 μg Cd/g dry food in larvae from the polluted locality (Fig. 2B). However, exposure of larvae from the unpolluted forest to both cadmium concentrations resulted in disappearance of isoform 3 and increased activity of isoform 2. In larvae from the polluted site isoform 3 also vanished at 100 μg Cd/g dry food while isoform 1 emerged (Fig. 2C).

3.1.3. Leucine aminopeptidase

Significant effects of dietary cadmium on the specific activity of LAP in the midgut of *L. dispar* larvae were registered, $F(5,53) = 16.38$, $p < 0.0001$. In caterpillars from the unpolluted locality enzyme activity was decreased after both Cd1 and Cd2 treatments in comparison with the control group ($p < 0.0001$, $p < 0.05$, respectively) (Fig. 3A). Inhibition by the lower metal concentration was more prominent compared to the higher one ($p < 0.001$). Control larvae from the polluted locality expressed lower enzyme activity than the control group from the unpolluted site ($p < 0.0001$). However, exposure to the Cd1 concentration increased LAP activity in the Ibar population over that for the control group ($p < 0.01$) and the equivalent group from the Kosmaj population ($p < 0.0001$).

All six LAP isoforms were detected in control larvae from the uncontaminated locality, among which isoform 1 was exclusively present in that group (Fig. 3B). The activity of isoform 2 was elevated in all cadmium treated groups of both populations, relative to the corresponding controls (Fig. 3C). Both cadmium concentrations in each population caused LAP isoform 3 to disappear. In larvae from the polluted site band 4 was intensified after exposure to both cadmium concentrations.

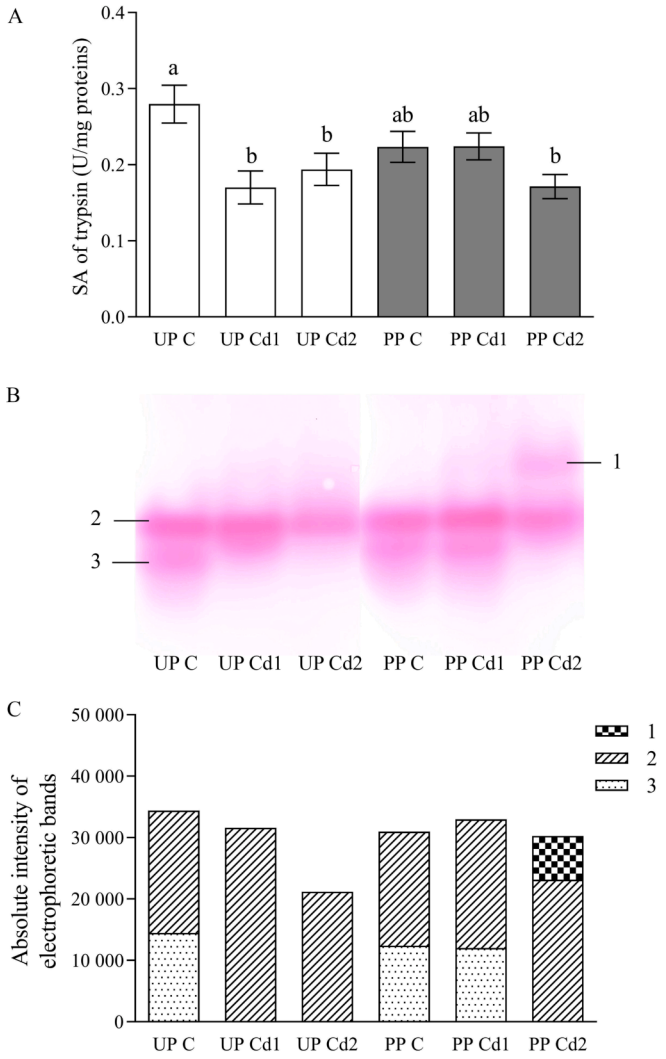


Fig. 2. Specific activity of trypsin (TRY) (A), native PAGE gel stained for TRY activity (B), and densitometric analysis of the bands (C) in the midgut of *L. dispar* larvae following chronic cadmium treatment. UP and PP – populations from the unpolluted and polluted sites, respectively; C - control, Cd1 and Cd2 - 50 and 100 μg Cd/g dry food, respectively. The bars show means±SE (n = 9–10 larvae per group). Different letters denote significant differences among the experimental groups (Tukey's post-hoc test, $p < 0.05$). The results shown in panels B and C were obtained from a pooled sample, whereas panel C represents the mean of three technical replicates.

