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Acetylcholinesterase (AChE) and heat shock proteins (Hsp70) of gypsy moth (*Lymantria dispar* L.) larvae in response to long-term fluoranthene exposure

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HIGHLIGHTS

- Long-term effects of PAH fluoranthene were examined in the fifth instar gypsy moth.
- Increased AChE activity was recorded in brain tissues at higher PAH concentrations.
- Elevated Hsp 70 levels were detected in brain tissues at lower PAH concentrations.
- Detected changes were suggested as useful indicator of PAH pollution.

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) may affect biochemical and physiological processes in living organisms, thus impairing fitness related traits and influencing their populations. This imposes the need for providing early-warning signals of pollution. Our study aimed to examine changes in the activity of acetylcholinesterase (AChE) and the concentration of heat shock proteins (Hsp70) in homogenates of brain tissues of fifth instar gypsy moth (*Lymantria dispar* L.) larvae, exposed to the ubiquitous PAH, fluoranthene, supplemented to the rearing diet. Significantly increased activity of AChE in larvae fed on the diets with high fluoranthene concentrations suggests the necessity for elucidation of the role of AChE in these insects when exposed to PAH pollution. Significant induction of Hsp70 in gypsy moth larvae reared on the diets containing low fluoranthene concentrations, indicate that changes in the level of Hsp70 might be useful as an indicator of pollution in this widespread forest species.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are complex organic compounds formed during incomplete combustion of organic materials, mainly connected with anthropogenic activities. They can be transported by air masses over long distances, and are detected in various environments throughout the world (Eadie et al., 1982; Fernández et al., 2002; Srogi, 2007; Wilcke, 2007). Numerous studies suggest harmful impact of PAHs on living beings (Šepič et al., 2003; Kummerová et al., 2006; Wang et al., 2007; Kim et al., 2013), which imposes the need for assessing their biological effects on organisms, and evaluation and prediction of impacts on populations and communities. Fluoranthene (Flann) is one of the most widespread compounds, often used as an indicator of the presence of other PAHs. It is among the dominant PAHs in leaves of various deciduous and coniferous tree species (Howsam et al., 2000; De Nicola et al., 2008; Tian et al., 2008; Bourotte et al., 2009), strongly affecting physiological mechanisms in woody plants of the forest ecosystem and in cultivated plant species (Berteigne et al., 1989; Váňová et al., 2009; Tomar and Jajoo, 2014). By changing the physiology and growth of host plants, pollutants may influence phytophagous species. Thus, we recently reported the harmful effects of different dietary concentrations of Flann on some life-history traits of the gypsy moth fifth instar. In addition,

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induction of the antioxidative enzymes, superoxide dismutase (SOD) and catalase (CAT), in response to chronic Flann stress was suggested as a potential indicator of PAH pollution (Mrdaković et al., 2015). Here we describe changes in the activity of AChE and concentration of Hsp70 in homogenates of brain tissues of the aforementioned gypsy moth larvae that had been long-term exposed to dietary Flann. The well known physiological processes of precisely defined developmental stages make this widespread insect species a good model organism in studies of the effects of environmental pollution. Also, continuous feeding by gypsy moth larvae, particularly in later instars, suggests their high bioaccumulation potential, which can be important, since phytophagous insects are a link between primary (e.g. leaves of host plants) and higher order members of food chains. Recent studies have demonstrated the effects of inorganic and organic pollutants on biochemical and physiological processes of gypsy moth larvae, while recorded changes in enzyme activity are suggested as biomarkers of environmental pollution (Mirčić et al., 2013; Vlahović et al., 2013; Ilijin et al., 2015).

AChE is an enzyme responsible for degradation of acetylcholine, major neurotransmitter in central nervous system of insects (Toutant, 1989). Insect AChE is a globular disulphide-linked dimer, existing in different allelic variations. Inhibition of its activity, e.g. by organophosphorous insecticides, may induce changes in the active site of the enzyme, leading to accumulation of acetylcholine within synapses and disrupted signal transmission (Gunning and Moores, 2001). Changes in AChE activity have been used as a biomarker of neurotoxicity, and described in insect species as the consequence of insecticide and biopesticide actions (Jensen et al., 1997; Senthil Nathan et al., 2008). Hsps or stress proteins are molecular chaperones involved in preventing and repairing damage to cellular components, induced by various stressors. By altering their pattern of expression, Hsps can respond to any disturbance of cellular homeostasis. This makes them suitable early warning bioindicators of environmental pollution (Feder and Hofmann, 1999; Morales et al., 2011).

This work is a continuation of our previous research (Mrdaković et al., 2015), aimed to assess whether the ubiquitous PAH, fluoranthene, is responsible for detected AChE activity and Hsp70 concentration in homogenates of brain tissues of treated gypsy moth larvae, and if so, whether the detected changes could be used as indicators of PAH pollution.

