Tissue-specific responses of *Lymantria dispar* L. (Lepidoptera: Erebidae) larvae from unpolluted and polluted forests to thermal stress

Larisa Ilijin, Anja Grčić, Marija Mrdaković, Milena Vlahović, Aleksandra Filipović, Dragana Matić and Vesna Perić Mataruga

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

*To whom correspondence should be addressed:

Larisa Ilijin

Department of Insect Physiology and Biochemistry, Institute for Biological Research “Siniša Stanković”,

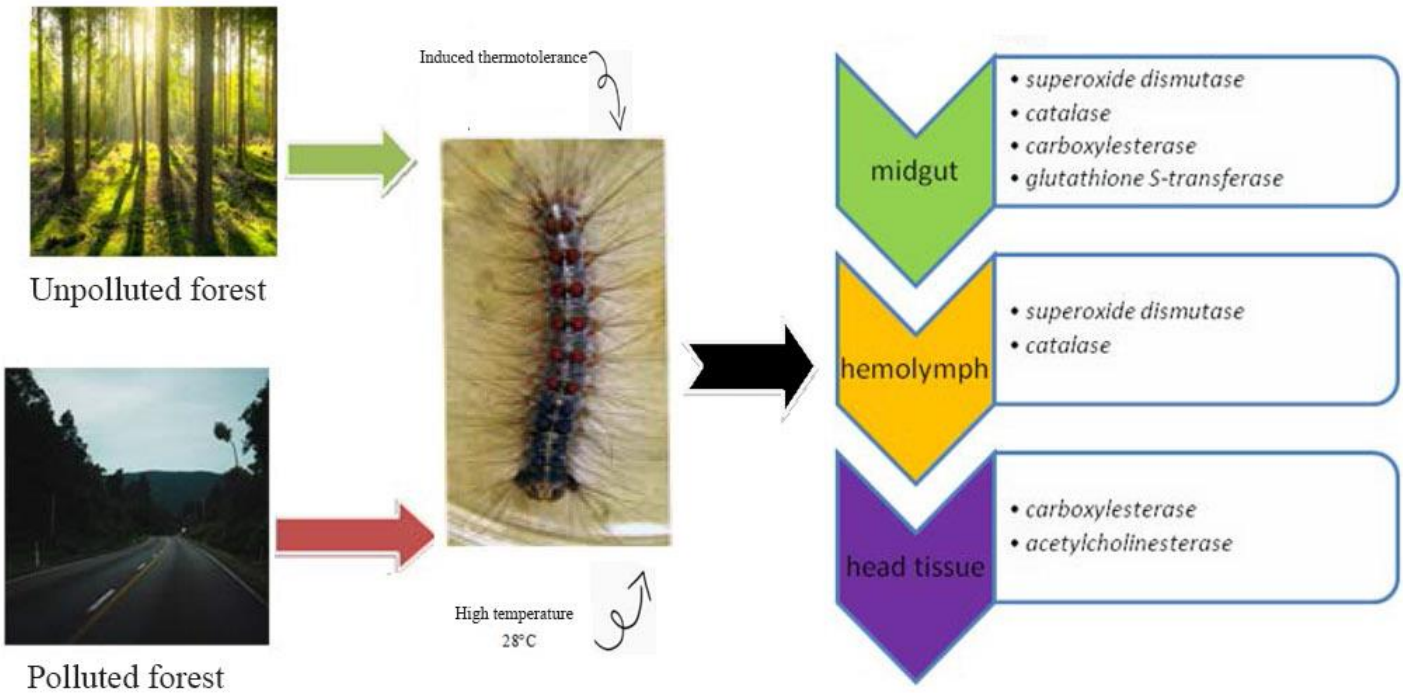
National Institute of Republic of Serbia, University of Belgrade,

Despot Stefan Blvd.142, 11060 Belgrade, Serbia

Phone: 381-11-2078-374 / mobile: 381-62-653-860

Fax: 381-11-2761-433

E-mail: lararid@ibiss.bg.ac.rs; bolejole@yahoo.co.uk


Induced thermotolerance
Unpolluted forest
Polluted forest
High temperature
28°C
midgut
• superoxide dismutase
• catalase
• carboxylesterase
• glutathione S-transferase
hemolymph
• superoxide dismutase
• catalase
head tissue
• carboxylesterase
• acetylcholinesterase

Highlights :

- *Lymantria dispar* larvae were exposed to thermal stress.
- Superoxide dismutase and catalase are sensitive to thermal stress.
- Carboxylesterase showed tissue specific responces.
- Glutathione S-transferases and acetylcholinesterase had origin dependent sensitivity.

Abstract

In this paper the effects of increased environmental temperature on the relative growth rate (RGR) and developmental time in 5th instar *L. dispar* larvae originating from unpolluted and polluted forests were analyzed. As indicators of the level of generated reactive oxygen species in thermal stress, we estimated midgut and hemolymph activity of the antioxidative enzymes, superoxide dismutase (SOD) and catalase (CAT), as well as the detoxifying enzymes glutathione S-transferase (GST), carboxylesterase (CaE) and acetylcholinesterase (AChE) from the midgut and brain tissue. We also examined the influence of induced thermotolerance as a species' ability to overcome the negative effects of this stressor. In larvae originating from the unpolluted forest, the midgut is the primary location of increased SOD and CAT activity and induced thermotolerance did not modified their activity in either tissue. In larvae from the polluted forest, in both tissues SOD activity was more sensitive to an increased temperature and induced thermotolerance than CAT. Carboxylesterase responded diversely to thermal stress depending on the analyzed tissue regardless the origin of larvae, while the activity of GST and AChE in tissue depended on the origin of larvae. Induced thermotolerance modified the activity of detoxifying enzymes in larvae originating from the polluted forest. Combining the selected parameters into an integrated biomarker response (IBR) the GST, CaE and AChE battery emerged as a potential biomarker for thermal stress in *L. dispar* larvae.

Key words: thermal stress, different larval tissues, antioxidative enzymes, detoxifying enzymes, larvae from unpolluted and polluted forests

1. Introduction

Temperature is an abiotic factor that plays a key role in insect growth, development, survival and abundance. As ectotherms they cannot regulate body temperature in a changing environment, and their energy metabolism is influenced by environmental temperature (Angilletta et al., 2002). Having in mind that during last 100 years global temperature has increased by approximately 0.6 °C, and is predicted to increase further by 1.4 °C before 2100 (Bale et al., 2002), it is crucial to understand the impact of a higher temperature on various insect species. Effects at different levels of organization (individual, population, community) and the physiological and ecological mechanisms of response to predicted events of climate change need investigation.

Intracellular redox state in each organism represents a delicate balance between reactive oxygen species (ROS) generated during metabolism and the activity of antioxidative system that scavenges them. These processes of reduction and oxidation occur simultaneously (redox cycle) and cannot happen independently. Under normal conditions there is a balance between generated ROS and the ability of an organism to detoxify them (Livingstone, 2001). In aerobes, generation of ROS like the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH) and other free radicals, accompanies respiration and numerous biochemical reactions in oxidative metabolism (Halliwell and Gutteridge, 2007). Thermal stress disturbs this regular redox cycle raises the level of ROS or otherwise causes oxidative stress (Ahmad and Pardini, 1990; Pardini, 1995). ROS include oxygen ions, free radicals and peroxides, both inorganic and organic, each of which is a potent oxidant that damages biological macromolecules (lipids, proteins, and nucleic acids) resulting in disruption of membranes and other cellular structures (Livingston, 2001). These disturbances can also trigger a series of events that could finally lead to apoptosis (Limón-Pacheco and Gonsebatt, 2009). To avoid or at least reduce this, insects have developed an antioxidant defense system consisting of enzymatic components (superoxide dismutase (SOD), catalase (CAT), glutathione reductase, ascorbate peroxidase) and nonenzymatic components (vitamins C and E, glutathione), which enable them to overcome the harmful effects of increased ROS level and protect their cells from oxidative stress (Hermes-Lima and Zenteno-Savin, 2002).

The first line of antioxidative defense includes SOD, that converts the superoxide anion (O_2^-) into oxygen (O_2) and H_2O_2. The H_2O_2 is then converted by CAT into O_2 and water (H_2O) (Wang et al., 2001). Like all metabolically active tissues, midgut has shown high activity of

antioxidative enzymes in studies on several phytophagous insect species (Ahmad and Pardini, 1990). Insects have also developed various detoxification mechanisms using glutathione S-transferases (GSTs), carboxylesterase (CaE) and acetylcholinesterase (AChE) as the second antioxidative defense line (Yu and Hsu, 1993; Yan et al., 2009; Strode et al., 2008). Surplus ROS causes lipid peroxidation, a process that can have a negative impact on lipid function (Ahmad, 1995). Lipids play an important, if not central, role in almost all physiological processes as signaling and regulatory molecules. GSTs are involved in detoxification of electrophilic xenobiotics through conjugation of these electrophiles to GSH enabling removal of the products of lipid peroxidation from cells (Fournier et al., 1992; Dubovskiy et al., 2008), and highest GST activity was found in Malpighian tubules, guts, fat body and nerve cord (Konno and Shishido, 1992).

Carboxylesterases (CaE) are important detoxifying enzymes able to hydrolyze various esters (Satoh and Hosokawa, 2006). They appear to be widely distributed in all insect tissues, having an active role in the regulation of hormone levels, general metabolism and mobilization of fats and energy from muscles (Jones and Bancroft, 1986). They are responsible for detoxification of many compounds generated in the attack of oxygen radicals on lipids and DNA, as well as after exposure of insects to organophosphate, carbamate, and pyrethroid insecticides (Hemingway et al., 2004). According Kapin and Ahmad (1980) *Lymantria dispar`s* midgut is the tissue richest in carboxylesterase activity, while brain and nerve cord have higher acetylcholinesterase activity. In hemolymph total activity of esterases is negligible.

In insects acetylcholinesterase (AChE) is the only member of the cholinesterase family (Hussein et al., 1983). It is a key enzyme in the nervous system, which regulates nerve impulse transmission by catalyzing hydrolysis of the neurotransmitter acetylcholine at the cholinergic synapse, blocking the nervous impulse. Insecticides and other types of environmental contaminants, possess great affinity towards binding to AChE, causing enzyme inhibition that leads to accumulation of acetylcholine and subsequent hyperpolarisation of the postsynaptic membrane. This disrupts nervous system function (Galloway and Handy, 2003). Published data indicate sensitivity of AChE to low temperature (Singh et al., 2013) but information on the effects of heat stress on this enzyme in insects is inconsistent.

Thermotolerance represents a species' ability to overcome the effects of climate changes (Laws and Belovsky, 2010; Schulte et al., 2011) and is a conservative characteristic of almost all living

organisms. There are two types of thermotolerance: basic without prior acclimation and acquired, i.e. developed after pretreatment at an elevated but sublethal temperature (Lindquist, 1986). Tolerance to a high temperature can be increased as a genetic adaptation in different geographical populations or in laboratory selected lines with an enhanced survival rate at high temperatures. The second mode is long or short term acclimatization of individual by rearing them at high, but sublethal temperatures for long or short period of time (Denlinger, 2009). The latter is acquired quickly, reaches a maximum within a few hours, and then decays slowly over several days. This rapid heat hardening secures protection from trauma at a very high temperature.

