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PII: S1532-0456(19)30275-3
DOI: https://doi.org/10.1016/j.cbpc.2019.108565
Article Number: 108565
Reference: CBC 108565

To appear in: *Comparative Biochemistry and Physiology, Part C*

Received date: 14 February 2019
Revised date: 25 June 2019
Accepted date: 29 June 2019

Please cite this article as: A. Filipović, M. Mrdaković, L. Ilijin, et al., Effect of fluoranthene on antioxidative defense in different tissues of Lymantria dispar and Euproctis chrysorrhoea larvae, Comparative Biochemistry and Physiology, Part C, https://doi.org/10.1016/j.cbpc.2019.108565

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Effect of fluoranthene on antioxidative defense in different tissues of *Lymantria dispar* and *Euproctis chrysorrhoea* larvae

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ABSTRACT

This study examined the effect of long-term exposure to environmentally relevant concentrations of dietary fluoranthene (6.7 and 67 ng / g dry food weight) on defense mechanisms of the polyphagous forest insects *Lymantria dispar* L. and *Euproctis chrysorrhoea* L. The activities and expression of isoforms of superoxide dismutase (SOD) and catalase (CAT), the activities of glutathione S-transferase (GST) and glutathione reductase (GR), and total glutathione content (GSH) were determined in the whole midgut and midgut tissue, while SOD and CAT activities were assessed in hemolymph of the larvae. The results showed significant changes of enzyme activities, with more pronounced responses in larval midgut tissues, and between-species differences in patterns of response. Significantly increased activity of SOD was recorded in the whole midgut and midgut tissue of *L. dispar* larvae, as well as in midgut tissue of *E. chrysorrhoea* larvae. Fluoranthene increased CAT activity in midgut tissue

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of *L. dispar* larvae, and in the whole midgut and midgut tissue of *E. chrysorrhoea* larvae. Different expression patterns were detected for enzyme isoforms in tissues of larvae exposed to dietary fluoranthene. Total GSH content and GST activity increased in *E. chrysorrhoea* larval midgut tissue. Significantly decreased SOD activity in hemolymph of *L. dispar* larvae, and opposite changes in CAT activity were recorded in the hemolymph of larvae of two insect species. The tissue-specific responses of enzymes to dietary fluoranthene, recorded in each species, enabled the larvae to overcome the pollutant induced oxidative stress, and suggest further assessment of their possible use as early-warning signals of environmental pollution.

Keywords: Fluoranthene · oxidative stress · antioxidative enzymes · midgut · hemolymph · *Lymantria dispar* L. · *Euproctis chrysorrhoea* L.

Introduction

Polycyclic aromatic hydrocarbons (PAHs), widely distributed organic pollutants, are emitted into the environment mainly as a result of anthropogenic activities like incomplete combustion of fossil fuels, emission of automobile exhaust fumes, domestic heating, cooking and tobacco smoking, etc. Sixteen PAHs, including fluoranthene, are described as priority pollutants by the US Environmental Protection Agency due to their recognized toxicity towards animals and humans. The four ringed PAH fluoranthene is a persistent and bioaccumulative compound which occurs in the atmosphere in both gas and particulate phases (ECHA, 2018). Fluoranthene is an important representative of the PAHs and is often used as a marker for total PAH exposure in industry and the environment. It is known as a pollutant with toxic and co-carcinogenic effects (Palmqvist et al., 2003; Srogi, 2007; Bauer et al., 2017).

Fluoranthene is present in forest ecosystems and strongly influences physiological processes and growth in higher plants (Berteigne et al., 1989; Oguntimehin et al., 2008), which can consequently affect the physiology of herbivores, including insects. Thus, through changes in nutritional quality or the defense ability of plants, pollutants

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can influence phytophagous insect species (usually by reducing their performance) and their population dynamics (Butler and Trumble, 2008; Holopainen, 2009). There are approximately 160,000 species of Lepidopteran insects in the world, many of which inhabit forest ecosystems. Fluoranthene is denoted as one of the dominant PAH pollutants in leaves of oak species (Howsam et al., 2000; De Nicola et al., 2008), suitable host plants for larvae of the selected insect species, *Lymantria dispar* and *Euproctis chrysorrhoea*.

Lymantria dispar L. and *Euproctis chrysorrhoea* L. (Lepidoptera, Erebidae) are widespread phytophagous forest insects with a broad range of host plants (Lance, 1983; Forestry Compendium, 2005). The larvae of both species hatch out and appear, respectively, in the spring and feed continually on buds and leaves of host plants. Outbreaks of these economically very important forest insects may occur at the same time. The enlargement of our knowledge about their physiological reactions to PAHs, their defense mechanisms and how they adjust to the presence of environmental xenobiotics is of great importance.

Feeding on PAH polluted leaves may influence the structure and functions of the insect gut. The object of our research, the midgut, is the largest part of the Lepidopteran alimentary tract and the main site where food is digested and absorbed but also a very important barrier to toxins/xenobiotics. Midgut epithelial cells secrete the peritrophic membrane, a unique acellular semi-permeable barrier that protects epithelia from damage by toxins in the diet (Barbehenn and Stannard, 2004). PAHs and their reactive metabolites can generate reactive oxygen species (ROS) which induce oxidative stress and damage to cellular macromolecules (Miller and Ramos, 2001). The midgut possesses a set of enzymes and non-enzymatic components that prevent ROS induced oxidative injuries. Thus, the enzyme superoxide dismutase (SOD) catalyzes the dismutation of superoxide anion radical into hydrogen peroxide, which can be decomposed by catalase (CAT) activity to water and oxygen. Hydrogen peroxide can also be eliminated by glutathione-dependent enzyme. Glutathione S-transferase (GST), an enzyme of phase II biotransformation, catalyzes the conjugation of electrophilic toxic molecules to glutathione (GSH), thereby increasing their solubility and elimination from

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the organism. It may also be considered part of an insect's antioxidant defense mechanism, since it removes hydroperoxides. Glutathione reductase (GR) does not act directly on ROS but enables glutathione-dependent enzyme action by converting oxidized glutathione to its active reduced form. The activities of antioxidative enzymes are tissue-specific and more prominent in metabolically active tissues. They depend on the developmental stages and can vary among different species. The non-enzymatic components include glutathione (GSH) which helps to maintain redox homeostasis. Finally, the peritrophic membrane is known as the "sacrificial antioxidant" since it is preferentially oxidized by reactive species to protect more important biomolecules (Ahmad and Pardini, 1990; Ahmad et al., 1991; Ahmad, 1992; Barbehenn et al., 2001; Barbehenn and Stannard, 2004).

Significant increases in antioxidative enzyme activity in response to oxidative stress was recorded in the insect midgut, while activity in hemolymph usually remained low (Ahmad 1992), although free radical species are produced in hemolymph (Toru 1994, 1995). Hemolymph is a transfer medium for xenobiotics and/or their metabolites, and contains cell elements known as hemocytes which play a role in immune defense. They may be damaged by xenobiotic induced oxidative and genotoxic effects. Components of antioxidative defense in hemolymph cells like SOD, CAT and GST, as well as non-enyzmatic antioxidants such as thiols and ascorbates, have a key role in elimination of ROS and maintenance of the optimal redox state (Büyükgüzel et al., 2010; Dubovskii et al., 2010).

