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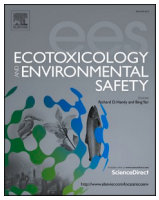

Effects of dietary fluoranthene on tissue-specific responses of carboxylesterases, acetylcholinesterase and heat shock protein 70 in two forest lepidopteran species

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ABSTRACT

In this study, responses of carboxylesterases, acetylcholinesterase, and stress protein Hsp70 were examined in the midgut and midgut tissue, and brain of fifth instar larvae of *Lymantria dispar* L. and *Euproctis chrysorrhoea* L. following chronic exposure to dietary fluoranthene. Specific carboxylesterase activity increased significantly in the midgut tissue of *E. chrysorrhoea* larvae treated with a lower fluoranthene concentration. The specific patterns of isoforms expression, recorded in larvae of both species, enable efficient carboxylesterase activity as a significant part of defense mechanisms. Increased Hsp70 concentration in the brain of *L. dispar* larvae points to a response to the proteotoxic effects of a lower fluoranthene concentration. Decreased Hsp70 in the brain of *E. chrysorrhoea* larvae in both treated groups can suggest induction of other mechanisms of defense. The results indicate the importance of the examined parameters in larvae of both species exposed to the pollutant, as well as their potential as biomarkers.

1. Introduction

Increasing environmental pollution is largely a consequence of various anthropogenic activities and affects, among others, plants and insects of terrestrial ecosystems. The effects of various types of pollutants on the fitness of herbivores have been recorded, which result from their influence on host plants, and data from numerous studies have pointed out that changes of plant nutritional quality caused by pollution, negatively influence phytophagous insects (reviewed in Butler and Trumble, 2008). Polycyclic aromatic hydrocarbons (PAHs) are organic pollutants that predominantly arise from anthropogenic activities. They can be transferred by air masses far from their places of origin and reach different parts of the environment (Laflamme and Hites, 1978; Ravindra et al., 2008; Quiroz et al., 2011; Stogiannidis and Laane, 2015; Yu et al., 2019). Sixteen PAHs, including fluoranthene, have been denoted by the United States Environmental Protection Agency (1982) as priority pollutants. These substances harmfully influence humans and the environment (Srogi, 2007; Sun et al., 2021). PAHs can be deposited in woodland areas and are considered the most abundant toxic compounds in forests (Belis et al., 2011). Adversely influencing the physiological processes and growth of different plant species (Berteigne et al., 1989; Wittig et al., 2003; Kummerovà et al., 2008; Desalme et al., 2013; Tomar and Jajoo, 2014), PAHs can also affect phytophagous insects. Examining these effects could provide informations on the defense mechanisms of phytophagous insects and how they can overcome stress caused by organic pollutants. Widespread polyphagous phytophagous insect species, whose developmental processes are well known and which can be easily reared under laboratory conditions, may be good model organisms for such examinations. For example, recent studies have shown tissue-specific responses of antioxidant enzymes in larvae of phytophagous lepidopteran species after chronic exposure to dietary PAHs, benzo [*a*]pyrene (BaP) and fluoranthene, respectively (Gavrilović et al., 2017; Filipović et al., 2019).

Induction of defense mechanisms, changes in specific activities of antioxidant and detoxification enzymes and their isoforms expression, and different concentrations and expression patterns of stress proteins, which can be seen in tissues and organs, enable insects to cope with the harmful effects of various xenobiotics (e.g., Terriere, 1984; Korsloot

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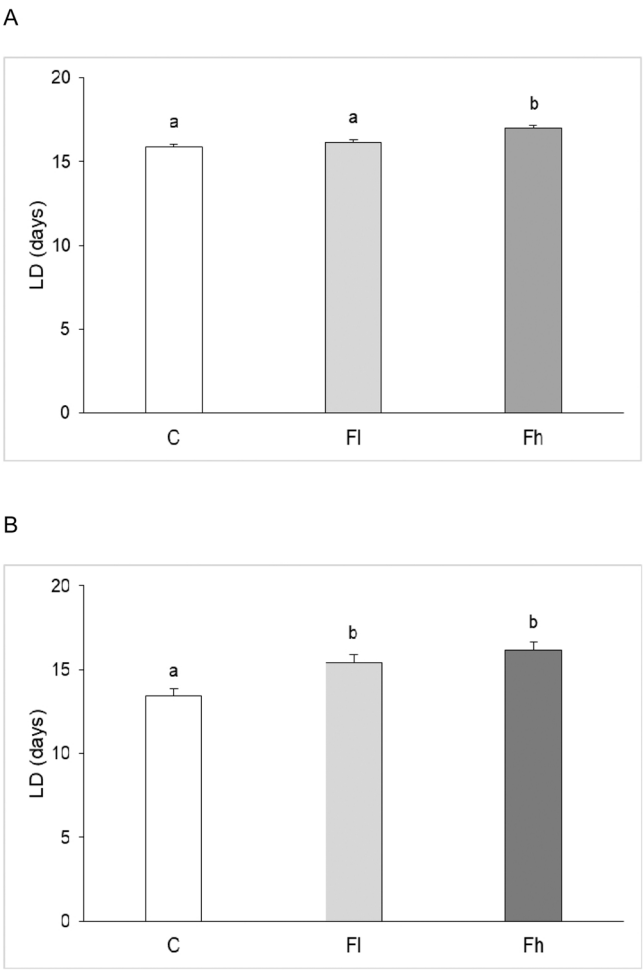

Fig. 1. Duration of development from molting into the third instar until 3rd day of the fifth instar of *L. dispar* (A), and *E. chrysorrhoea* (B) larvae exposed to lower (Fl) and higher (Fh) concentration of dietary fluoranthene, control group (C). Data are presented as mean ± SEM. Values marked with different letters differ significantly ($P < 0.05$).

et al., 2004; Després et al., 2007). In addition, they can be detected before obvious effects at the level of the organism, and some of these responses could be used as biomarkers of pollution.

Carboxylesterases (CarEs) are enzymes that catalyze the hydrolysis of carboxyl esters. They are characterized by numerous isoforms, and besides endogeneous physiological functions, CarEs have important roles in the metabolism and detoxification of various xenobiotics, such as secondary plant compounds in the grain aphids (Zhang et al., 2013), or insecticides in the tortricide moth pest species (Navarro-Roldan et al., 2019), etc. Considering the CarEs responsivity to PAH exposure, they can be potential biomarkers of the presence of these environmental pollutants (Wheelock et al., 2008; Solé et al., 2020).

