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Effects of fluoranthene on digestive enzymes activity and relative growth rate of larvae of lepidopteran species, *Lymantria dispar* L. and *Euproctis chrysorrhoea* L.

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ABSTRACT

Fluoranthene is one of the most abundant polycyclic aromatic hydrocarbon pollutants in the environment and it may accumulate in plant leaves which are the main food source for phytophagous insect species. The aim of this study was to establish the effects of dietary fluoranthene on specific activities of digestive enzymes and expression of their isoforms in the midgut, and the relative growth rates of *Lymantria dispar* and *Euproctis chrysorrhoea* larvae. Exposure to fluoranthene led to significantly decreased trypsin activity in the midgut of larvae of both species. Leucine aminopeptidase activity decreased significantly in the midgut of L. *dispar* larvae exposed to the lower concentration of fluoranthene, but that enzyme activity showed the opposite trend in *E. chrysorrhoea* larvae. There was no pollutant induced changes in lipase activity in L. *dispar*, while elevated enzyme activity was recorded in the midgut of *E. chrysorrhoea* larvae exposed to the lower concentration of fluoranthene. Different patterns of expression of enzyme isoforms were noticed. Relative growth rates of both species significantly decreased in fluoranthene treated larvae. These responses indicate to the significance of relationships between physiological changes and fitness-related traits in L. *dispar* and *E. chrysorrhoea* larvae affected by pollutant, and contribute to understanding the mechanisms of their adjustment to stressful conditions.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) represent a group of complex organic compounds composed of two or more fused benzene rings. They are widely distributed in the environment, and, although there are natural sources of PAHs such as volcanic activities and forest fires, they mainly result from anthropogenic activities (Finlayson-Pitts and Pitts, 1986; Srogi, 2007). There is much public concern about these pollutants due to their environmental persistence, potential for bioaccumulation, and numerous harmful effects for humans and the environment. Fluoranthene, a widely spread four-ringed PAH, is a pollutant with toxic and co-carcinogenic effects (Saunders et al., 2003; Šepič et al., 2003; Bauer et al., 2017) and one of the U.S. Environmental Protection Agency's 16 priority pollutant PAHs. Plants are exposed to the PAHs from the atmosphere or polluted soils, from where they can be uptake by plant shoots or roots, respectively. Direct pathway of PAH transfer, from the air to leaves, by deposition on the leaf cuticle or by uptake through stomata, is considered the major, for contamination of plants growing on unpolluted soils. Characteristics such as surface and presence of the hairs as well as, leaf components, waxes and lipids, have a significant role in the uptake and accumulation of lipophilic PAH compounds in leaves (in: Srogi, 2007; Desalme et al., 2013). The negative actions of fluoranthene on higher plants (Kummerová et al., 2006; Kummerová and Kmentová, 2004; Oguntimehin et al., 2008) may significantly influence phytophagous species.

Lymantria dispar L. and *Euproctis chrysorrhoea* L. (Lepidoptera, Erebidae), are polyphagous phytophagous pest insects. Larvae of L. *dispar* feed on over 500 plant species within 73 families (Lance, 1983; Liebhold et al., 1995), while larvae of *E. chrysorrhoea* feed on 26 genera of trees and shrubs within 13 plant families (Forestry Compendium, 2005). Larvae of both species, especially in older instars, consume large quantities of food. Feeding on PAH contaminated food may affect

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functions of the insect gut, but phytophagous insects can overcome harmful effects of ingested xenobiotics by activating a wide range of digestive, antioxidative and detoxification enzymes (Broadway, 1997; Zhu-Salzman et al., 2005; Desprès et al., 2007).

Digestion and absorption of ingested food take place mainly in the midgut, where epithelial cells are responsible for the synthesis of digestive enzymes, processes of transport between the lumen and hemolymph, maintenance of pH and potassium ion gradients (Terra and Ferreira, 2012). Various enzymes are involved in the processes of digestion of particular nutrients necessary for insect growth, development and reproduction (Terra et al., 1996).

Proteases are important enzymes in the alimentary tract of insects, as they catalyze the hydrolysis of peptide bonds in proteins. In the midgut of Lepidoptera protease activity mainly originates from serine proteases, such as trypsin which hydrolyzes internal peptide bonds of the polypeptide chain on the carboxyl side of basic L-amino acids. They contribute up to 95% of the total digestive activity (Terra and Ferreira, 1994; Srinivasan et al., 2006; Tabatabaei et al., 2011; Yao et al., 2012). Aminopeptidases are metalloenzymes that remove amino acid residues from the N-terminus of peptide chains (Valaitis, 1995). By hydrolyzing partially digested oligopeptides, insect midgut aminopeptidases play an important role in the intermediary stage of protein digestion (Lomate and Hivrale, 2010). Lipases are a group of enzymes that catalyze the hydrolysis of dietary lipids, supplying necessary fatty acids to larvae (Christeller et al., 2011). Enzymes are present in multiple molecular forms, known as isozymes. Characterized by the same catalytic activity, this allows more flexibility, adaptability and precision in carrying out specific metabolic functions (Zeidler, 2000). Some effects of plant allelochemicals, and insecticides and heavy metals as important environmental pollutants, on insect digestive enzymes have already been described (Lazarević and Perić-Mataruga, 2003; Mrdaković et al., 2013; Vlahović et al., 2015; Zou et al., 2019; Chen et al., 2021). However, data concerning PAHs are mainly related to various aquatic species, whose habitats are markedly affected by anthropogenic activities (e.g. Charron et al., 2013; Vignet et al., 2014; Caruso et al., 2016). Nevertheless, to the best of our knowledge, studies on the influence of PAHs on digestive enzymes of phytophagous insects are still scarce (e.g. Grčić, 2020), although there are data about some effects of these pollutants on enzyme activities in other organisms. Recently, changes in the activity of digestive enzymes in response to phenanthrene have been reported in the earthworm, *Eisenia fetida* (Shi et al., 2020). Sun et al. (2020) showed that fluoranthene alone or in a mixture with another PAH, inhibited α-amylase activity through action at the active site of the enzyme. However, effects of fluoranthene on digestive enzymes activity in phytophagous insects have not been examined as far as we know.

