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Prothoracicotropic hormone-producing neurosecretory neurons and antioxidative defense in midgut of *Lymantria dispar* in trophic stress

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Abstract: As a very invasive insect species, *Lymantria dispar* is adaptable and sensitive to a changing environment. In insects the neuroendocrine system first reacts to stress by production of prothoracicotropic neurohormones (PTTH) that control ecdysteroid synthesis (morphogenetic and stress hormones). In this article, we report changes in the L2' brain neurosecretory neurons that synthesize PTTH in *L. dispar* larvae after feeding on locust tree leaves (*Robinia pseudoacacia*), an unsuitable host plant. Groups of larvae (n = 20 per experimental group) were offered this in comparison with oak leaves (*Quercus robur*), a suitable control diet, for 3 days after molting into the fourth instar. L2' neurons and their nuclei were enlarged and the amount of neurosecretory product in the cytoplasm was increased (15.5%) after consumption of locust tree leaves in comparison to the control. Furthermore, activities of the following antioxidative defense components were estimated: superoxide dismutase (SOD), catalase (CAT), and amount of glutathione in the midgut. Higher SOD activity (13.85 ± 0.9 U/mg prot.) and glutathione amount (0.56 ± 0.06 µMGSH/g tissue) but unchanged CAT activity was found in the midgut of larvae offered locust tree leaves when compared to the control.

Key words: Oak leaves, locust tree leaves, survival, superoxide dismutase, catalase, glutathione

1. Introduction

As a gradogenic polyphagous and invasive species, the gypsy moth (*Lymantria dispar* L.) encounters different environmental challenges. Considering that its host range is estimated at more than 500 plant species within 73 families (Liebhold et al., 1995), the gypsy moth is characterized by a variety of adaptations that ensure survival and reproduction in heterogeneous and stressful environments. Problems with gypsy moth overcrowding are not solved yet (Demir et al., 2009, 2012). Plants from the genus *Quercus* are accepted as the most suitable food and the most susceptible to *L. dispar* population eruptions (Maksimović, 1987; Liebhold et al., 1995). In the Republic of Serbia, large areas of forest and orchards were attacked (171,914 ha) by gypsy moths in 2012 according to the State Enterprise for Forest Management of Serbia (http://www.srbijasume.rs/). Other parts of Europe are also at risk of gypsy moth invasion and degradation of forest ecosystems. This pest insect is one of the most serious insect defoliators of North American forests and urban landscapes according to the US Forest Service (http://www.fs.fed.us/). Gypsy moth defoliation results in loss of growth, mortality of oak species, and shifts in species composition in mixed-oak forests (McGraw et al., 1990). Young oak leaves contain low amounts of flavonoids in spring when *L. dispar* attacks them (Salminen et al., 2004). A high population density of gypsy moths induces changes in leaf chemistry during defoliation, i.e. flavonoid content increases, leading to suppression of the insect immune response, and their resistance to viruses is lowered (Martemyanov et al., 2012). One of the very rare species of plant that is an unsuitable host for gypsy moths is the locust tree, *Robinia pseudoacacia* (Barbosa and Krischik, 1987). A nonfamiliar host plant for *L. dispar*, *R. pseudoacacia* is a widespread species in its native habitat in southeastern North America. It was introduced to Europe in 1601 (Chapman, 1935). Today, it has spread throughout western, central, eastern, and southern Europe and has become a major invasive species with a significant impact on native plant communities. Despite the large number of polyphagous species of *Lepidoptera* and the long period after the introduction of *R. pseudoacacia* to Europe, so far only a few native lepidopteran species have adapted to it (Kulfan, 2012). The unfavorable effects of locust leaves on gypsy moth larvae can be attributed to the presence of phenolic defensive compounds (Barbosa

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and Krischik, 1987). Chromatographic separation of an ethanolic extract of *R. pseudoacacia* leaves enabled isolation of much quercetin, robitin, myricetin, and other allelochemicals (Nasir et al., 2005). These compounds, especially flavonoids and tannins, can induce metabolic stress, including production of free radicals and a state of oxidative stress in the larval gut (Perić-Mataruga et al., 1988, 1997, 2001a, 2006b; Mrdaković et al., 2011, 2013).

