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Benzo[*a*]pyrene-induced changes in carboxylesterase, acetylcholinesterase and heat shock protein 70 of *Lymantria dispar* (Lepidoptera: Lymantriidae) from unpolluted and polluted forests

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Abstract: Plant vegetation accumulates polycyclic aromatic hydrocarbons (PAHs) among which benzo[*a*]pyrene (B[*a*]P) is recognized as being very toxic, including cancerogenic. *Lymantria dispar* L. larvae are sensitive to changes in the environment, providing potential signs of pollutant presence. We examined the chronic effects of two concentrations of B[*a*]P on the activity of carboxylesterase (CaE), acetylcholinesterase (AChE) and heat shock protein 70 (Hsp70) levels in the brain tissue of two populations of *L. dispar* larvae, originating from unpolluted and polluted habitats. We found that the relative growth rate was significantly lower in both populations and that only larvae from polluted forests were sensitive to low B[*a*]P concentrations, exhibiting a significant increase in brain tissue CaE activity and Hsp70 concentration. AChE activity showed no changes in response to B[*a*]P exposure in either population. Examined biochemical parameters indicate that their sensitivity to chronic treatment with B[*a*]P was highly dependent on the pre-exposure history of *L. dispar* larvae, suggesting that they could be promising biomarkers of B[*a*]P and PAH pollution in forest ecosystems.

Keywords: *Lymantria dispar* L.; brain tissue; benzo[*a*]pyrene; esterase; Hsp70; polycyclic aromatic hydrocarbons (PAHs); environmental pollution

INTRODUCTION

Industrial development and urbanization give momentum to dramatic modification of environmental conditions, forcing nature to adapt to the unstoppable forces of anthropogenic influence. One of the more recent negative anthropogenic contributions to environmental contamination are the polycyclic aromatic hydrocarbons (PAHs), which are mostly generated by incomplete combustion of fossil fuels (particularly by automobiles) and wood, production of coke and charcoal, metal smelting, petroleum refining and petroleum spills [1]. PAHs are very persistent organic contaminants and ubiquitous in various environments across the world [2,3]. They are identified as pollutants of global concern as they pose a great risk to human health and wildlife, with some of them being recognized as carcinogens and mutagens, including benzo[*a*]pyrene (B[*a*]P) [4]. Also, PAHs have recently been included in official air quality standards [5], pointing to the necessity of their monitoring. About 40% of PAH emissions into the environment are scavenged by vegetation via dry and wet deposition [6], and due to the large surface area and high content of lipids, foliage is considered the main access point for the accumulation of these xenobiotics [7]. Significant concentrations of high molecular weight PAHs, especially B[*a*]P, were detected in the leaves of two oak species – *Quercus robur* and *Q. ilex* [8,9]. These deciduous trees are preferable host plants for the widespread polyphagous, herbivorous insect larvae, *Lymantria dispar*, that due to their vast feeding capacity possess a great potential for bioaccumulation of environmental toxicants [10]. B[*a*]P and its metabolites that covalently bind to macromolecules in insect tissues are readily available for trophic transfer to the

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predators of *L. dispar* larvae, and thus via several levels of the food chain, they pose an indirect threat to humans. The necessity for monitoring the impact of xenobiotic concentrations on environment quality has promoted numerous studies in search for biomarkers in living systems that can serve as indicators of biological sublethal changes resulting from individual exposure to toxicants [11]. In our previous studies of the physiological responses of *L. dispar* to various types of stressors, we observed significant changes in biochemical parameters, which points to their potential use as indicators of adverse environmental changes [12,13]. Many investigations have shown the negative effects of PAHs on insect development and reproduction [14,15]. *L. dispar* is an insect species that is a very suitable model system for this type of research, considering the uncomplicated manipulation of individuals in laboratory conditions, their short generation time, precisely defined developmental stages and well-known physiological processes. Furthermore, the nervous system of *L. dispar* has proven to be very sensitive to stressogenic or environmental stimuli and a primary activator of the stress response mechanisms [16].

Carboxylesterases (CaE) or esterases are enzymes in the carboxyl/cholinesterase gene family that hydrolyze different types of esters [17]. They appear to be widely distributed in all insect tissues, and after acetylcholinesterase, they are the most abundant enzymes in the brain and nervous system [18]. In insects, CaE are implicated in many endogenous functions, such as regulation of juvenile hormone titer, general metabolism and mobilization of fats and energy related to fat catabolism in muscles [19]. Also, as a component of the defense system of insects, CaE are frequently implicated in insect resistance to organophosphate, carbamate and pyrethroid insecticides [20]. Since the early stage of PAH metabolism in insects activates oxygenation reactions and CaE activity, these enzymes are considered to be an indicator of sublethal PAH action [21]. Acetylcholinesterase (AChE) belongs to the family of cholinesterases that are specialized carboxylic ester hydrolases, with the main function of hydrolyzing the neurotransmitter acetylcholine at the cholinergic synapse, thereby blocking the nerve impulse. Organophosphate and carbamate pesticides possess a great affinity towards binding to AChE, causing enzyme inhibition and leading to the accumulation of the neurotransmitter acetylcholine in the synapse, resulting in a disruption to the nervous system functioning [22]. The sensitivity of AChE activity to insecticides has been used as a biomarker of neurotoxicity in insects [23], and recently there have been reports supporting the inhibitory effects of AChE by other xenobiotics, such as PAHs, pointing to the potential of the enzyme to serve as an indicator of exposure to these pollutants [24,25]. Heat shock proteins (Hsp) act as molecular chaperones that participate in protein folding and unfolding and are essential in the cellular response to a variety of damaging conditions. Among several Hsp families, based on sequence homology and typical molecular weight, members of the Hsp70 family appear to be synthesized in the general response to stress [26]. They act as integrators of diverse aspects of protein damage and are considered very useful in biomonitoring by complementing other tests that are more specific measures of toxic action. These proteins have been successfully used to assess the effects of different environmental stressors, including PAHs, on invertebrates [27,28].