3.2. Specific activities and isoform pattern of acid phosphatases after cadmium exposure

3.2.1. Total acid phosphatases

Cadmium intake significantly influenced the activity of total ACP in the midgut of *L. dispar* caterpillars, $F(5,52) = 11.30$, $p < 0.0001$. An increase in enzyme activity was registered in the population from the unpolluted area after exposure to both Cd concentrations ($p < 0.01$) (Fig. 4A). In addition, these groups treated with Cd1 and Cd2 concentrations showed higher activity of total ACP in comparison with equivalent larvae from the polluted site ($p < 0.001$, $p < 0.05$, respectively).

Five ACP isoforms in larval midguts were detected in this study (Fig. 5A). In the control group from the uncontaminated locality isoforms 2, 3 and 4 were registered, but their activities were extremely low. Control larvae from the polluted site expressed isoforms 2, 3 and 1 expressed. Cadmium strongly induced the activity of isoform 4 in all treated groups from both localities (Fig. 5B). Isoform 4 was the least abundant in the group of Ibar larvae fed with 50 μg Cd/g dry food. Isoform 5 also appeared in both populations in response to each cadmium concentration, but activity levels were higher in larvae from the unpolluted forest.

3.2.2. Non-lysosomal acid phosphatases

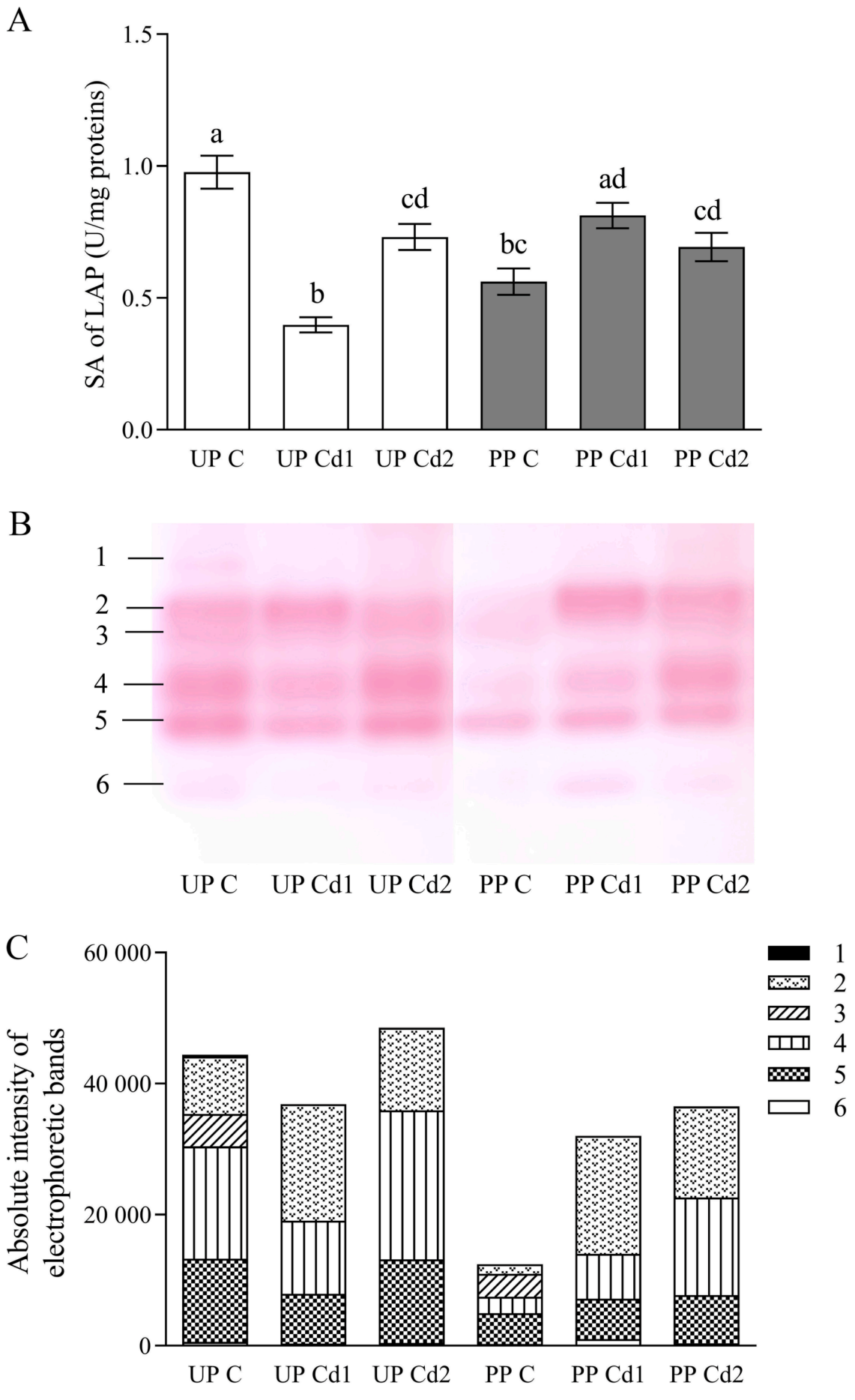
Activity of the non-lysosomal ACP fraction was significantly altered after cadmium treatment, $F(5,54) = 11.66$, $p < 0.0001$. Both 50 and 100 μg Cd/g dry food increased enzyme activity in larvae from the uncontaminated forest ($p < 0.05$, $p < 0.01$, respectively) (Fig. 