Changes in the activity of antioxidative and detoxifying enzymes and expression of their different isoforms indicate the physiological state of insects in stressful conditions, and may serve as early-warning signals of exposure to various pollutants (Stone et al., 2002; Ilijin et al., 2015; Gavrilović et al., 2017). As species with defined and well known development, with continual larval feeding and significant bioaccumulation potential, *L. dispar* and *E. chrysorrhoea* may be suitable organisms for environmental biomonitoring studies. In addition, our previous studies indicated the sensitivity of gypsy moth larvae to the effects of environmental pollutant (Mirčić et al., 2013; Vlahović et al., 2013; Matić et al., 2016; Perić-Mataruga et al., 2019).

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The aim of this study was to investigate whether environmentally relevant concentrations of fluoranthene affect the activity of antioxidative (SOD, CAT, and GR) and phase II biotransformation (GST) enzymes and total GSH concentration in the midgut and midgut tissue, as well as SOD and CAT activities in the hemolymph of *L. dispar* and *E. chrysorrhoea* larvae originating from natural populations. We examined if there are tissue-specific responses, as well as differences between species in response to the fluoranthene treatments. This study provides the first comparison between the activities of the defense components of *L. dispar* and *E. chrysorrhoea* when exposed to a dietary organic pollutant.

Materials and Methods

Insect Rearing

L. dispar and *E. chrysorrhoea* are highly polyphagous univoltine forest species. Development of *L. dispar* larvae is characterized by five (males) and six (females) instars, and a diapause which fully differentiated pharate first instar larvae spend within eggs (Leonard, 1968). The number of *E. chrysorrhoea* larval instars varies between five and eight, and young (second and third instar) larvae enter diapause inside communal winter nests (Elkinton et al., 2006; Frago et al., 2009). Larvae of both species appear in the spring, synchronized with the appearance of new leaves and buds of their host plants, and the majority of growth occurs during this period of their life cycles. Larvae eat large amounts of leaves, particularly in later instars, which is important for growth, but also for accruing reserves needed for non-feeding adult stages. During feeding, larvae may be exposed to allelochemicals and various pollutants, e.g. PAHs, which are ingested with host plant leaves. *L. dispar* egg masses were collected in November, from a mixed oak forest near the city of Majdanpek (National Park “Djerdap”, East Serbia), and kept in a refrigerator at 4°C until April, when they were enabled to hatch by transfer to 23 ± 0.5°C and a 12^{h} photoperiod. *E. chrysorrhoea* winter nests were collected in February from a mixed oak forest near the city of Prijepolje (Southwest Serbia), and also kept at 4°C until late March, when the collected nests interwoven with old leaves were placed on branches of wild plum with buds, at room temperature. Newly emerged larvae were then moved to 26 ± 0.5°C and a 16^{h} photoperiod, i.e. laboratory conditions

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favorable for initiation of feeding activity and post-diapausing molting (Kelly et al., 1989; Frago et al., 2009). The localities from which *L. dispar* egg masses and *E. chrysorrhoea* winter nests were collected are considered unpolluted. Majdanpek forest locality belongs to the largest National park in Serbia "Djerdap", while the forest locality near the city of Prijepolje is part of a protected natural area (Institute for Nature Conservation of Serbia, www.zzps.rs). Larvae of both species were reared on an artificial high wheat germ (HWG) diet (O'Dell et al., 1985). They were randomly assigned to control groups and groups given the HWG diet supplemented with fluoranthene. Control larvae received the fluoranthene-free HWG diet (C), while the experimental groups were fed either 6.7 ng fluoranthene / g dry food weight (Fl) or 67 ng fluoranthene / g dry food weight (Fh). The lower fluoranthene concentration was selected according to the amount previously recorded in leaves of several tree species (Howsam et al., 2000), among others, in leaves of suitable host plants for both *L. dispar* and *E. chrysorrhoea* larvae. The higher concentration is in the range of those detected in leaves of various plant species from rural and urban areas (e.g. Alfani et al., 2001; Tian et al., 2008). In addition, our previous research on the effects of a wide range fluoranthene concentrations revealed the most prominent influence of the chosen concentrations (6.7 and 67 ng of fluoranthene) on larval mass, relative growth rate, and enzyme activities in gypsy moths (Mrdaković et al., 2015). The food supplemented with fluoranthene was prepared by mixing the HWG diet with fluoranthene dissolved in reagent-grade acetone. The portions were kept for 4^{h} in a fume hood until the acetone had evaporated. Larvae were checked daily for molting, and equal amounts of fresh food placed every second day. Larvae were reared until the 3^{rd} day of the fifth instar when they were sacrificed, and enzyme activities and total GSH concentrations determined in larval tissues. Sample sizes of the experimental groups of *L. dispar* larvae for whole midgut were: N = 22-30 (C), N = 15-28 (Fl) and N = 23-28 (Fh); for midgut tissue: N = 7-9 (C), N = 9-10 (Fl) and N = 9-14 (Fh); for hemolymph: N = 10 (C), N = 10-11 (Fl) and N = 10 (Fh). Sample sizes of the experimental groups of *E. chrysorrhoea* larvae for whole midgut were: N = 20-28 (C), N = 14-24 (Fl) and N = 24-29 (Fh); for midgut tissue: N = 8-10 (C), N = 9-12 (Fl) and N = 9-10 (Fh); for hemolymph: N = 10 (C), N = 11 (Fl) and N = 9-11 (Fh).

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Preparation of homogenates

L. dispar and *E. chrysorrhoea* larvae from all experimental groups were sacrificed on ice. Midguts were removed. Whole midguts and midguts with removed peritrophic membranes (referred to as midgut tissue) were washed several times with ice-cold saline solution and kept at -20°C until homogenization. Hemolymph was collected and also kept at -20°C until needed. The whole midguts and midgut tissues were weighed and diluted (1 g of tissue : 5 ml of buffer) with an ice cold 0.25 M sucrose buffer (0.05 M Tris-HCl, 1 mM EDTA; pH 7.4). They were homogenized individually on ice at 2000 rpm for 3×10 s with 15 s pauses, using an Ultra Turrax homogenizer (IKA-Werke, Staufen, Germany) and then sonified for 3×15 s with 15 s pauses in an ultrasonic homogenizer (HD 2070, Bandelin, Berlin, Germany). The sonicated homogenates were centrifuged at 105,000×g for 100 min at 4°C (Beckman L7-55 Ultracentrifuge) and the resulting supernatants were used for the enzymatic assays. An aliquot of the sonicated homogenates was mixed with 10% sulfosalicylic acid and centrifuged at 10,000×g for 20 min at 4°C (5417R, Eppendorf, Hamburg, Germany) for determination of total GSH concentration in the supernatants.

Hemolymph samples were sonicated individually in ice cold buffer pH 7 containing 1.15 % KCl, 25 mM K_2HPO_4, 5 mM PMSF and 2 mM DDT (ultrasonic homogenizer Bandelin HD 2070), and centrifuged at 10,000×g for 15 min at 4°C (Eppendorf centrifuge 5417R). SOD and CAT activities were determined in the resulting supernatants.