We examined the effects that long-term exposure to relatively low concentrations of B[*a*]P in insect host plants [8] can have on several biochemical parameters: CaE, AChE and Hsp70 from the brain tissue of *L. dispar* larvae originating from unpolluted and polluted environments. Also, we focused our attention on the possible differences in the responses of two insect population, with regard to variations in their pre-exposure history and the phenotypic plasticity of biochemical parameters in response to B[*a*]P exposure, which can differ among different genotypes but can also serve as potential bioindicators of PAH exposure.

MATERIALS AND METHODS

Insect rearing and B[*a*]P treatments

Lymantria dispar egg masses were collected in November 2013 from two localities, a mixed oak forest in Majdanpek, Serbia: 44°25'17" N, 21°56'06" E, 180 km from Belgrade; and Bor, Serbia: 44°04'29.57" N, 22°05'45.28" E, 245 km from Belgrade. As stated [13], these locations are different in terms of the heavy industry facilities found in close proximity. The Majdanpek forest belongs to the largest national park in Serbia, the Đerdap National Park, and is considered

free of industrial pollution. The Bor forest is contaminated by various types of pollutants from the mining industry and smelter complex located next to it. On this locality, PAH concentration showed very high levels in the gas phase [29] and in the roots of blackberry [30]. *L. dispar* larvae originating from Majdanpek forest are from an unpolluted forest and represent the unpolluted population (UP), whereas those from Bor forest belong to a population from a polluted forest, and designated as the polluted population (PP).

Collected egg-masses were kept at 4°C until hatching in April 2014 and were then grown in plastic dishes (V=200 mL) at 23°C with a 12:12 h light:dark photoperiod. The larvae were fed on a diet specially optimized for *L. dispar* [31] to which two concentrations of B[*a*]P were added as follows:5 ng B[*a*]P/g dry food weight (5 ng of B[*a*]P was mixed with 1 g of dry diet, without any liquid), and 50 ng B[*a*]P/g dry food weight. Acetone was used to dissolve B[*a*]P to specified concentrations and was then mixed into the artificial diet. The concentration of acetone was 3% of the total diet volume. Diet mixtures were poured into wide, shallow plastic plates and left at 25°C for 4 h until the residual acetone evaporated. Two control groups of larvae were used to test the possible interference of acetone on enzyme activities and Hsp70 expression. Larvae in one control group were fed on the same artificial diet, but without acetone and B[*a*]P, while larvae in the other control group were fed the artificial diet with only acetone. There were no significant differences between these two control groups throughout the experiment, so we treated the second group as the control. All larvae were provided with the same amount of B[*a*]P-spiced food, B[*a*]P-free food or artificial food free of B[*a*]P and acetone was replaced every 48 h post hatching until death on the 3rd day of the 5th larval instar (on average after 31.67±0.33 days). There were no significant changes in the mortality rate between the experimental groups. The two concentrations of B[*a*]P were chosen based on the recorded content of B[*a*]P in the leaves of *Quercus* species [9] that are the preferred host plants of *L. dispar*.

Preparation of homogenates

The brain tissues were dissected out from the head capsules on ice, pooled in experimental groups (n=25 brain tissues per group) and diluted with distilled water (1:9/w:V). The brains of larvae were homogenized on ice at 5000 rpm during three 10-s intervals, separated by 15-s pauses (MHX/E Xenox homogenizer, Germany). Homogenates were centrifuged at 10000 *g* for 10 min at 4°C in an Eppendorf 5417R centrifuge (Germany). The supernatants were used for the enzyme assays, Western blotting and indirect non-competitive enzyme-linked immunosorbent assay (ELISA). The protein concentration was determined according to Bradford [32] with bovine serum albumin (BSA) as standard.

Estimation of relative growth rate

To estimate the effects of two B[*a*]P concentrations on the individual performance of *L. dispar* larvae, the relative growth rate (RGR) was measured, from molting into the 3th instar until the 3rd day of the 5th instar. RGR was calculated as $RGR=(\ln W_t - \ln W_0)/t$, where W_0 and W_t are the weights of the larvae at the beginning and end of the examined period and t is the interval in days.