Acetylcholinesterase (AChE) catalyzes the hydrolysis of a major insect neurotransmitter, acetylcholine. It is one of the most important enzymes in the nervous system, and some studies indicated inhibition of the AChE activity by pollutants, including PAHs (Toutant, 1989; Lionetto et al., 2013).

Heat-shock proteins (Hsps), ubiquitous in organisms, have significant roles in the correct folding of proteins, their localization, degradation of other proteins, etc. Hsps are important for cellular homeostasis and respond to cytotoxic effects of various environmental stressors, among others, to the impact of PAHs (Feder and Hofmann, 1999; Moreira-de-Sousa et al., 2018). Hsp70 is highly conserved and, in various stressful conditions, the most abundantly induced family of Hsps (Gupta et al., 2010).

We have previously described the influence of the PAH fluoranthene added to food on larvae of phytophagous insect species *L. dispar* L. and *E. chrysorrhoea* L. (Lepidoptera: Erebidae). Besides recorded changes in life-history traits, changes in the activity of antioxidant enzymes in various tissues and digestive enzyme activities in the midgut point to the induction of mechanisms that allow larvae to overcome detrimental pollutant effects and also suggest some of the enzymes as potential biomarkers of pollution (Mrdaković et al., 2015; Filipović et al., 2019, 2021). *L. dispar* and *E. chrysorrhoea* are widely distributed, polyphagous forest species. For both, preferred hosts are various oak species, but they can also attack many other plants. They are characterized by continuous larval feeding, especially in later instars, the main period of larval growth, when they eat large amounts of leaves (Kelly et al., 1989; Klapwijk et al., 2013), with which they can also uptake various xenobiotics. Fluoranthene is one of the most abundant PAH compounds detected in leaves of different plants species from rural and urban areas, respectively (Fellet et al., 2016; De Nicola et al., 2017; Huang et al., 2018). Its harmfull effects were shown in different plant (Berteigne et al., 1989; Kummerová et al., 2006) and animal species (Saunders et al., 2003; Rodrigues et al., 2013), as well as in vitro in human cell lines and tissues (Ito et al., 2004; Wang et al., 2007). However, data on fluoranthene effects on phytophagous insect species are still rare.

In the present study, we aimed to examine whether chronic exposure of *L. dispar* and *E. chrysorrhoea* larvae to environmentally relevant concentrations of fluoranthene, added in a diet, influences the above-mentioned enzymes that are part of defending mechanisms and the Hsp70 that responds to the cytotoxic effects of xenobiotics. As a continuation of previous research on the impact of fluoranthene on these economically important species, the present study can further contribute to the knowledge of how they respond to the presence/action of organic pollutants. The peritrophic membrane, a semipermeable barrier secreted by epithelial cells, protects midgut epithelium from damage by toxic compounds ingested with food, and from reactive species (Barbehenn and Stannard, 2004). We have previously observed more pronounced changes in the activity of antioxidant enzymes, as well as glutathione S-transferase (phase II biotransformation enzyme), in the midgut tissues than in whole larval midguts (Filipović et al., 2019). In this study, we also examined pollutant effects on CarEs activities in the whole midguts and midgut tissues (without peritrophic membranes), and on CarEs and AChE activities and Hsp70 expression in the brains of *L. dispar* and *E. chrysorrhoea* larvae. The insect brain is a part of the nervous system that is characterized by quick responses to stress, and regulates and coordinates the physiological reactions of an organism in stressful conditions (Perić-Mataruga et al., 2017). Since the exposure to stressful conditions often needs allocation of resources toward defense, which can affect life-history traits, we determined the duration of development of *L. dispar* and *E. chrysorrhoea* larvae, from molting into the third instar until the 3rd day of the fifth instar.

2. Material and methods

2.1. Experimental groups and rearing conditions

As described previously (Filipović et al., 2019, 2021), the egg masses of *L. dispar* were collected in November from a mixed oak forest near the city of Majdanpek (East Serbia), and the winter nests of *E. chrysorrhoea* were collected in February from a mixed oak forest near the city of Prijepolje (Southwest Serbia), and kept at 4 °C until April and late March, respectively. Then, the egg masses of *L. dispar* were transferred at a temperature of 23 ± 0.5 °C and a 12 L:12D photoperiod for hatching and further development of larvae. The winter nests of *E. chrysorrhoea* were placed first at room temperature, and larvae that soon appeared were transferred at a temperature of 26 ± 0.5 °C and a 16 L:8D photoperiod for further development. Larvae of each species were randomly assigned to experimental groups reared on a high wheat germ artificial