Enzyme assays

Protein concentration was determined according to Bradford (1976), with bovine serum albumin as the standard. SOD activity was determined according to Misra and Fridovich (1972). This method is based on the ability of SOD to prevent adrenaline autoxidation in an alkaline medium. The intensity of adrenaline autooxidation was measured spectrophotometrically as changes in absorption at λ = 480 nm for 10 min at 25°C. SOD activity is expressed as the amount of enzyme causing 50% inhibition of adrenaline autooxidation, in units per mg of protein. The activity of CAT was obtained by measuring the decomposition of H_2O_2 at λ = 240 nm at 25°C, for a period of 3 min (Claiborne,

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1984), and expressed as the amount of enzyme that catalyzed the reduction of 1 µmol of H_2O_2 per min. GST catalyzes the conjugation of 1-chloro-2.4-dinitrobenzene (CDNB) with the SH groups of GSH. The amount of derived CDNB-GSH complex was measured spectrophotometrically at λ = 340 nm at 25°C, for a period of 3 min (Habig et al. 1974) and expressed in nanomoles GSH per minute per milligram protein. The activity of GR was measured at λ = 340 nm over 3 min at 25°C using the method of Glatzle et al. (1974). One unit of GR activity was defined as the amount of enzyme that oxidizes 1 nmol of NADPH per min. Total GSH concentration was determined at λ = 412 nm at 30°C, for 4 min, by the method of Griffith (1980) and expressed in nmol/g tissue. In every assay, two blanks, three controls for noncatalytic activity, and three replicates for each experimental group were included.

Electrophoresis

SOD activity was detected on 12% nondenaturing polyacrylamide gel (Laemmli, 1970) at 100 V, 20 mA, for 3 h at 4°C, with 10 mg of protein placed in each lane. After electrophoresis, the gel was washed with deionized water and soaked in the dark in 50 mM carbonate bicarbonate buffer (pH 10.2) containing 1 mM EDTA, 0.05 mM riboflavin, 0.1 mM nitroblue tetrazolium and 0.3% tetramethylethylenediamine (TEMED) for 30 min at room temperature. The gel was briefly washed again and exposed to daylight until bright, transparent bands of SOD activity appeared (Salin and McCord, 1975). CAT activity was detected on 8% nondenaturing polyacrylamide gel (Stuber et al., 1988) under the same conditions as for SOD. After electrophoresis the gel was rinsed with water twice and incubated in the dark for 20 min in 50 mM phosphate buffer (pH 7.8) containing 0.01 M H_2O_2, at room temperature. Next, the gel was soaked in a mixture of 2 % $FeCl_3$ and 2 % $K_3[Fe(CN)_6]$ and exposed to daylight until bright yellow bands of CAT activity appeared (Aebi, 1983; Davis, 1964). All gels were scanned with a CanoScan LiDE 120 scanner.

Statistical analyses

Statistical analyses were performed using the Statistica 10.0 program. Mean values and standard errors were calculated for enzymes activities and total GSH concentrations.

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Following the assessment of normality (Shapiro-Wilk test) and homogeneity of variance (Levene`s test), one-way ANOVA followed by the Unequal N HSD test were applied on log-transformed values of the traits (Sokal and Rohlf, 1981). The level of significance for all comparisons was set at P lower than 0.05. Canonical discriminant analysis (CDA) was used to evaluate differences between the two species for enzyme activities and GSH concentrations in larval whole midguts and midgut tissues, after long-term exposure to dietary fluoranthene at 6.7 ng and 67 ng / g dry weight of the diet. Cluster analysis (Unweighted pair-group average; City-block Manhatten distances) was performed on Canonical scores to examine similarities between the parameters and the stressor effects.

Results

Enzyme activities in the whole midguts of *L. dispar* and *E. chrysorrhoea* larvae

Differences in enzyme activity and GSH concentration in homogenates of whole midguts of *L. dispar* larvae between the control group and those exposed to two dietary concentrations of fluoranthene (Fl and Fh) are presented in Fig. 1a. SOD activity was increased in both groups of larvae receiving fluoranthene [$F_{(2,74)}$ = 4.489, P = 0.0145] compared to the control group. Three enzyme isoforms were detected by native electrophoresis; two isoforms (I1 and I3) were expressed in whole midgut of larvae from all three groups, while one SOD isoform (I2) was detected only in whole midgut of larvae ingesting each dose of fluoranthene. CAT activity was much higher in larvae exposed to the higher fluoranthene concentration than that recorded for those given the lower amount [$F_{(2,72)}$=5.415; P=0.0064], but differences in comparison to the control group did not achieve statistical significance. The native PAGE zymogram of CAT activity revealed one isoform expressed in whole midguts of larvae from all three groups. No significant differences were observed for GST activity and GSH concentration between the groups of larvae exposed to dietary fluoranthene and the control groups. GR activity decreased in larvae exposed to the lower fluoranthene concentration [$F_{(2,\ 57)}$= 3.984, P= 0.0240] when compared with that detected in the control group (Fig. 1a).

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Enzyme activities and GSH concentration in homogenates of whole midguts from *E. chryssorhoea* larvae are presented in Fig. 1b. Compared to control group values, only CAT activity was significantly raised and solely in the group of larvae given the higher dietary fluoranthene concentration [$F_{(2,76)}$=6.408; P=0.0027]. Thus, SOD, GST and GR activities, as well as GSH concentration were not changed significantly. Zymography detected single isoforms of SOD and CAT in whole midguts of *E. chrysorrhoea* larvae from all three groups (Fig. 1b).

Canonical discriminant analyses and cluster analysis of the measured parameters in the whole midgut of *L. dispar* and *E. chrysorrhoea*, clearly differentiated between the species after treatment with both concentrations of fluoranthene (Fig. 3a and 3b). The first canonical function for Fl (Root 1) accounted for 93% of total heterogeneity indicating separation of *L. dispar* from *E. chrysorrhoea*. The second canonical function (Root 2) in the analysis for Fl accounted for 4.9% of total heterogeneity. Parameters which most contributed to the separation were the activities of GST and GR, and the concentration of GSH (Fig. 3a).

The first canonical function for Fh (Root 1) accounted for 94.5% of total heterogeneity, and indicates that *L. dispar* differed from *E. chrysorrhoea* in stress response to the higher concentration of fluoranthene. The second canonical function (Root 2) in the analysis for Fh accounted for 4.1% of total heterogeneity. Parameters which most contributed to the separation were again GST and GR activities and GSH concentration (Fig. 3b).

Enzyme activities in midgut tissues of *L. dispar* and *E. chrysorrhoea* larvae

The activities of SOD [$F_{(2,29)}$=17.871, P=0.0000] and CAT [$F_{(2,25)}$=24.490, P=0.0000] were markedly higher in the midgut tissues of *L. dispar* larvae exposed to both fluoranthene concentrations, in comparison to control larvae. Single SOD and CAT isoforms were detected in the larval midgut tissues from all three groups. GST and GR activities and GSH concentration in the midgut tissues did not differ significantly between larvae exposed to dietary fluoranthene and those from the control group (Fig. 2a).

