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Biomarkers of Oxidative Stress and Acetylcholinesterase Activity in the Blood of Grass Snake (*Natrix natrix* L.) during Prehibernation and Posthibernation Periods

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ABSTRACT

*This work examined the enzymatic (superoxide dismutase-CuZn SOD, catalase-CAT, glutathione peroxidase-GSH-Px, glutathione reductase-GR, and the biotransformation phase II enzyme glutathione-S-transferase-GST) and non-enzymatic (total glutathione-GSH and lipid peroxides-TBARS concentrations) biomarkers of oxidative stress and acetylcholinesterase (AChE) activity in the blood of the grass snake (*Natrix natrix *L.) during prehibernation and posthibernation. The animals were collected in October (prehibernation) and April (posthibernation) at the nature reserve Obedska Bara (OB) and industrial region Pančevački Rit (PR) in Serbia. In posthibernation, decreased CAT activity and TBARS concentration in specimens from PR, and decreased GR and AChE activities, and TBARS concentration in specimens from OB were observed, whereas GR and GST activities and GSH concentration were significantly elevated in the specimens from PR. In prehibernation, CAT activity and GSH concentration were increased, while GSH-Px, GR, GST and AChE activities and TBARS concentration were decreased in the specimens from PR when compared to animals from OB. During the posthibernation, the activity of CuZn SOD was decreased, while GST and AChE activities were increased in the specimens from PR when compared to the specimens from OB. These differences represented an adaptive mechanism to oxidative stress induced by tissue reoxygenation during arousal from hibernation and could be modulated by environmental pollution.*

Key words: Grass snake, oxidative stress, hibernation, acetylcholinesterase, antioxidant enzymes

INTRODUCTION

Reactive oxygen species (ROS) are the products of aerobic metabolism that have physiological roles at low concentrations. Oxidative stress occurs when the production of ROS exceeds the capacity of enzymatic and non-enzymatic antioxidants. Overproduction of ROS has deleterious effects as they can lead to damage of cell structures, proteins, DNA and lipids (Valko et al. 2007). Oxidative stress can occur during normal physiological activities such as exercise, arousal from hibernation, starvation, etc. (Bagnyukova et al. 2003). ROS can be also produced intracellularly by different xenobiotics that exist in the environment and due to the status of antioxidant that give information about the ability to resist environmental stress (Pavlović et al. 2010; Messina et al. 2014). In addition, some pharmaceutical products can also be found in aquatic ecosystems and affect the antioxidant defense system of aquatic organisms (Bartoskova et al. 2013). Components of the antioxidant system serve to neutralize the deleterious effects of ROS. The principal components of this system are the antioxidant enzymes, superoxide dismutase