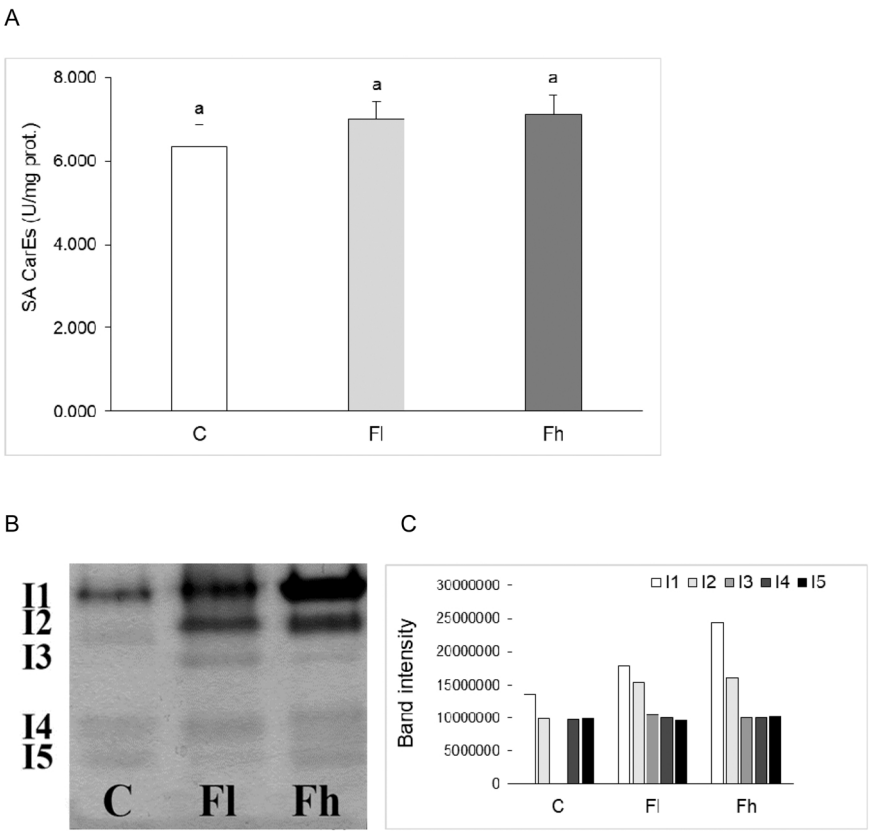

Fig. 2. The specific activity (A), and native polyacrylamide gel (B) with densitometric analysis of bands intensity (C) of CarEs in the whole midguts of fifth instar *L. dispar* larvae exposed to lower (Fl) and higher (Fh) concentration of dietary fluoranthene, control group (C). Data are presented as mean ± SEM. The numbers indicate CarEs isoforms (I1-I5).

diet (HWG) (O`Dell et al., 1985) containing a lower fluoranthene concentration (Fl group – 6.7 ng/g dry weight of food), and the HWG diet containing a higher fluoranthene concentration (Fh group – 67 ng/g dry weight of food). The control groups (C group) were reared on a fluoranthene-free HWG diet. The lower fluoranthene concentration corresponded to the one previously recorded in leaves of several tree species (Howsam et al., 2000) including oak leaves, as suitable host plants for both insect species, while the higher one was in the range of PAH concentrations reported in other plant species (Tian et al., 2008; De Nicola et al., 2015; De Nicola et al., 2017). Larvae were monitored daily to check for molting, and were provided with fresh food every second day. Larvae were reared until the 3rd day of the fifth instar and then sacrificed. The experimental procedures were in compliance with Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković"- National Institute of the Republic of Serbia, University of Belgrade.

2.2. Duration of development of L. dispar and E. chrysorrhoea larvae

In order to estimate the influence of two concentrations of dietary fluoranthene on the life-history trait of larvae, we measured the duration of larval development, from molting into the third instar until 3rd day of the fifth instar (LD, in days), in both species. Sample sizes of the experimental groups were N = 77–92 for *L. dispar* and N = 68–83 for *E. chrysorrhoea* larvae.

2.3. Preparation of homogenates for detection of enzyme activity

Larvae of both species were immobilized on ice, sacrificed, and their brains and midguts were isolated. Whole midguts and midguts with removed peritrophic membranes (referred to as midgut tissues) were weighed and individually homogenized (Ultra Turrax homogenizer, IKA-Werke, Staufen, Germany) on ice in sucrose buffer pH 7.4. The final tissue concentration was 200 mg/ml. Then, the samples were sonified with an ultrasonic homogenizer (HD 2070 Bandelin, Berlin, Germany). The homogenates were centrifuged at 105,000*g* for 100 min at 4 °C (Beckman L7–55 Ultracentrifuge). The activity of CarEs was determined from obtained supernatants. Sample sizes for determination of enzyme activity in whole midguts of *L. dispar* larvae from the control and treated groups were N = 27–29, and in whole midguts of *E. chrysorrhoea* larvae from the control and treated groups were N = 24–28. Sample sizes for enzyme activity determination in larval midgut tissues from the control and treated groups of both species were N = 10.

The brain tissues of larvae were isolated from head capsules, pooled per experimental group (n = 25–30) and diluted with ice-cold deionized water (1:9/w:V). They were homogenized (MHX/E Xenox homogenizer, Germany) on ice, and homogenates were centrifuged at 10,000*g* for 10 min at 4 °C (Eppendorf 5417 R centrifuge, Germany), aliquoted and stored at − 20 °C until use. The activities of CarEs and AChE were determined from obtained supernatants. Data points represent the mean of three replicates (homogenized brain tissues, pooled by experimental group). The concentrations of protein were determined according to Bradford (1976), by using bovine serum albumin as a standard.

2.4. Spectrophotometric assay of carboxylesterase activity

The activity of CarEs in the homogenates of whole midguts and midgut tissues and brain tissues homogenates were determined by the continuous spectrophotometric assay according to Main et al. (1961), with *o*-nitrophenyl butyrate as a substrate. The changes in absorbance

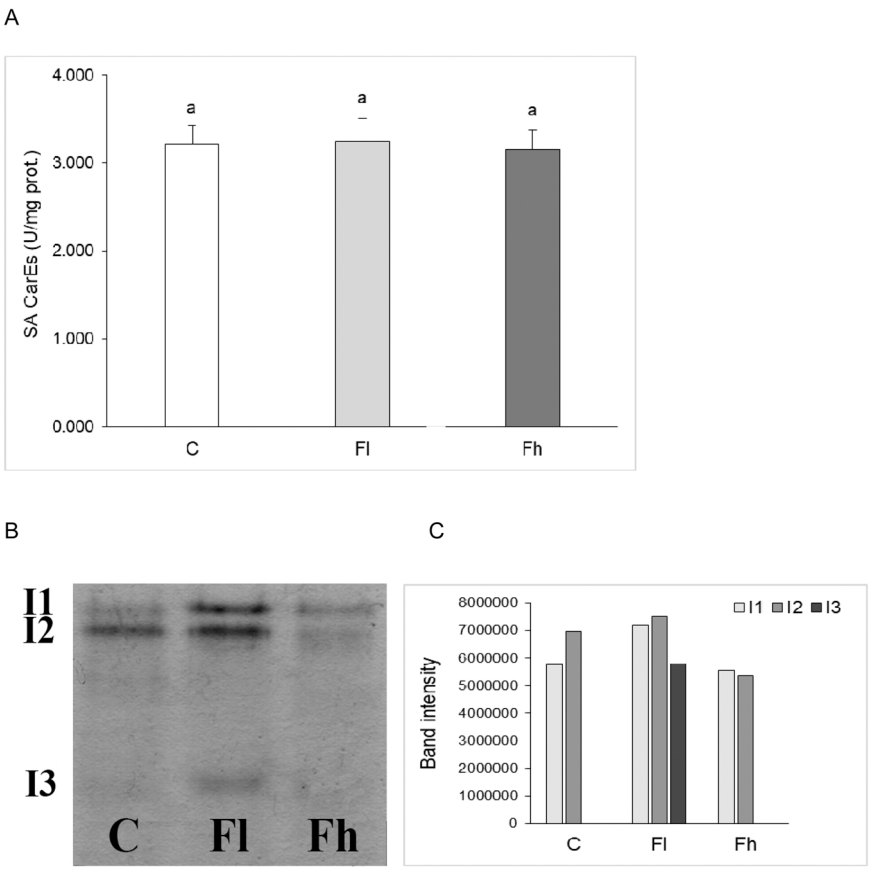

Fig. 3. The specific activity (A), and native polyacrylamide gel (B) with densitometric analysis of bands intensity (C) of CarEs in the whole midguts of fifth instar *E. chrysorrhoea* larvae exposed to lower (Fl) and higher (Fh) concentration of dietary fluoranthene, control group (C). Data are presented as mean ± SEM. The numbers indicate CarEs isoforms (I1-I3).