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Large increases of SOD [$F_{(2,25)}$=11.376, P=0.0003] and CAT [$F_{(2,27)}$=17.356, P=0.0000] activities were detected in the midgut tissues of *E. chrysorrhoea* larvae exposed to the lower fluoranthene concentration, in comparison to control larvae. Two SOD isoforms (I1 and I2) and one CAT isoform were detected on the native PAGE zymogram. Significantly raised activities of GST [$F_{(2,26)}$=33.440; P=0.0000] and much higher concentrations of GSH [$F_{(2,27)}$=55.180, P=0.0000] were observed in the midgut tissues of *E. chrysorrhoea* larvae receiving both the low and high concentrations of dietary fluoranthene. While no significant differences in GR activity were recorded between the groups of larvae exposed to dietary fluoranthene and the control group, higher enzyme activity was found in midgut tissues of larvae given the low dose in comparison to that recorded for those ingesting the larger amount of fluoranthene [$F_{(2,25)}$=9.855, P=0.0007] (Fig 2b).

Canonical discriminant analysis and cluster analysis of enzyme activities and GSH concentration in the midgut tissues of *L. dispar* and *E. chrysorrhoea* larvae revealed separation of the two species on exposure to dietary fluoranthene (Fig. 3c and 3d). However, differentiation was less marked for midgut tissue than for whole midgut. The first canonical function for Fl (Root 1) accounted for 66.9% of total heterogeneity. The second canonical function (Root 2) in the analysis for Fl accounted for 29.4% of total heterogeneity. GST activity and GSH concentration were the parameters that contributed most to the separation (Fig. 3c).

Separation of the reaction of the two species to the higher concentration of dietary fluoranthene along Root 1 carried 78.3% of the heterogeneity. The second canonical function (Root 2) in the analysis for Fh accounted for 19.5% of total heterogeneity. SOD and CAT activities and GSH concentration were the main contributors to the separation (Fig. 3d).

SOD and CAT activities in hemolymph of *L. dispar* and *E. chrysorrhoea* larvae

SOD activity was diminished in hemolymph of *L. dispar* larvae ingesting the higher concentration of fluoranthene ($F_{(2,27)}$=5.790, P=0.0081) compared to that detected in hemolymph from control larvae. Greatly increased CAT activity in hemolymph of both

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groups given fluoranthene [$F_{(2,28)}$=59.930, P=0.0000] was recorded in comparison with the control group (Fig. 4a).

The activity of SOD in hemolymph of *E. chrysorrhoea* larvae did not change significantly on exposure to fluoranthene but CAT activity decreased in both groups of treated larvae [$F_{(2,27)}$=18.490, P=0.0000] when compared to the control larvae (Fig. 4b).

Discussion

The effects of fluoranthene have been studied in aquatic organisms, but examinations related to the impact on terrestrial insect species are scarce (Bach et al., 2005; Schuller et al., 2007; Baas et al., 2010). The acute toxicity in *Daphnia magna* following exposure to fluoranthene and UV light is thought to be due to production of singlet oxygen or free radicals (Wernersson and Dave, 1998), while induction of detoxifying enzymes in *Aedes aegipty* larvae (Poupardin et al., 2008) and increased activity of antioxidative enzymes in *L. dispar* larvae (Mrdaković et al., 2015) were shown to be responses to fluoranthene exposure. In the present study significant changes of antioxidative enzyme activity were for the first time recorded in whole midguts and midgut tissues (without the peritrophic membrane) of *L. dispar* and *E. chrysorrhoea* larvae exposed to dietary fluoranthene. This was expected considering that the midgut is the principal site of defense against the harmful effects of dietary xenobiotics in lepidopteran larvae. Moreover, reallocation of resources towards induction of defense mechanisms was probably the cause of reduced growth of larvae exposed to dietary fluoranthene (unpublished results). The significantly elevated SOD activity recorded in both whole midgut and midgut tissues, as well as differently expressed enzyme isoforms in whole midguts of control, and of *L. dispar* larvae ingesting fluoranthene-supplemented diets, indicate increased production of superoxide anion radicals and their conversion to hydrogen peroxide. Elevated SOD activity was previously reported in *L. dispar* larvae exposed to trophic stress and the effects of allelochemicals, dietary cadmium and the PAH, benzo[a]pyrene (B[a]P), (Perić-Mataruga et al., 1997, 2015; Mirčić et al., 2013; Ilijin et al., 2015). As CAT is a scavenger of hydrogen peroxide, increased SOD activity is usually followed by increased CAT activity (Halliwell and Gutteridge, 2007). However, we did not detect significant changes of CAT activity in whole midguts of *L. dispar* larvae, although the

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enzyme response differed between the two fluoranthene concentrations applied. A significant role of the ascorbate-recycling system in antioxidant defense was shown in the gut lumen of *Orgya leucostigma* larvae fed on prooxidant rich food (Barbehenn et al., 2001). On the other hand, although ROS should be eliminated in the gut lumen by reaction with some components of the diet, by low molecular weight antioxidants, as well as by peritrophic membranes, peroxides may diffuse into midgut epithelial cells and induce serious harm (Ahmad, 1992; Barbehenn et al., 2001; Krishnan and Kodrík, 2006). Prevention of such damage could be the reason for the considerably higher activity of CAT recorded in midgut tissues of *L. dispar* larvae given fluoranthene in their food.

Although we recorded no changes of SOD activity in whole midgut of *E. chrysorrhoea* larvae, CAT activity was significantly increased in larvae exposed to the higher concentration of dietary fluoranthene. Namely, an increased level of peroxides was the outcome noted by others (Halliwell and Gutteridge, 2007). Directly regulated CAT activity by peroxide concentration has previously been reported in 6th instar larvae of *L. dispar* given cadmium in their diet and in the midgut of 5th instar *L. dispar* larvae ingesting high PAH concentrations (Mirčić et al., 2013; Mrdaković et al., 2015; Ilijin et al. 2015). Significantly higher activities of SOD and CAT were recorded in the midgut tissues of *E. chrysorrhoea* larvae given the lower dietary fluoranthene concentration. Similar, hormetic-like effects, have been shown in insects and other organisms exposed to low concentrations of insecticides and heavy metal (Veliki and Hackenberger, 2012; Qu et al. 2015; Liu et al. 2019). However, we did not detect inhibitory effects of the higher fluoranthene concentration on SOD and CAT activities in the midgut tissues of *E. chrysorrhoea* larvae compared to the control group. These enzymes are considered to be a physiological team important for functionality of insect antioxidant defense (Blagojević and Grubor-Lajšić, 2000). Although there were no significant changes in the activity of SOD and CAT, expression of certain isoforms can be an energetically favorable mode by which *E. chrysorrhoea* larvae from the Fh group cope with fluoranthene induced superoxide anion radicals and hydrogen peroxide in their midgut tissues. On the other hand, peroxides may also be decomposed by ascorbate

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peroxidase action, and/or induction of GST isoforms with peroxidase activity (Mathews et al., 1997; Perić-Mataruga et al., 1997).