Several studies have pointed to actions of PAHs on fitness-related traits of phytophagous insects (Mrdaković et al., 2015; Ilijin et al., 2015; Grčić et al., 2019). To overcome the harmful effects of a pollutant, insects redirect a significant part of their energy resources towards metabolism and defense, instead of development, resulting in reduced growth and reproduction (Van Straalen and Hoffmann, 2000). Fitness-related traits form a link between effects at the biochemical level of an individual and the population as a whole (Hyne and Maher, 2003). Changes in fitness traits can provide important information about possible effects of pollutants on the population of particular insects and mechanisms of adaptation.

The present study extends our previous findings about coping mechanisms of insects affected by a pollutant. We have already shown changes in activities of antioxidative and phase II biotransformation enzymes in L. *dispar* and *E. chrysorrhoea* larvae exposed long-term to dietary fluoranthene (Filipović et al., 2019). In this study we examined the effects of environmentally relevant concentrations of dietary fluoranthene on the activity of some digestive enzymes (trypsin, leucine aminopeptidase, and lipase) and expression of their isoforms in the larval midgut, as well as on the relative growth rates of L. *dispar* and *E. chrysorrhoea* larvae, originating from natural populations. To our knowledge, this is the first report to compare digestive enzymes activities and fitness-related traits between L. *dispar* and *E. chrysorrhoea* larvae long-term exposed to fluoranthene.

2. Materials and methods

2.1. Insect rearing and fluoranthene treatments

As previously stated (Filipović et al., 2019), *L. dispar* egg masses were collected in November, from a mixed oak forest near the city of Majdanpek (East Serbia), while *E. chrysorrhoea* winter nests were collected in February, from a mixed oak forest near the city of Prijepolje (Southwest Serbia), localities that were considered unpolluted. *L. dispar* egg masses were kept at 4 °C until April, when they were transferred to a temperature of 23 ± 0.5 °C with a photoperiod of 12 L:12D to hatch. *E. chrysorrhoea* winter nests were also kept at 4 °C until late March, when they were placed on wild plum branches with buds, at room temperature. Larvae that appeared were transferred to a temperature of 26 ± 0.5 °C, and a photoperiod of 16 L:8D. Larvae of each species were randomly assigned to one of three groups and fed on high wheat germ artificial diet (O'Dell et al., 1985). Diets for the two experimental groups contained fluoranthene at 6.7 ng/g dry food weight (Fl group) or 67 ng/g dry food weight (Fh group). The control groups (C) for both species were fed a fluoranthene-free diet. The lower concentration of fluoranthene was chosen according to that previously reported in leaves of several tree species (Howsam et al., 2000), including leaves of suitable host plants for both species. The higher concentration was shown to correspond to concentrations of PAHs previously detected in leaves of various plant species (e.g. Alfani et al., 2001; Tian et al., 2008). Also, when we tested the effects of different fluoranthene concentrations on life history traits and antioxidative enzyme activities in L. *dispar* larvae, we found that the chosen amounts (6.7 and 67 ng) led to notable changes (Mrdaković et al., 2015).

Fluoranthene was dissolved in reagent-grade acetone to the specified concentrations and then blended into the artificial diet. The mixture was distributed in plastic containers and kept for 4 h in a fume hood until the acetone had evaporated. Larvae were checked daily for molting and were provided with equal amounts of fresh food every 48 h. Larvae were reared until the 3rd day of the fifth instar when they were sacrificed.

2.2. Sample preparation and assays of enzyme activity

Larvae of both species were sacrificed on ice, the midguts were removed and kept at −20 °C until homogenization. Each midgut was weighed, and diluted with 0.15 M NaCl at the final concentration of 100 mg/mL. Midguts were then homogenized individually, using an Ultra Turrax homogenizer (IKA-Werke, Staufen, Germany), followed by centrifugation (5417R Eppendorf, Hamburg, Germany) at 10000*g* for 10 min at 4 °C. The obtained supernatants were used in further enzymatic assays.

Trypsin (TRY) activity was determined according to Erlanger et al. (1961) and Valaitis (1995) by using Nα−benzoyl-DL-arginine 4-nitroanilide hydrochloride as the substrate. Leucine aminopeptidase (LAP) activity was determined by a similar method (Erlanger et al., 1961) and L-leucine *p*-nitroanilide was used as the substrate. Enzyme activities were measured in duplicate, including controls without enzyme and controls without substrate. Lipase activity was determined using a modified method of Arreguín-Espinosa et al. (2000), adapted for lipase of L. *dispar* (Mrdaković et al., 2008), with *p*-nitrophenyl caprylate as the substrate.

Specific enzyme activity is expressed as unit per mg of midgut protein. Protein concentration was determined according to Bradford (1976), using bovine serum albumin as the standard. Sample sizes of the experimental groups of L. *dispar* larvae were: N = 10 (C), N = 9–11 (Fl) and N = 10 (Fh); Sample sizes of the experimental groups of *E. chrysorrhoea* larvae were: N = 10 (C), N = 9–10 (Fl) and N = 9–10 (Fh).

2.3. Native electrophoresis

Trypsin activity was detected on 10% nondenaturing polyacrylamide gel (modified method of Erlanger et al., 1961), at 100 V at 4 °C, with 5 μg of protein placed in each lane. After electrophoresis, the gel was washed with deionized water for 10 min and soaked in 50 mM glycine buffer (pH 10) for 20 min. At the same time, nitrocellulose membrane was incubated in 2 mM substrate Nα−benzoyl-DL-arginine 4-nitroanilide hydrochloride for 50 min at room temperature. The membrane was placed on the gel and left in the wet-chamber at 37 °C for 1 h. Then, the membrane was soaked in 0.1% $NaNO_2$ dissolved in 1 M HCl for 2.5 min, washed with 1% urea, and incubated in 0.05% 1-naphthylamine dissolved in 47.5% ethanol, until pink bands of trypsin activity appeared. The reaction was stopped by washing with distilled water.