CaE and AChE assays

CaE activity was determined according to Main et al. [33] using the spectrophotometric assay with *p*-nitrophenyl butyrate (15 mM) hydrolysis and recording the absorbance increase at 414 nm. Two replicates were used for each experimental group, 2 blanks and 2 noncatalytic probes. The reaction was performed in 50 mM Na-phosphate buffer (pH 7,5). The incubation time was 4 min at 30°C and the enzyme reaction was monitored with a UV mc2 spectrophotometer (SAFAS, Monaco City, Monaco). One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze 1 μmol of *p*-nitro-phenyl butyrate per min per mg protein.

AChE activity was determined spectrophotometrically according to the method of Ellman et al. [34] using acetylthiocholine iodide (0.25 M) as the artificial substrate. After hydrolysis, the thiocholine that in the presence of the dithiobis-nitrobenzoate (DTNB) ion generated the yellow 5-thio-2-nitrobenzoate anion was quantified by its absorbance at 406 nm. Enzyme kinetics were monitored at 25°C for 3.5 min with an UV mc2 spectrophotometer (SAFAS, Monaco City,

Monaco). All samples were measured in triplicate (homogenized brain tissues pooled in each group, n=25). Blanks were run without brain tissue to correct for the absence of enzymatic activity. The rate of enzyme activity was expressed as 1 μmol of substrate hydrolyzed per min per mg protein.

Native electrophoresis

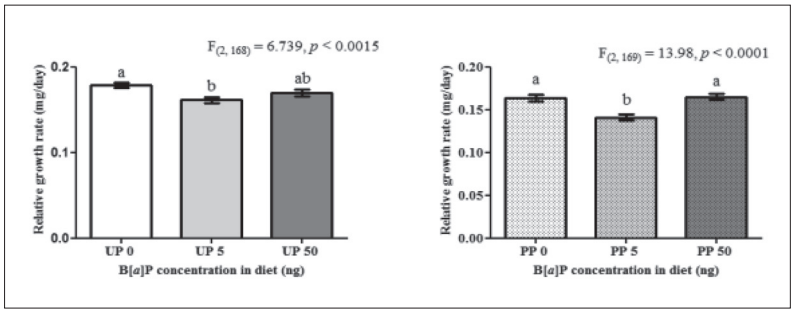
Zymography detection of CaE required electrophoretic separation of *L. dispar* brain homogenates (15 μg of protein per lane) on an 8% native polyacrylamide gel [35] at a constant current of 20 mA for 3 h at 4°C. For CaE visualization, the gel was soaked and shaken in a mixture of 1,1 mM *a*-naphthyl acetate, 1,1 mM *β*-naphthyl acetate and 1,2 mM Fast Blue B salt, previously dissolved in 20 mM phosphate buffer (pH 7.2) (modified from the method of Gottlieb [36]). After a few min at room temperature (25°C), pink lines corresponding to CaE activity appeared on a transparent background. Direct staining by the thiocholine method (modification of Karnovsky and Roots [37]) was used to localize AChE activity in the gel. Electrophoretic separation of brain tissue homogenates (35 μg of protein per lane) was carried out on a 10% nondenaturing polyacrylamide gel at a constant current of 20 mM for 3 h at 4°C. After electrophoresis the gel was rinsed with distilled water and incubated overnight at room temperature (25°C) in staining solution. The regents of the solution were dissolved in the 0.1 M phosphate buffer (pH 6) in the following order: 0.1 M acetylthiocholine iodide, 0.1 M sodium citrate, 30 mM $CuSO_4$ and 5 mM potassium ferricyanide. The next day, the gel was thoroughly rinsed with distilled water. Sites of AChE activity appeared as transparent bands against a brown background. All gels were scanned with a CanoScan LiDE 120, Japan. For the purpose of qualitative image analysis, scans of all gels were converted to black and white, but only the scans of AChE zymography gels were inverted into negative. The protein band area and relative optical density in the region of the CaE and AChE activity were analyzed using ImageJ 1.42q program (NIH, USA).

Hsp70 detection methods

The concentration of Hsp70s in the brain homogenates of *L. dispar* larvae was determined by Western blotting and indirect ELISA. An indirect noncompetitive ELISA was used to quantify the concentrations of Hsp70s in the homogenates of larval brain tissues. Samples were diluted with carbonate-bicarbonate buffer (pH 9.6) and coated on a microplate (Multiwell immuno plate, NAXISORP, Thermo Scientific, Denmark) overnight at 4°C. Coated samples were first incubated with monoclonal anti-Hsp70 mouse IgG1 (dilution 1:5000) (clone BRM-22, Sigma Aldrich, USA) for 12 h at 4°C, and then for 2 h at 25°C with secondary anti-mouse IgG1 (gamma-chain)-HRP conjugate (dilution 1:5000) antibodies (Sigma Aldrich, USA). Chromogenic substrate 3,3',5,5'-Tetramethylbenzidine (TMB) was used as a visualizing reagent and absorption of the end product was measured on a microplate reader (LKB 5060-006, Austria) at 450 nm. Serial dilutions of standard Hsp70 (recombinant Hsp70, 50 ng/mL) were used to calculate the Hsp70 concentrations, expressed as ng/mg protein. Each data point represents the mean of three replicates (n=25, homogenized brain tissues pooled for each group). The homogenates of brain tissues were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) on a 12% gel [35]. Protein transfer from the gel to the nitrocellulose membrane (Amersham Prothron, Premium 0.45 mm NC, GE Healthcare Life Sciences, UK) was left overnight at 40 V and 4°C. Hsp70 expression patterns in larval brain tissue were detected using the primary monoclonal anti-Hsp70 mouse IgG1 (dilution 1:5000) (clone BRM-22, Sigma Aldrich, USA) and secondary anti-mouse IgG1 (gamma-chain)-HRP conjugate antibodies (dilution 1:5000) (Sigma Aldrich, USA). Protein bands were visualized using a chemiluminescence (ECL kit, Amersham). Relative band densities of the Hsp70 protein band areas were analyzed using ImageJ 1.42q program (NIH, USA).