4B). In the population from the polluted area only exposure to the higher cadmium concentration resulted in elevated activity ($p < 0.05$). Both cadmium treated groups from Kosmaj (Cd1, Cd2) had higher activity levels when compared to equivalent larvae from the site near the Ibar Highway ($p < 0.01$, $p = 0.05$, respectively).

3.2.3. Lysosomal acid phosphatases

Exposure of *L. dispar* larvae to cadmium did not significantly affect the lysosomal ACP fraction in their midgut, $F(5,50) = 1.321$, $p = 0.2707$ (Fig. 4C).

3.3. Integrated biomarker response (IBR) to cadmium exposure

A concentration-dependent increase of the IBR index was evident in *L. dispar* larvae from the unpolluted forest after exposure to cadmium. In larvae from the polluted locality, the IBR value was strongly elevated only after treatment with the higher metal concentration (Fig. 6, Table 1).



(caption on next page)

Fig. 3. Specific activity of leucine aminopeptidase (LAP) (A), native PAGE gel stained for LAP activity (B), and densitometric analysis of the bands (C) in the midgut of *L. dispar* larvae after chronic dietary cadmium ingestion. UP and PP – populations from the unpolluted and polluted sites, respectively; C - control, Cd1 and Cd2 - 50 and 100 μg Cd/g dry food, respectively. The bars show means±SE (n = 9–10 larvae per group). Different letters denote significant differences among the experimental groups (Tukey's post-hoc test, $p < 0.05$). The results shown in panels B and C were obtained from a pooled sample, whereas panel C represents the mean of three technical replicates.