LAP activity was detected on nondenaturing polyacrylamide gel according to the same modified procedure of Erlanger et al. (1961) employing L-leucine p-nitroanilide dissolved in dimethylformamide as the specific substrate. Veronal/HCl buffer (50 mM; pH 7.8–8.0) was used for gel incubation and LAP activity was visualized on membranes in the same way as described for trypsin.

Lipase activity was detected on nondenaturing polyacrylamide gel, according to Diaz et al. (1999), at 100 V and 4 °C, with 5 μg of protein placed in each lane. After electrophoresis, the gel was washed with deionized water and incubated in 100 mM phosphate buffer (pH 7), for 10 min at 30 °C. 4-Methylumbelliferyl butyrate (0.4 mM) was added to the mixture and after 2 min the gel was exposed to UV light and fluorescent bands of lipase activity appeared.

Differences in bands intensities were analyzed using ImageJ 1.42q software (NIH, Bethesda, MD).

2.4. Estimation of relative growth rate

In order to determine the effects of fluoranthene on individual performance of L. *dispar* and *E. chrysorrhoea* larvae, we measured relative growth rate from molting into the third instar until the 3rd day of the fifth instar (RGRt), and during 3 days of the fifth instar ($RGR_{5/3}$). Relative growth rates were calculated as $RGR = (\ln LWt - \ln LW_0) / t$, where LW_0 and LWt represent the weight of larvae at the beginning and the end of monitored period, while t is the period in days. Sample sizes of the experimental groups of L. *dispar* larvae were N = 77–91, and those of the experimental groups of *E. chrysorrhoea* larvae were N = 69–83.

2.5. Statistical analyses

Mean values ± standard errors were calculated for specific activity of digestive enzymes and relative growth rates of L. *dispar* and *E. chrysorrhoea* larvae. Significance of fluoranthene effects on enzymes activity was revealed by one-way ANOVA, followed by Tukey's test. Analyses of variance were carried out on log-transformed values of digestive enzymes activity (Sokal and Rohlf, 1981). The influence of fluoranthene on relative growth rates of larvae was analyzed using Kruskal-Wallis ANOVA followed by multiple comparisons of mean ranks for all groups (Siegel and Castellan, 1988). P values lower than 0.05 were considered statistically significant. Canonical discriminant analysis (CDA) was used to evaluate differences between the two species for digestive enzyme activities after long-term exposure to dietary fluoranthene. Cluster analysis (Unweighted pair-group average; City-block Manhatten distances) was performed on Canonical scores to evaluate similarities between the parameters and the stressor effects. All analyses were performed in Statistica, version10 (StatSoft, Inc.).

3. Results

3.1. Lymantria dispar digestive enzymes

Specific activity of digestive enzymes in midgut homogenates of L. *dispar* fifth instar larvae from the control group and those exposed to dietary fluoranthene (Fl and Fh) are presented in Fig. 1. The specific activity of TRY was significantly lower [$F_{(2,26)} = 19.363$; $p = 0.0000$] in both groups exposed to fluoranthene, when compared to the control group. We identified two enzyme isoforms by electrophoresis. Isoform I1 was observed in all groups, with band intensity declining in fluoranthene treated larvae relative to the control group. Isoform I2 was observed only in the control group (Fig. 1a). Decreased specific activity of LAP [$F_{(2,27)} = 6.595$; $p = 0.0046$] was detected in the group of larvae exposed to the lower concentration of fluoranthene, when compared to the control group. Detection of LAP activity on native polyacrylamide gel revealed three isoforms. Isoform I1 was observed in all groups, while isoforms I2 and I3 appeared in the control and the group of larvae exposed to the higher fluoranthene concentration. Densitometric analysis showed decreased band intensity of isoform I1 in the group of larvae ingested lower concentration of fluoranthene in comparison to that for the control group (Fig. 1b).

There were no statistically significant differences in specific lipase activity in the midgut of L. *dispar* larvae exposed to dietary fluoranthene [$F_{(2,28)} = 0.614$; $p = 0.5484$] (Fig. 1c). Two lipase isoforms (I1 and I2) were detected on native PAGE zymogram in all three groups. A concentration-dependent increase in isoform 1 band intensity was detected in the groups of larvae exposed to dietary fluoranthene in comparison to the control group, whereas band intensity of isoform 2 did not differ between treatments (Fig. 1c).

3.2. Euproctis chrysorrhoea digestive enzymes

Specific activity of digestive enzymes in midgut homogenates of *E. chrysorrhoea* fifth instar larvae from the control group and those exposed to different concentrations of dietary fluoranthene (Fl and Fh) are shown in Fig. 2. Long-term exposure of *E. chrysorrhoea* larvae to dietary fluoranthene significantly influenced specific enzymes activity.

Namely, fluoranthene treatment had a marked effect on specific activity of TRY [$F_{(2,27)} = 13.348$; $p = 0.0001$], where the higher concentration (Fh) led to a significant decrease of enzyme activity (Fig. 2a). Native electrophoresis of TRY activity revealed the presence of four isoforms. Isoform I2 was detected only in the control group, while the other three appeared in all three groups of larvae. The most dominant TRY isoform was I4. Its band intensity was slightly lower in larvae given the higher concentration of fluoranthene relative to the control group. This trend was the same for the less expressed TRY isoforms (Fig. 2a).