Different populations of the same species can respond diversely to the same change in the environment, depending on previous contact with various environmental stressors and possible development of genetic and phenotypic adaptations under prolonged exposure. Many studies indicate that warming increases the toxicity of pollutants (climate – induced toxicant sensitivity), while some of them indicate the opposite, i.e. that pollutants can alter the ability of an organism to cope with an elevated environmental temperature (toxicant-induced climate change sensitivity) (de Beeck et al., 2016).

L. dispar is polyphagous insect with over 500 host plant species and widespread in temperate climate zone. *L. dispar* is easily manipulated and has a short generation time, precisely defined developmental stages, and well-known physiological processes. All of these characteristics make it a convenient model system in investigation of thermal stress in insects. The sensitivity of antioxidative defense system in *L. dispar* caterpillars reared on artificial diet was detected upon exposure to several environmental stressors (Mirčić et al., 2013; Perić Mataruga et al., 2014; Gavrilović et al., 2017; Filipović et al., 2019). Differences in oxidative stress parameters were observed among caterpillars collected from unpolluted and polluted forests (Perić Mataruga et al., 2019).

The aim of this work was to analyze tissue-specific sensitivity of the responses of antioxidative (SOD and CAT) and detoxifying (GST, CaE and AChE) enzymes, to high temperature and induced thermotolerance in 5^{th} instar *L. dispar* larvae originating from egg masses collected from unpolluted and polluted forests and reared on artificial diet and controlled conditions. Using larvae originating from two differently polluted natural populations will indicate the importance of knowing the history of population exposure to other environmental stressors when monitoring

forest ecosystems. Combining the selected parameters into an integrated biomarker response (IBR) we tested the sensitivity of the responses of two differently adapted *L. dispar* populations and estimated biomarker potential for environmental warming. Obtained results can enlarge our knowledge about stress responses in phytophagous insects as a pool of potential biomarkers of changes in forest environmental pollution in order to maintain forest health and stability in the future.

2. Material and methods

2.1 Insect rearing and temperature treatment

L. dispar egg masses were collected in the autumn (November) at two sites: Kosmaj forest (unpolluted) and Lipovica forest (polluted). Kosmaj is a mountain 40 km south east of Belgrade (coordinates 44°27′56″N 20°33′56″E), far from intensive anthropogenic pollution and is a part of the circle of protected green areas round Belgrade. With over 557 plant species, 300 fungi, 171 animal and numerous insect species among which 4 are new to fauna of Serbia, Kosmaj was declared as a protected area (Official Gazette of the city of Belgrade, 2005). Such areas are considered as unpolluted since it is prohibited by legal regulations to construct industrial facilities, agricultural and other economic facilities and facilities of communal, traffic and energy infrastructure whose construction or work may negatively affect the quality of air, water, land, wildlife, forests, or have other significant effects on the environment. The area has remained over 70% forested, mostly by mixed oak woods (Stajić et al., 2018). Lipovica forest is located 20 km south of downtown Belgrade (coordinates 44°38′11″N 20°24′12″E) and borders State Road 22, commonly known as the Ibar Highway an IB-class road, one of the most frequently used in Serbia. Different pollutants are present along heavy traffic road and the area most affected is the band within 300 to 500 meters of the road. With more than 18,000 vehicles daily passing through, this forest is considered as polluted. The most common species of trees in both forests are Hungarian oak (*Quercus frainetto*) and Turkey oak (*Quercus cerris*) (Vukin, 2017).

After collection the egg masses were kept in a refrigerator at 4 °C until spring (March), when they were set for hatching. Newly hatched larvae were grown in transparent plastic dishes (V = 200 mL) at 23 °C with a 12:12 h light: dark photoperiod and relative humidity of 60%. After hatching in each transparent Petri dish (V = 200 mL) 10 first instar larvae were transferred and they were reared together until third larval instar. Five third instar larvae were reared together in

the same Petri dish and after molting into the fourth instar each larvae was kept individually until 3rd day of 5th instar, when they were sacrificed. Larvae were fed on an artificial diet designed for *L. dispar* (O'Dell et al., 1985). Food was replaced every 48 h throughout the whole time of the experiment.

For each of eight experimental groups 200 eggs were set for hatching and treatments were the same for larvae originating from the unpolluted (**UP**) and polluted (**PP**) forests:

Each experimental group contained between 50 and 60 larvae	
UP23 and PP23	larvae reared at 23 °C from hatching to sacrifice
UP23In and PP23In	larvae reared at 23 °C from hatching until the first day of 4th instar, and then exposed to 28 °C for 24 h (induced thermotolerance). Afterwards they were returned to 23 °C until the third day of 5th larval instar.
UP28 and PP28	larvae reared at 23 °C from hatching until the first day of 5th larval instar, and then exposed to 28 °C for 72 h.
UP28 In and PP28In	larvae reared at 23 °C from hatching until the first day of 4th instar, and then exposed to 28 °C for 24 h (induced thermotolerance). They were then returned to 23 °C until the first day of 5th larval instar and exposure to 28 °C for 72 h.

Larvae were reared at 23 °C as an optimal temperature for development. The highest measured summer temperature (2007-2010) in serbian Quercus forests at similar elevation was 28.4 ℃, the lowest was 19.6℃, while average summer temperature was 26.3 ℃ (Babić, 2014). We then established variable temperature regimens that included a brief (24 hours), daily (72 h) exposures to 28 °C.

2.2. Estimating life history traits

Life history traits, such as relative growth rate (RGR) and larval developmental time were monitored in each experimental group (n = 15). Larvae were weighed on the third day after moulting into the 3rd, 4th and on the third day of the 5th instar. RGR was calculated as $RGR=(\ln W_t - \ln W_0)/t$, where W_0 and W_t are the weights of the larvae at the beginning and end of the examined period and t is the interval in days. We monitored individual larval development from hatching until molting into the 5th instar (days).

2.3. Preparation of homogenates

On the third day of the 5th instar *L. dispar* larvae were sacrificed on ice (n = 8 - 12 larvae per group for each enzyme assay). Midguts were excized, weighed individually, rinsed with

physiological saline, and homogenized on ice (final tissue concentration 50 mg / ml) in ice-cold saccharose buffer 0.25 M (0.05 M Tris–HCl, 1mM EDTA, pH 7.4), at 2,000 rpm using an UltraTurrax homogenizer (2000 Upm; IKA-Werke, Staufen, Germany). They were then sonified (Model HD2070, Bandelin, Berlin, Germany) for 3 x 15 s, with 15 s pauses. The homogenates were spun for 100 min at 105,000 g at 4 °C in a Beckman L7-55-Ultracentrifuge (Nyon, Switzerland). The resulting supernatants were used for determination of SOD, CAT and GST activity. Hemolymph was collected from individual larvae (n = 8 - 12 larvae per group for each enzyme assay) in plastic tubes containing a few small crystals of phenylthiourea (PTU), and then diluted fivefold with ice cold buffer (pH 7) containing 1.15% KCl, 25 mM K_2HPO_4, 5 mM PMSF and 2 mM DDT, after which they were sonicated three times for 10 s, with 10 s pauses, individually with a Bandelin HD 2070 ultrasonic homogenizer (Berlin, Germany). Homogenates were then centrifuged at 10,000 g for 15 min at 4 °C in an Eppendorf Centrifuge 5417R (Hamburg, Germany). The obtained supernatants were used for enzyme assays.

Brain tissue was dissected out from the head capsules on ice and due to the small size of the samples, larval brain tissues were pooled within each experimental group (n = 30 brains per group). Pooled brain tissue was diluted with distilled water (1:9/w:v) and homogenized on ice at 5,000 rpm, in three 10 s intervals, separated by 15 s pauses (MHX/E Xenox homogenizer, Germany). Homogenates were centrifuged at 10,000 g for 10 min at 4 °C in an Eppendorf 5417R centrifuge (Hamburg, Germany). The supernatants were used for the enzyme assays.

2.4. Spectrophotometric assays of enzyme activity

Protein concentration was measured by the Bradford micromethod (Bradford, 1976) using bovine serum albumin (BSA) as the standard.

The activity of SOD was determined according to Misra and Fridovich (1972). Measurement is based on the enzyme's ability to prevent adrenaline autoxidation in an alkaline medium. Conversion of adrenaline into adrenochrome releases superoxide anion radicals which accelerate the autoxidation reaction. Adrenaline autoxidation rate was determined spectrophotometrically at λ = 480 nm (Shimadzu UV-1800, UV Spectrophotometer). Two blanks, three controls for noncatalytic activity (adrenaline probe dissolved in carbonate buffer pH 10.2) and two replicates for each experimental group were used. SOD activity was expressed as the amount of enzyme causing 50% inhibition of adrenaline autoxidation per mg of protein (U / mg protein).

The method of Beutler (1982) was used to determine CAT activity. This spectrophotometric assay measures H_2O_2 (10 mM) hydrolysis by recording the absorbance decrease at 230 nm (Shimadzu UV-1800, UV Spectrophotometer). Two replicates for each experimental group, two blanks and three noncatalytic probes were measured. CAT activity was expressed in nanomoles as the amount of dissolved H_2O_2 reduced per minute per milligram protein (mM / min / mg protein).

Glutathione S-transferase (GST) catalyzes the conjugation of 1-chloro-2.4-dinitrobenzene (CDNB) with the SH groups of GSH. The yield of derived CDNB-GSH complex was measured spectrophotometrically at λ = 340 nm (Shimadzu UV-1800, UV Spectrophotometer) at 25 °C, for a period of 3 min (Habig et al., 1974) and expressed in nanomoles GSH per minute per milligram protein, using two replicates, two blanks and two non-conjugate probes.

Carboxylesterase activity (CaE) was determined according to Main et al. (1961) by measuring the hydrolysis of *p*-nitro-phenyl butyrate (15 mM) and recording the absorbance increase at 414 nm (Shimadzu UV-1800, UV Spectrophotometer). Two replicates were included for each experimental group, as well as two blanks and two noncatalytic probes. One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze 1 μmol of *p*-nitro-phenyl butyrate per minute per milligram of protein.

Acetylcholinesterase (AChE) activity was determined spectrophotometrically according to the method of Ellman et al. (1961), using acetylthiocholine iodide (0.25 M) as the substrate. Substrate hydrolysis yields thiocholine which can react with the dithiobisnitro-benzoate (DTNB) ion to yield the yellow 5-thio-2-nitrobenzoate anion This is quantified by its absorbance at 406 nm (Shimadzu UV-1800, UV Spectrophotometer). All samples were measured in triplicate (homogenized brain tissues pooled by each group). Enzyme activity was expressed as 1 μmol of substrate hydrolyzed per minute per mg of protein.

2.5. Integrated biomarker response (IBR)

IBR was evaluated according to Beliaeff and Burgeot (2002). The value of each biomarker (Xi) was determined by the formula Y_i = (Xi - mean)/SD, where Yi is the standardized biomarker response and means and SD were obtained from all values of the selected parameters. The next step described Z_i, as $Z_i = Y_i$ or $Z_i = -Y_i$, depending on whether the temperature treatment caused induction or inhibition of the selected biomarker. After the minimum value of Z_i was found for each biomarker (min), the scores (S_i) were computed as $S_i = Z_i + |min|$. Scores for biomarkers

were used as radius coordinates for each biomarker in star plots. For the four biomarker combination of antioxidative enzymes, ranged clockwise as follows: SOD activity in midgut, CAT activity in midgut, SOD activity in hemolymph and CAT activity in hemolymph; individual areas A_i of the star plot were calculated according to the formula: $A_i = S_i * S_{i+1}/2$, where S_i and S_{i+1} represent individual biomarker scores and their successive star plot radius coordinates. Star plot areas for the three biomarker assembly, positioned in the order: AChE activity in brain tissue, CaE activity in brain tissue and GST activity in midgut; were obtained from the formulas: $A_i = \frac{S_i}{2*\sin\beta}(S_i * \cos\beta + S_{i+1} * \sin\beta)$, $\beta = \tan^{-1}\left(\frac{S_{i+1}*\sin\alpha}{S_i - S_{i+1}*\cos\alpha}\right)$, $\alpha = 2\pi/n$ radians (*n* is the number of biomarkers). The IBR values for both biomarker combinations were calculated as follows: $IBR = \sum_{i=1}^{n} A_i$, where A_i is the area represented by two consecutive biomarkers on the star plot. Excel software (Microsoft, USA) was used to calculate IBR values and to generate star plots.