A dominant role for the neuroendocrine system in mechanisms of stress response in insects has been indicated (Janković-Hladni, 1991; Perić-Mataruga et al., 2001b; Gruntenko et al., 2005; Krishnan and Kodrik, 2012). Different stressors modify the activity of neurosecretory neurons (NSNs). Moreover, their responses are selective and depend on the type and intensity of stressor and environmental conditions (Chernysh, 1991; Janković-Hladni, 1991). Neurohormones influence the release of lipids (adipokinetic hormones) and carbohydrates (hypertrehalosemic hormone) from the fat body depots, stimulate the uptake of carbohydrates (hypoglycemic hormone), regulate carbohydrate homeostasis (small form of prothoracicotropic neurohormones), stimulate (diuretic hormone) or suppress (antidiuretic hormone) water excretion, and regulate steroid production [large form of prothoracicotropic neurohormone (PTTH)] (Gade and Goldsworthy, 2003). Analysis of PTTH in *Lepidoptera* revealed that these peptide neurohormones appear in several isoforms (Ishizaki and Suzuki, 1994). Two forms were described: a large PTTH of molecular mass 24–30 kDa (Kawakami et al., 1990) and a small form or bombyxin of Mr 5–19 kDa (Ishizaki and Suzuki, 1994). The large form of PTTH is synthesized in 2 pairs of dorsolateral protocerebral NSNs and is released from their axons into the corpus allatum (Dai et al., 1994; Ilijin et al., 2012b). This neurohormone was identified in *L. dispar* larvae by Kelly et al. (1991). The L2' protocerebral dorsolateral NSNs in *L. dispar* synthesize the large form of PTTH (Ilijin et al., 2012b). The hormone regulates synthesis of ecdysteroids through the prothoracic gland by elevating cyclic AMP and the calcium-dependent pathway (Agui et al., 1980). Ecdysone is synthesized from dietary cholesterol derived from food and, after a number metabolic steps, the substance is released from the gland. Conversion of ecdysone to the physiologically active form, 20-hydroxyecdysone (20-HE), occurs primarily in fat bodies or midgut cells, but many other tissues are known to contain ecdysone 20-monooxygenase, a key enzyme for conversion of ecdysone to 20-HE (Mizoguchi et al., 1990; Dai et al., 1994; Gade and Goldsworthy, 2003; Gade and Marco, 2006).

Ecdysteroids are known to be most responsible for eliciting the molting process and modulating stress responses in insects (Chernysh, 1991; Gruntenko et al., 2005). PTTH contributes to the processes of stress adaptation by regulating morphophysiological changes and development. Different stressors, such as trophic stress, temperature, intoxication, photoperiod, proprioceptive, mechanoreceptive stimuli, and many others, alter the ecdysteroid secretion regulated by PTTH (Chernysh, 1991; Rauchenbach, 1991). One of the most important members among insect steroid hormones, 20-HE shows potent antioxidative activity and minimizes oxidative stress after prooxidative stimuli (Krishnan, et al. 2007; Krishnan and Kodrik, 2012).