Dietary fluoranthene significantly affected LAP specific activity [$F_{(2,26)} = 21.265$; $p = 0.0000$] in the midgut of *E. chrysorrhoea* larvae. With the lower concentration of dietary fluoranthene there was a significant increase in specific LAP activity in comparison to both control larvae and those exposed to the higher fluoranthene concentration (Fig. 2b). Native PAGE zymogram of LAP activity showed three different isoforms. The dominant isoform, I1, was expressed in the midguts of larvae from all three groups. Isoform I2 was detected in the groups given each concentration of fluoranthene, while isoform I3 was seen only in the group ingesting the higher amount of fluoranthene. Isoform I1 band intensity was slightly increased in experimental group Fl but had decreased in experimental group Fh relative to the control group (Fig. 2b).

The presence of fluoranthene in the rearing diet influenced lipase activity [$F_{(2,26)} = 30.284$; $p = 0.0000$] in the midgut of *E. chrysorrhoea* larvae (Fig. 2c). Thus, significantly increased specific lipase activity was recorded in the group of larvae ingesting the lower concentration of dietary fluoranthene. We identified two isoforms, I1 and I2, by electrophoresis in all three groups. Densitometric analysis showed increased band intensities of both isoforms in the group exposed to the lower concentration of fluoranthene, in comparison to the control group (Fig. 2c).

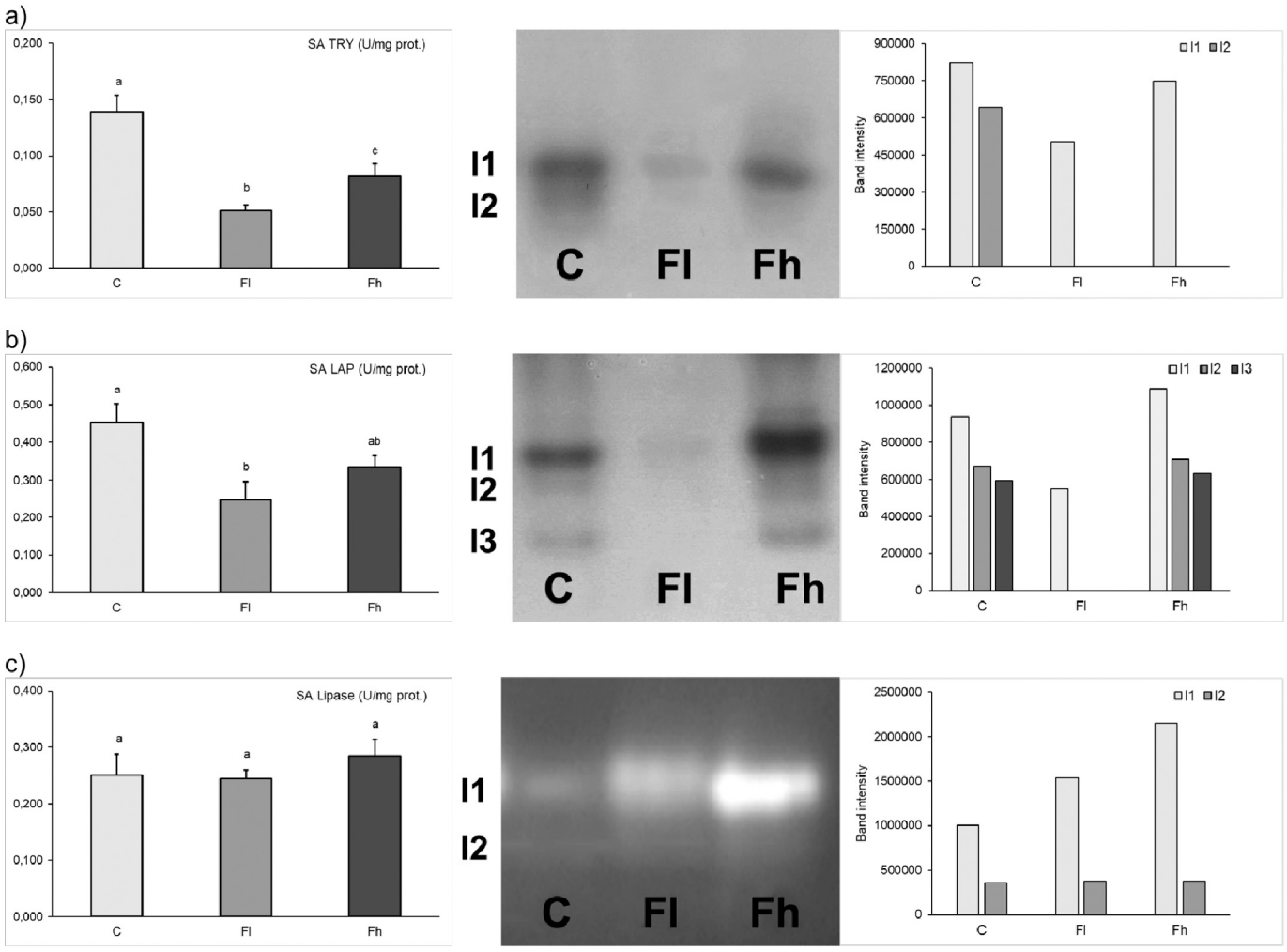

Fig. 1. Specific activities, and native polyacrylamide gels with densitometric analyses of bands intensities of: trypsin (a), leucine aminopeptidase (b), and lipase (c) in the midgut of *Lymantria dispar* larvae exposed to lower (6.7 ng/g dry wt) (Fl), and higher (67 ng/g dry wt) (Fh) concentration of dietary fluoranthene. The bars represent mean values (±SE). Values marked with different letters differ significantly, ($P < 0.05$). The numbers indicate enzymes isoforms (I).

3.3. Relative growth rates

Relative growth rates (RGRt and $RGR_{5/3}$) of L. *dispar* and *E. chrysorrhoea* larvae long-term exposed to fluoranthene-supplemented diets (Fl, Fh groups), as well as the control groups (C) are given in Fig. 3. Significantly decreased RGRt [$H(2,251) = 12.301$; $p = 0.0021$] and $RGR_{5/3}$ [$H(2,250) = 16.616$; $p = 0.0002$] were recorded in L. *dispar* larvae from groups Fl and Fh (Fig. 3a). Also, significant decreases of RGRt [$H(2,222) = 39.993$; $p = 0.0000$] and $RGR_{5/3}$ [$H(2,221) = 24.821$; $p = 0.0000$] were detected in *E. chrysorrhoea* larvae from groups Fl and Fh (Fig. 3b).

