Environmental Pollution 288 (2021) 117706

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Sensitivity of midgut physiological parameters of *Lymantria dispar* L. larvae to benzo[*a*]pyrene in populations with different multigeneration contact to environmental pollutants☆

Anja Grčić *, Larisa Ilijin, Dragana Matić, Aleksandra Filipović, Marija Mrdaković, Dajana Todorović, Vesna Perić-Mataruga

Department of Insect Physiology and Biochemistry, Institute for Biological Research "Siniša Stanković" National Institute of the Republic of Serbia, University of Belgrade, Despot Stefan Blvd.142, 11060, Belgrade, Serbia

ARTICLE INFO

Keywords:
Phosphatase
benzo[*a*]pyrene
Carboxylesterase
Hsp70
Lymantria dispar
PCA

ABSTRACT

Accumulation of organic pollutants in the environment calls for sensing physiological parameters adequate to indicate the presence of contaminants and their effects on ecosystems. Evidence points to the importance of insect adaptations in their habitats for the assessment of sensitive biomarkers so we examined the influence of origin and multigenerational adaptations of the *Lymantria dispar* larvae to chronic benzo[*a*]pyrene (B[*a*]P) treatment under laboratory conditions. The main aim was to compare reactions of larvae from unpolluted and polluted forests using alkaline phosphatase (ALP), acid phosphatase (ACP), and carboxylesterase (CE) specific activities in the midgut, including electrophoretic isoform patterns; midgut expression levels of Hsp70, larval development time (DT), and midgut mass (MM), after chronic exposure to 5 and 50 ng of B[*a*]P/g dry food weight. The biomarker potential of these parameters regarding larval pre-exposure history to pollution was estimated by principal component analysis (PCA). B[*a*]P treatment resulted in inhibition of ALP activity, a rise of CE activity, and reduction of MM in larvae from the unpolluted forest, while the population from the polluted forest showed significant elevation of Hsp70 expression in the midgut, prolonged DT, and reduction of MM. PCA confirmed variations in responses of the selected parameters regarding population origin. The obtained results provide insight into insect population variability concerning physiological responses to pollutants. It is indicative that all investigated physiological parameters of *L. dispar* larvae showed origin-dependent responses to long-term presence of B[*a*]P, which may be of great importance in ecotoxicological research.

1. Introduction

Over the last few decades, intense anthropogenic influence has led to a significant increase in pollution of the biosphere. This can greatly be attributed to organic pollutants like polycyclic aromatic hydrocarbons (PAH), especially benzo[*a*]pyrene (B[*a*]P). B[*a*]P is considered a prototype of this class and is the most studied PAH, primarily due to its wide range of toxic effects observed in laboratory animals and the human population (Miller and Ramos, 2001). Dominant sources of B[*a*]P are anthropogenic activities, primarily industrial production of coke and petroleum products, aluminium and iron but also during common practices like heating and traffic circulation (IARC, 2012; Shen et al., 2011; Liu et al., 2008). For 2012 the average annual concentration of B[*a*]P in the atmosphere above Europe was 0.5 ng/m^3 in rural, and 2.7 ng/m^3 in urban areas, while the atmospheric burden of B[*a*]P rose to 100 mg/m^3 in industrial zones (Guerreiro et al., 2016; EMEP/EEA Air Pollutant Emission Inventory Guidebook, 2019). B[*a*]P ranks eighth on the list of the most important poisons and belongs to group 1-proven carcinogens for humans (ATSDR, 2017; IARC, 2012).

In the atmosphere over 90% of PAH is adsorbed on aerosol particulate matter of diameter smaller than 2.5 μm, wherein B[*a*]P and other high molecular weight PAHs predominate (Gao et al., 2015). B[*a*]P reaches the ground by wet and dry atmospheric deposition where it mostly deposits on vegetation (40% of PAHs) due to high affinity for lipids and waxes within green parts of plants (Wang et al., 2008; Alfani et al., 2001). Vegetation is an important sink for B[*a*]P and a crucial link

☆ This paper has been recommended for acceptance by Wen Chen.

* Corresponding author.
E-mail address: anja.gavrilovic@ibiss.bg.ac.rs (A. Grčić).

https://doi.org/10.1016/j.envpol.2021.117706
Received 2 April 2021; Received in revised form 1 June 2021; Accepted 1 July 2021
Available online 5 July 2021

for its bioaccumulation in animals through which it becomes an integral part of human nutrition.

Investigations have shown significant B[*a*]P concentrations in the foliage of deciduous trees, especially in oak (*Quercus*) species (Howsam et al., 2001; Wang et al., 2008), that are the preferred host plants for gypsy moth larvae (*Lymantria dispar* L., Lepidoptera) (Liebhold et al., 1995). *Lymantria dispar* is a phytophagous polyphagous insect species that inhabit wide forest areas of the northern hemisphere. Thanks to its vast appetite and great adaptability to various host plants, *L. dispar* larvae can accumulate significant amounts of pollutants which makes them a suitable model system concerning the adverse effects of xenobiotics on the environment, as seen in our previous studies (Matić et al., 2016; Perić-Mataruga et al., 2017; Ilijin et al., 2015). Great importance is paid to biomonitoring the biological responses of organisms to changes in environmental quality. Molecular biomarkers stand out as particularly sensitive agents that have found wide application in ecotoxicological studies, both in laboratory and field conditions (Paniagua-Michel and Olmos-Soto, 2016). Depending on the history of contact with pollutants, different populations of the same species may exhibit diverse biomarker responses due to the development of genetic and phenotypic adaptations. Our previous studies revealed that *L. dispar* larvae originating from polluted localities showed different, tissue-specific responses of some biochemical parameters to chronic treatments with sublethal B[*a*]P and cadmium concentrations when compared to those from unpolluted sites (Matić et al., 2016; Gavrilović et al., 2017; Grčić et al., 2019). Therefore we presume that it is necessary to examine and establish selection of several physiological parameters in *L. dispar* larvae, wherein each set is suitable for different forest environments and the level of pollution they are burdened with. That way biomonitoring of individual locations would be simplified and more relevant. By using the multivariant statistical method of principal component analysis (PCA), we hope to elucidate the amount of influence that chronic B[*a*]P treatment has on several variables that represent potential biomarkers for pollution by this PAH, depending on the insect population origin.

Generally, detoxification of xenobiotics including B[*a*]P in Lepidoptera is mediated by various enzymes - firstly in phase I, cytochrome P 450 monooxygenase, carboxylesterase, and reductase are producing toxic electrophilic metabolites, that are further converted to hydrophilic products by the action of the glutathione S-transferases and uridine diphosphate glucuronosyltransferases in phase II and, finally in phase III, ATP binding cassette and major membrane transporters actively pump conjugated xenobiotic out of the cell (Dawkar et al., 2013). Apart from these many other enzymes and macromolecules are involved in B[*a*]P metabolism and remediation of its adverse effects, directly or indirectly.

Insect midgut alkaline and acid phosphatases (ALP and ACP) catalyze the hydrolysis of various phosphomonoesters, provide a pool of inorganic phosphates, regulate reactions of transphosphorylation and actively participate in the digestion of food (Terra et al., 1996). Changes in ALP activity are related to stress response and resistance to infections and insecticides (Wang et al., 2011; Rauschenbach et al., 2007). ACP is involved in many metabolic processes, including ion transport, water resorption, excretion, growth, and metamorphosis of insects (Terra and Ferreira, 2012). Studies have shown a significant negative effect of plant secondary metabolites on ALP and ACP activity in Lepidoptera, which had adverse effects on nutrient digestion and fitness traits (Senthil-Nathan, 2013; Basiouny et al., 2010). Many of these metabolites have complex polycyclic chemical structures, quite similar to B[*a*]P (Senthil-Nathan, 2013; Senthil-Nathan et al., 2005), so it can be assumed that this PAH will also interact with phosphatases in the midgut, making them a potential biomarker.

Carboxylesterases (CE) form a class of a/β hydrolases that catalyze the hydrolysis of carboxylic esters into alcohols and acids. They are the most common esterase class in *L. dispar* larvae tissues, where they are involved in the digestion of food, modulation of metabolism, growth, and reproduction (Montella et al., 2012). CE constitutes an integral part of the phase I detoxification system and plays an important role in the development of resistance to multiple types of insecticides (Hemingway et al., 2004). Thus, Farcy et al. (2013) observed that the presence of B[*a*]P increased the activity of CE in shells of *Mytilus edulis* (Mytilida). The authors concluded that CE are probably involved in B[*a*]P metabolism, which is why it would be beneficial to examine further the potential of this enzyme for use in ecotoxicological studies.

Heat shock proteins (Hsp) act as molecular chaperones in the process of folding proteins into native conformations, degradation of damaged proteins, and preventing their denaturation (Morimoto et al., 1994). Hsp70 is closely connected to stressful conditions showing sensitivity to chemical contaminants, which is why it has been widely used in ecotoxicological studies as a marker of proteotoxicity (Gupta et al., 2010). It was found that PAHs such as B[*a*]P strongly induce Hsp70 gene expression in *Eisenia fetida* (Haplotaxida) (Zhang et al., 2017), *Oniscus asellus* (Isopoda) (Köhler et al., 1999), and *Chironomus tentans* (Diptera) (Lee et al., 2006).

The chronic influence of chemical toxicants like B[*a*]P poses a challenge for insects in terms of redistribution of energy resources between maintaining physiological functions at optimal levels and activating energy-demanding defense systems. To ensure survival, insects consume most of their metabolic energy on processes related to transformation and excretion of B[*a*]P, as well on protective systems crucial for remediation of its toxic effects (Van Straalen and Hoffmann, 2000). In the insect, *Creontiades dilutus* (Diptera), chronic exposure to B[*a*]P harmed larval development and adult reproduction (Du et al., 2014). Also, Ilijin et al. (2015) found that long-term feeding of *L. dispar* larvae with B[*a*]P led to a significant reduction in body mass and prolonged development time.

The focus of our current research was to investigate chronic effects of dietary treatment with B[*a*]P on some midgut biochemical parameters such as enzyme activity, including isoform expression of ALP, ACP, and CE, as well as the level of Hsp70 expression, in *L. dispar* larvae from two populations. One was derived from an unpolluted oak forest and the other from a polluted oak forest. These parameters were selected based on their presumable roles in B[*a*]P detoxification process and the fact they are quite responsive to environmental stress, and yet there are very little or no data about PAH and B[*a*]P influence on them. Also, we examined some fitness-related traits including larval development time and midgut mass. We paid special attention to whether these parameters are important elements for B[*a*]P detoxification and whether their responses differ regarding larval pre-exposure history, as well phenotypic plasticity in the response to B[*a*]P exposure in the laboratory. The goal was to determine if the selected parameters are sensitive enough to the presence of B[*a*]P if their responses differ between the two populations, and if so, which of them might be used as biomarkers regarding the level of environmental pollution.