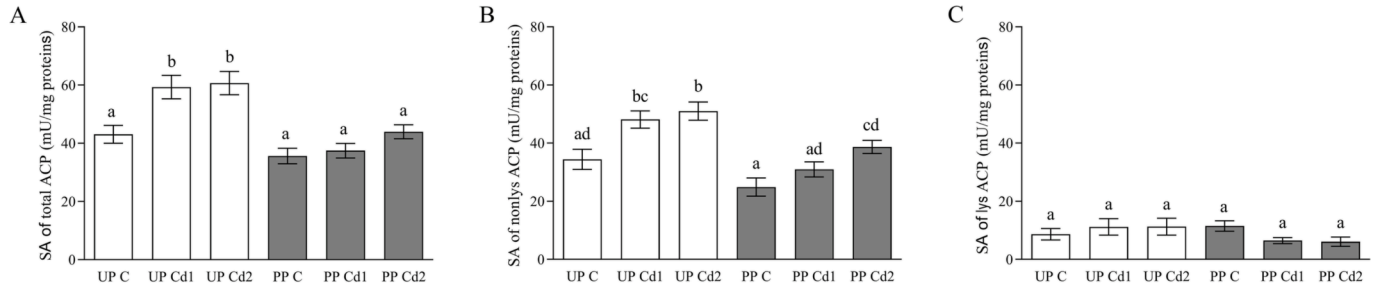


Fig. 4. Specific activities of total acid phosphatases (ACP) (A), non-lysosomal ACP (B), and lysosomal ACP (C) in the midgut of *L. dispar* larvae after chronic cadmium exposure. UP and PP – populations from the unpolluted and polluted sites, respectively; C - control, Cd1 and Cd2 - 50 and 100 μg Cd/g dry food, respectively. The bars show means±SE (n = 8–10 larvae per group). Different letters denote significant differences among the experimental groups (Tukey's post-hoc test, $p < 0.05$).

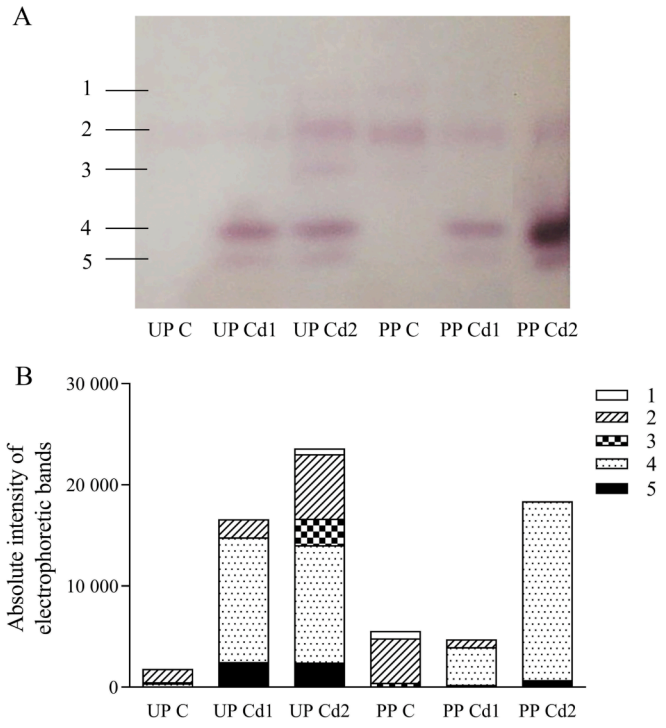


Fig. 5. Native PAGE gel stained for activity of acid phosphatases (ACP) (A) in the midgut of *L. dispar* caterpillars after chronic Cd exposure, and densitometric analysis of the bands (B). UP and PP – populations from the unpolluted and polluted sites, respectively; C - control, Cd1 and Cd2 - 50 and 100 μg Cd/g dry food, respectively. The results shown in panels A and C were obtained from a pooled sample, whereas panel B represents the mean of three technical replicates.