were monitored at λ = 414 nm (UV mc2, SAFAS, Monaco) for 4 min. One unit of CarEs specific activity was defined as μmol of *o*-nitrophenyl butyrate hydrolyzed per minute, per mg of protein.

2.5. Detection of midgut carboxylesterase activity by native polyacrylamide gel electrophoresis

Carboxylesterase isoforms in the homogenates of whole midguts and midgut tissues were detected according to Loxdale et al. (1983). The samples (10 μg protein per line) were added to 7.5 % native PAGE. After separation at 4 °C, the gel was soaked in 20 mM Na-phosphate buffer pH 7.2, 1.1 mM α-naphthyl acetate and 1.2 mM Fast Blue B. After incubation at room temperature for 40 min, bands that indicated enzyme activity appeared. The intensity of detected bands was estimated using ImageJ software (National Institute of Health, USA).

2.6. Spectrophotometric assay of acetylcholinesterase activity

The activity of AChE in the homogenates of brain tissues was determined spectrophotometrically using acetylthiocholine iodide as a substrate (Ellman et al., 1961). Thiocholine, a product of substrate hydrolysis catalyzed by AChE, reacts with the dithiobis-nitrobenzoate and generates the 5-thio-2-nitrobenzoate anion which can be quantified by its absorbance at λ = 406 nm (UV mc2, SAFAS, Monaco). One unit of AChE specific activity was defined as μmol of acetylthiocholine iodide hydrolyzed per min, per mg of protein.

2.7. Preparation of homogenates for Hsp70 detection in the larval brain

The brain tissues isolated from head capsules were diluted with 0.9 % NaCl (1:9/w:V). The pools were homogenized on ice (MHX/E Xenox homogenizer, Germany), and homogenates were centrifuged at 10,000*g* for 10 min at 4 °C (Eppendorf 5417 R centrifuge, Germany).

2.8. Methods for Hsp70 detection

Western blot and indirect ELISA were used to detect Hsp70 in homogenates of larval brain tissues. The homogenates (20 μg protein) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on a 12 % gel (Laemmli, 1970), and proteins were transferred to the nitrocellulose membrane, at 40 V and 4 °C, overnight. The primary monoclonal anti-Hsp70 mouse IgG1 (clone BRM-22, Sigma Aldrich, diluted 1:5000) and the secondary anti-mouse IgG1 – horseradish peroxidase (HRP) conjugate antibodies (Sigma Aldrich, diluted 1:10, 000) were used to detect the expression patterns of Hsp70. Protein bands were visualized by ECL (Enhanced Chemiluminescence, Amersham).

In order to quantify the concentrations of Hsp70 in homogenates of larval brain tissues, an indirect non-competitive ELISA was used. The samples (10 μg of protein per well) were diluted with carbonate-bicarbonate buffer pH 9.6, coated on microplate (Multiwell Immunoplate, Thermoscientific, Denmark), overnight at 4 °C. They were incubated in the presence of primary monoclonal anti-Hsp70 mouse IgG1 antibody (diluted 1:5000), followed by incubation in the presence of secondary anti-mouse IgG1 – HRP conjugated antibody (diluted 1:10,000). Absorption was measured on a microplate reader (LKB 5060–006), at λ = 450 nm. Serial dilutions of standard Hsp70 were used for determination the concentrations of Hsp70, which are expressed as ng/mg of protein.

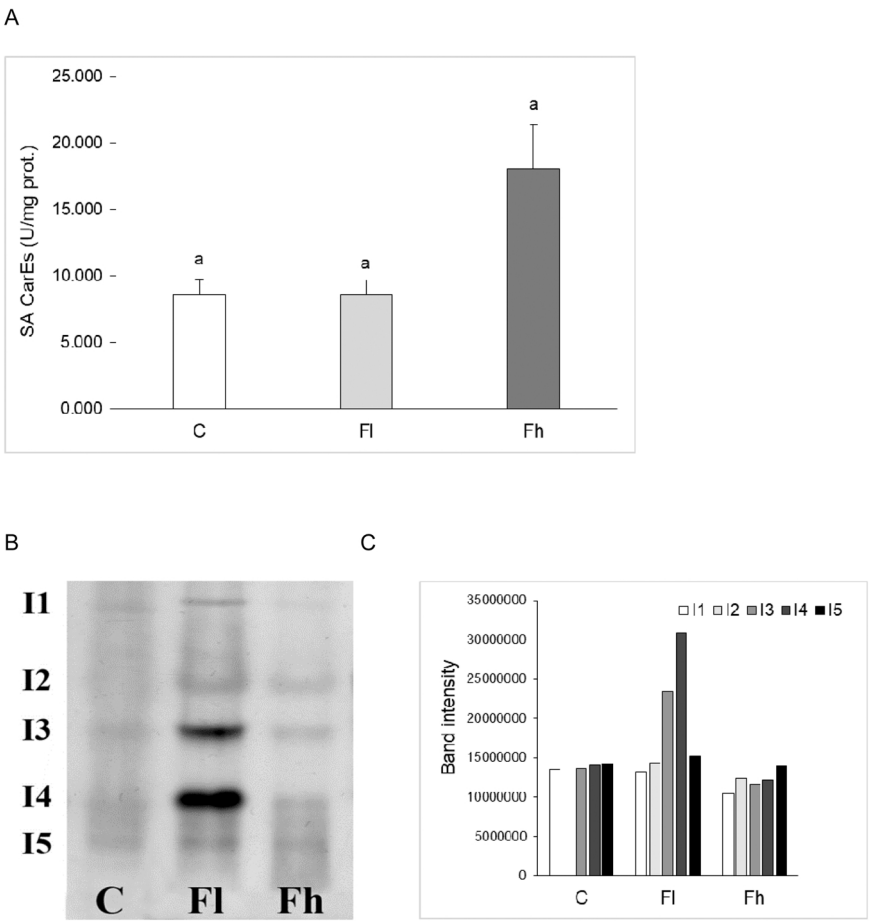

Fig. 4. The specific activity (A), and native polyacrylamide gel (B) with densitometric analysis of bands intensity (C) of CarEs in the midgut tissues of fifth instar *L. dispar* larvae exposed to lower (Fl) and higher (Fh) concentration of dietary fluoranthene, control group (C). Data are presented as mean ± SEM. The numbers indicate CarEs isoforms (I1-I5).