3.4. CDA and cluster analysis

Canonical discriminant analysis (CDA) and cluster analysis of digestive enzyme activities in the midguts of L. *dispar* and *E. chrysorrhoea* larvae revealed separation of the two species after treatment with both concentrations of fluoranthene (Fig. 4). The first canonical function for the lower fluoranthene concentration Fl (Root 1) accounted for 73% of total heterogeneity, while the second canonical function (Root 2) accounted for 27% of total heterogeneity. The parameter that contributed most to the separation along Root 1 was leucine aminopeptidase, while on Root 2 it was trypsin (Fig. 4a). Separation of the response of the two species to the higher concentration of dietary fluoranthene along Root 1 carried 87.1% of the heterogeneity. The second canonical function (Root 2) in the analysis for Fh accounted for 11.9% of total heterogeneity. The parameters that led to the observed separation were lipase along Root 1 and trypsin along Root 2 (Fig. 4b).

4. Discussion

The present study revealed significant changes of digestive enzymes activity in the midguts of *L. dispar* and *E. chrysorrhoea* larvae, in response to ingestion of environmentally relevant concentrations of fluoranthene. In order to acquire necessary nutrients, larvae of both species consume large amounts of leaves of their host plants. Conversion of ingested nutrients and provision of the energy resources required for growth and metabolism, are ensured through efficient digestive enzymes activity. The presence of toxins in food may markedly affect the activity of these enzymes, so insects may respond by changing their activity levels and/or by synthesis of less sensitive or insensitive isoforms. Pollutant actions on digestive enzymes can have an impact on their synthesis or secretion (Dedourge-Geffard et al., 2013). Thus, the potential importance of these enzymes as biomarkers of exposure to toxicants has been pointed out (Lagadic et al., 1994; Hyne and Maher, 2003; Lai et al., 2011). Moreover, harmful effects of xenobiotics on digestive enzymes activity may influence the growth and development of organisms.

In this study we reported that chronic exposure to fluoranthene induced changes in trypsin and leucine aminopeptidase specific activities in the midgut of L. *dispar* larvae. Both enzymes exhibited the same

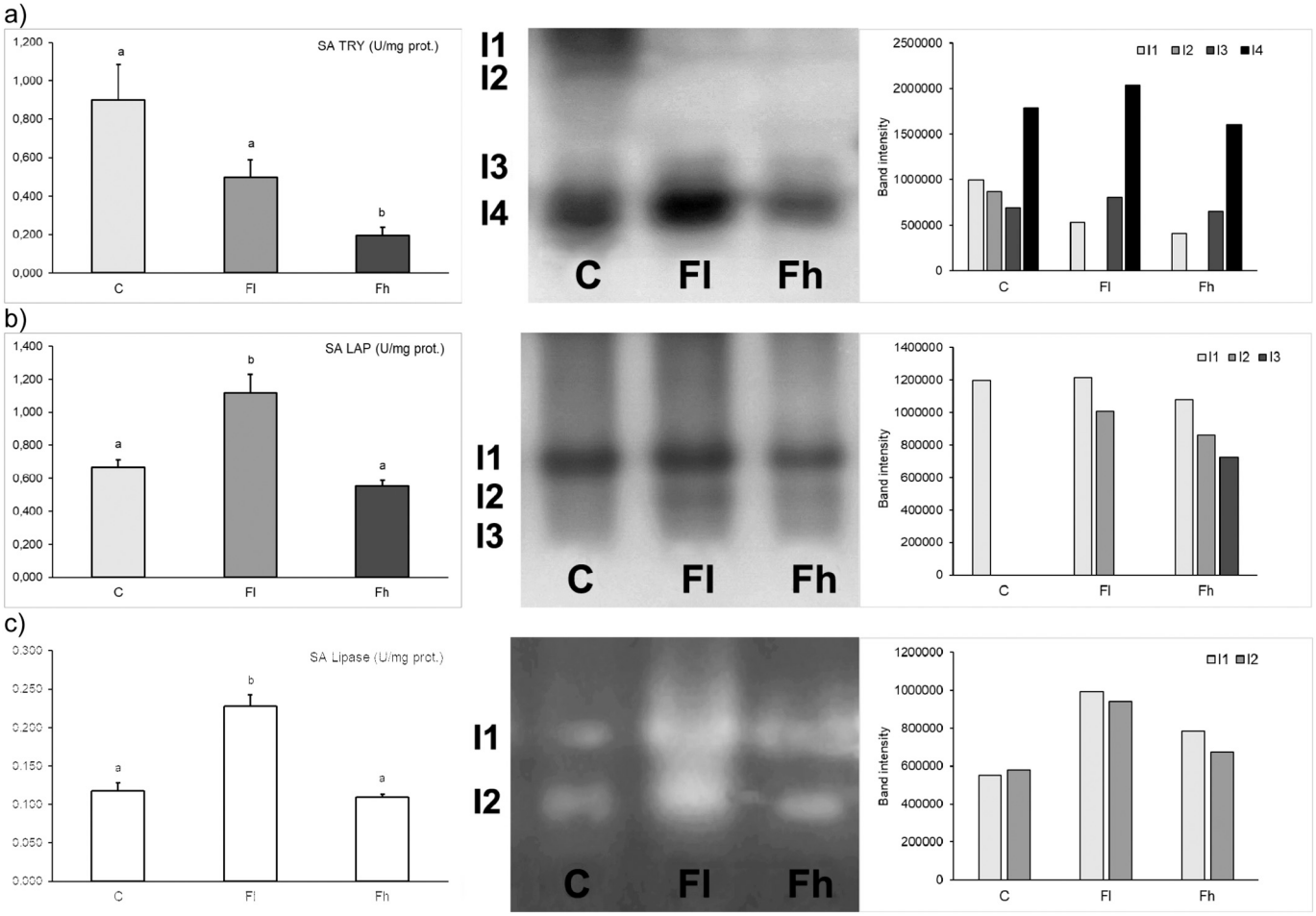

Fig. 2. Specific activities, and native polyacrylamide gels with densitometric analyses of bands intensities of: trypsin (a), leucine aminopeptidase (b), and lipase (c) in the midgut of *Euproctis chrysorrhoea* larvae exposed to lower (6.7 ng/g dry wt) (Fl), and higher (67 ng/g dry wt) (Fh) concentration of dietary fluoranthene. The bars represent mean values (±SE). Values marked with different letters differ significantly, ($P < 0.05$). The numbers indicate enzymes isoforms (I).