Statistical analyses

The normality of data distribution was estimated by the D'Agostino and Pearson omnibus and Shapiro-Wilk normality test. The statistical analyses of the data were carried out using one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test. The significance of the results was determined at $p<0.05$. The data were expressed as the means±standard error of the mean (SEM).



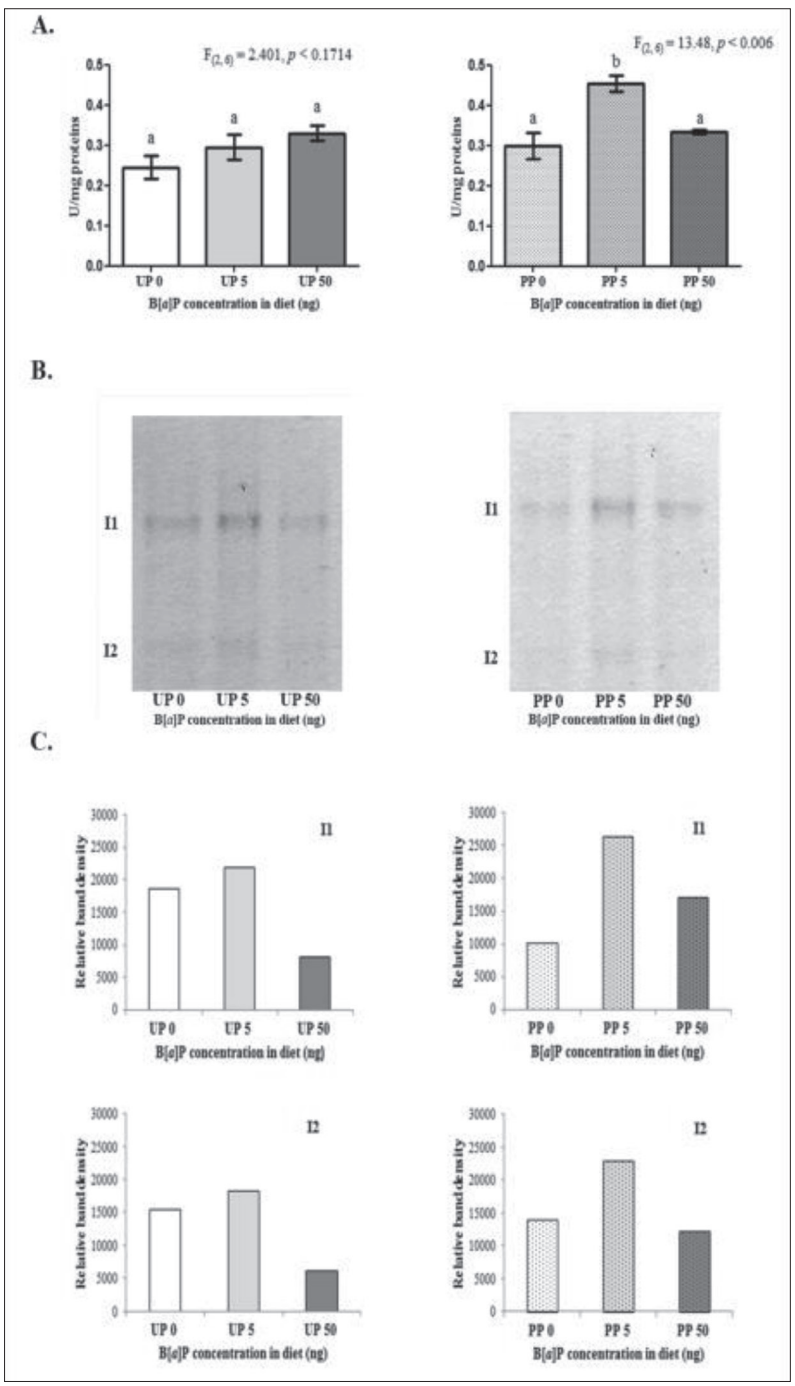


Fig. 1. RGR from molting to the 3rd instar until the 3rd day of the 5th instar of *L. dispar* larvae from unpolluted (UP) and polluted (PP) populations fed with an artificial diet supplemented with 5 ng B[*a*]P/g DW and 50 ng B[*a*]P/g DW of benzo[*a*]pyrene (B[*a*]P). The control group of larvae was fed with an artificial diet without B[*a*]P (0 ng). Data are expressed as the mean±SEM, mg/day. Values marked with different letters indicate significant differences between groups (Tukey's *post hoc* test, p<0.05).