2. Materials and methods

2.1. Insect rearing and experimental conditions

L. dispar egg masses were collected in November 2013 at two localities in Serbia - a mixed oak forest in Đerdap National Park 180 km from Belgrade (44°25′17″ N, 21°56′06″ E); and a mixed oak forest in Bor District, 245 km from Belgrade (44°04′29.57″N, 22°05′45.28″ E). The Đerdap National Park forest is considered free of industrial pollution. The Bor forest is contaminated by various types of pollutants, byproducts produced in the mining industry and the nearby smelter complex. In this locality, very high concentrations of PAHs and B[*a*]P were measured in the gas phase (Živković et al., 2015) and vegetation (Alagić et al., 2016). *L. dispar* larvae originating from the unpolluted Đerdap forest formed the unpolluted population (UP), whereas those from the Bor forest were designated as the polluted population (PP).

L. dispar egg-masses were kept at 4 °C until hatching in April 2014,

after which they were grown in plastic containers (V = 200 mL) at 23 °C with a 12 h light/12 h dark photoperiod. Based on information about B[*a*]P content in oak leaves, the *Quercus* species preferred by *L. dispar* larvae as a host plant, two concentrations of B[*a*]P were selected. The larvae were offered an artificial diet optimized for *L. dispar* (O'Dell et al, 1984) containing one of two concentrations of B[*a*]P. Namely, 5 ng or 50 ng of B[*a*]P was mixed with 1 g of dry diet, without any liquid, using a stock solution of B[*a*]P in acetone (UP 5 ng and UP 50 ng – larvae from the unpolluted forest exposed to 5 and 50 ng of B[*a*]P/g dry food weight; PP 5 ng and PP 50 ng – larvae from the polluted forest exposed to 5 and 50 ng of B[*a*]P/g dry food weight). Diet mixtures were poured into wide plastic boxes and left at 25 °C for 4 h so that any residual acetone could evaporate. In each population, two control groups were formed to test the possible interference of acetone. Larvae in one control group were fed with an artificial diet free from acetone, while larvae in the other received an artificial diet containing acetone (3% of total diet volume). Neither of these control groups contained added B[*a*]P. As no significant differences were recorded between the two control groups, the second one was designated as the control (UP 0 ng and PP 0 ng). All groups of larvae were offered the same amount of food that was replaced every 48 h post-hatching until death on the 3rd day of the 5th larval instar (on average, after 32 days). No significant changes in the mortality rate between the experimental groups were observed.

2.2. Preparation of midgut homogenates

L. dispar larvae were anesthetized on ice for 1 min, after which they were killed by decapitation (10–12 larvae per group). The gut was dissected and the midgut was separated from the foregut and hindgut. For the enzyme assays, midguts were homogenized separately in ice-cold extraction buffer at pH 7.4 (0.25 M sucrose, 0.05 M Tris HCl, and 1 mM EDTA) with an Ultra Turrax homogenizer (IKA-Werke) for three intervals of 15 s with 15 s pauses between them, at 5000 rpm. Tissue dilution was tenfold (1 g of tissue/9 mL of buffer). Midgut homogenates were then sonicated thrice, for 15 s with 15 s pauses in between, using a Bandelin HD 2070 ultrasonic homogenizer. After that, the homogenates were centrifuged at 37,000 *g* for 30 min at 4 °C (Beckman L7-55 ultracentrifuge) and the supernatants were saved for enzyme analysis at −20 °C. Midguts used for Hsp70 detection were also homogenized separately, but in ice-cold 0.9% NaCl mixed with protease inhibitors to a final concentration of 2 mM phenylmethylsulfonyl fluoride (PMSF) and 20 μM E64. The tissue was diluted fivefold (1 g of tissue/4 mL of buffer). Homogenization was performed in three 10 s repetitions, at 20,000 rpm, with 15 s pauses (Ultra Turrax homogenizer, IKA-Werke), followed by centrifugation at 10,000*g* for 10 min at 4 °C (Eppendorf 5417R centrifuge). The supernatants were separated and used for Western blot analysis. Protein content was determined in all midgut homogenates according to Bradford (1976) using bovine serum albumin as the standard.

2.3. Spectrophotometric assays of enzyme activity

The activity of ALP was determined spectrophotometrically by a modified method of Nemec and Socha (1988). It is based on the hydrolytic release of *p*-nitrophenol from *p*-nitrophenyl phosphate (pNPP) under alkaline conditions. The reaction mixture contained 0.1 M Tris HCl buffer pH 8.6, 5 mM $MgCl_2$, midgut homogenate, and 5 mM *p*-nitrophenyl phosphate. Incubation time was 30 min at 30 °C after which the reaction was stopped with 0.5 M NaOH. The absorbance was measured at 405 nm. The same modified method of Nemec and Socha (1988) was employed for determination of ACP activity but under acidic conditions (0.1 M citrate buffer pH 5.6) and a prolonged incubation time of 60 min. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol per minute per mg of total protein. In these assays, ten midgut homogenates per group were tested, for every sample. Blank and non-catalytic probes were included.

CE activity was determined by the continuous spectrophotometric assay according to Main et al. (1961) in which the amount of *p*-nitrophenol liberated from 15 mM *p*-nitrophenyl butyrate during 4 min at 30 °C is measured at 414 nm. The reaction was performed in 50 mM Na-phosphate buffer pH 7.5. One unit of CE activity was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol per minute per mg of total protein, under the assay conditions. For every experimental group, ten midgut homogenates were assayed in two replicates with blank and non-catalytic probes included.

2.4. Detection of enzyme activities after native polyacrylamide gel electrophoresis

The modified method of native polyacrylamide gel electrophoresis (PAGE) (Allen et al., 1963) was applied for the detection of ALP and ACP isoform patterns. The separation was carried out on 12% gels at a constant voltage of 100 V at 4 °C by the method of Laemmli (1970). ALP was visualized as dark blue lines by immersing gel into a staining mixture containing 0.13% α-naphthyl phosphate and 0.1% Fast Blue B, dissolved in 0.1 M Tris HCl buffer pH 8.6, at room temperature. For the detection of ACP isoforms, nitrocellulose membranes were prepared by incubation in 50 mM acetate buffer (pH 5.2) containing 0.13% α-naphthyl phosphate, at room temperature. The gel was incubated for 10 min in the same buffer at 30 °C, after which the prepared membrane was placed on it and left in a dark, humid chamber at 30 °C for 60 min. The membrane was then dipped in 0.3% Fast Blue B dye dissolved in acetate buffer until dark blue lines corresponding to ACP activity appeared.

Zymography of CE involved electrophoresis of midgut homogenates on 8% native gel at a constant voltage of 100 V for 3 h at 4 °C (Laemmli, 1970). For visualization of CE isoforms, the gel was soaked and shaken in a mixture of 1.1 mM α-naphthyl acetate, 1.1 mM β-naphthyl acetate, and 1.2 mM Fast Blue B salt dissolved in 20 mM phosphate buffer at pH 7.2 (modified from the method of Gottlieb, 1974). After a few minutes at room temperature, pink lines appeared on a transparent background, revealing the CE isoform patterns.

For all native PAGE experiments, 10 μg of protein per lane was loaded on the gels (same quantity for all experimental groups). Gels were scanned with a CanoScan LiDE 120, Japan. For qualitative and densitometric image analysis, scans of all gels were converted to black and white. The isoform band area and relative optical density in the regions of the ALP, ACP, and CE activities were analyzed using the ImageJ 1.42q program (NIH, USA).

2.5. Hsp70 detection by western blot

The midgut homogenates were separated by sodium dodecyl sulfate (SDS) PAGE on a 12% gel (Laemmli, 1970). Proteins were transferred from the gel to the nitrocellulose membrane (Amersham Prothron, Premium 0.45 mm NC, GE Healthcare Life Sciences, UK) overnight at a constant voltage of 40 V and 4 °C. Hsp70 expression in larval midguts was detected using primary monoclonal anti-Hsp70 mouse IgG1 (dilution 1:5000) (clone BRM-22, Sigma Aldrich, USA) and secondary anti-mouse IgG1 (gamma-chain)-HRP conjugated antibodies (dilution 1:5000) (Sigma Aldrich, USA). Protein bands were visualized by chemiluminescence (ECL kit, Amersham). Three midgut homogenates were analyzed by Western blot for every experimental group, and all samples loaded on the gels contained the same amount of total protein (70 μg) per lane. Relative band densities of the Hsp70 protein band areas were analyzed using the ImageJ 1.42q program (NIH, USA).

2.6. Estimation of L. dispar larvae fitness-related traits

The development time of larvae (DT) in days was monitored (n = 55–60 per group) from hatching until molting into the 5th instar. After sacrifice, dissected midguts (n = 17–22 per group) were washed with ice-cold deionized water, slightly tapped on paper, and then midgut

mass (MM) was determined.

2.7. Statistical analyses

Depending on the number of samples, D'Agostino-Pearson and Shapiro-Wilk tests were used to check the normality of data distribution. Extreme values were determined by Grubb's test (G-test). For parameters with a normal distribution (specific enzyme activity of ALP, ACP, and CE, including semi-quantitative analysis of Hsp70 expression) parametric statistics were applied - one-way analysis of variance (ANOVA) and Tukey's post hoc test. Non-parametric statistics, Kruskal-Wallis tests ANOVA, and Dunn's post hoc test were used to analyze the results for the fitness-related traits (DT and MM). Statistical significance of the results was determined at probability (p) < 0.05. The data were expressed as means ± standard error of the mean (SEM), except for Hsp70 expression where the error bars represented the standard deviation (SD). GraphPad Prism 8 software (GraphPad Software, Inc.) was used for these analyses. Principal Component Analysis (PCA) was performed using the statistical package Origin Pro Version 2020, OriginLab Corporation, Northampton, MA, USA. This was done to discriminate B[*a*]P treatments by integration of physiological parameters according to principal axes. The physiological parameters – MM, DT, ALP, ACP, and CE were the variables used in PCA. All principal components were obtained using the standardized values of all variables for individual samples corresponding to each dataset. Standardization of all variable results was accomplished using the equation: $Z_{ij} = \frac{x_{ij} - \overline{x_j}}{SD_j}$, where Z_{ij}, represents new standardized data; x_{ij} – individual data for variable j in sample unit i; $\overline{x_j}$ - sample mean for variable j; and SD_j - sample standard deviation for variable j.