Oxidative stress is caused by an imbalance between the production of reactive oxygen species (ROS) and the ability of a biological system to detoxify readily (Halliwell and Gutteridge, 2007). Generation of ROS accompanies oxygen metabolism in aerobes in diverse biochemical reactions. During the respiration process about 2% of molecular oxygen is permanently transformed into the superoxide anion radical ($O_2^{\cdot-}$), the principal generator of ROS and consequently other free radicals (Halliwell and Gutteridge, 2007). Prooxidant plant allelochemicals (phenolic compounds), such as flavonoids and tannins, are an exogenous source of oxidative stress acting against phytophagous pest insects. Phenolics can participate in 4 major types of bonds, hydrophobic, hydrogen, ionic, and covalent (Halliwell, 1996), to create toxic compounds. Almost any oxidation of phenolics in the gut can generate $O_2^{\cdot-}$, because the reactive semiquinone can donate an electron to molecular oxygen. In the insect gut, tissue toxic phenoxyl radicals are formed via oxidative processes owing to their ability to initiate free radical chain reactions in the membrane and the propensity to cross-link with a variety of molecules. The amount of oxidation of phenolics depends on the prevailing physicochemical conditions in the gut, including pH, redox potential, and antioxidative potential. The midgut of phytophagous insects, including *L. dispar*, is a highly oxidizing environment. Various indicators of oxidative stress in the gut tissue of the lepidopteran larvae of *Spodoptera litoralis* (Krishnan and Sehnal, 2006) and *Helicoverpa zea* (Felton et al., 1992) given a diet with phenol allelochemicals were found to increase. However, some insects, including *L. dispar*, possess a suite of antioxidative enzymes and nonenzymatic components that protect their cells from oxidative radicals (Ahmad, 1992; Perić-Mataruga et al., 1997; Mirčić et al., 2013). Superoxide anion radicals generated during oxidative stress in insects are rapidly converted by cytosol copper/zinc-containing superoxide dismutase (CuZnSOD) to H_2O_2, which can be decomposed by catalase activity to water and oxygen (Ahmad and Pardini, 1990; Fridovich, 1995; Perić-Mataruga et al., 1997). Overproduction of H_2O_2 is strongly oxidative and mediates hydroxy radical (OH) generation via Fenton and/or Haber–Weiss

reactions. A most important nonenzymatic cellular antioxidant is glutathione (GSH) (Ahmad and Pardini, 1990; Perić-Mataruga et al., 1997; Blagojević and Grubor-Lajšić, 2000). It is the most abundant nonprotein thiol in insect cells (Halliwell, 1996) and has a number of metabolic functions. One of the most important is protection of cells against oxidants and other xenobiotics. Protective antioxidative reactions in insects are regulated in a complex manner. Lately there was new information about a strong role for insect hormones in modulating the antioxidative defense strategy: adipokinetic hormones, glucagon, juvenile hormones, ecdysteroids, etc. (Krishnan et al., 2007; Alquicer et al., 2009; Krishnan and Kodrík, 2012; Grubor-Lajšić et al., 2013).

The objective of the present study was to investigate the responses of L2' NSNs of *L. dispar* (synthesis of PTTH) and correlations with the first line of enzymatic antioxidative protection [superoxide dismutase (SOD) and catalase (CAT) activity and GSH content] in the midgut of fourth instar larvae that had been fed for 3 days on *R. pseudoacacia* leaves, an unsuitable host plant.

2. Materials and methods

2.1. Insect rearing

Lymantria dispar egg masses were collected in an oak (*Quercus rubra*) forest (locality "Kosmaj", 80 km from Belgrade) and kept in a refrigerator at 4 °C from October to March, when they were set for hatching. After hatching, *Lymantria dispar* caterpillars were reared on oak leaves in transparent petri dishes (diameter of 10 cm, depth of 1.5 cm, volume of 117.8 cm^3) at 23 °C with a 16 h light/8 h dark photoperiod. Larvae were reared on suitable oak leaves until hatching of the fourth instar and then caterpillars were randomly assigned to the following experimental groups: 1) Control group fed suitable oak leaves (n = 20) and 2) group fed unsuitable locust tree leaves (n = 20). Survival was evaluated daily in each experimental group.