No changes in the activity of GST and total GSH concentration were recorded either in whole midgut or in midgut tissues of *L. dispar* larvae upon exposure to ingested fluoranthene, compared to the control groups, suggesting involvement of other mechanisms in pollutant metabolism. A similar response was observed in whole midgut from *E. chrysorrhoea* larvae, whereas GST activity and GSH concentration were elevated in midgut tissues of larvae given both concentrations of dietary fluoranthene. Increased GST activity has been reported in several insect species in response to PAH pollution. Poupardin et al. (2008) showed that treatment with fluoranthene raised GST activity in the mosquito *Aedes aegypti*. Gavrilović et al. (2017) recorded elevation of GST activity in *L. dispar* larval midgut in response to B[a]P treatment. GST removes toxic, hydrophobic compounds like PAHs by catalyzing their conjugation with GSH (Marrs, 1996; Lei et al., 2003). On the other hand, GST can also be considered as an antioxidant enzyme, as it removes hydroperoxides (Singh et al., 2001; Krishnan and Kodrik, 2006) or electrophilic substrates which are products of oxidative metabolism (Chasseaud, 1979). Although an increase in GST activity implies intensified GSH conjugation and its elimination, total GSH concentration in midgut tissues of *E. chrysorrhoea* larvae was elevated, which points to the significance of the many defense roles of this compound. Also, considering its importance, GR activity should be raised, but was not observed in the larval midgut tissues. Namely, GSH concentration may be elevated in different ways. For example, it can be recycled in the gut lumen or synthesized in salivary glands (Barbehenn et al., 2001). GSH is a tripeptide (Glu-Cys-Gly) found in most animal cells (Meister, 1994) and can act directly or passively as an antioxidant. It scavenges a wide variety of free radicals, can reduce H_2O_2 (Davies, 2000) and reacts with $^1O_2$, $O^{2\cdot-}$ and $HO^{\cdot}$ (Lesser, 2006). Moreover GSH is an important cofactor in reactions of glutathione-dependent enzymes and acts as a chain breaker for free radical reactions (Lesser, 2006). Allen et al. (1984) found increased total GSH concentration in houseflies exposed to paraquat as a result of adaptation to oxidative stress. Perić-Mataruga et al. (1997, 2015) recorded higher GSH concentrations in the midgut of *L. dispar* larvae fed unsuitable locust leaves, or treated with ghrelin. Apart

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from its antioxidative properties, GSH was shown to play a significant role in developmental processes of *Ostrinia nubilalis* larvae (Grubor-Lajišić et al., 1997).

GR has a pivotal role in maintaining normal functioning of GSH-dependent enzymes because it catalyzes the reduction of GSSG back to GSH (Wang et al. 2001). We detected a significant decrease in GR activity in whole midgut of *L. dispar* larvae given the lower concentration of dietary fluoranthene, although GST activity and total GSH concentration were not changed. Decreased GR activity has previously been demonstrated in two species of cereal aphids exposed to o-dihydroxyphenols (Lukasik and Golawska, 2007). Pritsos et al. (1988) also recorded diminished GR activity in larvae of *Papilio polyxenes* exposed to quercetin, and pointed out that GR activity may depend on both metabolic processes and non-enzymatic oxidation. It is unknown in which way fluoranthene inhibits GR activity, but Elliot et al. (1992) proposed that it was inhibited by reactive intermediates of allelochemicals interfering with the active site of the enzyme and leading to inactivation. However, this was less effective when SOD activity was induced. Another possibility is inhibition of GR activity due to complex formation with an allelochemical that can bind to the enzyme through covalent or hydrogen bonds. Considering the elevated SOD activity detected in the whole midgut of *L. dispar* larvae fed fluoranthene, we can only speculate that the reduced GR activity was a consequence of binding to the pollutant, or more likely, to one of its metabolites.

Detected enzyme activities and total GSH concentration were more pronounced in midgut tissues than in whole midguts of the larvae, and more enzyme activities were changed in the midgut tissues of *E. chrysorrhoea* larvae. Expression of the enzyme isoforms also differed between the species and was tissue-specific. Thus, native electrophoresis revealed more bands in regions of SOD activity in whole midgut than in midgut tissues of *L. dispar* larvae, while the situation was opposite for *E. chrysorrhoea* larvae. CDA confirmed between-species divergence, i.e. differences in response of the examined parameters in whole midgut and midgut tissues of *L. dispar* and *E. chrysorrhoea* larvae receiving low and high dietary fluoranthene levels.

Interestingly, we also found alterations in antioxidative enzyme activities in hemolymph of *L. dispar* and *E. chrysorrhoea* larvae upon exposure to dietary fluoranthene.

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Hemolymph cells play a key role in insect immune defense. They are significant indicators of stress, with phagocytic and encapsulation processes as defense responses (Dubovskii et al., 2010; Jiang et al., 2010). PAHs have been shown to act as immunotoxic agents, and chronic exposure to these pollutants may alter immune responses (Guo et al., 2011). Changes in hemocyte number and viability, reduction of their phagocytic ability, and increased levels of superoxide anions, were recorded in marine invertebrates exposed to PAHs (Gopalakrishnan et al. 2011; Giannapas et al. 2012). Furthermore, low molecular mass PAHs were shown to induce oxidative stress in hemocytes by generation of free radicals during PAH biotransformation within the lysosomes of hemocytes, disturbing their integrity and membrane fluidity (Giannapas et al., 2012). It has been noted that exposure of *Pecten maximus* to the PAH phenanthrene leads to oxidative stress. This was observed as a significant decrease in the amount of total GSH and increased levels of lipid peroxidation in the hemolymph (Hannam et al., 2010). In the present study, reduced SOD activity in the hemolymph of *L. dispar* larvae exposed to the higher concentration of dietary fluoranthene may suggest induction of enzyme isoforms that efficiently convert superoxide anion radicals into hydrogen peroxide. Similarly, decreased SOD activity in the hemolymph of *Chlamys ferrari* was shown to be a consequence of exposure to higher concentrations of the PAHs, B[a]P and benzo(k)fluoranthene (Pan et al. 2006). Hydrogen peroxide would have been further transformed by the increased CAT activity in both groups of larvae given dietary fluoranthene. Higher SOD and CAT activities have been recorded in the hemolymph of *L. dispar* exposed to B[a]P, which were presumed to be associated with activation of the larval immune system (Gavrilović et al. 2017). That implied generation of superoxide anion radicals in hemolymph cells (Ahmad et al., 1991). Exposure of *E. chrysorrhoea* larvae to both dietary fluoranthene concentrations resulted in reduction of CAT activity in hemolymph, which suggests involvement of other components or mechanisms of defense against the effects of pollutants and their metabolites. Involvement of non-enzymatic antioxidants, thiols and ascorbate was recently demonstrated in hemolymph of *Galleria mellonella* larvae during the process of encapsulation (Grizanova et al., 2018). The inhibition of CAT activity was shown to be provoked by superoxide anion radicals and conversion of the enzyme to inactive forms

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(Kono and Fridovich, 1982). Pan et al. (2006) suggested that changes in the activity of antioxidative enzymes in hemolymph are indicators of detoxification ability and point to the level of damage to the whole organism.

Our study revealed significant effects of environmentally relevant concentrations of fluoranthene on antioxidative enzyme activities in different tissues of larvae of two insect species. Tissue-specific responses of the analyzed parameters to dietary fluoranthene, and between-species differences in patterns of response, for the first time recorded in these forest species, represent an important step in clarifying how they adjust to stress induced by an organic pollutant. In addition, changes in the activity of antioxidative enzymes, as a response to dietary fluoranthene, point to their biomarker potential in assessment of environmental pollution.