RESULTS

L. dispar relative growth rate

In both populations of *L. dispar* larvae a significant decrease in the RGR was recorded but only in those reared on the diet supplemented with the lower concentration of B[*a*]P, 5 ng/g dry weight (DW) (Fig. 1) (one-way ANOVA, p<0.05). Relative to the control groups, 50 ng/g DW of B[*a*]P did not affect the RGR of the treated larvae.

CaE and AChE activities in *L. dispar* brain tissue

B[*a*]P supplemented in diet had different effects on the two populations of *L. dispar* larvae as regards brain CaE activity (Fig. 2A). UP larvae showed

Fig. 2. **A** – CaE activity in brain tissue of 5th instar *L. dispar* larvae from unpolluted (UP) and polluted (PP) populations fed with an artificial diet supplemented with 5 ng B[*a*]P/g DW and 50 ng B[*a*]P/g DW of benzo[*a*]pyrene (B[*a*]P). The control group of larvae was fed with an artificial diet without B[*a*]P (0 ng). Data are expressed as the mean±SEM, U/mg protein. Values marked with different letters indicate significant differences between groups (Tukey's *post hoc* test, p<0.05). **B** – Activity staining for CaE on an 8% polyacrylamide gel for UP and PP. I1 and I2 are two CaE isoforms. **C** – Densitometric analysis of polyacrylamide gels showing the relative levels of both CaE isoform activities compared with the control group in UP and PP.

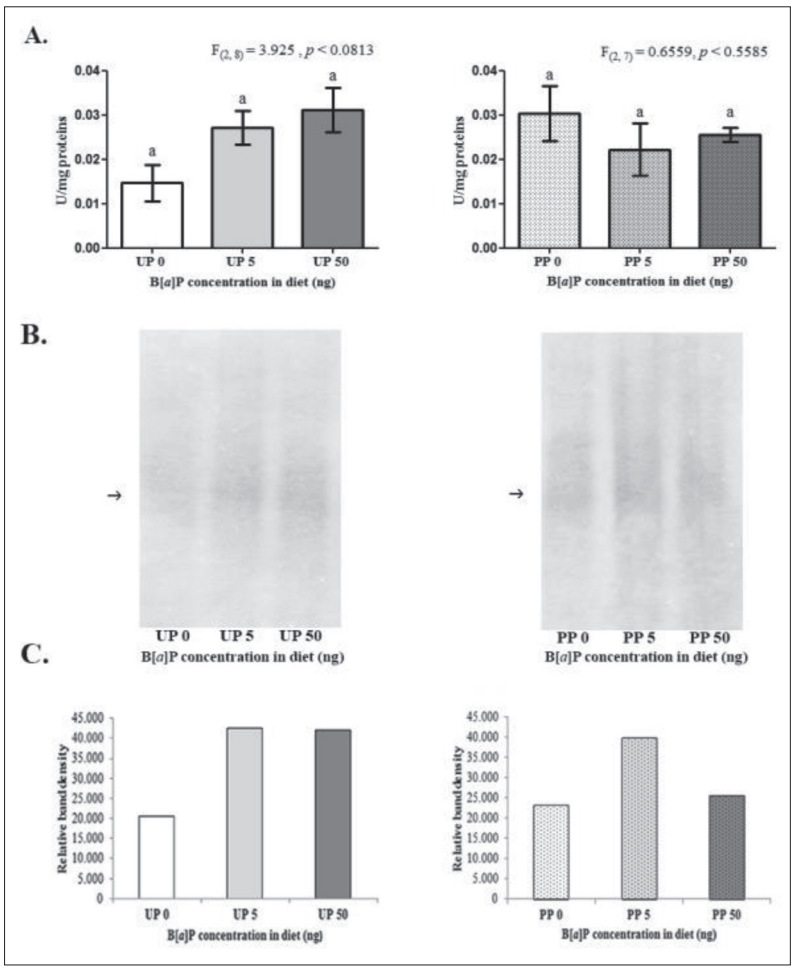


Fig. 3. **A** – AChE activity in brain tissue of the 5th instar *L. dispar* larvae from unpolluted (UP) and polluted (PP) populations fed with an artificial diet supplemented with 5 ng B[*a*]P/g DW and 50 ng B[*a*]P/g DW of benzo[*a*]pyrene (B[*a*]P). The control group of larvae was fed with an artificial diet without B[*a*]P (0 ng). The data are expressed as the mean±SEM, U/mg protein. Values marked with different letters indicate significant differences between groups (Tukey's *post hoc* test, p<0.05). **B** – Activity staining for AChE on a 10% polyacrylamide gel for UP and PP. The arrow (→) points to AChE activity of isoform I1. **C** – Densitometric analysis of polyacrylamide gels showing the relative levels of AChE isoform activities compared with the control group in UP and PP.

no changes in CaE activity after exposure to the two B[*a*]P concentrations. A significant rise in brain CaE activity was observed in the group of larvae from PP fed on 5 ng/g DW of B[*a*]P, relative to the control group (one-way ANOVA, p<0.05). The higher concentration of B[*a*]P (50 ng/g DW) did not cause a significant change in enzyme activity. Detection of CaE activity on a native polyacrylamide gel uncovered two isoforms, I1 and I2 (Fig. 2B), with I1 being dominant in all experimental groups. It appears that the lower concentration of B[*a*]P (5 ng/g DW) provoked a stronger expression of I1 and I2 isoforms in both *L. dispar* popula-