2.2. Histochemistry preparation and analysis

On the third day, the fourth instar caterpillars (n = 20 per experimental group) were sacrificed by decapitation and head capsules were fixed in Bouin's fixative for 24 h (Merck, Darmstadt, Germany). The histological procedure was carried out according to Panov (1980). Head capsules were dissected and brain complexes were extracted and then rinsed in 70 % ethanol (Hemos, Belgrade, Serbia), dehydrated in a graded series of ethanol (Hemos), impregnated in xylene (Hemos), and embedded in paraffin wax (59 °C, Merck). Serial 3.5-µm sections of brain complexes were cut for histochemistry (microtome, 820 Spencer) and collected on 0.2% gelatin/0.05% chrome alum (Sigma-Aldrich) coated slides (6 slides per brain complex). After drying for 48 h at 37 °C, the sections were deparaffinized in xylene, rehydrated with 10 mM phosphate buffered saline (Sigma-Aldrich), and stained by the paraldehyde fuchsin technique of Panov (1980). NSNs were stained dark purple/paraldehyde fuchsin-positive, and on the basis of morphological characteristics, L2' NSNs were easily selected (1 pair per brain). Neurosecretory products in NSN cytoplasm were stained dark purple and nucleoli were light pink (Panov, 1980). The activity of protocerebral L2' NSNs was determined by combined monitoring of the following cytological parameters: the relative size of NSNs and their nuclei (expressed as the mean values of smallest and largest diameters, µm) and the amount of neurosecretory product in the cytoplasm of neurons. All parameters were analyzed using Leica QWin image analysis software. The relative quantity of neurosecretory product via cytoplasmic optical density in the L2' PTTH-secreting NSNs was analyzed with National Institutes of Health (NIH) software Image J 1.42q (NIH, Bethesda, MD, USA) by means of digital image processing technique. The relative amounts of neurosecretory product in cytoplasm of L2' PTTH-secreting NSNs in caterpillars fed locust tree leaves were estimated in comparison with L2' neurons in caterpillars fed oak leaves (control), postulated as 100% intensity of optical density.

For further details, see our previous publications (Perić-Mataruga et al., 2001, 2006b; Ilijin et al., 2012a, 2012b, 2014).

2.3. Preparation of homogenates

After the caterpillars were sacrificed, the midguts were dissected on ice and washed several times with ice-cold physiological saline (0.9% NaCl). Midguts were pooled by weight (2 per homogenate) and homogenized in a buffer of 0.25 M sucrose, 0.05 M Tris-HCl, 1 mM EDTA, pH 7.4 (1:10 w/v) according to Rossi et al. (1983) and were sonicated according to Takeda et al. (1982). For determination of the total amount of GSH, part of the sonicated homogenate used to precipitate proteins with 5% sulfosalicylic acid and the total amount of GSH was measured after centrifugation at 5000 rpm for 10 min. The rest of the sonicated homogenate was centrifuged at 10,500 × *g* for 90 min and the activities of SOD and CAT were determined in the supernatant.

SOD activity was determined according to Misra and Fridovich (1972). This method is based on the ability of SOD to prevent adrenaline autoxidation in an alkaline medium. The adrenaline conversion into adrenochrome is followed by the release of superoxide anion radicals that lead to the acceleration of the autoxidation reaction. Adrenaline autoxidation rate was determined spectrophotometrically through the absorption change at a wavelength of 480 nm at 25 °C. SOD activity was expressed as the amount of enzyme causing a 50% inhibition of adrenaline autoxidation in units per milligram protein.

CAT activity was determined according to the method of Beutler (1982) using spectrophotometric determination of dissolution of the standard concentration of H_2O_2 (10 mM) at 230 nm. Activity was expressed in nanomoles as the amount of dissolved H_2O_2 reduced per minute per milligram protein.

Determination of the total concentrations of GSH (reduced GSH and oxidized GSSG) was conducted according to the method described by Griffith (1980). Sample proteins were precipitated in the homogenates by sulfosalicylic acid. The method is based on a recycling procedure where the oxidation of GSH with DTNB [producing 5.5-dithiobis(2-nitrobenzoic acid)] and its reduction by glutathione reductase with NADPH are conducted reciprocally. The rate of formation of 2-nitro-5-thiobenzoic acid was monitored spectrophotometrically at 412 nm, and the concentration of total GSH was calculated in accordance to the standard and expressed per grams of wet mass of tissue. Protein concentration was determined according to Bradford (1976) using bovine serum albumin as the standard.