3. Results

3.1. ALP and ACP activities in L. dispar midgut

The B[*a*]P supplemented diet had different effects on the two populations of *L. dispar* larvae concerning midgut ALP activity (Fig. 1. A). A statistically significant decrease in its activity was found only in the larvae originating from the unpolluted forest as a consequence of long-term exposure to the low concentration of B[*a*]P (UP 5 ng), relative to the control group ($p < 0.05$). No changes after B[*a*]P treatments were noticed in the population from the polluted forest compared to the control group ($p > 0.05$). Native electrophoresis detected different numbers of ALP isoforms in the two control groups, i.e. four in group UP 0 ng (I1–I4) and five in group PP 0 ng (I1–I5) (Fig. 2. A). In the larval population derived from the unpolluted forest, the low concentration of B[*a*]P led to the greatest inhibition of ALP isoforms (UP 5 ng), relative to all other experimental groups. I3 and I4 were dominant in all UP groups, while in the PP larvae, I4 and I5 had the same role. The population of larvae originating from the polluted forest showed a dose-dependent reduction in the intensity of isoform patterns after chronic treatment with B[*a*]P (Fig. 2. B).

ACP activity in *L. dispar* midgut was not significantly affected by the dietary B[*a*]P treatments, in either population, $p > 0.05$ (Fig. 1. B). Nevertheless, in the population from the polluted forest, a significant difference was observed between larvae receiving the high concentration of B[*a*]P when compared to the group given the lower B[*a*]P dose ($p < 0.05$). Five ACP isoforms (I1–I5) were detected by zymography in the

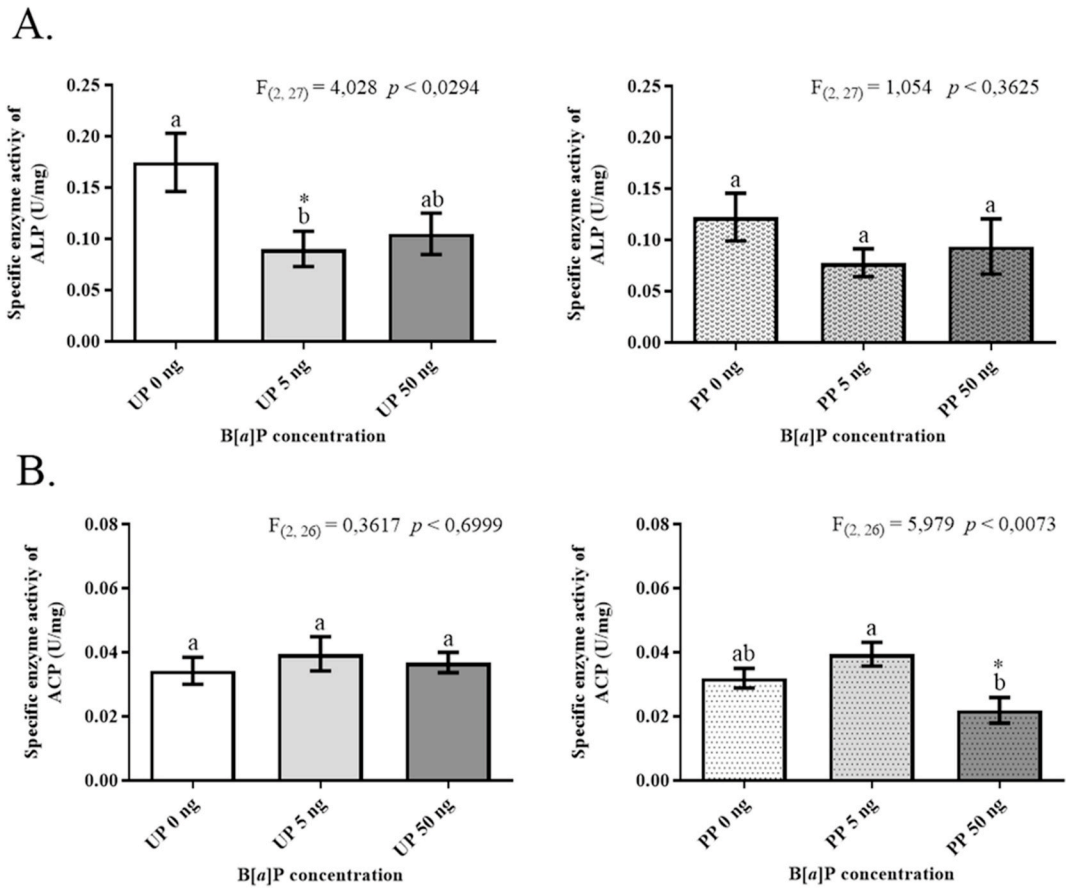

Fig. 1. The specific activity of ALP (**A**) and ACP (**B**) in the midgut of *L. dispar* larvae after chronic B[*a*]P exposure. UP and PP – populations from the unpolluted and polluted forest, respectively; 0 ng - control, 5 ng and 50 ng – 5 and 50 ng B[*a*]P/g dry food, respectively. The bars show means ± SE, (n = 8–10 larvae per group). Different letters denote significant differences among the experimental groups (Tukey's post-hoc test, * - statistical significance at $p < 0.05$).

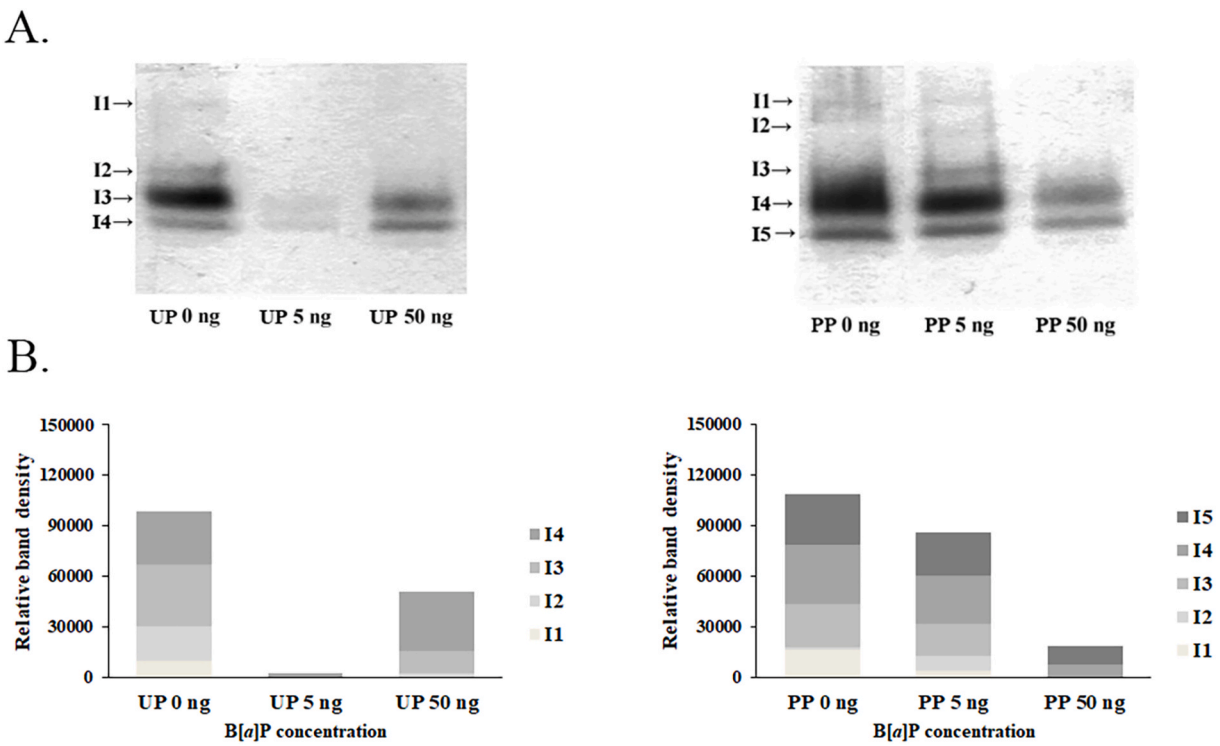

Fig. 2. Native PAGE gels stained for ALP activity in the midgut of *L. dispar* larvae after chronic B[*a*]P exposure (**A**) and densitometric analysis of the bands (**B**). The numbers indicate ALP isoforms (I1–I5). UP and PP – populations from the unpolluted and polluted forest, respectively; 0 ng - control, 5 ng and 50 ng –5 and 50 ng B[*a*]P/g dry food, respectively.

control groups from both populations of larvae (UP 0 ng and PP 0 ng) Fig. S1 (Suppl. 1. A). Expression of all isoforms faded with the rise of B[*a*]P concentration resulting in total inhibition of isoforms I1–I3 (UP 50 ng) Fig. S1 (Suppl. 1. B). The polluted population of larvae showed stable expression of three ACP isoforms (I3–I5) regardless of treatment, while that of I1 and I2 intensified under the influence of B[*a*]P Fig. S1 (Suppl. 1. B).

3.2. CE activity in L. dispar midgut

A significant increase of CE activity was found in the midgut of *L. dispar* larvae originating from the unpolluted forest after chronic intake of the high B[*a*]P concentration (UP 50 ng), relative to the control, $p < 0.05$ (Fig. 3. A). No statistically significant change in the activity of these enzymes was found in the larvae from the contaminated forest, $p > 0.05$ (Fig. 3. A). Numerous CE isoforms were revealed by native electrophoresis in both populations of larvae. Experimental groups from the unpolluted forest were characterized by constant expression of all isoforms (I1–I6), with a discrete elevation of the total activity in the B[*a*]P treated groups (Fig. 3. B). According to zymography, the polluted population of larvae was more sensitive to the xenobiotic, showing notable inhibition in the activity of almost all detected CE isoforms (I1–I6) after application of the high B[*a*]P concentration (PP 50 ng) (Fig. 3. C). This experimental group also possessed a special feature, i.e. one CE isoform more than the other groups, a seventh isoform (I7) found only there.

3.3. Effects of B[a]P on midgut Hsp70 levels

L. dispar larvae from the polluted forest (PP) showed a significant dose-dependent elevation of Hsp70 expression after the chronic B[*a*]P treatments in comparison to the relevant control group ($p < 0.01$) (Fig. 4). A rising trend in Hsp70 concentration was recorded in the unpolluted population (UP) under the B[*a*]P influence, but not significant ($p > 0.05$) (Fig. 4).

3.4. Fitness-related traits

The long-term presence of B[*a*]P in food did not affect the DT of larvae originating from the unpolluted forest. In contrast, for the *L. dispar* population from the polluted forest, feeding with the low B[*a*]P concentration (PP 5 ng) significantly prolonged the overall DT relative to the control, $p < 0.01$ (Fig. 5. A).

MM of larvae was significantly affected by dietary B[*a*]P in both populations, showing a great decrease in values (UP 5 ng, UP 50 ng, PP 5 ng and PP 50 ng) in comparison to the relevant control groups, $p < 0.01$ (Fig. 5. B).