2.9. Statistical analyses

The results are presented as means ± standard errors of mean (SEM). The normality of data distribution and homogeneity of variance were checked by Kolmogorov-Smirnov and Shapiro-Wilks tests and the Levene test, respectively. Significant differences in the mean values of examined parameters between experimental groups were estimated by one-way ANOVA followed by Tukey's test. Analysis of variance was performed on log-transformed values of specific activity of midgut CarEs (Sokal and Rohlf, 1981). The duration of larval development was analyzed by Kruskal-Wallis ANOVA and multiple comparisons of mean ranks for all experimental groups (Siegel and Castellan, 1988). P values lower than 0.05 were considered statistically significant.

3. Results and discussion

The results of this research indicate the significant role of the examined parameters, CarEs, AChE, Hsp70, components of defending mechanisms, in response to stress caused by chronic exposure to fluoranthene, an organic pollutant, in two important forest lepidopteran species. The induction of defending mechanisms in insects exposed to various xenobiotics, and the allocation of energy and matter resources towards defense (de novo biosynthesis, alterations in biochemistry, changes in signal transduction, etc.) may significantly influence their fitness, e.g., development time, mass, growth rate. In the present study prolonged development time from the third instar until 3rd day of the fifth instar was noted in *L. dispar* larvae upon chronic treatment with a higher concentration of dietary fluoranthene ($p < 0.001$) (Fig. 1A). In *E. chrysorrhoea* larvae, a significantly prolonged duration of development was found in both groups treated with fluoranthene ($p < 0.001$) (Fig. 1B). Similarly, Fan et al. (2020) detected the influence of PAHs on the development time and growth of *Hermetia illucens* larvae, i.e., at some PAH concentrations applied in the artificial diet, the larval development was deleyed and the larval growth slowed. On the other hand, chronic exposure to dietary B[*a*]P did not significantly influence the duration of development of *L. dispar* larvae originating from an unpolluted forest (Grčić et al., 2021). The study of the chronic effect of dietary cadmium on *L. dispar* larvae revealed a prolonged duration of the fourth instar, as well as total developmental time from hatching until 3rd day of the fifth instar (Matić et al., 2016). The prolonged duration of larval development could enable the acquisition of more resources necessary for growth and to cope with stressful conditions, but also for providing conditions needed for the next stages of the life cycle that do not feed.

Studies on CarEs in insects are mainly related to their responses to allelochemical and insecticide actions (Lindroth and Weisbrod, 1991; Chen et al., 2017), and their roles in the development of resistance to insecticides (Hilliou et al., 2021; Li et al., 2022). The results of Benito-Murcia et al. (2022) revealed the sensitivity of CarEs isoforms in honey bees upon chronic exposure to organophosphates, suggesting their potential biomarker role. Carboxylesterase activity and expression of isoforms are tissue-dependent and can vary between organisms and in various species (Satoh and Hosokawa, 1995; Sanchez-Hernandez and Wheelock, 2009). Carboxylesterases may also be induced by exposure to PAHs. In the present study, we reported no significant influence of dietary PAH fluoranthene on the specific activity of CarEs in the whole

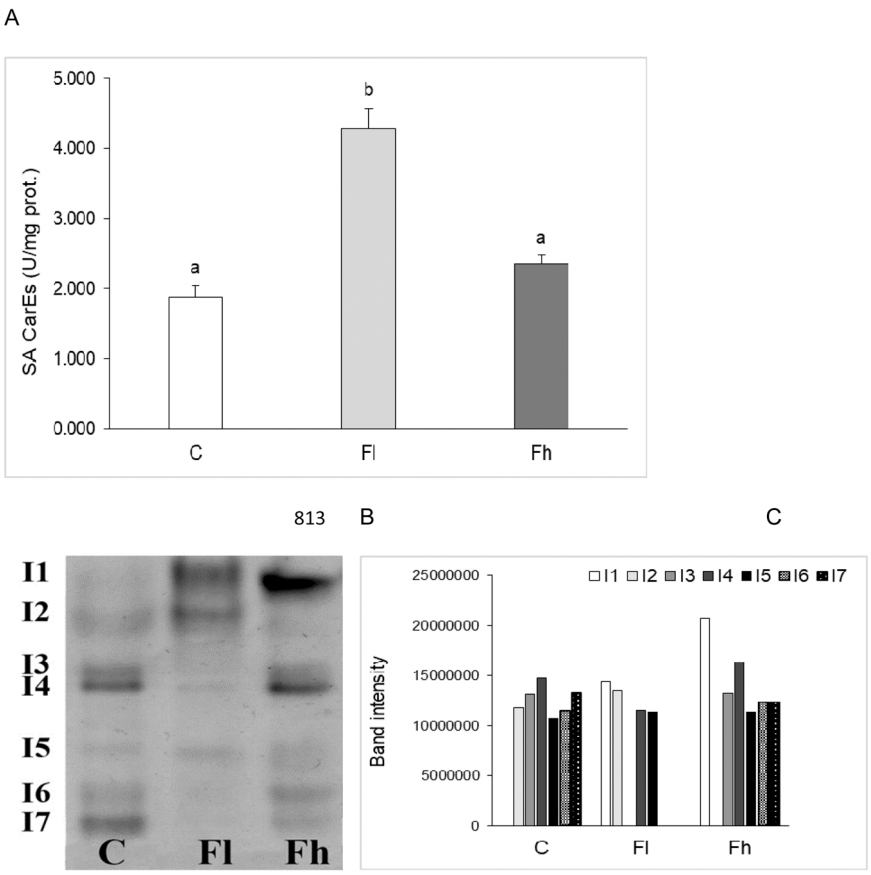

Fig. 5. The specific activity (A), and native polyacrylamide gel (B) with densitometric analysis of bands intensity (C) of CarEs in the midgut tissues of fifth instar *E. chrysorrhoea* larvae exposed to lower (Fl) and higher (Fh) concentration of dietary fluoranthene, control group (C). Data are presented as mean ± SEM. Values marked with different letters differ significantly ($P < 0.05$). The numbers indicate CarEs isoforms (I1-I7).