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(SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR), and the biotransformation phase II enzyme glutathione-S-transferase (GST) (Van der Oost et al. 2003).
Glutathione (GSH), the nonenzymatic component of the antioxidant system, plays a key role in the cellular defence and serves as a reservoir for the amino acid cysteine. Mitochondrial GSH depletion leads to cell death and could ultimately determine cell vulnerability to oxidant attack (Kovačević et al. 2008). Lipid peroxidation results from the oxidation of polyunsaturated fatty acids during oxidative stress and the concentrations of lipid peroxides are reliable biomarkers of oxidative stress (Van der Oost et al. 2003). Among numerous biomarkers used in environmental studies, acetylcholinesterase activity (AChE) is commonly used to assess the neurotoxicity (Durieux et al. 2011).
Metabolic rate depression is an adaptive strategy of hibernation that extends the survival time of animals, supporting months or even years of dormancy (Storey and Storey 1990). However, arousal from hibernation stimulates respiration and can cause oxidative stress (Banyukova et al. 2003). Therefore, antioxidant defences are important during the increased tissue oxygenation. Organisms use strict genetic control mechanisms to overcome these physiological extremes (Andrews 2007). Hibernators must maintain metabolic homeostasis at a very low body temperature and then return to a state that requires increased oxygen consumption and is associated with ROS generation. Hermes-Lima and Storey (1993) observed an increase in total antioxidant capacity during physiological stress of anoxia and freezing of snake species *Thamnophis sirtalis parietalis*. They suggested that the antioxidant system was an important part of the biochemical machinery that allowed the survival under anoxic or freezing conditions.
Contrary to the mammals, hibernation in reptiles has been studied to a lesser extent. Low winter temperatures are thermally challenging for ectothermal species such as snakes. Most temperate zone snakes spend unfavorable weather conditions during the winter in the hibernaculum, underground shelters, where they remain inactive (Bauwens 1981). Photoperiod and temperature changes affect the endocrine glands and are critical factors that regulate hibernation. According to Thapliyal and Sharan (1980), O_2 uptake by the brain is related to initiation, maintenance and termination of hibernation in the water snake *Natrix piscator*.
N. natrix L. is a semi-aquatic, medium-sized, oviparous, diurnal snake with a range that extends from extreme northern Africa to near the Artic Circle in Scandinavia (Gregory and Isaac 2004). Hibernation of *N. natrix* usually begins in October and lasts until April. They begin to hibernate when the environmental temperature drops below 6°C for several days. Water snakes are important components of ecosystems that use both aquatic and terrestrial resources. They are long-lived organisms at the top of the food chain. These features render them potential bioindicator species. Campbell and Campbell (2001) reported that snakes were generally ignored in ecological risk assessment studies and that they were the least studied group of vertebrates regarding environmental contaminants (Hopkins 2000).
The aim of the present study was to compare the expression of selected markers of oxidative stress and neurotoxicity in the blood of the grass snake *N. natrix* during the periods of prehibernation and posthibernation. Investigations were made for the enzymatic (superoxide dismutase-CuZnSOD, catalase-CAT, glutathione peroxidase-GSH-Px, glutathione reductase-GR, and the biotransformation phase II enzyme glutathione-S-transferase-GST), and nonenzymatic (total glutathione-GSH and lipid peroxides-TBARS concentrations) biomarkers of oxidative stress, as well as the biomarker of neurotoxicity, acetylcholinesterase (AChE) activity in the blood of snake during prehibernation (October) and posthibernation (April) periods in the specimens from two localities: nature reserve Obedska Bara (OB) and industrial region Pančevački Rit (PR). The study also established possible modulation of investigated blood parameters in respect to environmental conditions.

MATERIAL AND METHODS

Site description and sample collection

Female specimens of the grass snake species *N. natrix* were caught manually in autumn (late October - prehibernation period) and spring (April - posthibernation period) at two localities: Obedska bara (OB) and Pančevački rit (PR), (Fig. 1). In late October, the snakes cease to feed, became lethargic and were ready to hibernate. Ten healthy adult females were caught at OB and 10

were caught at PR in late October, while five and nine specimens were caught at OB and PR in April, respectively. Animal capture was approved by the Serbian Ministry for Energy, Development and Environmental Protection (Permissions Nos: 353-01-640/2012-03 and 353-01-77/2013-08). The animals were handled in accordance with the guidelines of the Animal Welfare Act of the Republic of Serbia.

The nature reserve Obedska Bara (OB) (44°44.8'08.37" N; 19°59'14.38" E) is one of the oldest protected areas in the world (since 1874). OB contains the abandoned bed of the Sava River that flows more southward. OB has been registered as a nature reserve by the Ramsar Convention on wetlands since 1977. This locality includes areas of special significance for birds of Europe. OB is linked to the Sava River only when water levels rise. Pančevački Rit (PR) (44°50'01.68" N; 20°29'48.43" E) is located in the southwestern part of Banat near Belgrade, between the rivers Danube and Tamiš. In the past, it was a wetland area often flooded by the Danube and Tamiš. Today it is a suburban region exposed to increased anthropogenic pressure, receiving extensive industrial and urban waste discharges. Consequently, it was exposed to a variety of pollutants communal and industrial waste from a factory for glue production, cattle fodder, dairy farm, sugar refinery, abattoir, glass and chemical industries and the Pančevo oil refinery.

Figure 1 - The geographical positions of the investigated localities, Obedska Bara (OB) and Pančevački Rit (PR) in Serbia.

Biochemical analysis

After decapitation, blood was immediately collected in heparinized test tubes (5000 U/mL heparin). Biomarkers of oxidative stress were assessed immediately after blood collection in order to avoid any possible modification of the results caused due to storage (Fazio et al. 2014).

Lipid peroxidation was measured by the thiobarbituric acid (TBA) reaction according to the method of Ohkawa et al. (1979). Red-color was produced by the reaction of TBA with malondialdehyde (MDA) and was measured at 532 nm. The results were expressed as nmol TBARS/mL blood.