3.5. Principal component analysis

The PCA was applied to establish criteria for the biological responses of *L. dispar* larvae to stress resulting from chronic dietary exposure to B[*a*]P. It was performed separately on data obtained for variables from unpolluted and polluted forests. The data covariance matrix consisted of standardized values for midgut ALP, ACP, and CE specific activity, together with DT and MM results for all experimental groups. For the population from the unpolluted forest, PCA resulted in a two-component model explaining 87.92% of total variability among the data. The first major component (PC1) covered 63.80% of the variability, and the second (PC2) 24.12%. The biplot of scores and the corresponding coefficients of the latent variables for both main components is presented in Fig. 6. A. It indicates the existence of three clusters of objects separated mostly along the PC2 axis that correspond to different dietary concentrations of B[*a*]P. The samples from the control group of larvae (UP 0 ng) are quite separate as a cluster, while those belonging to the B[*a*]P treatments partially overlap (UP 5 ng and UP 50 ng). The coefficients of latent variables showed that the variable CE had the greatest positive influence along the PC2 direction and largely defined the

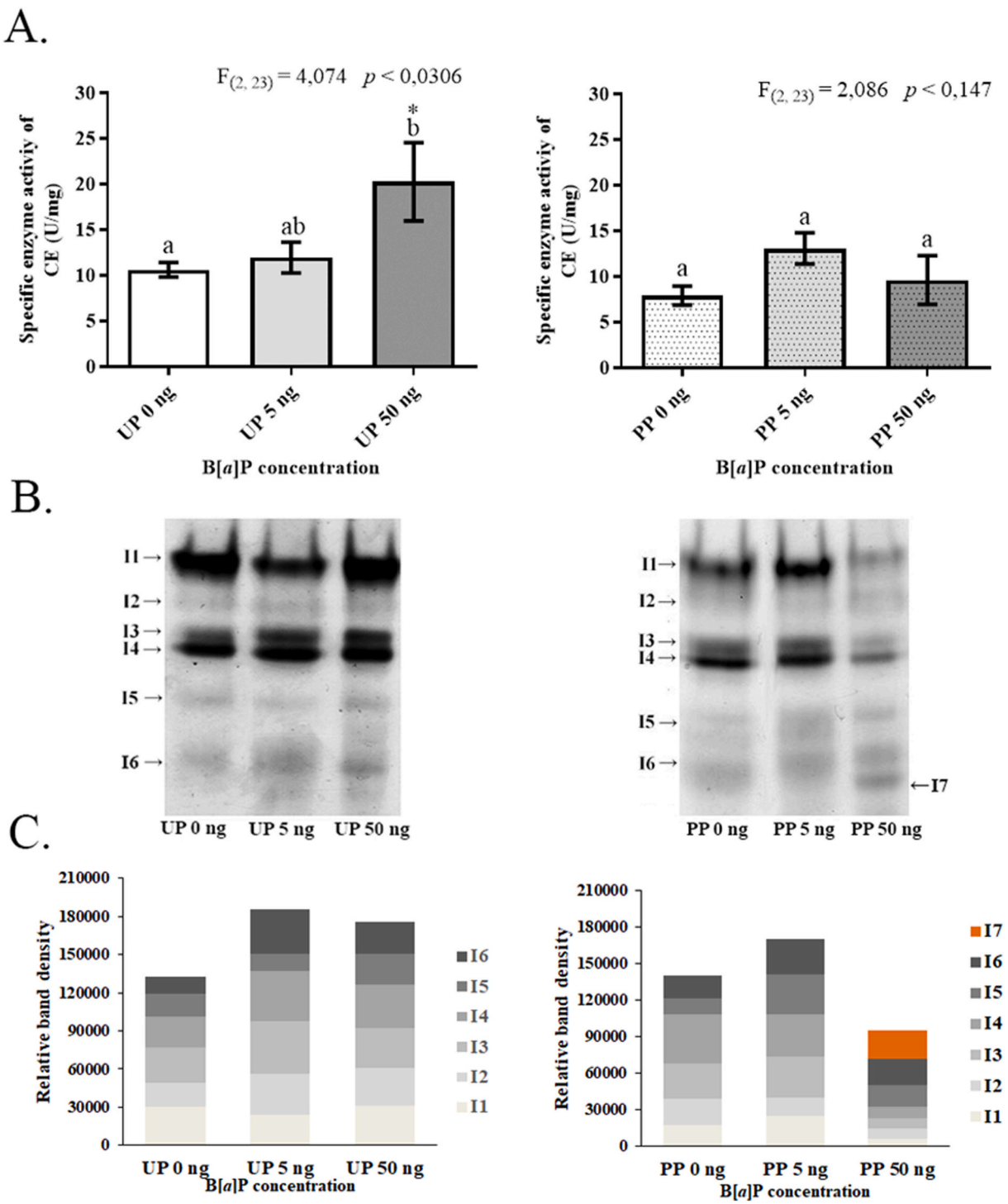

Fig. 3. The specific activity of CE in the midgut of *L. dispar* larvae after chronic B[*a*]P exposure (**A**), native PAGE gels stained for CE activity in the midgut of *L. dispar* larvae after chronic B[*a*]P exposure (**B**), and densitometric analysis of the bands (**C**). The numbers indicate CE isoforms (I1–I7). UP and PP – populations from the unpolluted and polluted forest, respectively; 0 ng - control, 5 ng and 50 ng – 5 and 50 ng B[a]P/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, * - statistical significance at $p < 0.05$).

clusters of B[*a*]P-treated samples, while the variables DT and MM had the greatest negative influence on PC2 and determined the control group (UP 0 ng). A major positive effect on distribution along the PC1 axis was achieved by variable ALP, followed by the DT variable coefficient, which contributed to notable separation of the control group.

In the samples from the polluted forest, PCA explained 89% of the total variance with a two-component model where PC1 included 64.50% and PC2 24.50% of the variability. Three clusters of objects were defined on the biplot showing the greatest separation between the control group (PP 0 ng) and the group treated with the low B[*a*]P concentration (PP 5 ng), primarily along the PC2 axis (Fig. 6. B). The latent variables that most influenced the distribution across PC2 were MM with the largest positive coefficient, while CE and DT had the largest negative ones. These variables defined the differences between the control group and those treated with B[*a*]P, especially with the lower concentration.

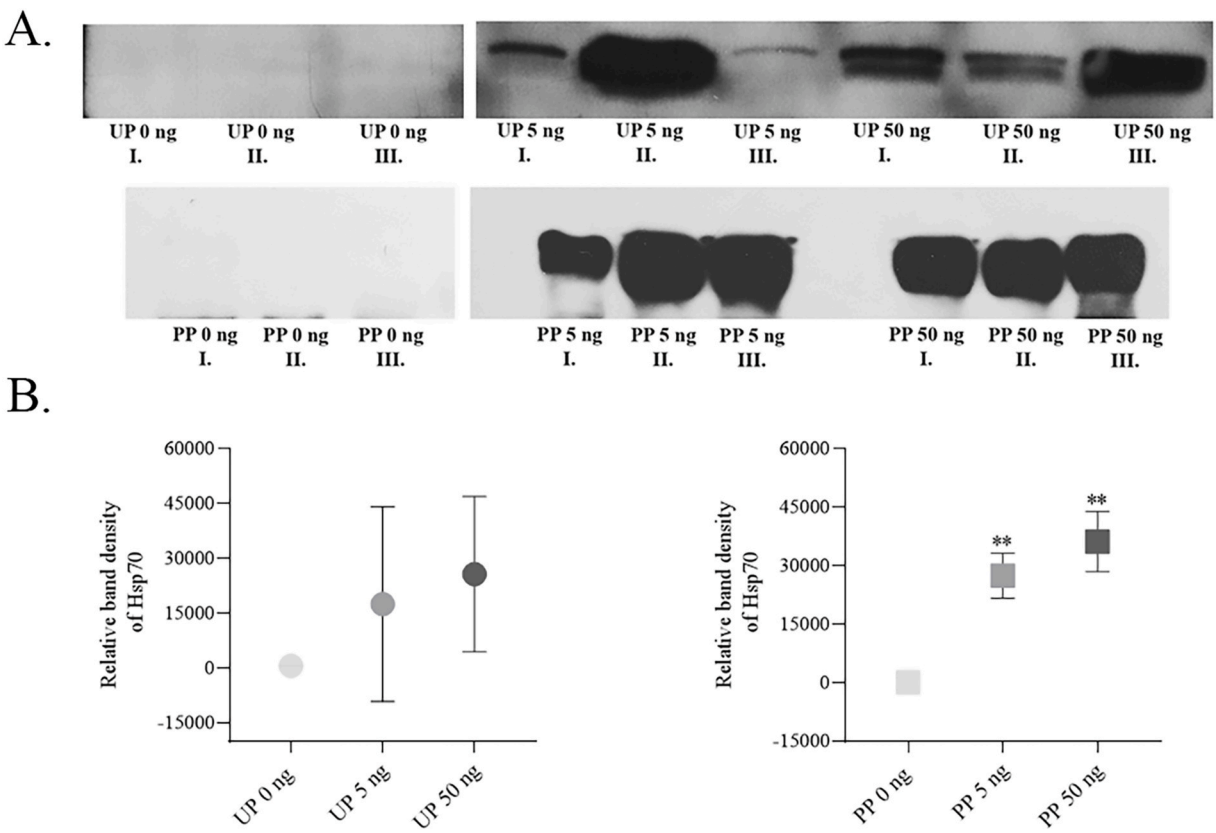

Fig. 4. Western immunoblots of Hsp70 expression in the midgut of *L. dispar* larvae after chronic B[*a*]P exposure (**A**) and densitometric analysis of the bands (**B**). UP and PP – populations from the unpolluted and polluted forest, respectively; 0 ng - control, 5 ng and 50 ng −5 and 50 ng B[*a*]P/g dry food, respectively. Graphs represent the results of one-way ANOVA and the bars show mean ± SD (n = 3). Two stars (**) denote significant differences among the experimental groups (Tukey's post-hoc test, $p < 0.01$).