midguts of fifth instar larvae of two lepidopteran species, *L. dispar* and *E. chrysorrhoea*. However, enzyme isoforms expressed species-specific sensitivity to the presence of dietary pollutant. Thus, long-term exposure to two concentrations of fluoranthene added to the artificial diet did not significantly influence CarEs specific activities in the whole midgut of *L. dispar* larvae ($p > 0.05$) (Fig. 2A). Five enzyme isoforms were detected by native electrophoresis. Isoform I3 was expressed in larvae of both treated groups, but not in the control group (Fig. 2B). Increased intensity of two isoforms was noticed in treated groups compared to the control group, and the intensity of dominant I1 isoform increased with pollutant concentrations (Fig. 2C). Different patterns of expression of CarEs isoforms presumably provide appropriate enzyme activity in the presence of pollutant in food. Similarly, there were no significant changes in the specific activity of CarEs in the whole midgut of *E. chrysorrhoea* larvae ($p > 0.05$) (Fig. 3A), and three enzyme isoforms were detected (Fig. 3B). Again, isoform I3 was expressed only in larvae of the group treated with fluoranthene, but with its lower concentration, in which the most intense expression of isoforms I1 and I2 was also observed (Fig. 3C). The increased expression intensity of enzyme isoforms in this group of larvae, with the expression of one more isoform, could also enable efficient functioning of CarEs in the whole midgut of *E. chrysorrhoea* larvae exposed to pollutant. Recent study has described the response of CarEs in mussels chronically exposed to PAHs pollution in their habitat, suggesting that enzyme activity may be a suitable parameter for organisms exposure to these pollutants (Solé et al., 2020). Differences in CarEs responses to PAH effects were noted in various species; acute exposure of the earthworm *Eisenia fetida* to fluoranthene and other 3- and 4-ringed PAHs led to significantly reduced CarEs activity (Nam et al., 2015). Conversely, significantly increased CarEs activity has been shown in dipteran larvae *Chironomus sancticaroli* upon acute treatments with high concentrations of the PAH phenanthrene (Richardi et al., 2018). Grčić et al. (2021) have detected significantly increased CarEs specific activity and expression of several enzyme isoforms in the midgut of *L. dispar* larvae after chronic treatment with dietary benzo[*a*]pyrene.

Regarding CarEs responses to dietary fluoranthene in midgut tissue, differences in specific activities and isoforms expressions were detected in larvae of two species. In *L. dispar* there were no significant differences in CarEs specific activity in the midgut tissue among control and groups of larvae treated with dietary fluoranthene ($p > 0.05$), but enzyme activity tended to increase in the group exposed to a higher concentration of fluoranthene (Fig. 4A). Five enzymes isoforms were detected, with isoform I2 observed in the midgut tissue of treated larvae but not in the control ones (Fig. 4B). The most expressed isoforms I3 and I4 were seen in larvae exposed to the lower fluoranthene concentration (Fig. 4C). On the other hand, significantly increased CarEs specific activity was recorded in the midgut tissue of *E. chrysorrhoea* larvae exposed to the lower concentration of dietary fluoranthene ($p < 0.001$) (Fig. 5A), and in which expression of three isoforms (of the seven detected) was inhibited (Fig. 5B and C), which could be more beneficial in the presence of a pollutant, in terms of the resources necessary for the induction of other enzymes previously shown to be included in the defense (Filipović et al., 2019). The detected midgut (the whole midgut, as well as midgut tissue) CarEs responses, i.e., specific patterns of isoforms expression in larvae of these species exposed to dietary PAH pollutant, indicate the importance of maintaining appropriate enzymes activity, considering CarEs detoxifying role, and also their potential as biomarkers of pollution.

Esterase activities were determined in different insect tissues and at various stages of insect life, e.g., in fifth instar *L. dispar* larvae, with

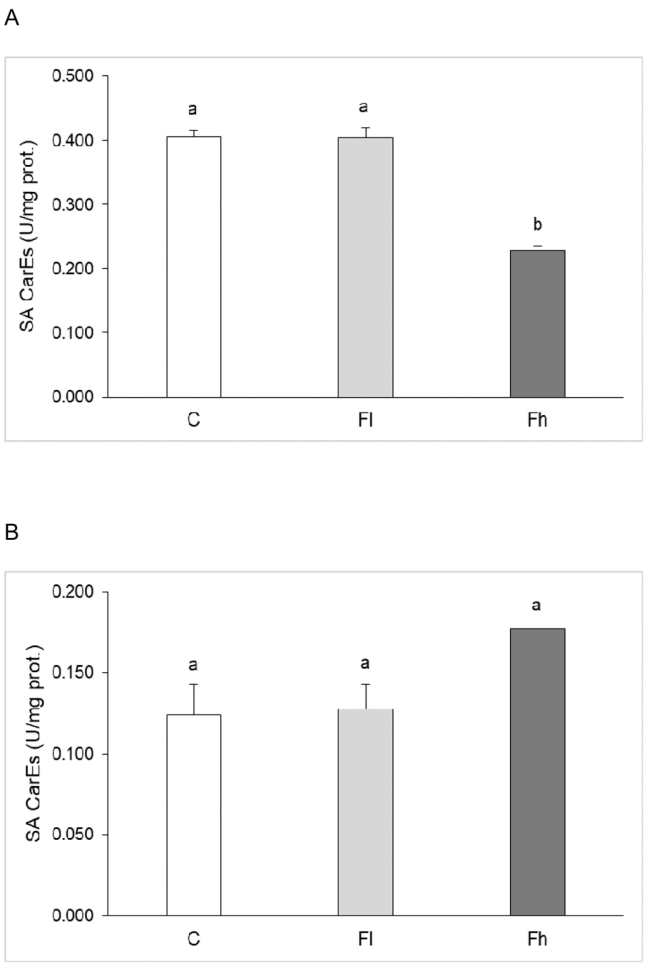

Fig. 6. The specific activity of CarEs in the brain tissues of fifth instar *L. dispar* (A), and *E. chrysorrhoea* (B) larvae exposed to lower (Fl) and higher (Fh) concentration of dietary fluoranthene, control group (C). Data are presented as mean ± SEM. Values marked with different letters differ significantly ($P < 0.05$).