2.6. Statistical Analysis

Statistical analyses were made in GraphPad Prism 6 (GraphPad Software, Inc., USA). The normality of data distribution was estimated using the D'Agostino and Pearson omnibus, as well as the Shapiro-Wilk normality test. Data were examined by one-way analysis of variance (ANOVA) and a *post hoc* multiple range test (Tukey's Multiple Comparison Test). The results were expressed as means ± standard error of the mean (SEM). For all comparisons, the probability of significance was set at $p < 0.05$.

3. Results

3.1. Life history traits

Relative growth rate of 5th instar *L. dispa*r larvae from the unpolluted forest increased upon exposure to a temperature of 28 °C without and with induced thermotolerance, while for larvae originating from the polluted forest this parameter was similar for all experimental treatments (**Fig. 1 A)**.

The thermal treatments did not influence larval development in individuals from the unpolluted forest, but in those originating from the polluted forest development was prolonged upon exposure to an increased temperature without and with induced thermotolerance in comparison to the control group. This was more pronounced in group PP28In with thermo tolerance (**Fig. 1B)**.

3.2. Tissue-specific changes in the activities of antioxidative enzymes superoxide dismutase and catalane

Midgut SOD activity (**Fig. 2A**) was increased after exposure of larvae from the unpolluted forest to an increased temperature with and without previous induction of thermotolerance. In larvae originating from the polluted forest, SOD activity was higher in midguts of larvae with induced thermotolerance at both experimental temperatures. The same change in activity of midgut CAT (**Fig. 2B**) was observed in both larval populations.

In hemolymph SOD activity (**Fig. 3A**) was increased in larvae originating from the unpolluted forest exposed to a higher temperature with and without induced thermotolerance. For larvae originating from the polluted forest SOD activity in hemolymph was raised only in group PP23In reared at 23 °C with induced thermotolerance and in group PP28 exposed to an increased temperature. CAT activity in hemolymph (**Fig. 3B**) was not altered in any group of larvae from the unpolluted forest, while in those from the polluted forest a decrease in CAT activity was recorded solely in group PP28In exposed to the high temperature with induced thermotolerance.

3.3. Tissue-specific activity of CaE and GST in larval midgut

Midgut CaE activity did not change upon exposure to the higher temperature or after induced thermotolerance in larvae from either forest (**Fig. 4A**).

In larvae from the unpolluted forest midgut GST activity was significantly elevated only in the group exposed to an increased temperature (**Fig. 4B**), while in those from the polluted forest the activity of this enzyme was similar under all temperature treatments.

3.4. Tissue-specific activity of CaE and AChE in larval brain tissue

CaE showed increased activity in larvae from the unpolluted forest upon exposure to all temperature treatments. In larvae originating from the polluted forest induced thermotolerance and return to 23 °C led to increased activity, while exposure to 28 °C induced a decrease in activity (**Fig. 5A**).

AChE activity in brain tissue was higher after temperature treatments at 28 °C in individuals originating from the unpolluted forest and in polluted forest larvae with induced thermotolerance returned to the increased temperature (**Fig. 5B**).

3.5. Integrated biomarker response (IBR) to temperature exposure

The antioxidative enzymes, SOD and CAT were more sensitive to the increased temperature of 28 ^{o}C and induced thermotolerance at this temperature in *L. dispar* larvae originating from the

unpolluted forest (**Table 1**). The highest scores on the IBR plot were shown by SOD in hemolymph (SODh) and midgut CAT (CATm) in the group of larvae with induced thermotolerance and returned later to increased temperature (UP28In) and in midgut SOD (SODm) and CAT (CATm) in the group exposed only to a higher temperature (UP28) (**Fig 6A**).

L. dispar larvae originating from the polluted forest showed higher IBR indexes after induced thermotolerance at both temperatures (**Table 1**). On the radial plot the highest scores were detected for CATh in the group with induced thermotolerance and exposed to increased temperature (PP28In) and for CATm in group PP23In with induced thermotolerance and returned to 23 °C (**Fig 6B**).

The IBR index calculated for the detoxifying enzymes CaE, GST and AChE had higher values in both groups of larvae from the unpolluted forest subjected to a higher temperature (**Table 2**). The highest scores on the radial plot were for midgut GST (GSTm) and brain CaE (CaEb) for the group exposed to a higher temperature and in the UP28In group for brain AChE (AChEb) (**Fig 7A**).

In larvae from the polluted forest a higher IBR index was also recorded for both groups subjected to an increased temperature (**Table 2**), and higher scores on the plot were observed for CaEb in these larvae after both temperature treatments with induced thermotolerance (**Fig 7B**).

4. Discussion

As an abiotic factor, environmental temperature has a profound effect on development of phytophagous insects, through its influence on leaf composition, nutritive quality and the presence of plant allelochemicals, besides a direct effect (Williams et al., 2003). Also, a raised temperature enhances the toxicant sensitivity of insects (de Beeck et al., 2016). In *L. dispar* larvae food consumption and utilization were found to increase with elevation of temperature (Lindroth et al., 1997). In our experiment this was the case for larvae originating from the unpolluted forest, where the temperature of 28 ^{o}C (with and without induced thermotolerance) elevated RGR but larval development pattern was unchanged. However, in the larvae originating from the polluted forest RGR remained unchanged and larval development was prolonged (**Fig 1**). In this case energy resources were probably used in other physiological processes to overcome the negative effects of thermal stress with consequent slower larval development.

Exposure to thermal stress generates elevated levels of ROS, leading to oxidation of proteins, peroxidation of membrane lipids, damage to nucleic acids, changes in metabolism and mutagenesis and can even induce cell death (LeBras et al., 2005).

As the first line of defense against oxidative stress energy resources are directed towards the SOD-CAT system. Midgut SOD and CAT levels in polyphagous herbivorous *L. dispar* larvae are very sensitive to toxic polycyclic aromatic hydrocarbons like benzo[*a*]pyrene and fluoranthene (Ilijin et al., 2015; Mrdaković et al.,2015; Gavrilović et al., 2017; Filipović et al., 2019), while cadmium ingestion did not affect midgut SOD and CAT activities (Mirčić et al., 2013). Increased activity of SOD was observed after trophic stress (Perić Mataruga et al., 2014), Protective roles of SOD and CAT during thermal stress were detected after exposure of *Ectomyelois ceratoniae* 5^{th} instar larvae (Farahani et al., 2020), oriental fruit fly *Bactrocera dorsalis* (Jia et al., 2011), and the oriental army worm *Mythimna separata* (Ali et al., 2016).

In this experiment, we observed that *L. dispar* larvae originating from the unpolluted forest, activate both midgut antioxidative enzymes to manage the increased levels of ROS generated upon exposure to the hotter environment (**Fig. 2**). Short term induced thermotolerance also improved the ability of larvae from this forest to overcome the effects of oxidative stress caused by a raised temperature, which was manifested by increased SOD and CAT activity. The advantage of induced adaptation is that the mechanism of tolerance is constantly activated, but functions only after a certain threshold is reached. The second benefit is that this mechanism is reversible. Namely, it is turned off when the insect returns to its optimal temperature range.

Our results showed **(Fig. 2)** that in larvae originating from the polluted forest, SOD and CAT activities were not significantly increased indicating that exposure to various environmental stressors, through several generations, had improved their ability to cope with the additional stress of a hotter environment. Basically, SOD activity was higher in midguts of *L. dispar* larvae from polluted locations than in those from unpolluted ones (Perić Mataruga et al., 2019), which is evidence of cross protection, i.e. induction of tolerance to one stressor by exposure to another. Thus, larvae originating from the polluted forest were sensitive to thermal stress only in the groups with induced thermotolerance. We presume that their adaptive mechanisms are constantly activated, with greater tolerance to elevated temperature arising from their population origin. However, they did recognize the temperature of 28 °C as stressful with timely ROS elevation,

which, probably represents a critical threshold for activation of antioxidative protective mechanisms (Schieber and Chandel, 2014).

Antioxidative enzyme activity varies among insect tissues and is the highest in tissues of high metabolic activity, like the midgut, while activity in hemolymph is low and remains that way in response to oxidative stress (Ahmad,1992). Pan et al. (2006) revealed that changes in antioxidative enzyme activity in hemolymph reflect damage to the whole organism. Increased activity of SOD and CAT in hemolymph as a response to induced oxidative stress by boric acid, was reported in *Galleria mellonella* (Hyršl et al., 2007), while treatment with eicosanoids, bioactive signaling lipids, resulted in elevated SOD and decreased CAT activity in hemolymph (Büyükgüzel et al., 2010). We found increased SOD and CAT activities in the hemolymph of *L. dispar* larvae exposed to benzo[*a*]pyrene (Gavrilović et al., 2017), while, SOD activity was decreased and CAT activity was elevated upon exposure to fluoranthene (Filipović et al., 2019). Exposure of both populations of *L. dispar* larvae to thermal stress induced a rise of SOD activity in hemolymph, but had no effect or inhibited CAT activity to a level below the control regardless of origin or thermotolerance (**Fig. 3**). There are two possible reasons for this. Firstly, other protective mechanisms or non-enzymatic components of the antioxidative system, such as thiols and ascorbate, are primarily engaged in hemolymph, as found for *Galleria mellonella* larvae (Grizanova et al., 2018). Secondly, accumulation of ROS in hemolymph and increased SOD activity in larvae originates from phagocytic activity by hemocytes, cells included in an insects` immune defense (Ahmad et al., 1991) with consequent inhibition of CAT activity.

We showed that different populations of *L. dispar* larvae possess disparate tissue sensitivities to thermal stress concerning antioxidative enzyme activity. In larvae originating from forests unburdened with pollution, midgut is the primary location of antioxidative activity in comparison to hemolymph but induced thermotolerance did not modify in either tissue. Larvae from forests impacted with various environmental stressors manifest a different mode of antioxidative action. In both examined tissues SOD activity was more sensitive to the increased temperature, than CAT activity. The set activity of this antioxidative enzyme is fairly dependent on population origin due to previous contact with pollutants for generations, in comparison to briefly induced thermotolerance.