4. Discussion

Human-made pollutants burden every part of the biosphere causing permanent changes in plants and animals. In Lepidoptera, alkaline and acid phosphatases have important roles in numerous metabolic processes through their primary functions of phosphomonoester hydrolysis and transphosphorylation (Terra and Fereira, 2012). Production of phosphate ions crucial in energy metabolism is of immense importance during stress. Vlahović et al. (2013, 2009) recorded inhibition of these enzymes in the midgut of *L. dispar* larvae under acute and chronic cadmium influence. In the present work, the low concentration of B[*a*]P had a significant inhibitory effect on ALP activity exclusively in larvae from the unpolluted forest (UP 5 ng), while ACP showed no significant changes in either population. Badiou-Bénéteau et al. (2013) found that *Apis mellifera* (Hymenoptera) derived from urban areas loaded with heavy metals expressed a higher level of ALP than populations from rural areas. Our results indicate that the pre-treatment history of contact with pollutants contributed to differences in the enzymatic response between the studied populations. The impact of PAHs on these insect digestive enzymes has not been investigated so far, so we could not find similar studies in the available literature for comparison. Nevertheless, Senthil-Nathan (2013) described negative effects of allelochemicals from the group of polycyclic triterpenoids (limonoids) on digestive enzymes of Lepidoptera, especially inhibition of ALP and ACP activity. Although the mechanism of ALP inhibition by limonoids is not known, based on the relative similarity in chemical structure between B[*a*]P and limonoids, we assume that B[*a*]P and/or its metabolites may act in the same way.

Typical for B[*a*]P metabolism is the production of toxic intermediates and a rise of reactive oxygen species, which have a high affinity for lipid molecules, especially those in lipid membranes. Peroxidation of lipids disrupts membrane structure, leads to inhibition of membrane-bound enzymes, and disorders cell function (Maria and Bebianno, 2011). Being mostly membrane enzymes on columnar epithelial cells, oriented towards the midgut lumen (Terra et al., 1996), ALP structure and catalytic activity are certainly impaired during peroxidation of membrane lipids. Bouchard and Bouchard-Madrell (2000) showed that ingestion of B[*a*]P caused significant lesions in the alimentary tract of *Gomphocerus sibiricus* (Orthoptera). Several other studies revealed degenerative effects of B[*a*]P on intestinal epithelial cells in aquatic vertebrates and invertebrates (Lowe et al., 1981; Yuen et al., 2007). In our population of larvae from the unpolluted forest, we found that, while chronic feeding with B[*a*]P inhibited ALP activity, it induced CE activity in the midgut. This indicates an active phase I of biotransformation, resulting in more polar and reactive B[*a*]P-intermediates that can damage macromolecules and disbalance redox status. Presumably, not all of the detoxification enzymes needed for efficient B[*a*]P metabolism are expressed at a level sufficient to manage the high content of toxic species in the larvae from the unpolluted forest. That is probably why the effects of B[*a*]P are reflected through the decline of digestive enzyme activities like ALP. The function of these enzymes was not significantly altered in the population of larvae from the polluted forest, probably due to generationally formed adaptations to the presence of pollutants.

In both L. *dispar* populations, native electrophoresis revealed three ALP isoforms, whose intensities were stronger in larvae from the polluted forest. Larvae from the unpolluted forest gave a dose-independent response so I1 and I2 were inhibited by the low dose of B[*a*]P (UP 5 ng). The exposure to a lower dose of B[*a*]P often gave a greater response of the tested parameters, compared to the higher dose –

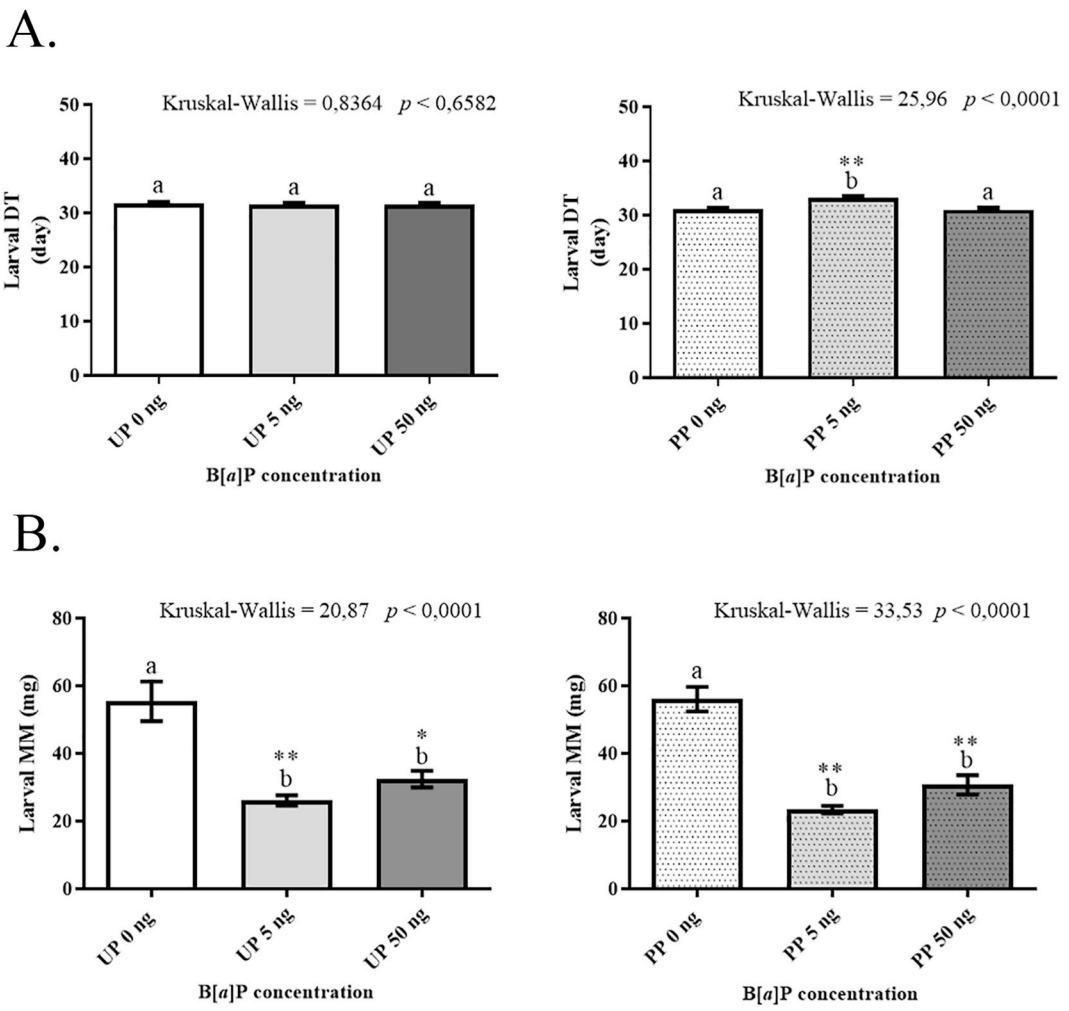

Fig. 5. The DT of *L. dispar* larvae (**A**) and the MM of *L. dispar* larvae (**B**) after chronic B[*a*]P exposure. UP and PP – populations from the unpolluted and polluted forest, respectively; 0 ng - control, 5 ng and 50 ng – 5 and 50 ng B[*a*]P/g dry food, respectively. The bars show means ± SE, (n = 55–60 larvae per group for DT and n = 17–22 larvae per group for MM). Different letters denote significant differences among the experimental groups (Tukey's post-hoc test; * - statistical significance at $p < 0.05$, and - ** at $p < 0.01$).

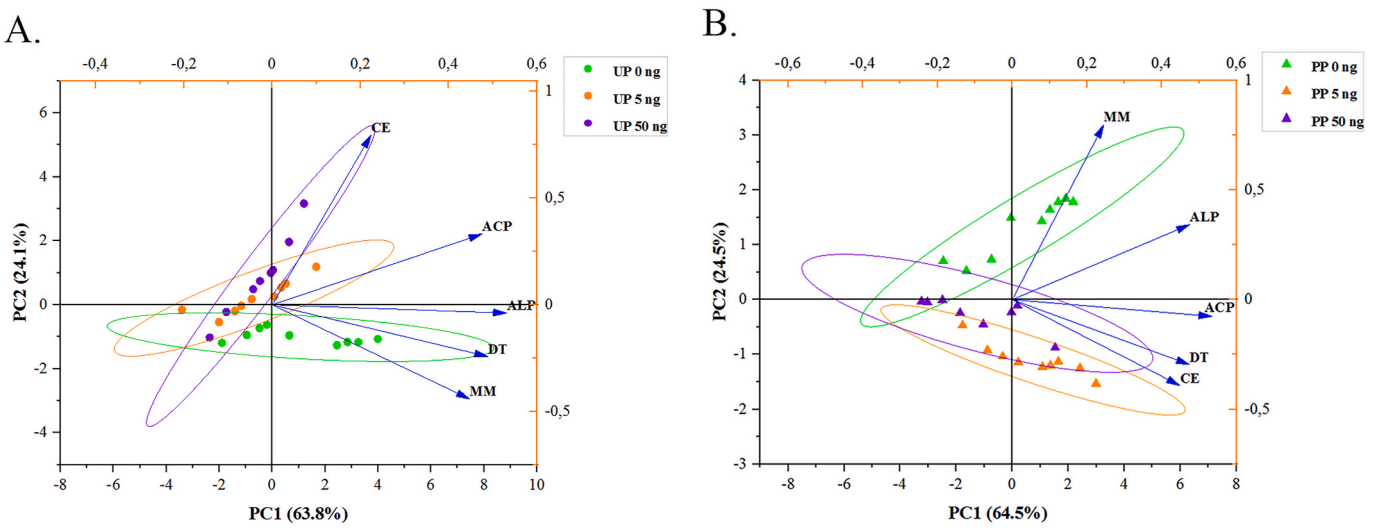

Fig. 6. Biplots of principal component analysis (PCA) of chronic B[*a*]P influence on various parameters in *L. dispar* larvae from the unpolluted – UP (**A**) and polluted -PP forest (**B**). 0 ng - control, 5 ng and 50 ng – 5 and 50 ng B[*a*]P/g dry food, respectively. ALP, ACP, CE, DT, and MM – latent variables.

the activity of ALP in the unpolluted, the DT in the polluted, and the MM in both populations of larvae. These biphasic responses are probably a phenomenon called hormesis, which is specified by low-dose stimulation and a high-dose inhibition or absence of the effect, and it is widely recognized as a general, real and reproducible phenomenon (Calabrese, 2005; Cutler, 2013). In addition, by exerting estrogenic and anti-estrogenic properties, benzo[*a*]pyrene belongs to a group of chemical compounds known as endocrine disruptors, for which is common to induce dose-independent biological responses (Lagarde et al., 2015; Lintelmann et al., 2003). Similar responses were observed for many physiological parameters in *Lymantria dispar* larvae, after exposure to benzo[*a*]pyrene (Ilijin et al., 2015, 2017) and fluoranthene (Filipović et al., 2019; Mrdaković et al., 2013). Although no significant changes in ACP activity were detected by spectrophotometric assay, the zymogram showed inhibition of three isoforms (I1–I3) in larvae from the unpolluted forest after treatment with the high dose of B[*a*]P (UP 50 ng). ACP activity was less affected by B[*a*]P-induced toxicity than ALP, presumably due to its primary localization within the cytoplasm of intestinal epithelial cells (Terra et al., 1996). This physically separates ACP from the midgut lumen, the main site of generation of reactive intermediates and oxygen radicals. Our results suggest a small share of these enzymes in B[*a*]P detoxification mechanisms. However, adverse effects of this xenobiotic are reflected in ALP activity of *L. dispar* larvae from the unpolluted population.