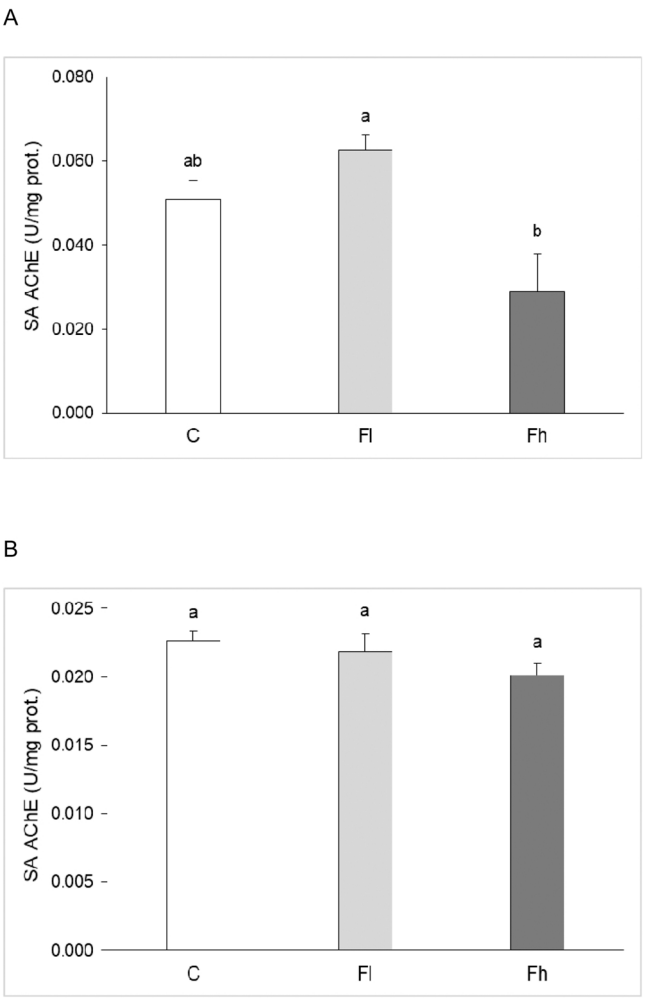

Fig. 7. The specific activity of AChE in the brain tissues of fifth instar *L. dispar* (A), and *E. chrysorrhoea* (B) larvae exposed to lower (Fl) and higher (Fh) concentration of dietary fluoranthene, control group (C). Data are presented as mean ± SEM. Values marked with different letters differ significantly ($P < 0.05$).

dominant CarEs activity in the midgut (Kapin and Ahmad, 1980), or in adult *Schistocerca gregaria* females of different ages, in which CarEs activity is marked as dominant, among others, in brain tissue, indicating the possibility of the enzyme's role in response to insecticide action (Rashad, 2008). Also, Yang et al. (2021) assume that CarEs activity in the brain of *Spodoptera frugiperda* larvae may be included in the fast development of insect resistance to pesticide actions. Tissue-dependent patterns of CarEs genes, including those expressed in the brain, were also revealed in *Locusta migratoria* (Zhang et al., 2014). Authors suggest a possible role of CarEs in protecting the locust brain from the harmful effects of toxicants. Increased CarEs activity has been detected in the brain tissue of fifth instar *L. dispar* larvae originating from polluted forest, after long-term exposure to dietary benzo[*a*]pyrene (Grčić et al., 2019). However, in the present study, we detected a reduction of brain CarEs activity in *L. dispar* larvae upon exposure to a higher concentration of dietary fluoranthene ($p < 0.001$) (Fig. 6A), while the pollutant presence did not influence brain CarEs activity in *E. chrysorrhoea* larvae ($p > 0.05$) (Fig. 6B). Also, AChE activity in the brain of *E. chrysorrhoea* did not change in treated larvae ($p > 0.05$) (Fig. 7B), indicating a protective role of other defense parameters and the importance of maintaining appropriate AChE function in the presence of pollutant. Assessment of the effects of several PAHs on *E. fetida* revealed significantly reduced CarEs activity at the concentrations of pollutants that did not influence AChE activity, which can indicate a protective role of CarEs, i.e., sequestering the xenobiotics, and thus protection of AChE in this species (Nam et al., 2015). As we found reduced AChE specific activity only in the brain of *L. dispar* larvae treated with the higher concentration of dietary pollutant, compared to those treated with the lower one ($p < 0.05$) (Fig. 7A), we could assume that the detected response of CarEs prevented more harmful influences of the dietary pollutant on AChE activity at this concentration.

Hsp70 participates in cell homeostasis and, as fast responds to many stressful factors, this stress protein could be used to assess environmental pollution (Moreira-de-Sousa et al., 2018). In the present study, a significant increase of Hsp70 concentration was detected in brain homogenates of *L. dispar* larvae treated with lower concentration of dietary fluoranthene ($p < 0.001$) which suggests a protective role of Hsp70 against pollutant effects, althought isoform expression pattern did not follow detected concentration changes (Fig. 8A). Induction of Hsps was shown in *Folosomia candida* exposed to two concentrations of phenanthrene (Nota et al., 2009). Monari et al. (2011) detected increased expression of Hsp70 in the digestive glands of *Chamelea gallina* following short- and long-term exposure to B[*a*]P. On the other hand, Hsp70 concentration in the brain of *E. chrysorrhoea* decreased significantly in groups of larvae exposed to dietary fluoranthene ($p < 0.001$), (Fig. 8B). This points to the species-specific responses of Hsp70 in brain tissues of larvae exposed to dietary fluoranthene, and may suggest a higher energy cost of the increase in Hsp70 concentration in *E. chrysorrhoea* larvae,