GST engages in antioxidative protection as a ROS scavenger, by removing the products of lipid peroxidation or hydroperoxides from cells and as a detoxifying enzyme by conjugating reactive intermediates to glutathione (Singh et al., 2001). It plays a vital role in prevention of stress induced damage in insects. Increased activity of this enzyme was detected upon exposure of *G. mellonela* to the allelochemical xanthotoxin (Büyükgüzel et al., 2010), in the midgut of *L. dispar* larvae after ingesting alkaloid and flavonoid enriched diets (Perić Mataruga et al., 1997), upon exposure of *L. dispar* larvae to benzo[*a*]pyrene (Gavrilović et al., 2017) and in *Blaptica dubia* individuals given fluoranthene orally (Mrdaković et al., 2019). No changes in GST activity were recorded in *L. dispar* larvae midgut after ingesting fluoranthene or cadmium (Filipović et al., 2019; Vlahović et al., 2016). Adults of several different insect species exposed to thermal stress at over 30 °C, showed elevation of lipid peroxidation followed by a significant increase of GST activity (Yang et al., 2010; Zhang et al., 2015). Our present findings indicate that neither population origin nor induced thermotolerance had any influence on GST activity in *L. dispar* larval midgut upon exposure to thermal stress (**Fig. 4B**). The exception was the group of larvae from the unpolluted forest exposed to 28 °C, where GST activity was significantly increased. We presume that this generated higher levels of ROS in their midgut which could not be effectively eliminated by the SOD-CAT antioxidative enzyme system alone and that lipid peroxidation was increased with subsequent elevation of GST activity. Enhanced of lipid peroxidation can be caused by hyperthermia and hypothermia and is a biological marker of oxidative stress in tissues (Del Rio et al., 2005).

Increased lipid peroxidation activates lipolytic detoxification enzymes such as carboxylesterases, which hydrolyse into acids and alcohols the esters generated during metabolism of diverse xenobiotics, endobiotic degradations, biocatalysis and drug metabolism. Presumably GST activity in the midguts of our larvae was sufficient antioxidative protection from thermal stress, so similar levels of activity of this enzyme were detected after all temperature treatments, regardless of induced thermotolerance or larval origin (**Fig. 4A**). However, CaE activity in brain tissue of *L. dispar* larvae from the unpolluted forest increased after both treatments with induced thermotolerance and at the temperature of 28 °C (**Fig. 5A**). In larvae from the polluted forest only induced thermotolerance and return to 23 °C increased the activity of this enzyme, while for all other thermal treatments activity remained near or below the control level (**Fig. 5A**). Temperature shock alters allatotropin and juvenile hormone secretion, decreases the activity of

JH degrading enzymes and induces supernumerary molts (Gruntenko et al., 2000). Carboxylesterases are also involved in juvenile hormone degradation in insects (Steiner et al., 2017), so the detected activation CaE in brain tissue of larvae from the unpolluted forest may assist in preventing negative effects of thermal stress on the normal developmental pattern. In the other population this enzyme change was missing, which could indicate that multigenerational exposure of larvae to various other pollutants made their development less sensitive to the thermal stress we exposed them to and did not induce a stress reaction.

In brain tissue AChE, the other esterase we analyzed, showed increased activity at the high temperature after induced thermotolerance in both populations, but at the increased temperature alone only in larvae originating from the unpolluted forest (**Fig. 5B**). AChE is found at a very high level in insects, since it participates in hydrolysis of acetylcholine, a common neurotransmitter in insect nervous systems. Inhibition leads to accumulation of acetylcholine after signal transduction and represents an early warning signal of neurotoxicity of an environmental pollutant (Badiou et al. 2008). The increased AChE activity found here indicated that elevated temperature with induced thermotolerance had neurotoxic effects in larvae from both forests.

The response of antioxidative and detoxification enzymes in *L. dispar* larvae exposed to thermal stress revealed the presence of tissue-specific responses, population adaptation differences and changes in enzyme activity upon induced thermotolerance. Using IBR analysis we combined all parameters to estimate possible applications of the studied biomarkers in thermal stress biomonitoring using *L. dispar* larvae as the indicator organism. For the population from the unpolluted forest, the SOD and CAT biomarker battery showed a higher IBR index in larvae exposed to 28 ^{o}C with and without induced thermotolerance than for the population from the polluted forest, which indicates different sensitivity to increased environmental temperature depending on the level of pollution. The CaE, GST and AChE battery showed the highest IBR indexes at increased temperature (with and without induced thermotolerance) in both populations, which suggests that these detoxifying enzymes possess good biomarker potential for indicating thermal stress.

5. Conclusion

The analyzed components of the antioxidative defense system in 5th instar *L. dispar* larvae expressed different sensitivities to temperature rises from 23 °C to 28 °C. Thus, SOD and CAT

activities in midgut and hemolymph were modified differently at this temperature, and were also influenced by larval origin. The detoxifying enzymes GST, CaE and AChE responded diversely to thermal stress depending on the analyzed tissue (CaE) and larval origin (GST and AChE). Induced thermotolerance led to increased enzyme activity in larvae from the polluted forest, while IBR analysis pointed to GST, CaE and AChE as a battery with biomarker potential for thermal stress in *L. dispar* larvae as bioindicator species.

Author statement

All animal 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 Republic of Serbia, University of Belgrade.

Funding information

This study was supported by the Serbian Ministry of Education, Science and Technological Development, Contract 451-03-68/2020-14/200007.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

CRediT authorship contribution statement

Larisa Ilijin: Study concept and design, Methodology, Data acquisition and interpretation, Writing the original draft.

Anja Grčić: Methodology, Data acquisition and analysis, Writing, Reviewing & Editing the original draft.

Marija Mrdaković: Study concept and design, Data interpretation.

Milena Vlahović: Conceptualization, Revising the article for intellectual content.

Aleksandra Filipović: Methodology, Approval of the final version.

Dragana Matić: Methodology, Data acquisition and analysis.

Vesna Perić Mataruga: Conceptualization, Revising the article, Supervision, Project administration

References

1. Ahmad, S., Pardini, R.S., 1990. Mechanisms for regulating oxygen toxicity in phytophagous insects. Free Radic. Biol. Med. 8, 401-413. doi: 10.1016/0891-5849(90)90107-t.
2. Ahmad, S., Duval, D.L., Weinhold, L.C., Pardini, R.S., 1991. Cabbage looper antioxidant enzymes:Tissue specificity, Insect Biochem. 21, 563–572. https://doi.org/10.1016/0020-1790(91)90111-Q.
3. Ahmad, S., 1992. Biochemical defence of pro-oxidant plant allelochemicals by herbivorous insects. Biochem. Syst. Ecol. 20, 269–296. https://doi.org/10.1016/0305-1978(92)90040-K.
4. Ahmad S, 1995. Oxidative stress from environmental pollutants. Arch. Insect. Biochem. Physiol. 29, 135–157. https://doi.org/10.1002/arch.940290205.
5. Ali, A., Rashid, M. A., Huang, Q. Y., Wong, C., Lei, C.-L., 2016. Response of antioxidant enzymes in *Mythimna separata* (Lepidoptera: Noctuidae) exposed to thermal stress. Bull. Entomol. Res. 107(03), 382–390. doi: 10.1017/S0007485316001000.
6. Anguilletta Jr, M.J., Niewarowski, P.H., Navas, C.A., 2002. The evolution of thermal physiology in ectotherms. J. Therm. Biol. 27, 249–268. https://doi.org/10.1016/S0306-4565(01)00094-8.
7. Babić V. 2014. Influence of ecological factors and stand characteristics on natural sessile oak (*Quercus petraea* agg. ehr.) forest regeneration on Fruška Gora mountain, Doctoral dissertation, Faculty of Forestry.
8. Badiou, A., Meled, M., Belzunces, L.P., 2008. Honeybee *Apis mellifera* acetylcholinesterase — A biomarker to detect deltamethrin exposure. Ecotoxicol. Environ. Saf. 69, 246-253. doi: 10.1016/j.ecoenv.2006.11.020
9. Bale, J. S., Masters, G. J., Hodkinson, I. D., Awmack, C., Bezemer, T. M., Brown, V. K., Butterfield, J., Buse, A., Coulson, J. C., Farrar, J., Good, J. E. G., Harrington, R., Hartley, S., Jones,T. H., Lindroth, R. L., Press, M. C., Symrnioudis, I., Watt, A. D., Whittaker, J. B., 2002. Herbivory in global climate change research: direct effects of rising temperature on insect herbivores. Glob. Chang. Biol. 8, 1-16. doi:10.1046/j.1365-2486.2002.00451.x.
10. Beliaeff, B., Burgeot, T., 2002. Integrated biomarker response: a useful tool for ecological risk assessment. Environ. Toxicol. Chem. 21, 1316–1322. https://doi.org/10.1002/etc.5620210629.

11. Beutler, E., 1982. Catalase. In: Beutler, E. (Ed), Red cell metabolism. A manual of biochemical methods. Grune and Stratton, New York, pp. 105-106.
12. Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein- dye binding. Anal. Biochem. 72, 248-254. https://doi.org/10.1016/0003-2697(76)90527-3.
13. Büyükgüzel, E., Hyršl, P., Büyükgüzel, K., 2010. Eicosanoids mediate hemolymph oxidative and antioxidative response in larvae of *Galleria mellonella* L. Comp. Biochem. Physiol. B, Mol. Int. Physiol. 156(2), 176–183.
14. de Beeck, O. L., Verheyen, J., Stoks, R., 2017. Integrating both interaction pathways between warming and pesticide exposure on upper thermal tolerance in high- and low-latitude populations of an aquatic insect. Environ. Pollut. 224, 714–721. https://doi.org/10.1016/j.envpol.2016.11.014.
15. Del Rio, D., Stewart, A.J., Pellegrini, N., 2005. A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. Nutr. Metab. Cardiovasc. Dis. 15, 316–328. doi: 10.1016/j.numecd.2005.05.003.
16. Denlinger, D.L., 2009. Cold/Heat Protection. In: Vincent, H. R., Ring, T. C. (Eds), Encyclopedia of insects. Academic Press, Cambridge, Massachusetts, pp. 204-209.
17. Dubovskiy, I.M., Martemyanov, V.V., Vorontsova, Y.L., Rantala, M.J., Gryzanova, E.V., Glupov, V.V., 2008. Effect of bacterial infection on antioxidant activity and lipid peroxidation in the midgut of *Galleria mellonella* L. larvae (Lepidoptera, Pyralidae). Comp. Biochem. Physiol. C 148, 1–5. doi: 10.1016/j.cbpc.2008.02.003.
18. Ellman, G.L., Courtney, K.O., Anders, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88-95. doi: 10.1016/0006-2952(61)90145-9.
19. Farahani, S., Bandani, A.R., Alizadeh, H., Goldansaz, S.H., Whyard, S., 2020. Differential expression of heat shock proteins and antioxidant enzymes in response to temperature, starvation, and parasitism in the Carob moth larvae, *Ectomyelois ceratoniae* (Lepidoptera: Pyralidae).PLoS ONE 15(1), e0228104. https://doi.org/10.1371/journal.pone.0228104.
20. Filipović, A., Mrdaković, M., Ilijin, L., Vlahović, M., Todorović, D., Grčić, A., Perić Mataruga, V., 2019. Effect of fluoranthene on antioxidative defense in different tissues of

Lymantria dispar and *Euproctis chrysorrhoea* larvae. Comp. Biochem. Physiol. C 224,108565. https://doi.org/10.1016/j.cbpc.2019.108565.