Acknowledgments This study was supported by the Serbian Ministry of Education, Science and Technological Development, Grant No. 173027.

Declarations of interest: none.

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Figure Legends

Fig. 1. The activities of SOD, CAT, GST and GR, total GSH concentration and the expression of SOD and CAT isoforms in whole midguts of 5th instar larvae of *Lymantria dispar* (a) and *Euproctis chrysorrhoea* (b) fed with an artificial diet containing low (6.7 ng /g dry wt) (Fl) and high (67 ng /g dry wt) (Fh) fluoranthene concentrations. Different letters indicate significant differences between groups ($P<0.05$). Figures present data as mean values ± standard error. Sample sizes of the experimental groups of *L. dispar* larvae were: N = 22-30 (C), N = 15-28 (Fl), and N = 23-28 (Fh); Sample sizes of the experimental groups of *E. chrysorrhoea* larvae were: N = 20-28 (C), N = 14-24 (Fl), and N = 24-29 (Fh).

Fig. 2. The activities of SOD, CAT, GST and GR, total GSH concentration and the expression of SOD and CAT isoforms in midgut tissues of 5th instar larvae of *Lymantria dispar* (a) and *Euproctis chrysorrhoea* (b) fed with an artificial diet containing low (6.7 ng /g dry wt) (Fl) and high (67 ng /g dry wt) (Fh) fluoranthene concentrations. Different letters indicate significant differences between groups ($P<0.05$). Figures present data as mean values ± standard error. Sample sizes of the experimental groups of *L. dispar* larvae were: N = 7-9 (C), N = 9-10 (Fl), and N = 9-14 (Fh); Sample sizes of the experimental groups of *E. chrysorrhoea* larvae were N = 8-10 (C), N = 9-12 (Fl), and N = 9-10 (Fh).

Fig. 3. Canonical discriminant and cluster analyses for the enzyme activities and total GSH concentration in *Lymantria dispar* and *Euproctis chrysorrhoea* larvae: **a**. 6.7 ng fluoranthene/g dry wt (Fl) in whole midgut; **b**. 67 ng fluoranthene/g dry wt (Fh) in whole midgut; **c**. 6.7 ng fluoranthene/g dry wt (Fl) in midgut tissues; **d**. 67 ng fluoranthene/g dry wt (Fh) in midgut tissues.

Fig. 4. The activities of SOD and CAT in hemolymph of 5th instar larvae of *Lymantria dispar* (a) and *Euproctis chrysorrhoea* (b) fed with an artificial diet containing low (6.7 ng /g dry wt) (Fl) and high (67 ng /g dry wt) (Fh) fluoranthene concentrations. Different

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letters indicate significant differences between groups ($P<0.05$). Figures present data as mean values ± standard error. Sample sizes of the experimental groups of *L. dispar* larvae were: N = 10 (C), N = 10-11 (Fl) and N = 10 (Fh). Sample sizes of the experimental groups of *E. chrysorrhoea* larvae were: N = 10 (C), N = 11 (Fl) and N = 9-11 (Fh).

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Highlights

- Larvae of *Lymantria dispar* and *Euproctis chrysorrhoea* were exposed to fluoranthene.
- Antioxidant defense parameters were assessed in the midgut and hemolymph of larvae.
- Tissue-specific enzymes responses were recorded in larvae of both species.
- Between-species difference in patterns of enzymes response was noticed.
- Detected changes suggest the ways these insects overcome stressful conditions.

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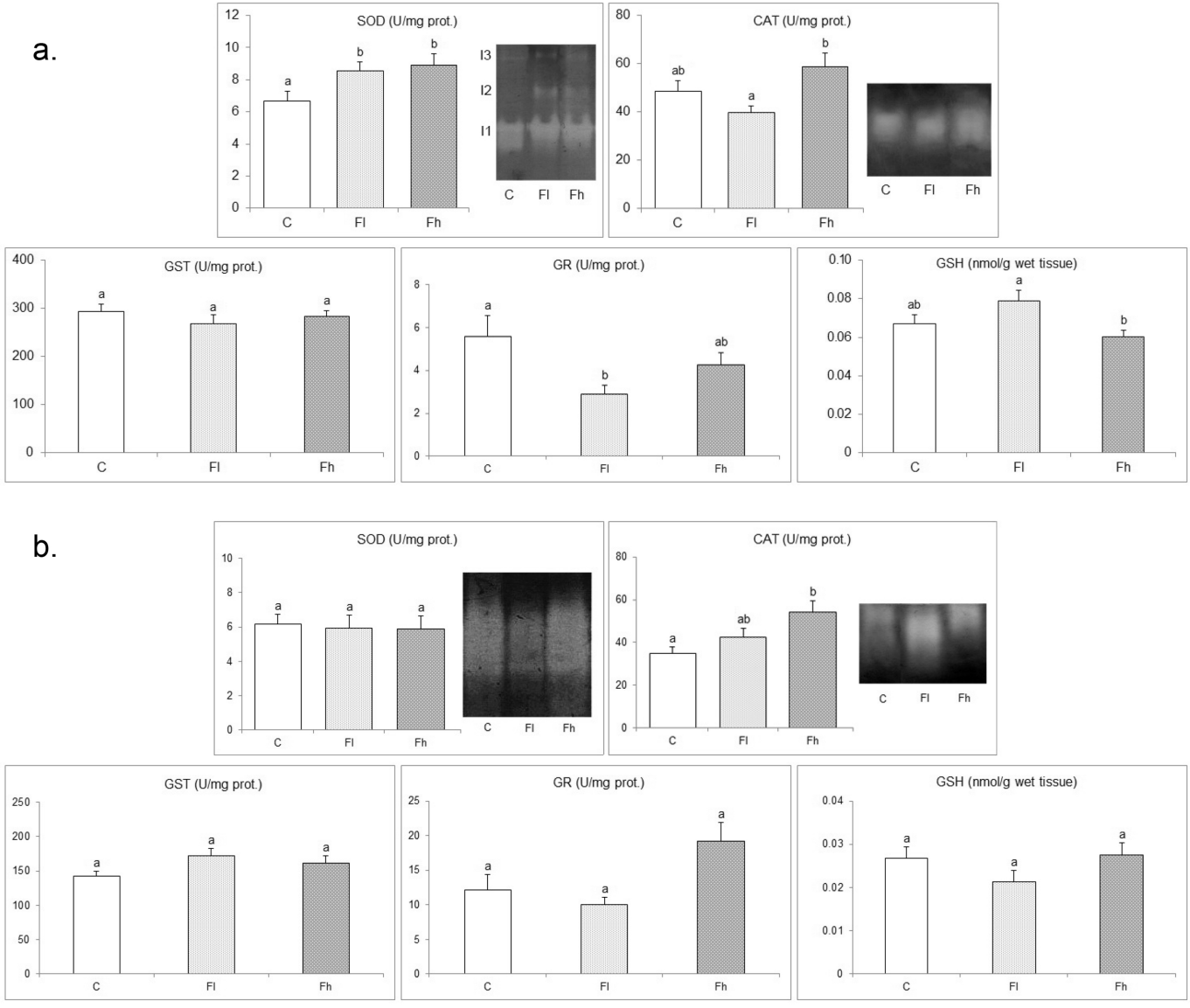