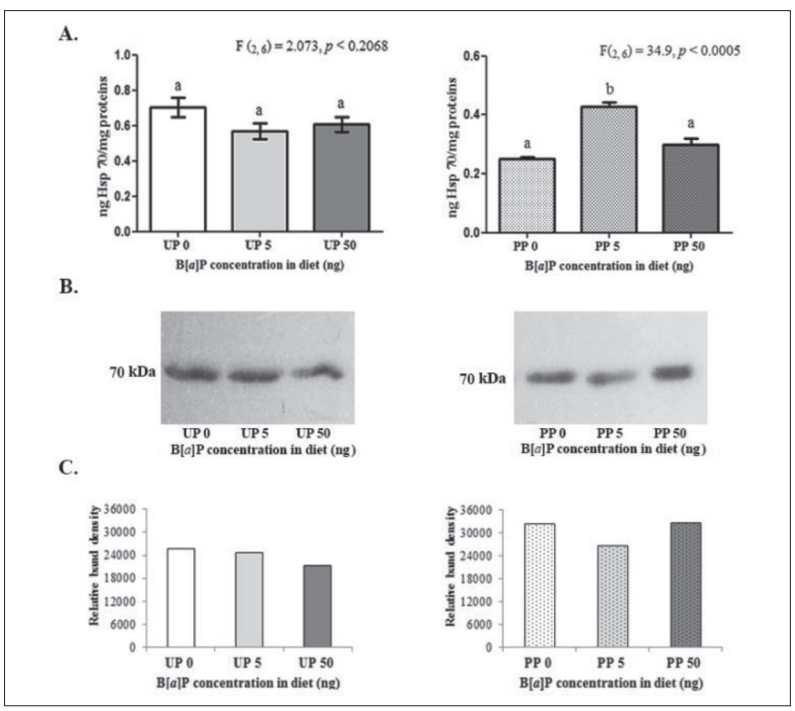


Fig. 4. A – Concentrations of Hsp70 and **B** – Western immunoblots of Hsp70 in brain tissue of 5th instar *L. dispar* larvae from unpolluted (UP) and polluted (PP) populations fed with an artificial diet supplemented with 5 ng B[*a*]P/g DW and 50 ng B[*a*]P/g DW of benzo[*a*]pyrene (B[*a*]P). The control group of larvae was fed with an artificial diet without B[*a*]P (0 ng). The data are expressed as the mean±SEM ng Hsp70/mg protein. Values marked with different letters indicate significant differences between groups (Tukey's *post hoc* test, p<0.05). **C** – Densitometric analysis of western immunoblots showing relative levels of Hsp70 expression compared with the control group in UP and PP.

tions when compared to the other B[*a*]P treatment and the control groups (Fig.2C).

AChE activity in *L. dispar* brain tissue was not significantly affected by the B[*a*]P dietary treatment (Fig. 3A) in either population. Native electrophoresis of brain tissue homogenates revealed the presence of the I1 isoform of AChE (Fig. 3 B). The relative intensity of this isoform corresponds to AChE activity in both populations of larvae, and there were no notable differences between the groups treated with different B[*a*]P concentrations and the control groups (Fig. 3C).

Concentration of Hsp70 in the brain of *L. dispar* larvae

The concentration of Hsp70 estimated by indirect ELISA was significantly increased only in brain tissue homogenates of PP *L. dispar*, fed on the lower concentration of B[*a*]P (5 ng/g DW) (one-way ANOVA, $p<0.05$). Hsp70 levels remained unchanged during the dietary treatment with B[*a*]P in the UP larvae, relative to the control group (Fig. 4A). Western blot analysis of Hsp70 revealed a single protein band at 70 kDa in all experimental groups of both UP and PP *L. dispar* populations (Fig. 4B). Relative estimation of band densities followed the concentrations of Hsp70 (Fig. 4C).

DISCUSSION

Exposure to PAHs poses a great risk to herbivorous insects that live and feed on tree leaves in contaminated forests. Intermediates of B[*a*]P metabolism that can bind to nucleic acids and proteins have been shown to be very toxic as they are highly mutagenic and/or carcinogenic for vertebrates and invertebrates, and studies have demonstrated the direct impact of B[*a*]P on life history traits such as development and growth in insects [38]. Stress provoked by xenobiotics like B[*a*]P trigger the biochemical defense mechanisms (changes in enzyme activities, Hsp synthesis, etc.) that enable insect survival [39]. Large amounts of metabolic energy are crucial for the successful coping with and elimination of toxicants, and the fitness-related traits of insects, development, growth and reproduction, are most commonly affected by this energy allocation [39]. As we described in our previous research [13], most energy resources are probably directed toward the activation of the SOD-CAT system as the first line of defense against oxidative stress. Our present results showed that only the lower concentration of B[*a*]P (5 ng/g DW) had a harmful effect on the relative growth rate of *L. dispar* larvae in both populations of insects. This disruption of larval growth, which is apparently inconsistent with the applied B[*a*]P concentration, seems to be the characteristic response for this type of compound in *L. dispar*. Previous investigation of B[*a*]P and fluoranthene influence on *L. dispar* larval development and growth showed that mainly a lower concentration of these PAHs caused the greatest harm to larval fitness [15,40]. It is possible that the B[*a*]P mechanism of action resembles the class of compounds called endocrine disruptors (EDCs), which elicit reverse responses of different parameters as the concentration of disruptors increases, resulting in an U-shaped or inverted U-shaped curve [41]. Tomas [42] also found that B[*a*]P indeed displays effects like EDCs, specifically as an antiandrogenic compound.