The metabolism of B[*a*]P in insects is directly connected to enzymes of the detoxification system. CE consists of a heterogeneous group of enzymes able to hydrolyze a wide range of substrates, with an active role in the degradation and elimination of many xenobiotics (Panini et al., 2016). Application of the high concentration of B[*a*]P provoked an increase of CE activity only in the midgut of larvae from the unpolluted forest (UP 50 ng). Data in the literature concerning B[*a*]P effects on CE are modest and inconsistent. In *Chironomus sancticaroli* (Diptera) larvae without prior contact with pollutants, brief exposure to B[*a*]P led to a marked rise of CE activity (Vicentini et al., 2017). Similar treatment of a laboratory strain of *Aedes aegypti* (Diptera) had no impact on CE activity (Riaz et al., 2009). Furthermore, it was found that natural populations of insects and marine invertebrates, from environments loaded with chemical contaminants, exhibited significantly higher expression of CE when compared to populations from unpolluted habitats (Callaghan et al., 1998; Farcy et al., 2013). The results of our experiment differed. Induction of midgut CE activity in larvae from the unpolluted population represents a possible acclimatization strategy to B[*a*]P intoxication, while individuals from the polluted forest, with relatively low expression levels of CE, probably employ different detoxification enzymes. This could be an example of a trade-off in CE activity for other detoxification mechanisms, more efficient for the organism under the particular environmental condition. Constant exposure to a stressful environment certainly led to an adaptive refinement of protective strategies, selected to manage and neutralize toxic effects of pollutants with the highest efficiency and the least energy cost. Moreover, lower expression of these enzymes was apparent in native electrophoretic profiles from the midgut, where isoform intensities were stronger for larvae from the unpolluted forest.

Callaghan et al. (1998) discovered that populations of *Culex pipiens* (Diptera) generationally selected with insecticides and organic pollutants were characterized by the higher specific activity of CE and fewer isoenzymes when compared to populations from an unpolluted environment. This pattern was most apparent for environmental contamination with insecticides. In contrast, in our experiment, native electrophoresis revealed more midgut CE isoforms in the larvae from the polluted forest, specifically after treatment with the higher B[*a*]P concentration (PP 50 ng), where seven CE isoforms were detected, while all other experimental groups had six. The seventh isoform of CE (I7) probably represents a specific adaptive trait contributing to the resistance mechanisms, by acting to reduce sensitivity to B[*a*]P. As such, it may be a potential indicator of chronic exposure to B[*a*]P, at least for populations from contaminated habitats.

During biotransformation of B[*a*]P an assortment of reactive metabolites and oxygen radicals are formed. They interact with structural and functional proteins causing proteotoxicity and damage to other macromolecules. Numerous studies have recorded a rise of Hsp70 concentration in insects and arthropods exposed to B[*a*]P and its crucial role in the protection and modulation of protein damage (Lee et al., 2006; Ilijin et al., 2017; Rhee et al., 2009). *L. dispar* larvae from the polluted forest showed pronounced dose-dependent increases in the expression of midgut Hsp70 after the B[*a*]P treatments (PP 5 ng and PP 50 ng). The same trend was found in larvae from the unpolluted forest but less consistent and striking than for the polluted population and without statistical significance. Thus, the variance around the mean value for Hsp70 band intensity indicated that the response of B[*a*]P treated larvae from the unpolluted forest was much more variable than that of the polluted population. This and the notably higher intensity of Hsp70 bands indicates that these larvae strongly employ Hsp70 as a means of defense against B[*a*]P toxicity, which is probably a result of selective pressure due to PAH and other chemical pollutants in their forest habitat. In both control groups (UP 0 ng and PP 0 ng) Hsp70 was barely detectable indicating a very low constitutive expression regardless of larval heritage. Considering the larvae from the polluted forest and the constant selective pressure they face, this finding suggests probable primary expression and high efficiency of other protective mechanisms that were not part of our experiment. Thus, Gavrilović et al. (2017) detected higher constitutive activity of GST, an important detoxification enzyme in the midgut of *L. dispar* larvae from the same polluted population, relative to the control group larvae from an unpolluted population. This very likely provided resilience to the larvae from the polluted forest.

Chronic nutrition of *L. dispar* larvae with B[*a*]P had the greatest negative impact on the midgut, because of its active metabolic function and the nature of the products produced. Constant loading with reactive species may exceed detoxification and antioxidant systems at some point causing proteotoxicity (Sanders and Martin, 1993), as seen in our case through high expression of Hsp70. Monari et al. (2011) also recorded increased production of Hsp70 in the digestive gland of the seashell *Chamelea gallina* (Venus) after long-term contact with B[*a*]P. Although the synthesis of Hsp70 proteins is an energetically expensive process, their protective functions are extremely valuable in larval midgut. Therefore, it is possible that with increasing exposure to B[*a*]P, allocation of energy resources from other adaptive strategies had gradually occurred.

During exposure to environmental stressors, the amount of available metabolic energy is of fundamental importance for insects. Induction and continuance of protective processes require a significant input of metabolic energy provided by allocation from functions that are non-essential for survival (Sokolova et al., 2012). We have found that MM was seriously affected by chronic B[*a*]P treatment in both *L. dispar* populations, while the larval DT was prolonged only in the polluted population, after the administration of the low B[*a*]P concentration (PP 5 ng). As we stated earlier, this hormesis-like phenomenon is relatively common for some biochemical parameters in insects faced with B[*a*]P and PAH and has been described previously (Grčić et al., 2019; Ilijin et al., 2015). Prolonged exposure of *M. edulis* (Mytilida) to a mixture of PAHs was observed to alter the structure of digestive cells, disrupt their synchronicity, and radically affect the structure of cellular lysosomes (Lowe et al., 1981). Most likely, B[*a*]P also exerts toxic effects on cells of the midgut epithelium of *L. dispar* larvae, probably impairing their structure and leading to MM reduction. Furthermore, increased expression of Hsp70 and induction of detoxification enzymes certainly affected the larval energy balance negatively. There are indications that B[*a*]P directly interferes with the electron transport chain, disrupting energy metabolism and adenosine triphosphate (ATP) production (Zhu et al., 1995). An extended period of increased energy needs, conditioned by activation of compensatory mechanisms, eventually draws from

energy depots and reduces larval fat body volume. It can be presumed that larvae from both populations suffered from energy deficit, which was reflected by their lower MM and larval mass as previously recorded (Ilijin et al., 2015). This could be the cause of the prolonged DT in the polluted population of larvae.

Molting of Lepidoptera larvae is completely dependant on achieving a certain, critical body mass because it directly controls secretion of the molting hormones (Davidowitz et al., 2003). Adaptive strategies of larvae from the polluted forest probably included detoxification mechanisms that were much more energy-demanding than those used by larvae from the unpolluted forest (Mouneyrac et al., 2011). This negatively affected larval mass and extended the time required to reach the 5th larval stage. Moreover, larvae from the polluted forest had a stronger expression of Hsp70 in the midgut, relative to those from the unpolluted forest, which increased the already high cost of protein production, requiring almost one-fifth of the available ATP stocks (Hand and Hardewing, 1996). It is likely that larvae without a pre-history of contact with pollutants resort to acclimatization strategies that are less of a burden for individuals, so there is no trade-off in terms of DT. The fitness characteristics of *L. dispar* larvae from the current experiment showed a fairly uniform response to the effect of relatively low concentrations of B[*a*]P, which is why they may potentially be very useful in environmental biomonitoring studies.

The method of PCA enabled detection of logical patterns in the responses of several *L. dispar* parameters to xenobiotic exposure in relation to the origin of the larval population. The biplots revealed that the clusters (experimental groups) showed dose-dependent separation in the population from the unpolluted forest, opposite to the pattern in the polluted population. Also, the analysis indicated that the most sensitive parameters under chronic B[*a*]P dietary treatment were CE, ALP, MM, and even DT in the *L. dispar* population from the unpolluted forest. The same biomarker potential was observed for DT, MM, and CE in larvae from the polluted forest.

5. Conclusion

Investigation of enzyme, stress, and fitness parameters in the midgut of 5th instar *L. dispar* larvae from two forest environments with different pollution levels, revealed different sensitivity of their responses to long-term B[*a*]P exposure. Thus, larvae from the unpolluted forest were characterized by a rise of CE activity and a decrease in ALP activity in their midgut and with a reduction of MM. The population from the polluted forest showed a significant elevation of midgut Hsp70 concentration, prolonged DT, and reduction in MM. PCA also indicated that the examined variables have diverse significance and biomarker potential for continuous biomonitoring of B[*a*]P presence, depending on the *L. dispar* pre-history of contact with pollutants.

Funding information

This study was supported by the Serbian Ministry of Education, Science and Technological Development, Contract 451-03-9/2021-14/200007.

Authors' contributions

Anja Grčić: Methodology, Investigation, Formal analysis, Writing - Original Draft, Visualization. Larisa Ilijin: Writing - Review & Editing, Conceptualization. Dragana Matić: Validation, Methodology. Aleksandra Filipović: Resources, Investigation. Marija Mrdaković: Data Curation, Visualization. Dajana Todorović: Supervision, Conceptualization. Vesna Perić Mataruga: Project administration, Funding acquisition.

Author agreement statement

We the authors of the manuscript entitled "Sensitivity of midgut physiological parameters of *Lymantria dispar* L. larvae to benzo[*a*]pyrene in populations with different multigeneration contact to environmental pollutants" declare that this manuscript is original, has not been published before, and is not currently being considered for publication elsewhere. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. We understand that the Corresponding Author is the sole contact for the Editorial process. She is responsible for communicating with the other authors about progress, submissions of revisions, and final approval of proofs.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2021.117706.

References

Alagić, S., Stankov-Jovanović, V., Mitić, V., Cvetković, J., Petrović, G., Stojanović, G., 2016. Bioaccumulation of HMW PAHs in the roots of wild blackberry from the Bor region (Serbia): phytoremediation and biomonitoring aspects. Sci. Total Environ. 562, 561–570. https://doi.org/10.1016/j.scitotenv.2016.04.063.

Allen, S.L., Misch, M.S., Morrison, B.M., 1963. Variations in the electrophoretically separated acid phosphatases of *Tetrahymena*. J. Histochem. Cytochem. 11, 706–719. https://doi.org/10.1177/11.6.706.

Alfani, A., Maisto, G., Prati, M.V., Baldantoni, D., 2001. Leaves of *Quercus ilex* L. as biomonitors of PAHs in the air of Naples (Italy). Atmos. Environ. 35, 3553–3559. https://doi.org/10.1016/S1352-2310(01)00087-5.