pattern of inhibition as a result of exposure to the lower dietary fluoranthene concentration, compared to the control. However, trypsin expressed higher sensitivity, and its activity was also significantly reduced in response to the higher fluoranthene concentration. Lowe et al. (1981) found that long-term exposure of mussels *Mitilys edulis* to mixed PAHs altered the structure of digestive cells, disrupted their synchronicity and affected cellular lysosomes structure. It is possible that fluoranthene has similar toxic effects on larval midgut epithelial cells, disturbing structural and functional features that can lead to reduced enzyme synthesis and/or secretion. Another possibility is that fluoranthene inhibit enzyme activity directly, by blocking the catalytic site or amino acid residues involved in binding and stabilization of the substrate. Similar mechanism of trypsin inhibition was described by Napoleão et al. (2012), who exposed *Aedes aegypti* larvae *to Myracrodruon urundeuva* leaf lectin. In addition, inhibition of trypsin and leucine aminopeptidase activities was reported in L. *dispar* larvae exposed to cadmium (Vlahović et al., 2015), while trypsin activity was reduced in the midgut of *Helicoverpa armigera* larvae fed on a diet containing plant inducible LAP (Lomate et al., 2013). It was found that phytophagous insects can overcome some harmful effects of host plants due to digestive enzyme plasticity, rapid and efficient modulation of their activity (Broadway, 1997; Lomate and Hivrale, 2011; War et al., 2013). Mrdaković et al. (2014) reported decreased trypsin activity in response to allelochemical stress, while increased lipase activity in a stressful environment was shown to be an adaptive response of L. *dispar* larvae.

Interestingly, *E. chrysorrhoea* larvae showed a different response of digestive enzymes to fluoranthene exposure. We observed decreased trypsin activity with the higher concentration of fluoranthene. On the other hand, leucine aminopeptidase activity was significantly increased in larvae exposed to the lower concentration of dietary fluoranthene. The increase of leucine aminopeptidase activity under fluoranthene exposure might lead to better utilization of food by enabling efficient digestion of oligopeptides. We also recorded a significant increase in lipase activity in *E. chrysorrhoea* larvae fed the lower concentration of fluoranthene compared to the control. Increased lipase activity was observed in other lepidopteran larvae exposed to nutritive and temperature stress (Janković-Tomanić, 2012; Sarate et al., 2012). This suggests that fluoranthene treated *E. chrysorrhoea* larvae have a greater need for lipid nutrients as an important source of energy. Higher lipase activity may provide more energy for activation of defense mechanisms that help larvae to overcome harmful effects of xenobiotics and is important for energy storage. High lipase activity may reflect the increased needs for dietary lipids of older larval instars in species with a non-feeding adult stage (Stockhoff, 1993 and references therein). Lavarías et al. (2006) observed raised lipase activity in the digestive gland of *Macrobrachium borellii* exposed to the soluble fraction of oil, i.e. a mixture of monocyclic aromatic hydrocarbons. They concluded that the presence of organic pollutants causes high energy consumption and constant mobilization of energy reserves.

Enzyme isoforms can be differentiated by their sensitivity to a particular toxicant and may respond in diverse ways that include inhibition or activation (Sanchez-Hernandez, 2011). Our results show that, depending on the fluoranthene concentration applied and the treated species, different enzyme isoforms showed differences in activity.