PAH metabolism in insects is connected to several important enzyme systems of detoxification – microsomal oxidases, glutathione S-transferases and carboxylesterases. Being a heterogeneous group of enzymes with the ability to hydrolyze different esters, CaE have a potential role in the degradation and elimination of various xenobiotics [43]. The results of the present study showed that the very low concentration of B[*a*]P (5 ng/g DW) significantly elevated brain CaE activity in the PP *L. dispar* larvae, while the higher B[*a*]P concentration had no effect. Induction of CaE activity was not recorded in UP larvae at either B[*a*]P concentration. Native polyacrylamide gels revealed that the CaE I1 isoform is most probably responsible for B[*a*]P metabolism in larvae brain tissue, especially after exposure to 5 ng/g DW B[*a*]P in both the UP and PP *L. dispar* populations. Callaghan et al. [44] proposed that quantitative differences in CaE activity between populations are an adaptive mechanisms against environmental xenobiotics. We presume that the higher CaE sensitivity to B[*a*]P in the polluted *L. dispar* population was the consequence of multigenerational population exposure and adaptation to various pollutant challenges found in their natural habitat. Unfavorable conditions probably led to the phenomenon of developing faster and higher levels of expression of detoxification enzymes such as CaE when confronted to xenobiotics, compared to insects originating from

clean forest locations. Esterase overexpression can be due to either gene amplification or upregulation, or a combination of both [43]. In our previous research, we showed a stronger induction of the midgut antioxidative enzymes, superoxide dismutase (SOD) and catalase (CAT) and the detoxification enzyme glutathione S-transferase (GST) in a polluted population than in ones from an unpolluted habitat, in response to B[*a*]P treatment [13]. Migula et al. [45] discovered increased CaE activity in red wood ants, *Formica polyctena*, from metal-polluted areas. Kapin and Ahmad [18] determined that most of CaE activity in *L. dispar* larvae is located in the midgut, while the nerve cord and the brain contain predominantly AChE. On the basis of obtained results, it seems that in unpolluted populations the applied B[*a*]P concentrations were too low to bypass the high concentration of CaE and other defense enzymes in the larval midgut epithelium, so that brain CaE isoforms were not activated.

The likely reason why we recorded only a significant change in CaE activity under chronic exposure to the lower B[*a*]P concentration (5 ng/g DW) in the polluted population may be due to the aggregation of enzyme molecules and decreased sensitivity to higher xenobiotic concentrations [46]. Also, the extended time of exposure to the higher B[*a*]P concentration (50 ng/g DW) probably caused direct and indirect inhibition of CaE activity due to the accumulation of reactive B[*a*]P intermediaries [47].

AChE is established as a major biomarker of insecticide and pesticide exposure [48]. Although Jett et al. [24] and Kang and Fang [25] demonstrated that a number of different PAHs, including B[*a*]P, inhibit AChE directly *in vitro*, the results of our study showed no significant changes in AChE activity under the dietary treatment with B[*a*]P (5 and 50 ng/g DW) in either of the two *L. dispar* populations. In all experimental groups, zymogram detection of AChE activity revealed discrete, barely visible lines on the gel corresponding to AChE, indicating moderate expression of this enzyme. It is very likely that this is a consequence of the high efficiency of the first line defense systems present in the midgut of *L. dispar* larvae [18,49] and the fact that PAHs are not natural, specific substrates for AChE. The concentrations of B[*a*]P present in host plant species for *L. dispar* larvae used in our experiment probably were not high enough to influence brain AChE activity in any way. Ilijin et al. [50] found that B[*a*]P present in low concentrations of 2 and 10 ng/g DW in insect diet did not provoke changes in AChE activity of *L. dispar* larvae from an unpolluted population, and neither did dietary intake of another PAH, fluoranthene, at low and high concentrations (6.7, 33,5 and 67 ng/g DW) [51].

Van Brummelen and Van Straalen [52] found, in their investigation of the uptake, elimination and tissue distribution of B[a]P in the isopod *Porcellio scaber*, that 14% of all absorbed B[*a*]P remains in the brain. Kapin and Ahmad [18] determined that the dominant esterase in the brain of *L. dispar* larvae is AChE while the content of CaE is much smaller. On the other hand, CaE are much more sensitive and responsive to B[*a*]P presence than AChE, even in low concentrations of the contaminant. Other studies have shown that other xenobiotics have increased the affinity for CaE over AChE, suggesting that CaE activity provides a more sensitive endpoint in the evaluation of environmental pollution [53,54]. The lack of inhibition of AChE activity in *L. dispar* larvae in response to B[*a*]P chronic effects suggests that changes in other biochemical parameters, such as CaE, are probably a more convenient indicator of PAH exposure in this polyphagous insect species.