21. Fournier, D., Bride, J.M., Poirie, M., Bergé, J.B., Plapp, F.W., 1992. Insect glutathione S-transferases. Biochemical characteristics of the major forms from houseflies susceptible and resistant to insecticides. J. Biol. Chem.. 267, 1840-1845.
22. Galloway, T.S., Handy, R., 2003. Immunotoxicity of organophosphorous pesticides. Ecotoxicology. 12, 345-363. doi: 10.1023/a:1022579416322.
23. Gavrilović, A., Ilijin, L., Mrdaković, M., Vlahović, M., Mrkonja, A., Matić, D., Perić Mataruga, V., 2017. Effects of benzo[a]pyrene dietary intake to antioxidative enzymes of *Lymantria dispar* (Lepidoptera: Lymantriidae) larvae from unpolluted and polluted forest. Chemosphere 179,10–19. doi: 10.1016/j.chemosphere.2017.03.083.
24. Grizanova, E.V., Semenova, A.D., Komarov, D.A., Chertkova, E.A., Slepneva, I.A., Dubovskiy, I.M., 2018. 2018. Maintenance of redox balance by antioxidants in hemolymph of the greater wax moth *Galleria mellonella* larvae during encapsulation response. Arch. Insect Biochem. Physiol. 98 (e21460), 1–13. doi: 10.1002/arch.21460.
25. Gruntenko, N.E., Khlebodarova, T.M., Vasenkova, I.A., Sukhanova, M.J., Wilson, T.G., Rauschenbach, I.Y., 2000. Stress-reactivity of a *Drosophila melanogaster* strain with impaired juvenile hormone action. *J. Insect Physiol.* 46(4), 451-456. doi: 10.1016/s0022-1910(99)00131-6.
26. Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.
27. Halliwell, B., Gutteridge, J.M.C., 2007. Free Radicals in Biology and Medicine. 4th ed. Oxford University Press, New York, USA.
28. Hemingway, J., Hawkes, N., McCarroll, L., Ranson, H., 2004. The molecular basis of insecticide resistance in mosquitoes. Insect. Biochem. Mol. Biol. 34(7), 653-665. doi: 10.1016/j.ibmb.2004.03.018.
29. Hermes-Lima, M., Zenteno-Savin, T., 2002. Animal response to drastic changes in oxygen availability and physiological oxidative stress. Comp. Biochem. Physiol.C 133, 537e556. doi: 10.1016/s1532-0456(02)00080-7.

30. Hyršl, P., Büyükgüzel, E., Büyükgüzel, K., 2007. The effects of boric acid-induced oxidative stress on antioxidant enzymes and survivorship in *Galleria mellonella.* Arch. Insect Biochem. Physiol. 66(1), 23–31. https://doi.org/10.1002/arch.20194.
31. Hussein, M.F., Mohamed, M.I., Selim, M.F., El Din, M.A., 1983. Ambient temperature effects on brain and spinal cord acetylcholinesterase activity of two poikilotherms. Comp. Biochem. Physiol. C. 75(2), 357-359.
32. Ilijin, L., Mrdaković, M., Todorović, D., Vlahović, M., Gavrilović, A., Mrkonja, A., Perić Mataruga, V., 2015. Life history traits and the activity of antioxidative enzymes in *Lymantria dispar* L. (Lepidoptera, Lymantriidae) larvae exposed to benzo(a)pyrene. Environ. Toxicol. Chem. 34(11), 2618-2624, doi: 10.1002/etc.3116.
33. Jia, F. X., Dou, W., Hu, F., Wang, J.J., 2011. Effects of thermal stress on lipid peroxidation and antioxidant enzyme activities of oriental fruit fly, *Bactrocera dorsalis* (Diptera: Tephritidae), Fla. Entomol. 94(4), 956-963. doi: 10.1007/s10493-014-9806-y.
34. Jones, B.R., Bancroft, H.R. 1986. Distribution and probable physiological role of esterases in reproductive, digestive and fat-body tissue of adult cotton boll weevil, *Anthonomus grandis*. Biochem. Genet. 24, 499-508.
35. Kapin, M.A. and S. Ahmad, 1980. Esterases in larval tissues of gypsy moth, *Lymantria dispar* (L.): Optimum assay conditions, quantification and characterization. Insect Biochem. 10: 331-7.
36. Konno, Y. and T. Shishido, 1992. Distribution of glutathione s-transferase activity in insect tissues. App. Entomol. Zool. 27: 391-397.
37. Laws, A.N. and G.E. Belovsky. 2010. How will species respond to climate change? examining the effects of temperature and population density on an herbivorous insect. Environ. Entomol. 39, 312–319. doi: 10.1603/EN09294.
38. Le Bras, M., Clement, M.V., Pervaiz, S., Brenner, C,. 2005. Reactive oxygen species and the mitochondrial signaling pathway of cell death. Histol. Histopathol. 20, 205–220. doi: 10.14670/HH-20.205.
39. Lindquist, S., 1986. The heat-shock response. Ann. Rev. Biochem. 55,1151-91.
40. Livingstone, D.R., 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar. Pollut. Bull. 42, 656–666. doi: 10.1016/s0025-326x(01)00060-1.

41. Limón-Pacheco, J., Gonsebatt, M.E., 2009. The role of antioxidants and antioxidant-related enzymes in protective responses to environmentally induced oxidative stress. Mutat. Res. Gen. Tox. En. 674, 137–147. doi: 10.1016/j.mrgentox.2008.09.015.
42. Main, A.R., Miles, K.E., Braid, P.E., 1961. The Determination of Human-Serum-Cholinesterase Activity with o-Nitrophenyl Butyrate. Biochem. J. 78, 769-776. doi: 10.1042/bj0780769.
43. Misra, H.P., Fridowich, I., 1972. The role of superoxide anion in the antioxidation of epinephrine and a simple assay for superoxide dismutase mimics. Biochem. J. 29, 2802-2807.
44. Mirčić, D., Blagojević, D., Perić Mataruga, V., Ilijin, L., Mrdaković, M., Vlahović, M., Lazarević, J., 2013. Cadmium effects on the fitness-related traits and antioxidative defense of *Lymantria dispar* L. larvae. Environ. Sci. Pollut. Res. 20(1), 209-218. https://doi.org/10.1007/s11356-012-1057-z.
45. Mrdaković, M., Ilijin, L., Vlahović, M., Todorović, D., Gavrilović, A., Mrkonja, A., Perić Mataruga, V., 2015. Effects of fluoranthene on the fitness-related traits and antioxidative defense in *Lymantria dispar* L. Environ. Sci. Pollut. Res. 22 (13), 10367–10374. https://doi.org/10.1007/s11356-015-4653-x.
46. Mrdaković, M., Ilijin, L., Vlahović, M., Filipović, A., Grčić, A., Todorović, D., Perić Mataruga, V., 2019. Effects of dietary fluoranthene on nymphs of *Blaptica dubia S. (Blattodea: Blaberidae).* Environ. Sci. Pollut. Res. 26(6), 6216-6222. https://doi.org/10.1007/s11356-019-04133-1.
47. O'Dell, T.M., Butt, C.A., Bridgeforth, A.W., 1984. *Lymantria dispar*. In: Singht, P., Moore, R., (Eds). Handbook of Insect Rearing, Vol.2. Elsevier, New York. pp 355-367.
48. Official Gazette of the city of Belgrade, 2005. 29, 36-37.
49. Pan, L.Q., Ren, J., Liu, J., 2006. Responses of antioxidant systems and LPO level to benzo(a)pyrene and benzo(k)fluoranthene in the haemolymph of the scallop *Chlamys ferrari*. Environ. Pollut. 141, 443–451. doi: 10.1016/j.envpol.2005.08.069.
50. Pardini, R.S., 1995. Toxicity of oxygen and naturally occurring redox-active pro-oxidants. Arch. Insect. Biochem. Physiol. 29,101-118. https://doi.org/10.1002/arch.940290203.

51. Perić Mataruga, V., Blagojević, D., Spasić, M., Ivanović, J., Janković-Hladni, M., 1997. Effect of the Host Plant on the Antioxidative Defence in the Midgut of *Lymantria dispar L.* Caterpillars of Different Population Origins. J. Insect Physiol. 43(1), 101–106. https://doi.org/10.1016/S0022-1910(96)00018-2.
52. Perić Mataruga, V., Vlahović, M., Mrdaković, M., Todorović, D., Matić, D., Gavrilović, A., Ilijin, L., 2014. Prothoracicotropic hormone-producing neurosecretory neurons and antioxidative defense in midgut of *Lymantria dispar* in trophic stress. Turk. J. Biol. 38, 403-411. doi:10.3906/biy-1309-57.
53. Perić Mataruga, V., Ilijin, L., Mrdaković, M., Todorović, D., Prokić, M., Matić, D., Vlahović, M., 2019. Parameters of oxidative stress, cholinesterase activity, Cd bioaccumulation in the brain and midgut of *Lymantria dispar* (Lepidoptera: Lymantriidae) caterpillars from unpolluted and polluted forest. Chemosphere 218, 416-424. doi: 10.1016/j.chemosphere.2018.11.112.
54. Satoh, T., Hosokawa, M., 2006. Structure, function and regulation of carboxylesterases. Chem. Biol. Interact. 162(3), 195–211. doi: 10.1016/j.cbi.2006.07.001.
55. Schieber, M., Chandel, N.S., 2014. ROS function in redox signaling and oxidative stress. Curr. Biol. 24, R453–R462. doi: 10.1016/j.cub.2014.03.034.
56. Schulte, P.M., Healy, T.M., Fangue, N.A., 2011. Thermal performance curves, phenotypic plasticity, and the timescales of temperature exposure. Integr. Comp. Biol. 51, 691–702. doi: 10.1093/icb/icr097
57. Singh, S.P., Coronella, J.A., Beneš, H., Cochrane, B.J., Zimniak, P., 2001. Catalytic function of *Drosophila melanogaster* glutathione S-transferase DmGSTS1-1 (GST-2) in conjugation of lipid peroxidation end products. Eur. J. Biochem. 268(10), 2912–2923. doi: 10.1046/j.1432-1327.2001.02179.x
58. Singh, A., Jaiswal, S.K., Sharma, B., 2013. Effect of low temperature stress on acetylcholinesterase activity and its kinetics in 5th instar larvae of *Philosamia ricini*. J. Biochem. Res. 1, 17–25.
59. Stajić S., Cvjetićanin R., Čokeša V., Miletić Z., 2018. Phytosociological characteristics of the community of submontane beech forest (*Helleboro odori-Fagetum moesiacae* Soo & Borhidi 1960.) on Mt. Kosmaj. Forestry 3-4; 71-87 (in serbian).