2.4. Data analysis

After the normality of data distribution had been tested, differences between groups were carried out by Student's t-test. P-values below 0.05 were regarded as significant. Data analysis was done by Statistic 6.0 software.

3. Results

3.1. Larval survival

Fourth instar gypsy moth (*L. dispar*) larvae showed a trend of lower survival (95%) when fed locust tree (*R. pseudoacacia)* leaves in comparison to those (97.89%) given an oak leaf diet (*Quercus rubra*).

3.2. L2' PTTH-producing neurosecretory neurons

L2' NSNs in the protocerebral part of the brain of fourth instar *L. dispar* after 3 days of feeding with 2 different diets are shown in Figure 1. The size of these dorsolateral protocerebral NSNs was increased in larvae fed unsuitable locust tree leaves (22.97 ± 0.33 μm) when compared with those fed oak leaves (20.01 ± 0.9 μm) ($P < 0.05$). Moreover, the nuclei in L2' NSNs were larger in the group offered locust tree leaves (11.93 ± 0.11 μm) than in the group fed oak leaves (9.23 ± 0.55 μm) at $P < 0.01$ (Figure 1). These neurons contained smaller amounts of a fine granulated neurosecretory product in the group fed oak leaves than in the group given locust tree leaves. The amount of the neurosecretory product was lower by 15.5% in L2' neurons from larvae given oak leaves than in those offered locust leaves (Figure 2). The nuclei of the L2' neurons had large centrally positioned and clearly visible nucleoli, which suggested intensive synthetic processes (Figure 2).

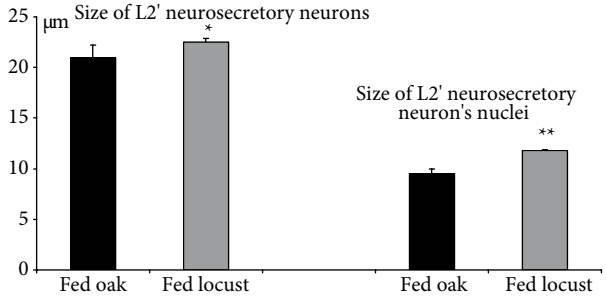

Figure 1. Size of L2' neurosecretory neurons and their nuclei in the brain of *Lymantria dispar* L. (fourth instar) that were fed on oak (*Quercus robur*) or locust (*Robinia pseudoacacia*) tree leaves for 3 days. *: $P < 0.05$, **: $P < 0.01$.

3.3. Antioxidative defense

SOD activity in the midgut of the larvae fed locust leaves (13.85 ± 0.9 U/mg prot.) was significantly higher than that in the control group fed oak leaves (9.08 ± 0.64 U/mg prot.) at $P < 0.05$ (Figure 3). CAT activity in the larval midgut tended to decrease (202.27 ± 20.45 nM H_2O_2 min^{-1} mg $prot.^{-1}$) in larvae fed locust leaves in comparison with larvae fed oak leaves (215.9 ± 0.88 nM H_2O_2 min^{-1} mg $prot.^{-1}$), but the difference between the groups given different diets was not statistically significant (Figure 4). The amount of GSH in the midgut of the *L. dispar* fourth instar larvae was significantly elevated in response to the locust leaf diet (0.56 ± 0.06 μMGSH/g tissue) when compared to the oak leaf diet (0.38 ± 0.06 μMGSH/g tissue) at $P < 0.01$ (Figure 5).