a.
SOD (U/mg prot.)
12
10
8
6
4
2
0
a
b
b
C
Fl
Fh
I3
I2
I1
C Fl Fh
CAT (U/mg prot.)
80
60
40
20
0
ab
a
b
C
Fl
Fh
C Fl Fh
GST (U/mg prot.)
400
300
200
100
0
a
a
a
C
Fl
Fh
GR (U/mg prot.)
8
6
4
2
0
a
b
ab
C
Fl
Fh
GSH (nmol/g wet tissue)
0.10
0.08
0.06
0.04
0.02
0
ab
a
b
C
Fl
Fh
b.
SOD (U/mg prot.)
10
8
6
4
2
0
a
a
a
C
Fl
Fh
C Fl Fh
CAT (U/mg prot.)
80
60
40
20
0
a
ab
b
C
Fl
Fh
C Fl Fh
GST (U/mg prot.)
250
200
150
100
50
0
a
a
a
C
Fl
Fh
GR (U/mg prot.)
25
20
15
10
5
0
a
a
a
C
Fl
Fh
GSH (nmol/g wet tissue)
0.04
0.03
0.02
0.01
0
a
a
a
C
Fl
Fh

Figure 1

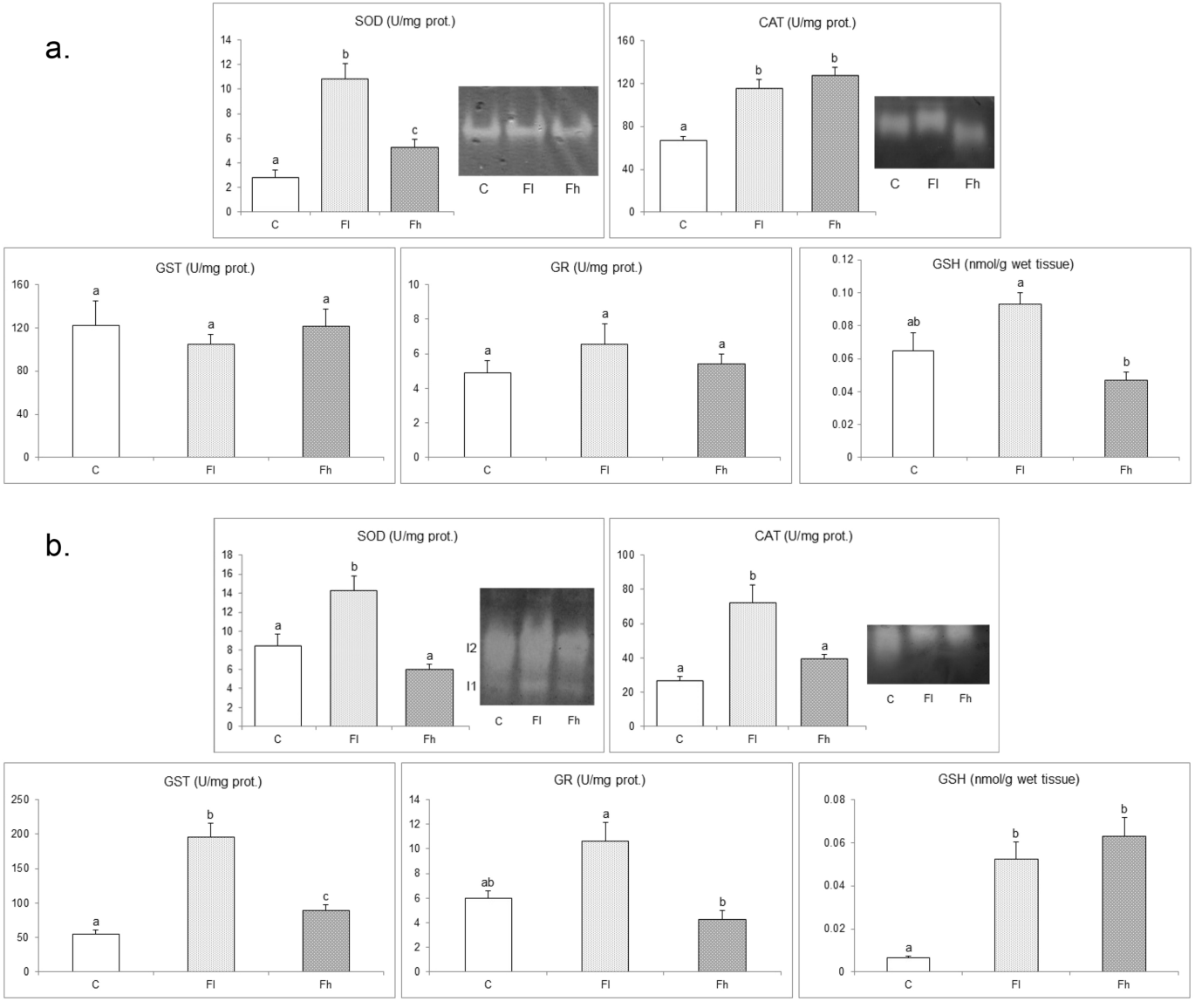

a.
SOD (U/mg prot.)
CAT (U/mg prot.)
GST (U/mg prot.)
GR (U/mg prot.)
GSH (nmol/g wet tissue)
C
Fl
Fh
b.
SOD (U/mg prot.)
CAT (U/mg prot.)
GST (U/mg prot.)
GR (U/mg prot.)
GSH (nmol/g wet tissue)
I2
I1