ATSDR, n.d, 2017. Agency for toxic substances and disease registry. ATSDR. Subst. Prior. List. URL. https://www.atsdr.cdc.gov/spl/.

Badiou-Bénéteau, A., Benneveau, A., Géret, F., Delatte, H., Becker, N., Brunet, J.L., Reynaud, B., Belzunces, L.P., 2013. Honeybee biomarkers as promising tools to monitor environmental quality. Environ. Int. 60, 31–41. https://doi.org/10.1016/j.envint.2013.07.002.

Basiouny, A.L., Hamadah, KhSh, Tanani, M.A., 2010. Efficacy of the wild plant *Fagonia bruguieri* (Zygophyllaceae) on acid and alkaline phosphatase activities in the desert locust *Schistocerca gregaria* (Orthoptera: acrididae). Egypt. Acad. J. Biol. Sci., C Physiol. Mol. Biol. 2, 1–10. https://doi.org/10.21608/eajbsc.2010.16255.

Bouchard, J., Bouchard-Madrell, C., 2000. Lesions provoquees chez le criquet *Gomphocerus sibiricus* par l'absorption de benzo[*a*]pyrene et de 3-mehylcholanthrene. Ann. sci. nat., Zool. biol. Anim. 21, 119–136. https://doi.org/10.1016/S0003-4339(00)01023-6.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1006/abio.1976.9999.

Calabrese, E.J., 2005. Paradigm lost, paradigm found: the re-emergence of hormesis as a fundamental dose-response model in the toxicological sciences. Environ. Pollut. 138, 378–411. https://doi.org/10.1016/j.envpol.2004.10.001.

Callaghan, A., Parker, P.J.A.N., Holloway, G.J., 1998. The use of variance in enzyme activity as an indicator of long-term exposure to toxicant-stressed environments in *Culex pipiens* mosquitoes. Funct. Ecol. 12, 436–441. https://doi.org/10.1046/j.1365-2435.1998.00200.x.

Cutler, C., 2013. Insects, insecticides and hormesis: evidence and considerations for study. Dose-Response 11, 154–177. https://doi.org/10.2203/dose-response.12-008.Cutler.

Davidowitz, G., D'Amico, J.L., Nijhout, H.F., 2003. Critical weight in the development of insect body size. Evol. Dev. 5, 188–197. https://doi.org/10.1046/j.1525-142X.2003.03026.x.

Dawkar, V., Chikate, Y., Lomate, P., Dholakia, B., Gupta, V., Giri, A., 2013. A review. Molecular insights into resistance mechanisms of Lepidopteran insect pests against toxicants. J. Proteome Res. 12 (11), 4727–4737. https://doi.org/10.1021/pr400642p.

Du, J., Li, Y., Huang, Z.C., You, J., 2014. Chronic toxicity thresholds for sediment-associated benzo[*a*]pyrene in the midge (*Chironomus dilutus*). Arch. Environ. Contam. Toxicol. 66, 370–378. https://doi.org/10.1007/s00244-014-0005-y.

EMEP/EEA Air Pollutant Emission Inventory Guidebook, 2019. European Environment Agency. Publications Office of the European Union, Luxembourg. https://doi.org/10.2800/293657.

Farcy, E., Burgeot, T., Haberkorn, H., Auffret, M., Lagadic, L., Allenou, J.P., Budzinski, H., Mazzella, N., Pete, R., Heydorff, M., Menard, D., Mondeguer, M.,

Menard, D., Mondeguer, F., Caquet, T., 2013. An integrated environmental approach to investigate biomarker fluctuations in the blue mussel *Mytilus edulis* L. in the Vilaine estuary, France. Environ. Sci. Pollut. Res. 20, 630–650. https://doi.org/10.1007/s11356-012-1316-z.

Filipović, A., Mrdaković, M., Ilijin, L., Vlahović, M., Todorović, D., Grčić, A., Perić-Mataruga, V., 2019. Effect of fluoranthene on antioxidative defense in different tissues of *Lymantria dispar* and *Euproctis chrysorrhoea* larvae. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 224, 108565. https://doi.org/10.1016/j.cbpc.2019.108565.

Gao, B., Wang, X., Zhao, X., Ding, X., Fu, X., Zhang, Y., He, Q., Zhang, Z., Liu, T., Huang, Z., Chen, L., Peng, Y., Guo, H., 2015. Source apportionment of atmospheric PAHs and their toxicity using PMF: impact of gas/particle partitioning. Atmos. Environ. 103, 114–120. https://doi.org/10.1016/j.atmosenv.2014.12.006.

Gavrilović, A., Ilijin, L., Mrdaković, M., Vlahović, M., Mrkonja, A., Matić, D., Perić-Mataruga, V., 2017. Effects of benzo[*a*]pyrene dietary intake to antioxidative enzymes of *Lymantria dispar* (Lepidoptera: *lymantriidae*) larvae from unpolluted and polluted forests. Chemosphere 179, 10–19. https://doi.org/10.1016/j.chemosphere.2017.03.083.

Gottlieb, L.D., 1974. Genetic confirmation of the origin of *Clarkia lingulata*. Evolution 28, 244–250. https://doi.org/10.2307/2407325.

Grčić, A., Ilijin, L., Mrdaković, M., Vlahović, M., Filipović, A., Đurašević, S., Perić-Mataruga, V., 2019. Benzo[*a*]pyrene-induced changes in carboxylesterase, acetylcholinesterase and heat shock protein 70 of *Lymantria dispar* (Lepidoptera: *lymantriidae*) from unpolluted and polluted forests. Arch. Biol. Sci. 71, 735–745. https://doi.org/10.2298/ABS190620056G.

Guerreiro, C.B.B., Horalek, J., de Leeuw, F., Couvidat, F., 2016. Benzo[*a*]pyrene in Europe: ambient air concentrations, population exposure and health effects. Environ. Pollut. 214, 657–667. https://doi.org/10.1016/j.envpol.2016.04.081.

Gupta, S.C., Sharma, A., Mishra, M., Mishra, R.K., Chowdhuri, D.K., 2010. Heat shock proteins in toxicology: how close and how far: a minireview. Life Sci. 86, 377–384. https://doi.org/10.1016/j.lfs.2009.12.015.

Hand, S.C., Hardewig, I., 1996. Downregulation of cellular metabolism during environmental stress: mechanisms and implications. Annu. Rev. Physiol. 58, 539–563. https://doi.org/10.1146/annurev.ph.58.030196.002543.

Hemingway, J., Hawkes, N., McCarroll, L., Ranson, H., 2004. The molecular basis of insecticide resistance in mosquitoes. Insect Biochem. Mol. Biol. 34, 653–665. https://doi.org/10.1016/j.ibmb.2004.03.018.

Howsam, M., Jones, K.C., Ineson, P., 2001. PAHs associated with the leaves of three deciduous tree species. II: uptake during a growing season. Chemosphere 44, 155–164. https://doi.org/10.1016/s0045-6535(00)00268-x.

IARC, 2012. Chemical agents and related occupations. In: IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, 100F, pp. 111–138.

Ilijin, L., Mrdaković, M., Todorović, D., Vlahović, M., Gavrilović, A., Mrkonja, A., Perić-Mataruga, V., 2015. Life history traits and the activity of antioxidative enzymes in *Lymantria dispar* L. (Lepidoptera, *Lymantriidae*) larvae exposed to benzo[*a*]pyrene. Environ. Toxicol. Chem. 34, 2618–2624. https://doi.org/10.1002/etc.3116.

Ilijin, L., Mrdaković, M., Vlahović, M., Matić, D., Gavrilović, A., Mrkonja, A., Perić-Mataruga, V., 2017. Acetylcholinesterase and heat shock protein 70 response in larval brain tissue of *Lymantria dispar* L. (Lepidoptera, *Lymantriidae*) upon chronic exposure to benzo[*a*]pyrene. Environ. Sci. Pollut. Res. 24, 20818–20823. https://doi.org/10.1007/s11356-017-9898-0.

Köhler, H.R., Knödler, C., Zanger, M., 1999. Divergent kinetics of hsp70 induction in *Oniscus asellus* (Isopods) in response to four environmentally relevant organic chemicals (B[*a*]P, PCB52, *g*-HCH, PCP): suitability and limits of a biomarker. Arch. Environ. Contam. Toxicol. 36, 179–185. https://doi.org/10.1007/s002449900458.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685. https://doi.org/10.1038/227680a0.

Lagarde, F., Beausoleil, C., Belcher, S.M., Belzunces, L.P., Emond, C., Guerbet, M., Rousselle, C., 2015. Non-monotonic dose-response relationships and endocrine disruptors: a qualitative method of assessment. Environ. Health 14, 13. https://doi.org/10.1186/1476-069X-14-13.

Lee, S.M., Lee, S.B., Park, C.H., Choi, J., 2006. Expression of heat shock protein and hemoglobin genes in *Chironomus tentans* (Diptera, *Chironomidae*) larvae exposed to various environmental pollutants: a potential biomarker of freshwater monitoring. Chemosphere 65, 1074–1081. https://doi.org/10.1016/j.chemosphere.2006.02.042.

Liebhold, A.M., Gottschalk, K.W., Muzika, R.M., Montgomery, M.E., Young, R., O'Day, K., Kelley, B., 1995. Suitability of North American Tree Species to the Gypsy Moth: A Summary of Field and Laboratory Tests. USDA Forest Service. General Technical Report. NE 211. https://doi.org/10.2737/NE-GTR-211.

Lintelmann, J., Katayama, A., Kurihara, N., Shore, L., Wenzel, A., 2003. Endocrine disruptors in the environment (IUPAC technical report). Pure Appl. Chem. 75, 631–681. https://doi.org/10.1351/pac200375050631.

Liu, G., Niu, Z., Niekerk, D., Xue, J., Zheng, L., 2008. Polycyclic aromatic hydrocarbons (PAHs) from coal combustion: emissions, analysis, and toxicology. Rev. Environ. Contam. Toxicol. 1921, 1–28. https://doi.org/10.1007/978-0-387-71724-1_1.

Lowe, D.M., Moore, M.N., Clarke, K.R., 1981. Effects of oil on digestive cells in mussels: quantitative alterations in cellular and lysosomal structure. Aquat. Toxicol. 1, 213–226. https://doi.org/10.1016/0166-445X(81)90016-3.

Main, A.R., Miles, K.E., Braid, P.E., 1961. The determination of human- serum-cholinesterase activity with o-nitrophenyl butyrate. Biochem. J. 78, 769–776. https://doi.org/10.1042/bj0780769.