4. Discussion

The nervous system/neurohormones and hormones that regulate ecdysteroids have been emphasized lately as important in balancing the redox status as well as the potential for antioxidative defense in insect tissues. Vertebrate steroid hormones such as estrogen and related components with a phenolic A ring (Jellinck and Bradlow, 1990; Liehr and Roy, 1998; McHugh et al., 1998) inhibit oxidative cascades by donating hydrogen radicals to the A-ring. Steroids in arthropods synthesized after the tropic effect of PTTH neurohormones have a similar function (Rees, 1995; Cai et al., 2002). The activity of PTTH-secreting NSNs, which regulate ecdysteroids, i.e. 20-HE content in the hemolymph, was higher in larvae fed unsuitable locust tree leaves (Figures 1 and 2). 20-Hydroxyecdysone is the most active form of ecdysteroid. Stress induces ecdysone 20-monooxygenase, an enzyme that catalyzes conversion of ecdysone to the physiologically active form, 20-HE, in *Drosophila virilis*. The activity of this enzyme correlated with the level of 20-HE (Chentsova et al., 2007), which may have improved the defense against free radicals and the prooxidative effects of plant compounds. Lower lipid

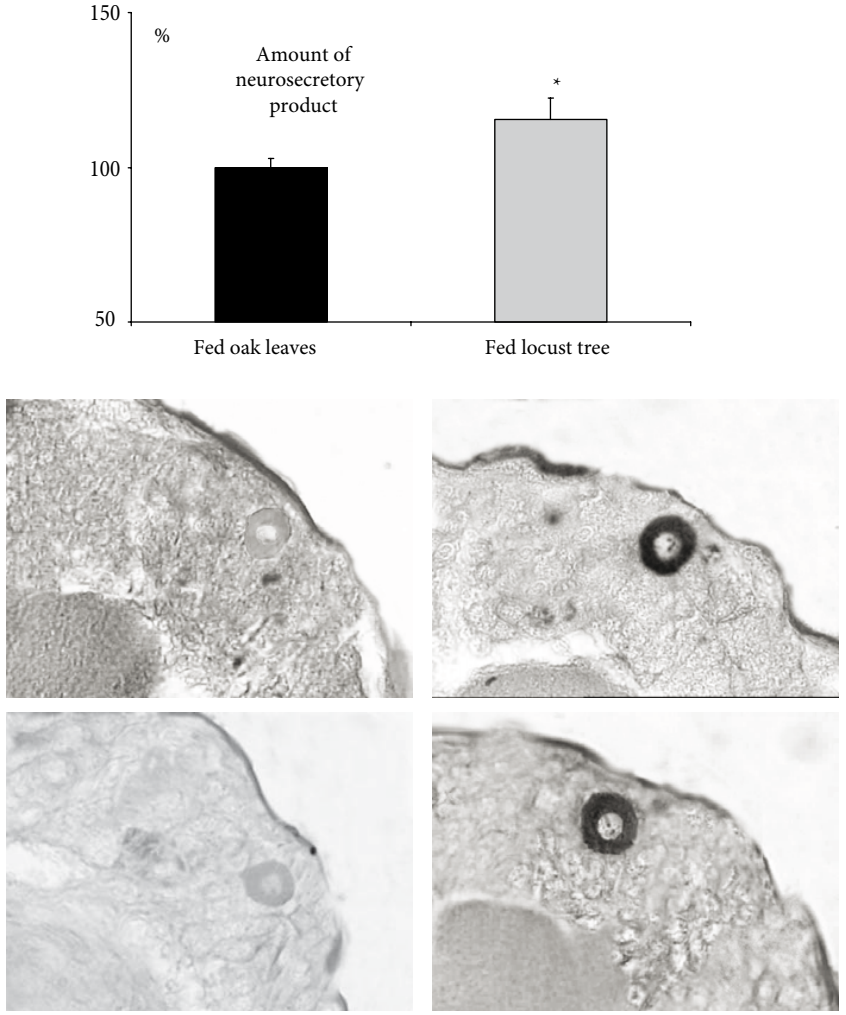

Figure 2. Transverse cross section of *L. dispar* brain (fourth instar) in the area of L2' protocerebral dorsolateral neurosecretory neurons. The relative amount of neurosecretory product in cytoplasm of L2' PTTH-secreting neurosecretory neurons in larvae fed on locust tree leaves are presented in comparison with neurons in larvae fed on oak leaves (control) (NIH software Image J 1.42q). *: $P < 0.01$.