60. Steiner, C., Bozzolan, F., Montagné, N., Maïbèche, M., Chertemps, T., 2017. Neofunctionalization of "Juvenile Hormone Esterase Duplication" in Drosophila as an odorant-degrading enzyme towards food odorants. Sci. Rep. 7(1). doi:10.1038/s41598-017-13015-w.
61. Strode, C., Wondji, C. S., David J.P., Hawkes, N. J., Lumjuan, N., Nelson, D.R., Drane D.R., Parakrama Karunaratne, S.H.P., Hemingway, J., Black, W.C. IV, Ranson, H., 2008. Genomic analysis of detoxification genes in the mosquito *Aedes aegypti*, Insect Biochem. Mol. Biol.38, 113–123. doi: 10.1016/j.ibmb.2007.09.007
62. Vlahović, M., Ilijin, L.**,** Mrdaković, M., Todorović, D., Matić, D., Lazarević, J., Mataruga, V.P., 2016. Glutathione S-transferase in the midgut tissue of gypsy moth (*Lymantria dispar*) caterpillars exposed to dietary cadmium. Environ. Toxicol. Pharmacol. 44,13-17. https://doi.org/10.1016/j.etap.2016.04.002.
63. Vukin, M., 2017. A quality assessment of an artificially-established white ash stand in the protected area of 'Lipovica forest - Dugi rt'. Forestry 3-4; 85-102.
64. Wang, Y., Oberley, L.W., Murhammer, D.W., 2001. Evidence of oxidative stress following the viral infection of two lepidopteran insect cell lines. Free Radic. Biol. Med. 31(11), 1448–1455. doi: 10.1016/s0891-5849(01)00728-6.
65. Williams, R.S., Lincoln, D.E., Norby, R.J., 2003. Development of gypsy moth larvae feeding on red maple saplings at elevated CO_2 and temperature. Oecologia 137(1), 114–122. doi: 10.1007/s00442-003-1327-z
66. Yan, S., Cui, F., Qiao, C., 2009. Structure, Function and Applicationsof Carboxylesterases from Insects for Insecticide Resistance. Protein Pept. Lett. 16, 1181-1188. doi: 10.2174/092986609789071243.
67. Yang, L.H., Huang, H., Wang, J.J., 2010. Antioxidant responses of citrus red mite, *Panonychus citri* (McGregor) (Acari: Tetranychidae), exposed to thermal stress. J. Insect Physiol. 56, 1871–1876. doi: 10.1016/j.jinsphys.2010.08.006.
68. Yu, S.Y., Hsu, E.L., 1993. Induction of detoxification enzymes in phytophagous insects: roles of insecticide synergists, larval age, and species. Arch. Insect Biochem. Physiol. 24, 21-32. https://doi.org/10.1002/arch.940240103.

69. Zhang, S., Fu, W., Li, N., Zhang, F., Liu, T.X., 2015. Antioxidant responses of *Propylaea japonica* (Coleoptera: Coccinellidae) exposed to high temperature stress. J. Insect Physiol. 73, 47–52. doi: 10.1016/j.jinsphys.2015.01.004.

Figure legends:

Figure 1. - (**A**) Relative Growth Rate (RGR) and (**B**) changes in the development dynamics in 5th instar *Lymantria dispar* larvae from unpolluted (**UP - I**) and polluted forest (**PP - II**) exposed to different temperature treatments. **UP 23 and PP23 -** larvae reared on 23°C from hatching to sacrification; **UP 23In and PP 23In -** larvae reared on 23°C from hatching until the first day of 4th instar, and then exposed to temperature of 28°C for 24h (induced thermotolerance). Afterwards they were returned to 23°C until the third day of 5th larval instar; **UP 28 and PP23 -** larvae reared on 23°C from hatching until the first day of 5th larval instar, and then exposed to temperature of 28°C for 72h; **UP 28 In and PP 28 In -** larvae reared on 23°C from hatching until the first day of 4th instar, and then exposed to temperature of 28°C for 24h (induced thermotolerance). Afterwards they were returned to 23°C until the first day of 5th larval instar, and then exposed to temperature of 28°C for 72h. Error bars indicate the standard error of the mean (SEM) for (n = 8 - 12). Different letters (a,b,c) indicate significant differences between groups (Tukey's multiple comparisons test, $P<0.01$).

Figure 2. - (**A**) Superoxide dismutase (SOD) and (**B**) catalase (CAT) activity (mean ± SE U/mg protein) in midgut tissue of 5th instar *Lymantria dispar* larvae exposed to different temperature treatments from unpolluted (**UP - I**) and polluted forest (**PP - II**). All abbreviations are the same as in **Figure 1**. Different letters (a,b,c) indicate significant differences between groups (LSD test, $P<0.01$).

Figure 3. - (**A**) Superoxide dismutase (SOD) and (**B**) catalase (CAT) activity (mean ± SE U/mg protein) in hemolymph of 5th instar *Lymantria dispar* larvae exposed to different temperature treatments from unpolluted (**UP - I**) and polluted forest (**PP - II**). All abbreviations are the same as in **Figure 1**. Different letters (a,b,c) indicate significant differences between groups (LSD test, $P<0.01$).

Figure 4. – (**A**) Carboxylesterase (CaE) and (**B**) glutathione S-transferase (GST) activity (mean ± SE U/mg protein) in midgut tissue of 5th instar *Lymantria dispar* larvae exposed to different temperature treatments from unpolluted (**UP - I**) and polluted forest (**PP - II**). All abbreviations are the same as in **Figure 1**. Different letters (a,b,c) indicate significant differences between groups (LSD test, $P<0.01$).

Figure 5. – (**A**) Carboxylesterase (CaE) and (**B**) acetylcholinesterase (AChE) activity (mean ± SE U/mg protein) in head tissue of 5th instar *Lymantria dispar* larvae exposed to different temperature treatments from unpolluted (**UP - I**) and polluted forest (**PP - II**). All abbreviations are the same as in **Figure 1**. Different letters (a,b,c,d) indicate significant differences between groups (LSD test, $P<0.01$).

Figure 6. – IBR index calculated for antioxidative enzymes superoxide dismutase (SOD) and catalase (CAT) for 5th instar *Lymantria dispar* larvae exposed to different temperature treatments from unpolluted forest (**A**) and polluted forest (**B**). **SODm** and **CATm** – midgut enzyme activity; **SODh** and **CATh** – hemolymph enzyme activity.

Figure 7. – IBR index calculated for detoxifying enzymes carboxylesterase (CaE), glutathione S-transferase (GST) and acetylcholinesterase (AChE) for 5th instar *Lymantria dispar* larvae exposed to different temperature treatments from unpolluted forest (**A**) and polluted forest (**B**). **GSTm**– midgut enzyme activity; **CaEb** and **AChEb** – brain enzyme activity.

Table 1. IBR index values for *L. dispar* larvae antioxidative enzymes SOD and CAT activity upon exposure to different temperature treatments

	Experimental treatments	IBR index
L. dispar larvae originated from unpolluted forests (**UP**)	UP23	1.449
	UP23In	1.866
	UP28	6.860
	UP28In	8.405
L. dispar larvae originated from polluted forests (**PP**)	PP23	0.002
	PP23In	4.307
	PP28	2.211
	PP28In	4.162

Table 2. IBR index values for *L. dispar* larvae detoxifying enzymes carboxylesterase (CaE), glutathione S-transferase (GST) and acetylcholinesterase (AChE) activity upon exposure to different temperature treatments

	Experimental treatments	IBR index
L. dispar larvae originated from unpolluted forests (**UP**)	UP23	0.000
	UP23In	0.565
	UP28	4.654
	UP28In	1.870
L. dispar larvae originated from polluted forests (**PP**)	PP23	0.173
	PP23In	0.517
	PP28	1.472
	PP28In	1.400

Figure 1

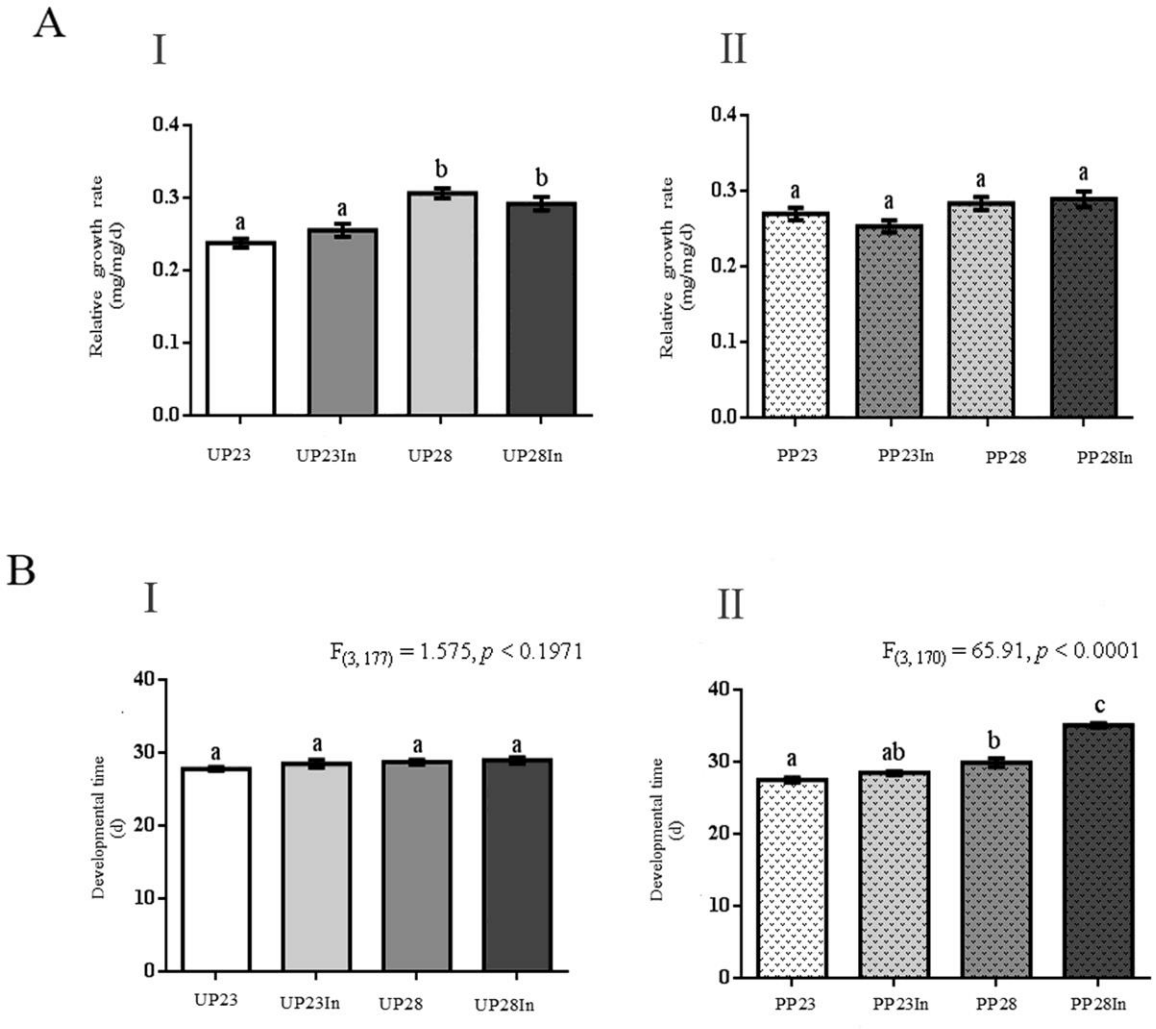

A
I
II
Relative growth rate (mg/mg/d)
0.4
0.3
0.2
0.1
0.0
a
a
b
b
UP23
UP23In
UP28
UP28In
a
a
a
a
PP23
PP23In
PP28
PP28In
B
I
II
$F_{(3, 177)} = 1.575, p < 0.1971$
$F_{(3, 170)} = 65.91, p < 0.0001$
Developmental time (d)
40
30
20
10
0
a
a
a
a
a
ab
b
c