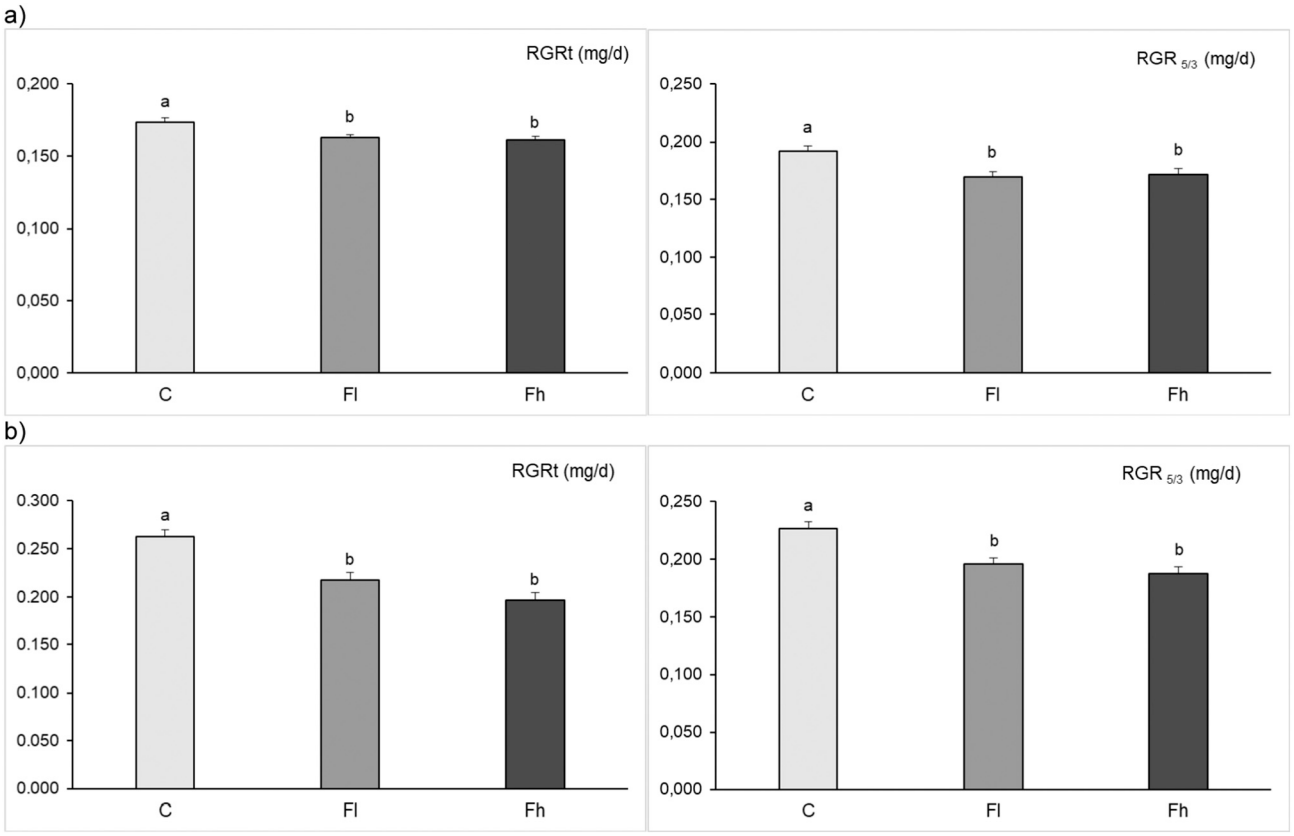

Fig. 3. Relative growth rate from the third instar until the 3rd day of the fifth instar (RGRt), and during 3 days of the fifth instar ($RGR_{5/3}$) of *Lymantria dispar* (a) and *Euproctis chrysorrhoea* (b) larvae exposed to lower (6.7 ng/g dry wt) (Fl), and higher (67 ng/g dry wt) (Fh) concentration of dietary fluoranthene. The bars represent mean values (±SE). Values marked with different letters differ significantly, ($P < 0.05$).

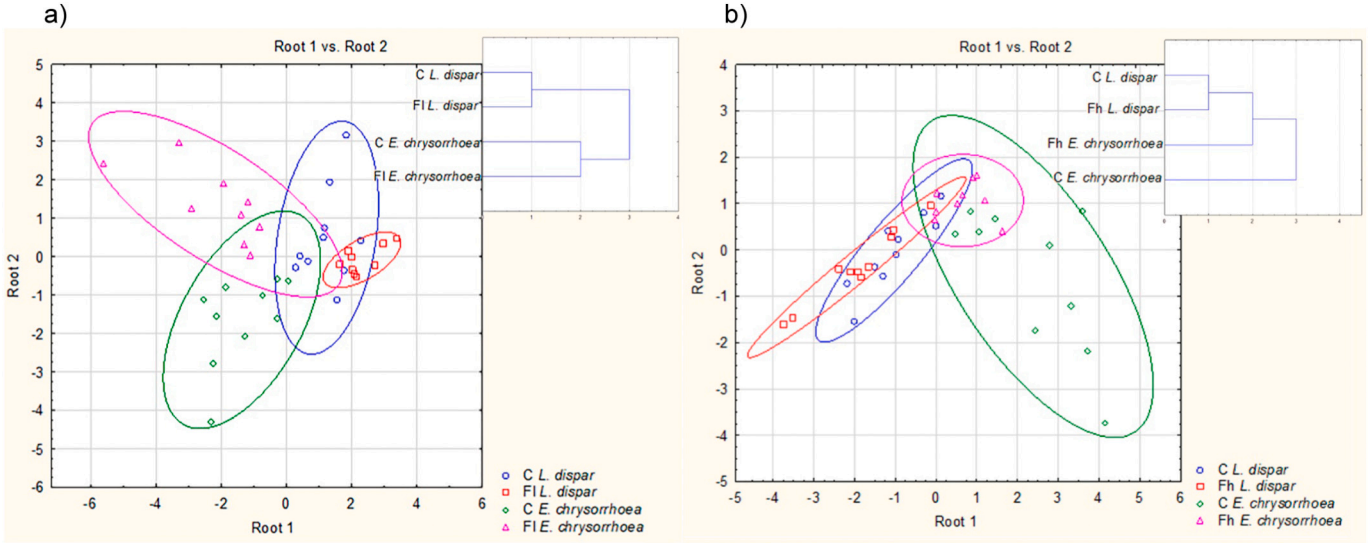

Fig. 4. Canonical discriminant and cluster analyses for digestive enzyme activities in the midguts of *Lymantria dispar* and *Euproctis chrysorrhoea* larvae exposed to lower (a) (Fl - 6.7 ng/g dry wt), and higher (b) (Fh - 67 ng/g dry wt) concentration of dietary fluoranthene.