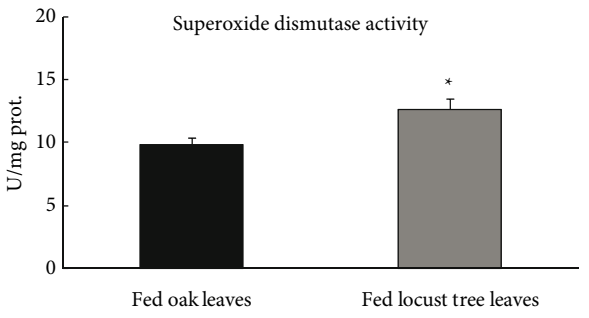

Figure 3. Activity of superoxide dismutase in the midgut of *Lymantria dispar* (fourth instar) fed on oak (*Quercus robur*) or locust (*Robinia pseudoacacia*) tree leaves for 3 days. *: $P < 0.01$.

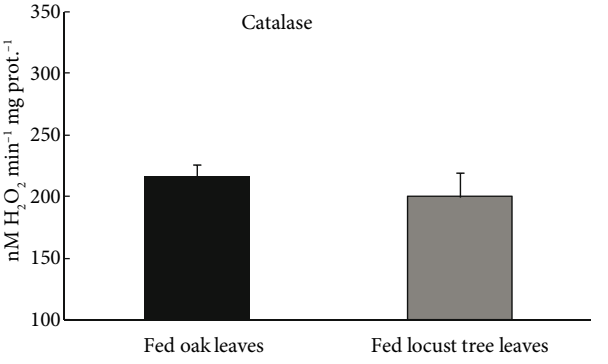

Figure 4. Activity of catalase in the midgut of *Lymantria dispar* (fourth instar) fed oak (*Quercus robur*) or locust (*Robinia pseudoacacia*) tree leaves for 3 days.

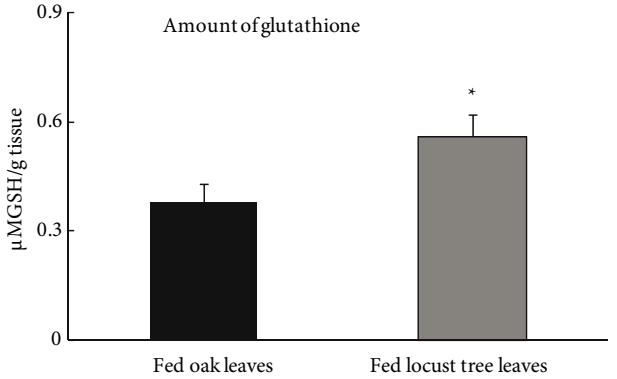

Figure 5. Glutathione content in the midgut of *Lymantria dispar* (fourth instar) fed on oak (*Quercus robur*) or locust (*Robinia pseudoacacia*) tree leaves for 3 days. *: $P < 0.01$.

peroxidation and protein carbonylation following 20-HE injection has been found with a subsequent decrease in oxidative radicals and minimization of oxidative stress due to paraquat in *Pyrrhocoris apterus* (Krishnan et al., 2007). The hormone affects the GSH redox shuttle and γ-glutamyl transpeptidase (GGT) activity, i.e. transfer of the γ-glutamyl moiety of GSH to an acceptor that may be an amino acid, a peptide, or water (forming glutamate). The γ-glutamyl cycle is thought to sustain the cellular GSH concentration. GGT plays a key role in this cycle, as a pathway for the synthesis and degradation of GSH and drug and xenobiotic detoxification (Karp et al., 2001; Roesijadi et al., 2007). It is interesting that, besides functional changes in PTTH-producing NSNs, our results showed more GSH in the midgut tissue of locust leaf-fed caterpillars than in the control group (Figure 5). GSH is an important antioxidant that acts by several mechanisms, including scavenging free radicals (Sies, 1999). Recovery of GSH following 20-HE injection is only partially due to the abrogation of oxidative radicals. The concentration of intracellular GSH depends on the availability of substrates for its synthesis and on the rate of depletion into the extracellular space, as there is only small or no uptake into cells. It is metabolized by membrane-bound γ-GTP, which removes the γ-glutamyl group, thereby rendering the remaining cysteinylglycine susceptible to cleavage by a membrane dipeptidase. The released cysteine can be transported into the cell and used as a substrate for GSH synthesis. The ameliorating effect of 20-HE on γ-GTP activity may be based on stabilization of its anchor in the plasma membrane (reduced lipid peroxidation) or on protection against direct oxidative damage, which contributes to the GSH recovery.