4. Discussion

Energy availability from food directly depends on digestion processes and metabolic pathways that essentially rely on the action of various enzymes. Detrimental effects of pollutants on the digestive system and consequently on energy metabolism can affect growth and reproduction of an individual. Toxicants may change hydrolytic features of the enzymes via direct interaction, but also by affecting their synthesis and secretion (Dedourge-Geffard et al., 2013). However, the resistance of some enzymes to metals may contribute to the capacity of an organism to cope with stress in contaminated environments (Zvereva et al., 2003). Therefore, according to Lai et al. (2011), digestive enzymes may reflect the metabolic status of an organism and its level of adaptation to the environment conditions.

The effects of long-term exposure of *L. dispar* to pollution near the Ibar highway were barely noticeable in the control larvae reared in the laboratory. Among all midgut enzymes only LAP activity differed from the control group originating from the unpolluted locality on Kosmaj mountain. However, differences in the sensitivity of protease and ACP activities to cadmium treatment between the populations were obvious. Midgut enzymes of caterpillars from the polluted site exhibited resistance to both metal concentrations except for non-lysosomal ACP activity at 100 μg Cd/g dry food and LAP activity at 50 μg Cd/g dry food. Isoform analysis revealed differential susceptibility of the isoforms to cadmium inhibition, and alterations in their expression upon exposure to the metal. We found their responses consistent to a large degree within each population as well as between populations. It should be emphasized that the isoforms in caterpillars from the contaminated locality confirmed enzyme resistance to the lower cadmium concentration, but most were sensitive to the higher level of metal.

The inhibition of protease activity (total proteases, TRY and LAP) that we registered in caterpillars from the unpolluted site confirms our previous finding of a reduction of enzyme activity at lower cadmium concentrations (10 and 30 μg Cd/g dry food) (Vlahović et al., 2015). De Coen and Janssen (1997) showed inhibition of TRY activity in *Daphnia magna* while Sahana and Joy (2016) detected inhibition of total protease activity in the collembolan *Cyphoderus javanus* Borner after exposure to cadmium. The mechanisms of protease activity inhibition by cadmium are not fully understood. Nevertheless, they probably include direct interaction due to the high affinity of cadmium for sulfhydryl groups or competition between cadmium and the metal in the active center of an enzyme. The study of Grover et al. (2016) proved in vitro inhibition of TRY-like protease activity isolated from the gut of *Helicoverpa armigera* (Lepidoptera). Leucine aminopeptidases of many insect species, including Lepidoptera, contain Zn ions in their structure (Cristofoletti et al., 2006; Terra and Ferreira, 2012), which may be replaced by cadmium ions resulting in decreased enzyme activity (Tamás et al., 2014; Tang et al., 2014). Toxic effects of cadmium on ribosomes, endoplasmic reticulum or mitochondria may disrupt protein synthesis (Planelló et al., 2007; Sokolova et al., 2012), including enzyme synthesis.

In the current study, TRY isoform 3 in the population from the unpolluted forest demonstrated high susceptibility to cadmium toxicity, vanishing completely at both metal concentrations. Inhibition of TRY