Figure 2

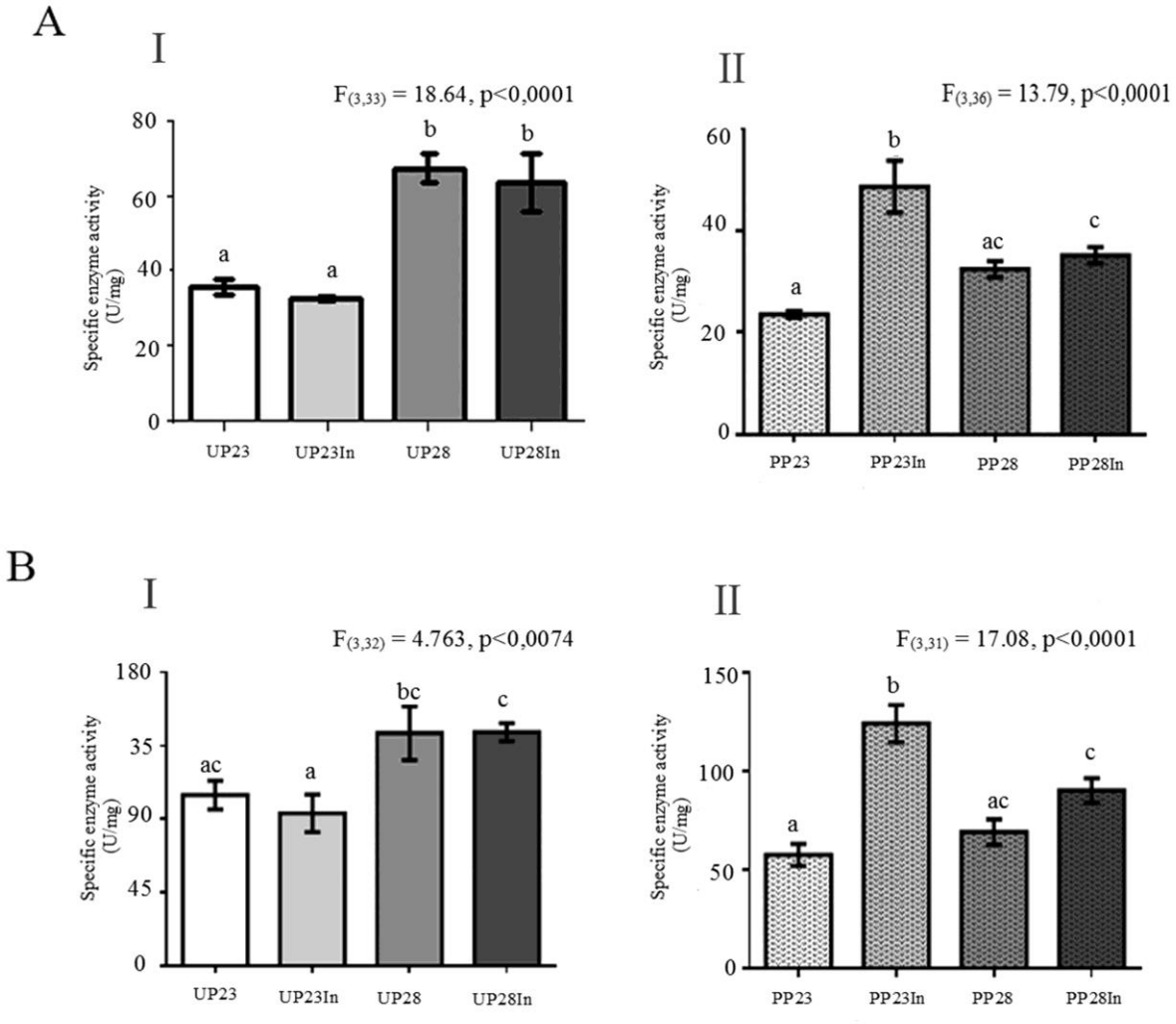

A
I
$F_{(3,33)} = 18.64, p<0,0001$
Specific enzyme activity (U/mg)
80
60
40
20
0
a
a
b
b
UP23
UP23In
UP28
UP28In
II
$F_{(3,36)} = 13.79, p<0,0001$
Specific enzyme activity (U/mg)
60
40
20
0
a
b
ac
c
PP23
PP23In
PP28
PP28In
B
I
$F_{(3,32)} = 4.763, p<0,0074$
Specific enzyme activity (U/mg)
180
135
90
45
0
ac
a
bc
c
UP23
UP23In
UP28
UP28In
II
$F_{(3,31)} = 17.08, p<0,0001$
Specific enzyme activity (U/mg)
150
100
50
0
a
b
ac
c
PP23
PP23In
PP28
PP28In

Figure 3

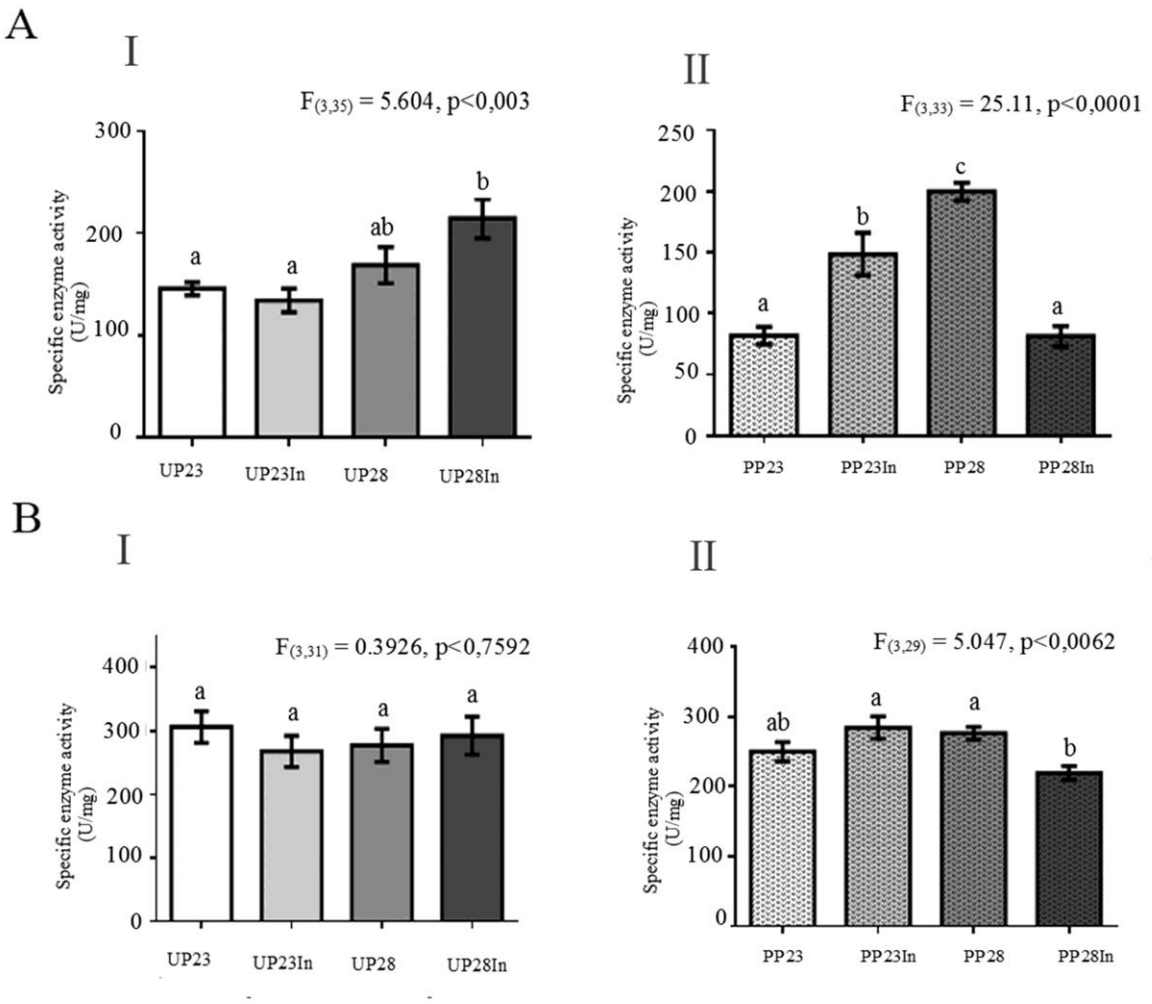

A
I
$F_{(3,35)} = 5.604, p<0,003$
Specific enzyme activity (U/mg)
300
200
100
0
a
a
ab
b
UP23
UP23In
UP28
UP28In
II
$F_{(3,33)} = 25.11, p<0,0001$
Specific enzyme activity (U/mg)
250
200
150
100
50
0
a
b
c
a
PP23
PP23In
PP28
PP28In
B
I
$F_{(3,31)} = 0.3926, p<0,7592$
Specific enzyme activity (U/mg)
400
300
200
100
0
a
a
a
a
UP23
UP23In
UP28
UP28In
II
$F_{(3,29)} = 5.047, p<0,0062$
Specific enzyme activity (U/mg)
400
300
200
100
0
ab
a
a
b
PP23
PP23In
PP28
PP28In

Figure 4

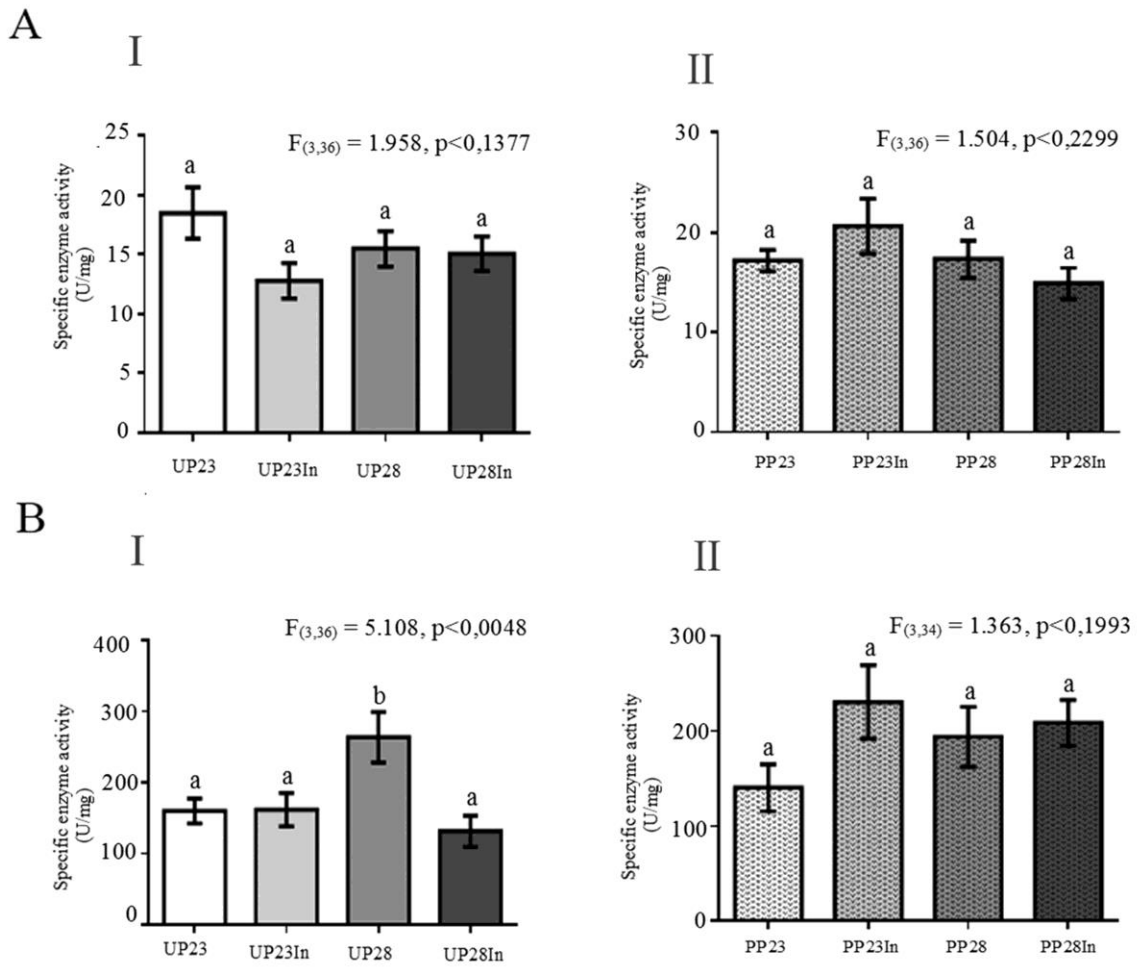

A
I
Specific enzyme activity (U/mg)
25
20
15
10
5
0
a
a
a
a
UP23
UP23In
UP28
UP28In
$F_{(3,36)}$ = 1.958, p<0,1377
II
Specific enzyme activity (U/mg)
30
20
10
0
a
a
a
a
PP23
PP23In
PP28
PP28In
$F_{(3,36)}$ = 1.504, p<0,2299
B
I
Specific enzyme activity (U/mg)
400
300
200
100
0
a
a
b
a
UP23
UP23In
UP28
UP28In
$F_{(3,36)}$ = 5.108, p<0,0048
II
Specific enzyme activity (U/mg)
300
200
100
0
a
a
a
a
PP23
PP23In
PP28
PP28In
$F_{(3,34)}$ = 1.363, p<0,1993