Higher mortality, prolonged development time including the fourth larval instar, lower pupal and larval mass, and digestive, antioxidative, and neuroendocrine reorganization were demonstrated previously as chronic effects of a locust tree diet on *L. dispar* (Perić-Mataruga et al., 1997; Lazarević et al., 2002). Here we showed a trend of higher mortality of *L. dispar* larvae given locust leaves for just 3 days. Ingested phenolic compounds become extensively oxidized, which produces ROS (Canada et al., 1990; Barbehenn et al., 2001; Barbehenn et al., 2009). Moreover, quinones, the oxidation products of phenols (Zheng et al., 1997; Barbehenn et al., 2006) may also be toxic and can cause the formation of gut lesions (Thiboldeaux et al., 1998; Perić-Mataruga et al., 2006a) and oxidative stress in the midgut tissues of *L. dispar* (Pecci, 2011).

Our results confirmed that SOD activity (Figure 3) was higher in the midgut of fourth larval instar fed locust tree leaves for 3 days. It is interesting that CAT activity tended to be lower in the individuals fed locust leaves (Figure 4), similar to chronic exposure of fourth larval instar to trophic stress by locust tree feeding (Perić-Mataruga et al., 1997). Considering that the phenol-induced stress results in increased formation of $O_2\bullet-$ and consequently reactive oxygen species (Canada et al., 1990; Barbehenn and Martin, 1994; Barbehenn et al., 2001), regulation by SOD and CAT is necessary. SOD works continuously to eliminate $O_2\bullet-$ with production of H_2O_2. CAT catalyzes the decomposition of H_2O_2 to water and oxygen. It is possible that CAT could effectively reduce H_2O_2 to low levels after the feeding of larvae with unsuitable locust tree leaves. We presume that the enzyme ascorbate peroxidase could be competing with the overproduction of H_2O_2 to compensate for the defect of CAT levels in order to eliminate and detoxify the elevated levels of H_2O_2. PTTH and ecdysteroids inhibit ROS and reactive nitrogen species production and modulate oxidative stress by inducing signal transduction pathways. They are also associated with increased expression of gene products involved in maintaining redox homeostasis, including methionine sulfoxide reductase (an antioxidant repair enzyme that reduces oxidized methionine to methionine), and may regulate conversion of oxidized proteins to their reduced state (Weissbach et al., 2005; Roesijadi et al., 2007). It is interesting that the expression of the SOD gene in some insects and mammals is responsive to ecdysone and is regulated at both transcriptional levels, although the molecular mechanisms of this regulation are poorly understood. We could also suppose that higher mortality was an aftermath of the prooxidant activity of phenols in the unsuitable locust leaves and consequently allocation of resources towards defense mechanisms and induction of antioxidative components. These are exhausting processes that can affect larval survival.

Our previous research demonstrated correlations between the prooxidative effect of locust tree leaves in chronic treatment (Perić-Mataruga et al., 2000) and activation of L2' NSNs (Perić-Mataruga et al., 1997,

2001). These neurohormones regulate steroid synthesis and their potential to alleviate oxidative damage through reorganization od antioxidative strategies (Krishnan et al., 2007).

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