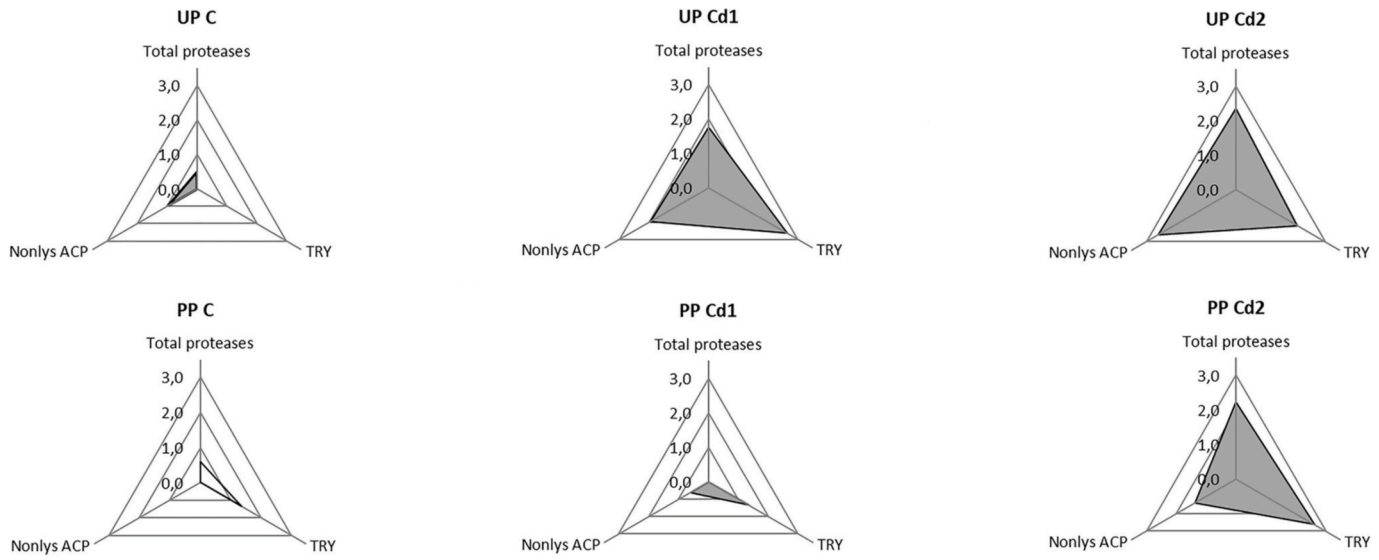


Fig. 6. Integrated biomarker response (IBR) star plots for *L. dispar* caterpillars from unpolluted (UP) and polluted (PP) localities after chronic cadmium exposure. C - control, Cd1 and Cd2–50 and 100 μg Cd/g dry food, respectively.

Table 1
The integrated biomarker response (IBR) of *L. dispar* larvae from the unpolluted (UP) and polluted (PP) localities to chronic cadmium exposure. C - control, Cd1 and Cd2 - 50 and 100 μg Cd/g dry food, respectively.

Group	UP C	UP Cd1	UP Cd2	PP C	PP Cd1	PP Cd2
IBR value	0.2	4.6	5.6	0.1	0.6	5.7

isoform 3 could have accounted for the decline of TRY activity registered in the enzyme assay. The isoform also disappeared in larvae from the polluted site receiving 100 μg Cd/g dry food, but probably was compensated for by the appearance of TRY isoform 1, which seemed to be cadmium-resistant. Similarly, LAP isoform 3 could have been replaced by LAP isoforms 2 and 4 that were overexpressed in larvae from the polluted site after exposure to the higher cadmium concentration. As Terra and Ferreira (2012) explained, insects may adapt to ingestion of plant serine protease inhibitors by increasing expression of some proteases or switching to isoforms resistant to inhibition. It has not been explained yet how an inhibitor induces synthesis of resistant TRY, but the process starts with expression of the full set of TRY isoforms (Brioschi et al., 2007).

The increase in total ACP activity detected in caterpillars from the uncontaminated site in response to cadmium resulted almost entirely from elevation of the activity of the non-lysosomal ACP fraction, whereas lysosomal ACP were not significantly affected in either population. Vlahović et al. (2013) previously identified lysosomal phosphatases in the *L. dispar* midgut as isoforms with low electrophoretic mobility. Our current study revealed that isoforms 1 and 2 with the lowest mobility on the gel exhibited low phosphatase activity and reacted weakly to cadmium treatment in both populations, which agrees with the result of the enzyme assay for the lysosomal ACP activity. Perhaps lysosomal ACP activity was initially elevated, but lysosomal membrane could have recovered later during chronic cadmium treatment due to activation of cellular defense mechanisms. Jayakumar et al. (2007) reported alterations in ACP activity in the gill and hepatopancreas of mussels during the later stages of chronic cadmium exposure. Considering that non-lysosomal ACP activity increased in larvae from both populations after cadmium exposure (although larvae from the polluted site responded only to 100 μg Cd/g dry food), those enzymes might be involved in the adaptive response to metal stress. Non-lysosomal ACP activity probably corresponded to isoforms 4 and 5 with high electrophoretic mobility, whose activities were elevated following cadmium treatment. Isoform 4 was particularly abundant in all groups exposed to the metal. Tsvetkov et al. (2003) are among the rare authors who have studied invertebrate ACP forms localized in the cytosol besides the forms found in lysosomes. In the liver of the snail, *Viviparus viviparous,* they identified lysosomal isoforms as those with low electrophoretic mobility in comparison to cytosolic isoforms that showed high mobility and enhanced induction of activity after cadmium exposure, similarly to our results. According to that study, both lysosomal and cytosolic ACP forms are involved in carbohydrate metabolism. A drastic increase in activity of the cytosolic fraction in response to cadmium exposure would result in inhibition of catabolic processes and accumulation of free carbohydrates and glycerol, which is probably part of the nonspecific adaptation syndrome. On the other hand, Reddy and Bhagyalakshmi (1994) and Suresh et al. (2016) related the increase in ACP activity in certain crustaceans exposed to cadmium with intensified phosphate catabolism that should improve poor energetic status in stress conditions.