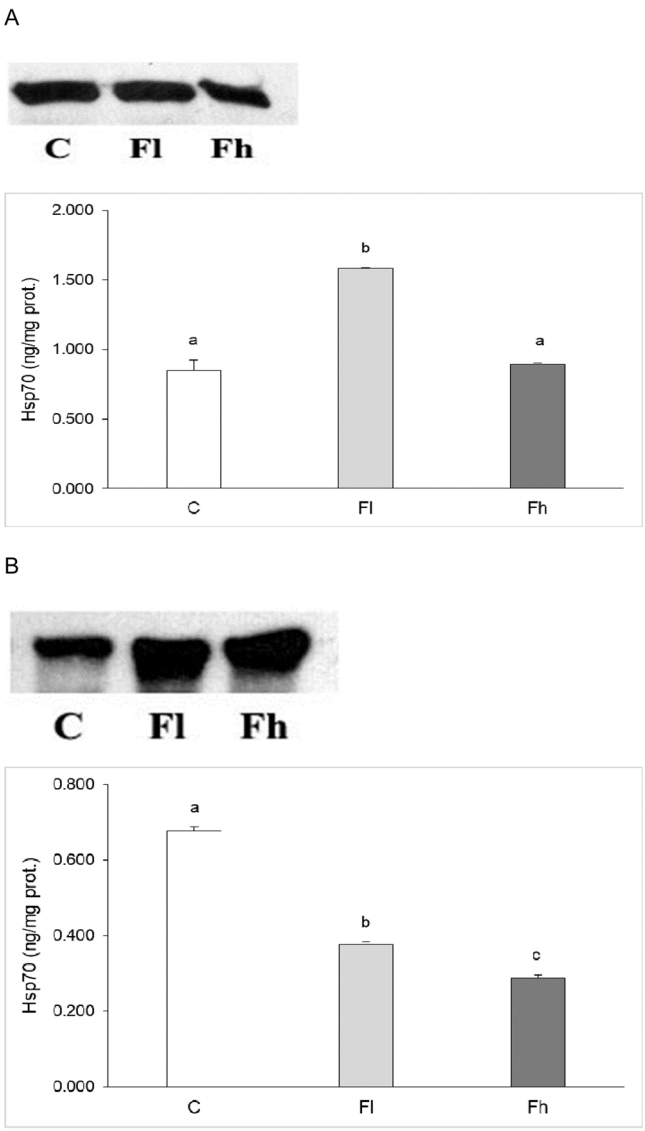

Fig. 8. Concentrations and Western immunoblots of Hsp70 in the brain tissues of fifth instar *L. dispar* (A), and *E. chrysorrhoea* (B) larvae exposed to lower (Fl) and higher (Fh) concentration of dietary fluoranthene, control group (C). Data are presented as mean ± SEM. Values marked with different letters differ significantly ($P < 0.05$).

especially in circumstances when the components of defending mechanisms are induced, as already mentioned (Filipović et al., 2019). Significantly reduced expression of stress protein (Hsp71) has been shown in *Hyalella azteca* adults exposed to fluoranthene (Werner and Nagel, 1997). Kafel et al. (2012) have reported decreased levels of Hsp70 in *Harpalus rufipes* adults exposed to petroleum product (used engine oil) that contain PAHs and other contaminants. Also, Hsp70 concentration was significantly reduced in the brain of *L. dispar* larvae exposed to dietary cadmium, high temperature, and to their combined effect (Perić-Mataruga et al., 2017). In stressful conditions, energy consumption, necessary for the induction of defending mechanisms may lead to decreased protein synthesis (Callow, 1991), while on the other hand redirection of resources to the synthesis or degradation of Hsps may disturb other vital processes (Feder and Hofmann, 1999). However, the intensity of expression of detected Hsp70 isoform in brain of *E. chrysorrhoea* larvae appear to increase at both tested concentrations of dietary fluoranthene (Fig. 8B). Differences that we recorded in the intensities of expression of Hsp70 isoforms and in changes of Hsp70 concentrations in brain tissues of larvae of both species may be due to localization both the constitutive (Hsp73) and inducible (Hsp72) forms by methods used in the experiment. Therefore, further analysis of Hsp70 should be done in larvae of *L. dispar* and *E. chrysorrhoea*, in order to distinguish the responses of Hsp70 isoforms to dietary stress (their expression patterns vs. changes in their concentrations), and their dependence on the applied stressors. Moreover, the results of Hsp70 analysis in gill extracts of *Mytilus edulis*, exposed to cadmium and tributyltin, showed a higher sensitivity of the ELISA test than Western blotting, that is, differences between control and exposed organisms were detected by the ELISA method, but not by immunoblotting (Pempkowiak et al., 2001). Besides, Fang et al. (2021) detected induction of stress-inducible and constitutive Hsp70 forms in *Bombyx mori* larvae exposed to temperature stress, and the expression of inducible form but inhibition of most of the constitutive forms upon exposure to insecticides.

4. Conclusions

The responses of examined parameters to the presence of dietary fluoranthene in *L. dispar* and *E. chrysorrhoea* larvae imposed the need for resources, which led to prolonged larval development. The CarEs isoforms in the whole midgut and midgut tissue of larvae, with recorded specific patterns of expression, enabled efficient enzyme activity, as part of their defending mechanisms in the presence of pollutant. Reduced CarEs, and also AChE activities in the brain of *L. dispar* larvae upon exposure to the higher concentration of fluoranthene, could suggest preventive response of CarEs, against more harmful pollutant effect on AChE activity. The significance of maintaining appropriate AChE function was highlighted in *E. chrysorrhoea* larvae, in which there were no changes in the enzymes activity. Differences in the concentrations of stress protein were detected in the larval brains of these species, with an increased Hsp70 concentration in the brain of *L. dispar* larvae as a response to the proteotoxic effect of lower fluoranthene concentration, and reduced Hsp70 concentration in the brain of *E. chrysorrhoea* larvae in both treated groups, probably due to the induction of other defense mechanisms and, hence, greater resource needs. The results of this research indicate a significant role of the examined parameters in different tissues of larvae of both species, following chronic exposure to an organic pollutant, as well as their potential as pollution biomarkers.

CRediT authorship contribution statement

Marija Mrdaković: Conceptualization, Formal analysis, Writing – original draft. **Aleksandra Filipović:** Methodology, Investigation, Formal analysis, Writing – review & editing. **Larisa Ilijin:** Conceptualization, Validation. **Anja Grčić:** Methodology, Investigation. **Dragana Matić:** Methodology, Validation. **Dajana Todorović:** Visualization. **Milena Vlahović:** Validation. **Vesna Perić-Mataruga:** Project administration, Supervision.

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.

Data Availability

We have shared the link to our data at the Attach File step. The datasets analyzed in this study can be found at: https://data.mendeley.com/datasets/6rvkv28nn6/1.

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