Although *E. chrysorrhoea* larvae had a greater number of midgut trypsin isoforms, in both species we observed inhibition of isoform I2 in the midguts of larvae exposed to fluoranthene. On the other hand, expression of the most dominant trypsin isoform I4 in the midgut of *E. chrysorrhoea* larvae exposed to the lower fluoranthene concentration, could indicate induction of a less sensitive isoform that enables efficient digestion of proteins. Also, we recorded differences in specific leucine aminopeptidase activity between the species. Exposure of larvae to the lower concentration of fluoranthene elevated leucine aminopeptidase activity in the midgut of *E. chrysorrhoea* larvae, but decreased it in the midgut of L. *dispar* larvae when compared to their control group values. Several studies pointed out important roles of differently expressed protease isoforms in lepidopteran species, as a response to nutritive stress, plant proteinase inhibitors and toxin actions (Chikate et al., 2013;

Hivrale et al., 2013; Azimi et al., 2020; Chauhan et al., 2021). Therefore, the explanation for the difference in specific leucine aminopeptidase activity between the species may be found in different expression pattern of leucine aminopeptidase isoforms. We observed inhibition of isoforms I2 and I3 in L. *dispar* larvae fed with the lower concentration of fluoranthene compared to the control. On the other hand, isoform I2 appeared in *E. chrysorrhoea* larvae given the lower concentration of fluoranthene, but not in the control group. We assume that expression of a less sensitive leucine aminopeptidase isoform in the midgut of *E. chrysorrhoea* larvae during fluoranthene exposure is the reason of the increased specific enzyme activity. Expression of lipase isoform in the midgut of *L. dispar* larvae was dependent on pollutant concentration. Intensity of expression of both lipase isoforms in the midgut of *E. chrysorrhoea* larvae was the highest in the group exposed to the lower fluoranthene concentration, which corresponds to detected specific activity of the enzyme, and probably enables more efficient digestion of dietary lipids in a stressful condition. Changes in enzyme isoform expression suggest an adaptive response of larvae to the presence of the toxicant. Isozyme variability might become a valuable tool for monitoring populations of organisms that are sensitive to chemical pollutants (Guttman, 1994; Yap et al., 2011). Canonical discriminant analysis revealed that digestive enzyme activities significantly contributed to differentiation between the species. Despite the diverse responses of digestive enzymes activity to fluoranthene exposure between L. *dispar* and *E. chrysorrhoea* larvae, fitness-related traits of both species were affected.

Induction of various defense mechanisms enables insects to survive stressful conditions caused by xenobiotics (Van Straalen and Hoffmann, 2000). In previous research (Filipović et al., 2019) we showed that a significant part of energy resources in L. *dispar* and *E. chrysorrhoea* larvae is directed towards activation of antioxidative and detoxifying enzymes, which represent the first line of defense against oxidative stress. In the present paper, we observed changes in the specific activities of digestive enzymes and patterns of isozyme expression resulting from stress provoked by ingestion of fluoranthene. Inhibition/stimulation of certain digestive enzymes can lead to metabolic imbalance, reduced larval growth or even mortality of insect larvae (Applebaum et al., 1961; Bhattacharyya et al., 2007; Babu and Subrahmanyam, 2010). Increased use of energy resources to maintain homeostasis in organisms under stress influences fitness-related traits. Thus, we recorded reduced relative growth rates of L. *dispar* and *E. chrysorrhoea* larvae exposed to dietary fluoranthene, from the third instar until the 3rd day of the fifth instar and over three days of the fifth instar. This period of larval stage is characterized by higher food consumption and hence their maximal weight gain, and also by a change in nutritional demands of larvae (Kelly et al., 1989; Stockhoff, 1993). As already mentioned, previously revealed induction of defending mechanisms in larvae of L. *dispar* and *E. chrysorrhoea* long-term exposed to fluoranthene treatments (Filipović et al., 2019), and the responses of digestive enzymes, reported for larvae of both species in the present study, probably led to higher resource allocation towards defense and metabolism in larvae exposed to pollutant, and to decreased relative growth rates. The last larval instars are characterized by changes related to metamorphosis, and imply the accumulation of resources necessary for the pupal and adult stage (Stockhoff, 1993; Ojeda-Avila et al., 2003). Decreased relative growth rates of *L. dispar* and *E. chrysorrhoea* larvae exposed to dietary fluoranthene could also be a consequence of reduced consumption rate, as it has been shown in *Manduca sexta* larvae which grow slowly when they were exposed to variation in diet components, while its compensatory feeding for protein was less pronounced in fourth and fifth instar, compared to the third one (Ojeda-Avila et al., 2003).

Although markedly lower instantaneous RGR of the last instars of several lepidopteran larvae has been reported by Tammaru and Esperk (2007), we noticed slightly higher values of $RGR_{5/3}$ than of RGRt in L. *dispar* larvae from all experimental groups, which was not detected in *E. chrysorrhoea* larvae. Great percent of larval nutrition is achieved in the last larval instars (Leonard, 1974), and higher $RGR_{5/3}$ of larvae of this species may be due to more intensive feeding during this period, allowing them to reach pupal stage faster, despite pollutant treatments.

Harmful effects of the PAHs, benzo[*a*]pyrene and fluoranthene, on relative growth rate of L. *dispar* larvae have already been demonstrated (Ilijin et al., 2015; Mrdaković et al., 2015; Grčić et al., 2019). Insect growth is affected by various factors that may change their physiology and behavior. Data indicate that larvae of Lepidoptera do not grow at their physiological maximum, but rather adjust growth to the optimal (Gotthard, 2008), depending on the different and often stressful environmental conditions they encounter.

The recorded changes of digestive enzymes activity and different expression of their isoforms enabled optimal growth of larvae under the given conditions. Examining the relationship between physiological changes and fitness-related trait in larvae of L. *dispar* and *E. chrysorrhoea* exposed to environmentally relevant concentrations of dietary fluoranthene, may help us to understand their mechanisms of adjustment to stressful conditions, and to predict possible effects of pollutant on their populations. Furthermore, detected changes in trypsin and leucine aminopeptidase activities and isoform expression indicate that these enzymes could be considered as potential biomarkers of fluoranthene pollution in the environment.

CRediT authorship contribution statement

Aleksandra Filipović: Investigation, Writing- Original draft preparation, Writing- Reviewing and Editing. **Marija Mrdaković:** Conceptualization, Methodology, Supervision. **Larisa Ilijin:** Resources, Formal analysis**. Anja Grčić:** Investigation, Formal analysis. **Dragana Matić:** Visualization, Software**. Dajana Todorović**: Visualization. **Milena Vlahović:** Validation. **Vesna Perić-Mataruga:** Project administration, Validation, Formal analysis.

Declaration of competing interest

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

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