Figure 5

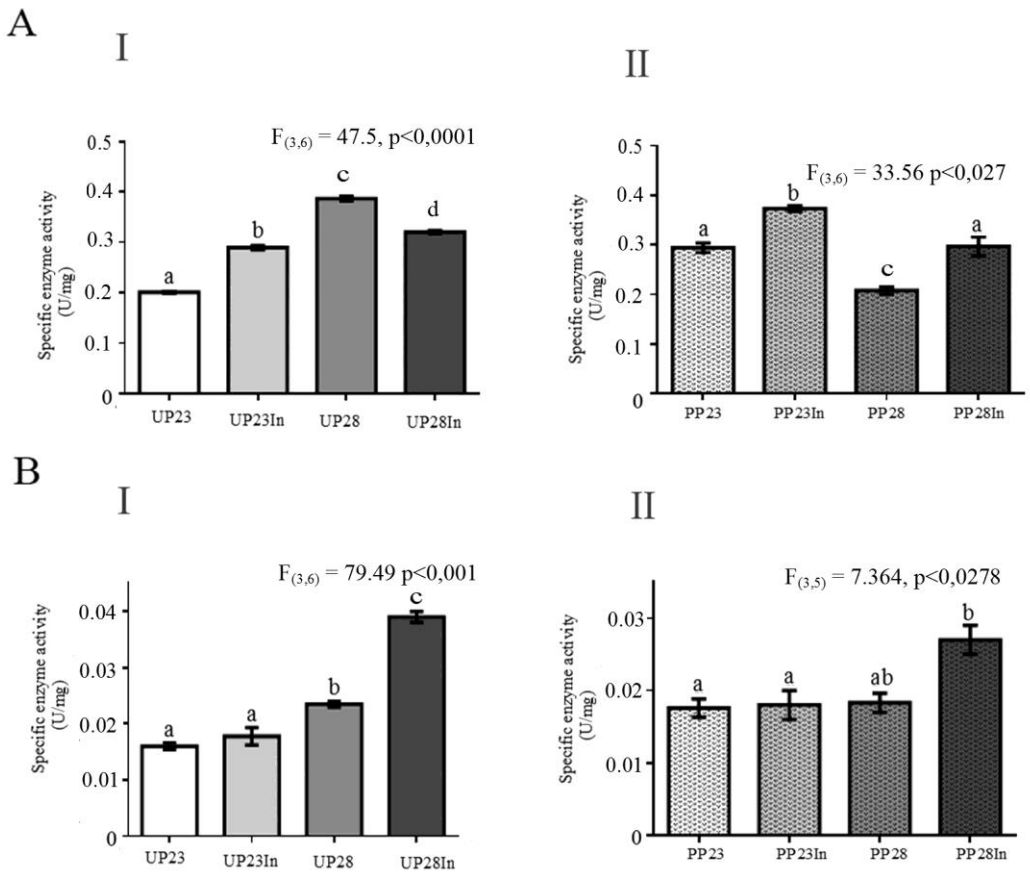
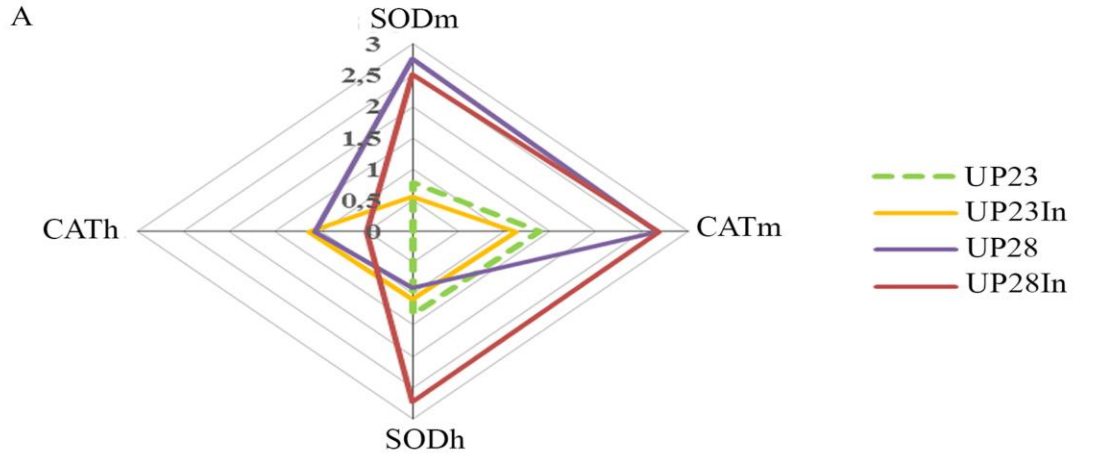

A
I
II
$F_{(3,6)}$ = 47.5, p<0,0001
$F_{(3,6)}$ = 33.56 p<0,027
Specific enzyme activity (U/mg)
0.5
0.4
0.3
0.2
0.1
0
a
b
c
d
UP23
UP23In
UP28
UP28In
PP23
PP23In
PP28
PP28In
B
I
II
$F_{(3,6)}$ = 79.49 p<0,001
$F_{(3,5)}$ = 7.364, p<0,0278
0.04
0.03
0.02
0.01
ab

Figure 6


A
SODm
3
2,5
2
1,5
1
0,5
0
CATh
CATm
SODh
UP23
UP23In
UP28
UP28In

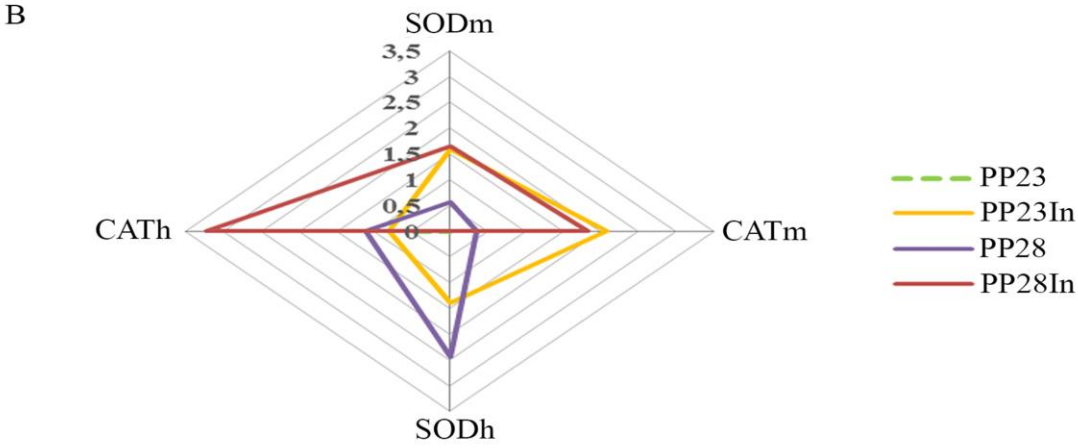
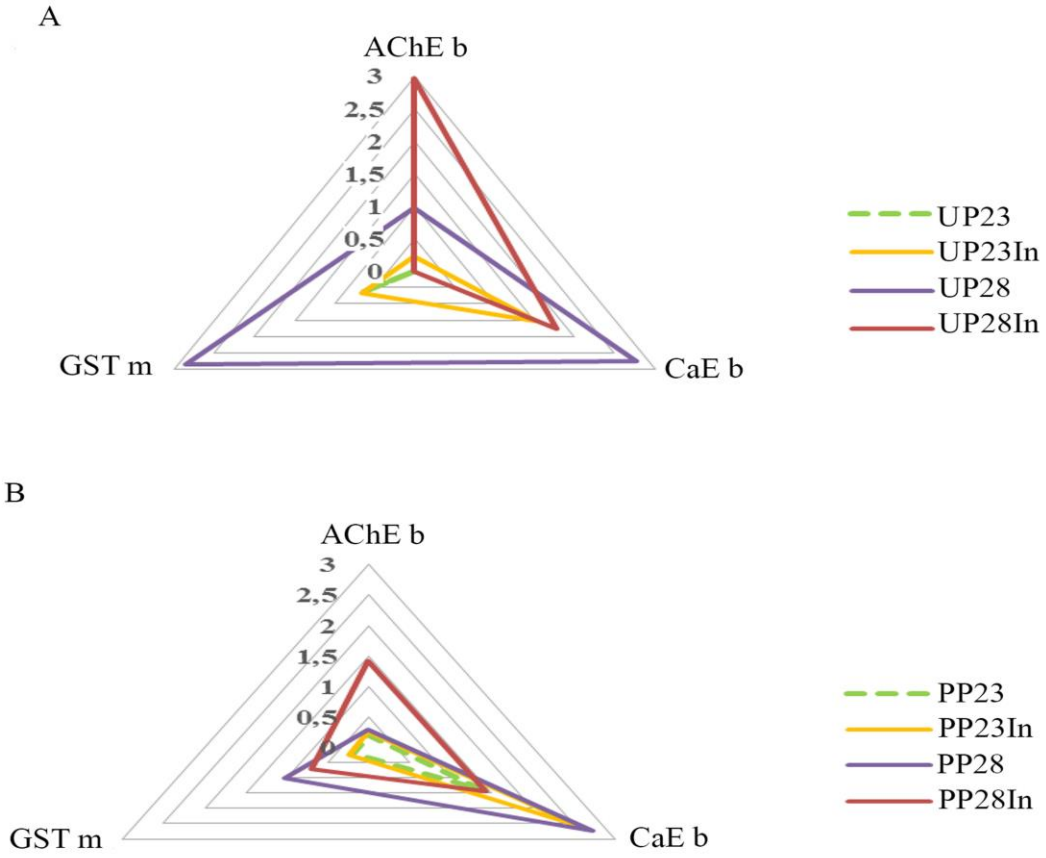

B
SODm
3,5
3
2,5
2
1,5
1
0,5
0
CATh
CATm
SODh
PP23
PP23In
PP28
PP28In

Figure 7


A
AChE b
3
2,5
2
1,5
1
0,5
0
GST m
CaE b
UP23
UP23In
UP28
UP28In
B
AChE b
3
2,5
2
1,5
1
0,5
0
GST m
CaE b
PP23
PP23In
PP28
PP28In