Figure 2

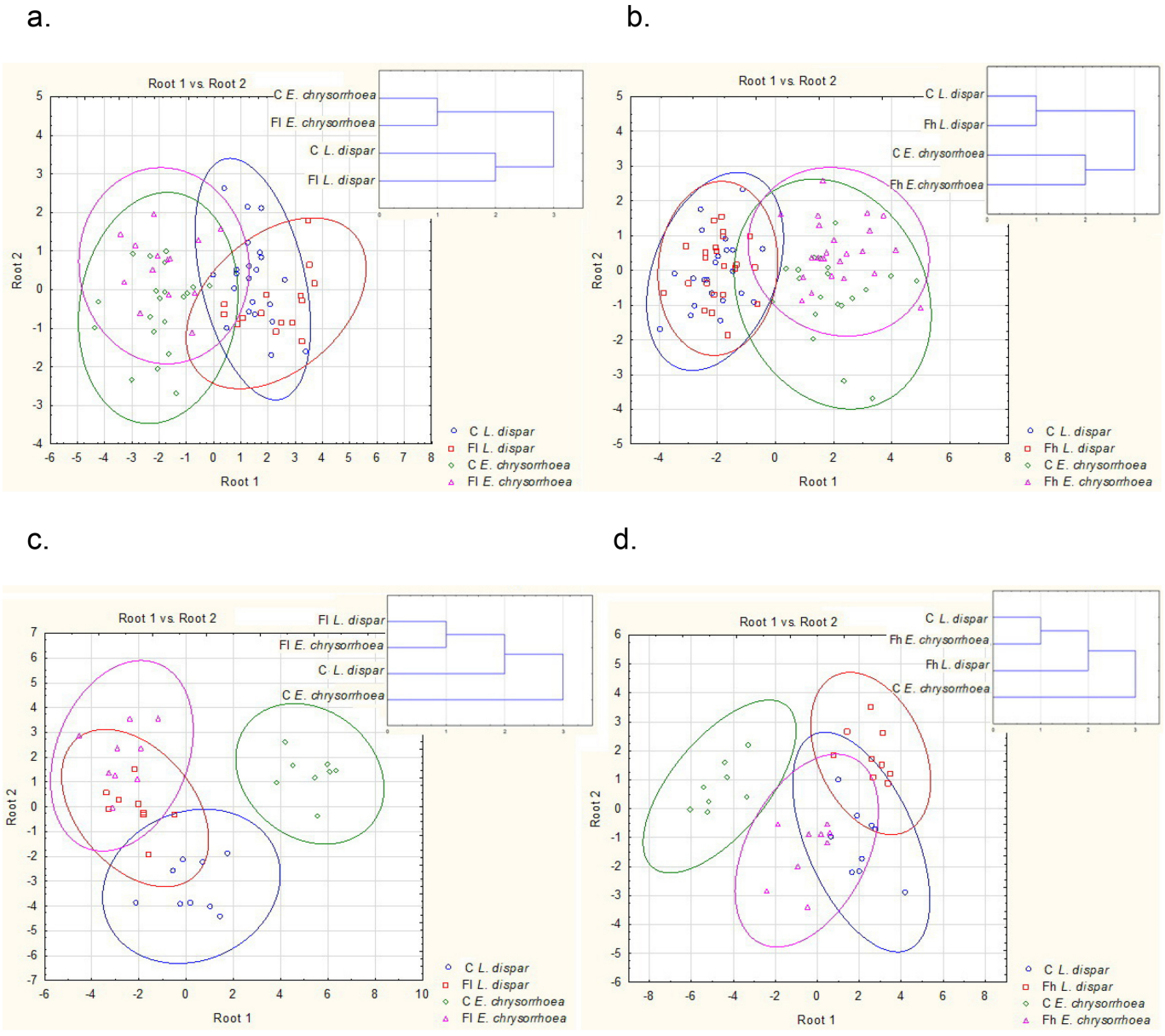
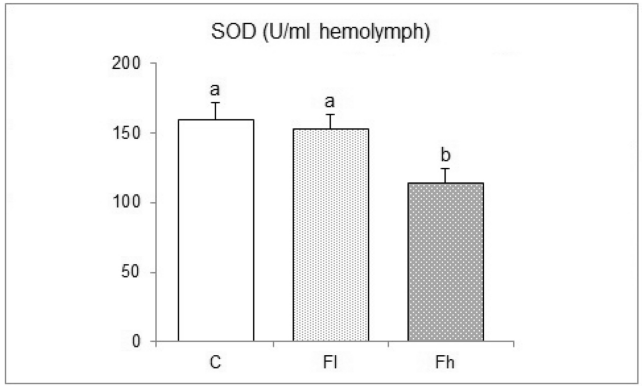
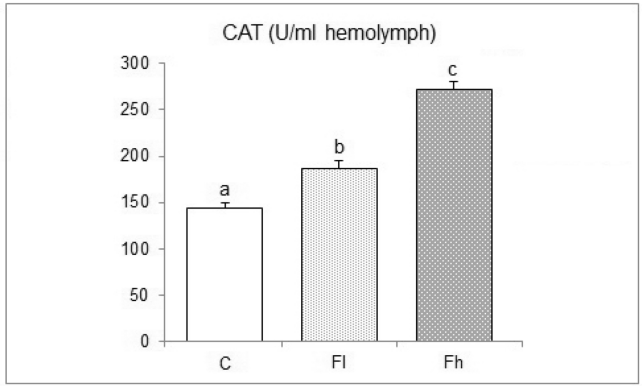

a.
Root 1 vs. Root 2
C E. chrysorrhoea
Fl E. chrysorrhoea
C L. dispar
Fl L. dispar
Root 2
Root 1
C L. dispar
Fl L. dispar
C E. chrysorrhoea
Fl E. chrysorrhoea
b.
Root 1 vs. Root 2
C L. dispar
Fh L. dispar
C E. chrysorrhoea
Fh E.chrysorrhoea
Root 2
Root 1
C L. dispar
Fh L. dispar
C E. chrysorrhoea
Fh E. chrysorrhoea
c.
Root 1 vs. Root 2
Fl L. dispar
Fl E. chrysorrhoea
C L. dispar
C E. chrysorrhoea
Root 2
Root 1
C L. dispar
Fl L. dispar
C E. chrysorrhoea
Fl E. chrysorrhoea
d.
Root 1 vs. Root 2
C L. dispar
Fh E. chrysorrhoea
Fh L. dispar
C E. chrysorrhoea
Root 2
Root 1
C L. dispar
Fh L. dispar
C E. chrysorrhoea
Fh E. chrysorrhoea

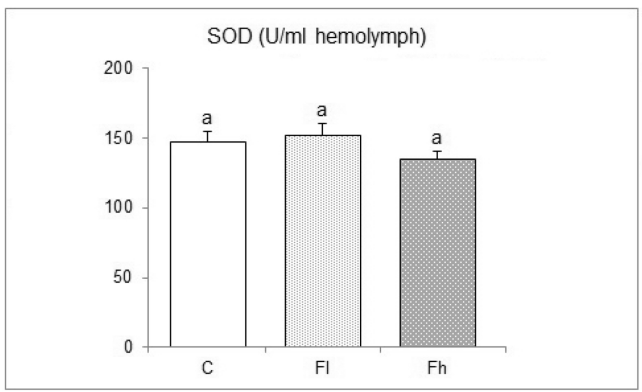
Figure 3

a.


SOD (U/ml hemolymph)
200
150
100
50
0
a
a
b
C
Fl
Fh


CAT (U/ml hemolymph)
300
250
200
150
100
50
0
a
b
c
C
Fl
Fh

b.


SOD (U/ml hemolymph)
200
150
100
50
0
a
a
a
C
Fl
Fh

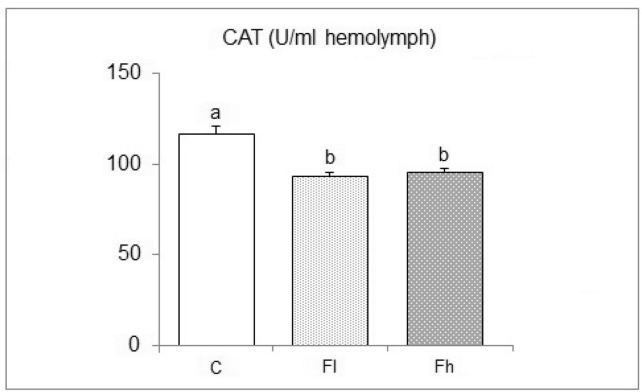

CAT (U/ml hemolymph)
150
100
50
0
a
b
b
C
Fl
Fh

Figure 4