Maria, V.L., Bebianno, M.J., 2011. Antioxidant and lipid peroxidation responses in *Mytilus galloprovincialis* exposed to mixtures of benzo[*a*]pyrene and copper. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 154, 56–63. https://doi.org/10.1016/j.cbpc.2011.02.004.

Matić, D., Vlahović, M., Kolarević, S., Perić Mataruga, V., Ilijin, L., Mrdaković, M., Vuković Gačić, B., 2016. Genotoxic effects of cadmium and influence on fitness components of *Lymantria dispar* caterpillars. Environ. Pollut. 218, 1270–1277. https://doi.org/10.1016/j.envpol.2016.08.085.

Miller, K.P., Ramos, K.S., 2001. Impact of cellular metabolism on the biological effects of benzo[*a*]pyrene and related hydrocarbons. Drug Metab. Rev. 33, 1–35. https://doi.org/10.1081/dmr-100000138.

Monari, M., Foschi, J., Rosmini, R., Marin, M.G., Serrazanetti, G.P., 2011. Heat shock protein 70 response to physical and chemical stress in *Chamelea gallina*. J. Exp. Mar. Biol. Ecol. 397, 71–78. https://doi.org/10.1016/j.jembe.2010.11.016.

Montella, I.R., Schama, R., Valle, D., 2012. The classification of esterases: an important gene family involved in insecticide resistance: a review. Memorias Inst. Oswaldo Cruz, Rio de Janeiro 107, 437–449. https://doi.org/10.1590/s0074-02762012000400001.

Morimoto, R.I., Tissieres, A., Georgopoulous, C., 1994. Progress and perspectives on the biology and heat shock proteins and molecular chaperones. In: Morimoto, R.I., Tissieres, A., Georgopoulous, C. (Eds.), The Biology of Heat Shock Proteins and Molecular Chaperones. Cold Spring Harbor Press, pp. 1–30. https://doi.org/10.1101/0.1-30.

Mouneyrac, C., Leung, P.T.Y., Leung, K.M.Y., 2011. Cost of tolerance. In: Triquet, C. Amiard, Rainbow, P.S., Romeo, M. (Eds.), Tolerance to Environmental Contaminants. CRC Press, Boca Raton, pp. 265–297.

Mrdaković, M., Perić-Mataruga, V., Ilijin, L., Vlahović, M., Janković-Tomanić, M., Mirčić, D., Lazarević, J., 2013. Response of *Lymantria dispar* (Lepidoptera: *lymantriidae*) larvae from differently adapted populations to allelochemical stress: effects of tannic acid. Eur. J. Entomol. 110, 55–63. https://doi.org/10.14411/eje.2013.007.

Nemec, V., Socha, R., 1988. Phosphatases and pteridines in Malpighian tubules: a possible marker of mosaic mutant in *Pyrrhocoris apterus* (Heteroptera, Pyrrhoridae). Acta Entomol. Bohemoslov. 85, 321–326.

O'Dell, T.M., Bell, R.A., Mastro, V.C., Tanner, J.S., Kennedy, J.F., 1984. Production of the gypsy moth, *Lymantria dispar*, for research and biological control. In: King, E.G., Leppla, N.C. (Eds.), Advances and Challenges in Insect Rearing. USDA-ARS, New Orleans, LA, pp. 156–166.

Panini, M., Manicardi, G.C., Moores, G.D., Mazzoni, E., 2016. An overview of the main pathways of metabolic resistance in insects. Invertebr. Surviv. J. 13, 326–335. https://doi.org/10.25431/1824-307X/isj.v13i1.326-335.

Paniagua-Michel, J., Olmos-Soto, J., 2016. Modern approaches into biochemical and molecular biomarkers: key roles in environmental biotechnology. J. Biotechnol. Biomater. 6, 1000216. https://doi.org/10.4172/2155-952X.1000216.

Perić-Mataruga, V., Petković, B., Ilijin, L., Mrdaković, M., Dronjak Čučaković, S., Todorović, D., Vlahović, M., 2017. Cadmium and high-temperature effects on brain and behavior of *Lymantria dispar*. L. caterpillars originating from polluted and less-polluted forests. Chemosphere 185, 628–636. https://doi.org/10.1016/j.chemosphere.2017.07.050.

Rauschenbach, I.Y., Bogomolova, E.V., Gruntenko, N.E., Adonyeva, N.V., Chentsova, N. A., 2007. Effects of juvenile hormone and 20-hydroxyecdysone on alkaline phosphatase activity in Drosophila under normal and heat stress conditions. J. Insect Physiol. 53, 587–591. https://doi.org/10.1016/j.jinsphys.2007.02.011.

Rhee, J.S., Raisuddin, S., Lee, K.W., Seo, J.S., Ki, J.S., Kim, I.C., Park, H.G., Lee, J.S., 2009. Heat shock protein (Hsp) gene responses of the intertidal copepod *Tigriopus japonicus* to environmental toxicants. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 149, 104–112. https://doi.org/10.1016/j.cbpc.2008.07.009.

Riaz, M.A., Poupardin, R., Reynaud, S., Strode, C., Ranson, H., David, J.P., 2009. Impact of glyphosate and benzo[*a*]pyrene on the tolerance of mosquito larvae to chemical insecticides. Role of detoxification genes in response to xenobiotics. Aquat. Toxicol. 93, 61–69. https://doi.org/10.1016/j.aquatox.2009.03.005.

Sanders, B.M., Martin, L.S., 1993. Stress-proteins as biomarkers of contaminant exposure in archived environmental samples. Sci. Total Environ. 140, 459–470. https://doi.org/10.1016/0048-9697(93)90043-6.

Senthil-Nathan, S., 2013. Physiological and biochemical effect of neem and other Meliaceae plants secondary metabolites against Lepidopteran insects. Front. Physiol. 4, 359. https://doi.org/10.3389/fphys.2013.00359.

Senthil-Nathan, S., Kalaivani, K., Murugan, K., Chung, P.G., 2005. The toxicity and physiological effect of neem limonoids on *Cnaphalocrocis medinalis* (Guenée) the rice leaf folder. Pestic. Biochem. Physiol. 81, 113–122. https://doi.org/10.1016/j.pestbp.2004.10.004.

Shen, H., Tao, S., Wang, R., Wang, B., Shen, G., Li, W., Su, S., Huang, Y., Wang, X., Liu, W., Li, B., Sun, K., 2011. Global time trends in PAH emissions from motor vehicles. Atmos. Environ. 45, 2067–2073. https://doi.org/10.1016/j.atmosenv.2011.01.054.

Sokolova, I.M., Frederich, M., Bagwe, R., Lannig, G., Sukhotin, A.A., 2012. Energy as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. Mar. Environ. Res. 79, 1–15. https://doi.org/10.1016/j.marenvres.2012.04.003.

Terra, W.R., Ferreira, C., Jordão, B.P., Dillon, R.J., 1996. Digestive enzymes. In: Lehane, M.J., Billingsley, P.F. (Eds.), Biology of the Insect Midgut. Chapman and Hall, London, UK, pp. 153–194.

Terra, W.R., Ferreira, C., 2012. 11 - biochemistry and molecular biology of digestion. In: Gilbert, L.I. (Ed.), Insect Molecular Biology and Biochemistry. Elsevier, pp. 365–418. https://doi.org/10.1016/B978-0-12-384747-8.10011-X.

Van Straalen, N.M., Hoffmann, A.A., 2000. Review of experimental evidence for physiological costs of tolerance to toxicants. In: Kammenga, J., Laskowski, R. (Eds.), Demography of Ecotoxicology. Wiley, New York, pp. 147–161.

Vicentini, M., Morais, G.S., Rebechi-Baggio, D., Richardi, V.S., Santos, G.S., Cestari, M. M., Navarro-Silva, M.A., 2017. Benzo[*a*]pyrene exposure causes genotoxic and

biochemical changes in the midge larvae of *Chironomus sancticaroli* Strixino and Strixino (Diptera: chironomidae). Neotrop. Entomol. 46, 658–665. https://doi.org/10.1007/s13744-017-0505-3.

Vlahović, M., Lazarević, J., Perić-Mataruga, V., Ilijin, L., Mrdaković, M., 2009. Plastic responses of larval mass and alkaline phosphatase to cadmium in the gypsy moth larvae. Ecotoxicol. Environ. Saf. 72, 1148–1155. https://doi.org/10.1016/j.ecoenv.2008.03.012.

Vlahović, M., Mataruga, V.P., Mrdaković, M., Matić, D., Lazarević, J., Nenadović, V., Ilijin, L., 2013. Enzymatic biomarkers as indicators of dietary cadmium in gypsy moth caterpillars. Environ. Sci. Pollut. Res. 20, 3447–3455. https://doi.org/10.1007/s11356-012-1292-3.

Wang, Y.Q., Tao, S., Jiao, X.C., Coveney, R.M., Wu, S.P., Xing, B.S., 2008. Polycyclic aromatic hydrocarbons in leaf cuticles and inner tissues of six species of trees in urban Beijing. Environ. Pollut. 151, 158–164. https://doi.org/10.1016/j.envpol.2007.02.005.

Wang, Z., Liu, S., Yang, B., Liu, Z., 2011. Characterization of soluble and membrane-bound alkaline phosphatase in *Nilaparvata lugens* and their potential relation to development and insecticide resistance. Arch. Insect Biochem. Physiol. 78, 30–45. https://doi.org/10.1002/arch.20437.

Yuen, B.B., Wong, C.K., Woo, N.Y., Au, D.W., 2007. Induction and recovery of morphofunctional changes in the intestine of juvenile carnivorous fish (*Epinephelus coioides*) upon exposure to foodborne benzo[*a*]pyrene. Aquat. Toxicol. 82, 181–194. https://doi.org/10.1016/j.aquatox.2007.02.010.

Zhang, L., Duan, X., He, N., Chen, X., Shi, J., Li, W., Xu, L., Li, H., 2017. Exposure to lethal levels of benzo[a]pyrene or cadmium triggers distinct protein expression patterns in earthworms (*Eisenia fetida*). Sci. Total Environ. 595, 733–742. https://doi.org/10.1016/j.scitotenv.2017.04.003.

Zhu, H., Li, Y., Trush, M.A., 1995. Characterization of benzo[*a*]pyrene quinone-induced toxicity to primary cultured bone marrow stromal cells from DBA/2 mice: potential role of mitochondrial dysfunction. Toxicol. Appl. Pharmacol. 130, 108–120. https://doi.org/10.1006/taap.1995.1015.

Živković, M., Jovašević-Stojanović, M., Cvetković, A., Lazović, I., Tasić, V., Stevanović, Ž., Gržetić, I., 2015. PAHs levels in gas and particle-bound phase in schools at different locations in Serbia. Chem. Ind. Chem. Eng. Q. 21, 159–167. https://doi.org/10.2298/CICEQ140206016Z.