2. Materials and methods

2.1. Insect rearing

As previously stated (Mrdaković et al., 2015), gypsy moth egg masses were collected from a mixed oak forest in “Djerdap” National Park, Eastern Serbia. Egg masses were kept in a refrigerator at 4 °C until hatching. Newly hatched larvae were randomly assigned to six experimental groups ($n = 15–25$) reared on diets (standard laboratory diet for gypsy moths, O'Dell et al., 1985) supplemented with increasing concentrations of fluoranthene (F1–F6), (Sigma Aldrich, St Louis, MO). Larvae were exposed to dietary Flann from hatching until the 3rd day of the fifth instar. Diets were mixed with different concentrations of Flann dissolved in reagent-grade acetone, placed in containers of the same volume, and kept in the fume hood for at least 4 h until evaporation of the acetone. The control group of larvae (C) was reared on the diet without added Flann. It has been shown that acetone used as a solvent did not affect significantly life processes and activity of enzymes, including insect head cholinesterase (Dauterman et al., 1962; Desaiah and Koch, 1977; Bellas and Thor, 2007). The same amount of fresh food was supplied every second day. The lowest Flann concentration (6.7 ng/g dry weight of the diet) was chosen according to the concentration already recorded in oak leaves (Howsam et al., 2000), which are the preferred food of gypsy moth larvae. The higher concentrations of Flann (33.5 ng, 67 ng, 335 ng, 670 ng, and 6.7 μg/g dry weight of the diet) correspond to PAHs concentrations reported in different plant species (e.g. Simonich and Hites, 1994; Tian et al., 2008).

2.2. Preparation of homogenates

Larvae were sacrificed 3 days after molting into the fifth instar; the brain tissues were isolated from head capsules, pooled by experimental group, homogenized on ice in 0.9% saline (hand-held homogenizer MHX/E Xenox, Germany), and then centrifuged at 10 000 g for 10 min, at 4 °C (Eppendorf centrifuge, 5417R, Germany). The resultant supernatants were used for the enzyme assay, for Western blot and the indirect non-competitive enzyme-linked immunosorbent assay (ELISA). Protein was determined spectrophotometrically using the BioRad DC protein assay kit (Richmond, CA, USA), with bovine serum albumin (BSA) as the standard (Bradford, 1976).

2.3. Acetylcholinesterase activity

Acetylcholinesterase (AChE) activity was determined spectrophotometrically according the method of Ellman et al. (1961), using acetylthiocholine iodide (ATChI) as artificial substrate. The reaction of dithiobisnitrobenzoate (DTNB) with thiocholine, the product of hydrolysis of ATChI catalyzed by AChE, generates yellow 5-thio-2-nitrobenzoate that can be quantified by its absorbance at 406 nm (UV mc2, SAFAS, Monaco). Acetylcholinesterase activity was calculated as U/mg protein. Each data point represents the mean of three replicates (homogenized brain tissues pulled by each group).

2.4. Hsp70 detection method

The same samples were used for both Western blotting and indirect ELISA. The homogenates of brain tissues were separated by SDS PAGE electrophoresis on 12% gel (Laemmli, 1970). Proteins were transferred overnight on to nitrocellulose membrane (Amersham Prothron, Premium 0.45 μm NC, GE Healthcare Lifescience), at 40–50 V and 4 °C. The primary monoclonal anti-Hsp70 mouse IgG1 that localizes both the constitutive and inducible forms of Hsp70 (clone BRM-22, Sigma Aldrich, diluted 1:5000) and secondary mouse anti-mouse Hsp70 horseradish peroxidase conjugate antiserum (Sigma Aldrich, diluted 1:10,000) were used to detect Hsp70 expression pattern in the homogenates of brain tissue of gypsy moth larvae. Bands were visualized using chemiluminescence (ECL kit, Amersham).

An indirect non-competitive ELISA was used to quantify concentrations of Hsp70s in the homogenates of larval brain tissues. Samples (15 μg per well) were diluted with carbonate-bicarbonate buffer (pH 9.6), coated on a microplate (Multiwell immunoplate, NAXISORP, Thermo Scientific, Denmark) and incubated overnight in the dark, at 4 °C in the presence of monoclonal anti-Hsp70 mouse IgG1 (clone BRM-22, Sigma Aldrich, diluted 1:5000) followed by the secondary mouse anti-mouse Hsp70 antiserum conjugated to horseradish peroxidase (HRP) (Sigma Aldrich, diluted 1:10,000). Absorption was measured on microplate reader (LKB 5060-006) at 450 nm. Serial dilutions of standard Hsp70 (recombinant Hsp70 50 ng/μL) were used to calculate the Hsp70 concentrations, expressed as ng/mg protein. Each data point represents the mean of two replicates (homogenized brain tissues pulled by each group).