The adverse effects of B[*a*]P metabolism are particularly pronounced in sensitive tissues like insect brain. Damaged proteins tend to aggregate in the cytoplasm of the cell, which consequently provokes increased expression of heat shock proteins (Hsp70). By promoting the refolding of misfolded proteins and by removing excessively damaged ones, Hsp70 contributes to the preservation of cell function and protects the cell from various stressors. Lee et al. [55] observed that PAHs and other types of chemical pollutants stimulate the expression of inducible and constitutive forms of Hsp70 in *Chironomus tentans.* An increase in Hsp70 expression was also recorded in aquatic invertebrates, oysters and mollusks, in response to exposure to PAHs and B[*a*]P [56]. Köhler et al. [27] reported a significant but transient induction of Hsp70 levels in the terrestrial isopod *Oniscus asellus* after exposure to B[*a*]P. The results of the present investigation revealed different Hsp70 expression patterns among two *L. dispar* populations after treatment with different B[*a*]P concentrations. Indirect noncompetitive ELISA revealed a significant increase in Hsp70 concentration only in

larval brains from PP, and only under the influence of a lower concentration of pollutant (5 ng/g DW B[*a*]P) relative to the control group. Chronic treatment with B[*a*]P did not cause any meaningful changes in Hsp70 levels of UP larvae. One protein band of Hsp70 was detected by Western blot analysis in all experimental groups of both populations of larvae, with discrete variations in band intensity. The obtained results are very similar to those recorded in larvae from the same populations (UP and PP) in terms of brain CaE activity and the mentioned response of midgut SOD, CAT and GST enzymes [13], which indicates that *L. dispar* larvae derived from a habitat burdened with pollutants demonstrate higher levels of synthesis and activity of vital, protective enzymes and proteins against the toxic influence of B[*a*]P. Perić-Mataruga et al. [16] also discovered differences in Hsp70 expression among two differently adapted *L. dispar* populations after exposure to temperature and heavy metal stress. Thus, larvae from the less polluted environment showed decreased amounts of Hsp70, while the polluted population exhibited a rise in Hsp70 concentration. It is possible that the larvae from UP also showed changes in Hsp70 levels at the beginning of the treatment with B[*a*]P, but since the stress protein system probably does not possess the capacity to withstand long-term exposure to a toxicant, and as its prolonged expression consumes too much of the cellular energy needed for housekeeping metabolism, there was no measurable response at the endpoint of the experiment. Beside the possibility of involvement of additional detoxification systems in the elimination of the higher B[*a*]P concentration (50 ng/g DW), there is evidence that the nonlinear response of Hsp70 expression to pollutant dose is the consequence of the properties of B[*a*]P. As we indicated before, it appears that B[*a*]P induces a higher response from biochemical parameters at lower than at higher concentrations, which is quite similar to the patterns characteristic for endocrine disruptors. This type of behavior was observed by Dinan et al. [57] during fluoranthene treatment of *Drosophila melanogaster* cells. Ilijin et al. [50] reported that among six tested dietary doses of B[*a*]P, the largest induction of Hsp70 expression was under the chronic influence of the lowest (2 and 10 ng/g DW) B[*a*]P concentration. An analogous situation was described in the research of Mrdaković et al. [51], where the lowest concentration of fluoranthene (6.7 ng/g DW) caused the greatest elevation of Hsp70 in the brain of *L. dispar* larvae. Also, Köhler et al. [27] found that high doses of B[*a*]P (0.5, 7.2 and 59.3 μg/g DW) induced a rise in Hsp70 in the isopod *Oniscus asellus* after one day of exposure, which was followed by a continuous decline in Hsp70. Authors have recommended that for highly lipophilic compounds such as B[*a*]P, Hsp70 could be a good indicator of long-term exposure to sublethal toxic effects. Based on the response of Hsp70 in our present study, we suggest that it can serve as a potential biomarker of chronic exposure to B[*a*]P concentrations that are recorded in the environment and correspond to those used in this research. However, this possibility is only valid for *L. dispar* populations that originate from habitats exposed to some kind of pollution. Further research is advisable when we have in mind that Hsp70 is generally sensitive to cytotoxicity, responsive to a wide range of chemicals, and is involved in numerous physiological functions that take place in insects under stress [58].

CONCLUSION

Both populations of *L. dispar* larvae (UP and PP) were sensitive to the adverse effects of B[*a*]P present in the diet in two different concentrations, expressed as significantly lower RGR with respect to the control groups. Examination of the responses of biochemical parameters, CaE and Hsp70 to B[*a*]P revealed an increase only in larvae from the polluted population. We did not detect any meaningful changes in AChE activity in response to B[*a*]P exposure. A very low B[*a*]P concentration (5 ng/g DW) provoked significant changes in brain CaE activity and Hsp70 level, reflecting their high inducibility in response to this xenobiotic. Although only the larvae population originating from polluted forests were sensitive, a more comprehensive examination of the dose-dependent response of these parameters is required to support the use of these biomarkers as indicators of B[*a*]P and PAH pollution in forest ecosystems.

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and interpreted the results. Marija Mrdaković, Milena Vlahović and Larisa Ilijin designed the study. Siniša Đurašević and Vesna Perić-Mataruga supervised the project and helped in work planning.

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