The IBR index indicated a strong influence of both cadmium concentrations on the *L. dispar* population from the unpolluted forest. Nevertheless, the poor IBR response of the other population to the lower cadmium concentration confirmed less sensibility of those caterpillars to cadmium. Insect proteases and phosphatases in the midgut have not been investigated in the light of such adaptations after long-term exposure to pollution. The resistance of the activities of midgut enzymes to the metal that we registered in larvae from the polluted locality was probably a consequence of their superior cellular defense mechanisms. Previously we reported doubly efficient elimination of cadmium via feces in larvae from the locality near the Ibar highway when fed with 50 μg Cd/g dry food compared to the equivalent group from Kosmaj (Vlahović et al., 2017). Thus, disruptive effects of cadmium on midgut enzymes and the whole cellular machinery could have been reduced due to elimination of cadmium from the gut. Moreover, our field study revealed enhanced antioxidant defense in caterpillars from the polluted forest compared to those from the unpolluted one (Perić-Mataruga et al., 2019). Higher metallothionein expression has been well documented as an adaptation in invertebrate populations inhabiting metal-polluted localities (Mustonen et al., 2014; Timmermans et al., 2005). Considering changes in the isoform patterns of midgut enzymes in response to cadmium, the results of the current study suggest that the two *L. dispar* populations share similar physiological adaptations, but the mechanisms underlying those adaptations were triggered at a higher cadmium concentration in the population from the polluted locality.

5. Conclusions

L. dispar fulfills several important ecological requirements for an adequate bioindicator such as sensitivity to cadmium, wide distribution, clear feeding habits, and well-known physiology and ecology of the species. Choosing a pest as a bioindicator is an additional advantage. The results of the current study suggested that *L. dispar* could be a relevant species for the assessment of the condition of terrestrial ecosystems concerning cadmium pollution. However, the differences in sensitivity between populations with different histories of exposure to pollution must be considered. The population from the contaminated site near the Ibar Highway exhibited lower sensitivity to chronic dietary cadmium intake compared to larvae from the unpolluted forest on Kosmaj mountain. Nevertheless, non-lysosomal ACP activity and the presence of ACP isoform 5 could be generally applied as indicators of cadmium pollution, except that in populations with a history of exposure they would be expected to respond only in the higher metal concentration range. On the other hand, the applicability of non-lysosomal ACP isoform 4 as a biomarker extends to a broad range of cadmium concentrations. The activities of TRY and total ACP might be suitable biomarkers in populations that have not been previously exposed to pollution. Total protease activity is not a sufficiently sensitive parameter for application in cadmium biomonitoring, whereas LAP activity lacks consistency in response to the metal. The IBR, that combines activities of total proteases, TRY and non-lysosomal ACP, is expected to be an adequate indicator for populations with no history of exposure, but less cadmium-susceptible for populations inhabiting contaminated localities.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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