To separate the plasma from the blood cells, fresh blood was centrifuged at 5000 rpm for 15 min. Plasma samples were used for GSH determination according to the method of Griffith (1980) and expressed as nmol/L plasma. GST activity towards 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate was measured as described by Habig et al. (1974). One unit of GST activity was defined as nmol GSH/min/mL of plasma. AChE activity was determined in the plasma according to Ellman et al. (1961) by measuring the continuous reaction of thiol with 5,5-dithiobis-(2-nitrobenzoic acid) DTNB. The product formed in the reaction was yellow 5-thio-2-nitrobenzoate (TNB) and was detected spectrophotometrically at 412 nm. AChE activity was expressed as μmol/min/L plasma.

Isolated red blood cells (RBCs) were washed three times with three volumes of 0.65% NaCl. The hemoglobin (Hb) concentration in erythrocytes (g Hb/100 mL) was estimated by the cyanmethemoglobin method (Drabkin and Austin 1935). CuZn SOD activity was measured in the hemolysates of washed RBCs from which Hb was previously removed by the method of Tsuchihashi (1923). CuZn SOD activity was measured by the epinephrine method (Misra and Fridovich 1972), based on the capacity of SOD to inhibit autoxidation of adrenaline to adrenochrome. One unit of SOD activity was defined as the amount of enzyme that caused 50% inhibition of the autoxidation of adrenaline.

CAT activity was determined according to Claiborne (1984) and expressed as μmol H_2O_2/min/g Hb. The method is based on H_2O_2 degradation by CAT contained in the examined samples. In this procedure, 30 mM H_2O_2 served as the substrate. According to McCord and Fridovich (1969), hemolysates containing about 50 g Hb/L were used for the determination of GSH-Px activities (Maral et al. 1977). The assay was based on the measurement of oxidation of nicotine amide dinucleotide phosphate (NADPH) at 340 nm, with t-buthyl-hydroperoxide as substrate and the activity was expressed as nmol NADPH/min/g Hb. The activity of GR was estimated by measuring the NADPH oxidation at 340 nm in the presence of oxidized glutathione (Glatzle et al. 1974) and expressed as nmol NADPH/min/g Hb. The method is based on the ability of GR to catalyze the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH), using NADPH as substrate in phosphate buffer (pH 7.4). The activities of antioxidant defence enzymes were measured using a Shimadzu UV-160 spectrophotometer with a temperature-controlled cuvette holder. All chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA).

Statistical analysis

The data have been expressed as the means ± Standard Error (S.E.). Significant differences between the samples were determined by the non-parametric Mann-Whitney U-test. The minimum significance level was $p<0.05$. Principal component analysis (PCA) served to detect the variables that significantly contributed to the differences of the investigated parameters during the pre- and posthibernation periods and at different localities. Analytical protocols described by Darlington et al. (1973) and Dinneen and Blakesley (1973) were used.

RESULTS

Snout-vent length (SVL) and body weight of the grass snakes from the OB and PR are given in Table 1. The hemoglobin concentration in the blood of the grass snake *N. natrix* from the two investigated localities is presented in Table 2. The hemoglobin concentration was significantly lower during the posthibernation period at both the localities ($p<0.05$).

Table 1 - Snout-vent length (cm) and body weight (g) of the grass snake (*Natrix natrix*) during the prehibernation and posthibernation periods. Data are expressed as the mean ± standard error of the mean.

		Prehibernation	Posthibernation
SVL[1] (cm)	OB[2]	78.36 ± 1.94	73.20 ± 4.35
	PR[3]	75.39 ± 0.92	73.28 ± 1.72
Body weight (g)	OB	209.26 ± 13.07	149.38 ± 24.84
	PR	157.32 ± 6.33	138.12 ± 10.02

Note: [1]SVL = snout-vent length, [2]OB = Obedska bara, [3]PR = Pančevački rit

Table 2 - The concentration of hemoglobin (g/100 mL) in the blood of the grass snake (*Natrix natrix*) during the prehibernation and posthibernation periods. Data are expressed as the mean ± standard error of the mean.
* $p<0.05$ represents a minimal significant level.

	OB[1]	PR[2]
Prehibernation	5.44 ± 0.41	6.37 ± 0.43
Posthibernation	3.65 ± 0.49 *	4.73 ± 0.29 *

Note: [1]OB = Obedska bara, [2]PR = Pančevački rit

The activity of CuZn SOD (Fig. 2A) was significantly decreased during the posthibernation period at PR ($p<0.05$), while no differences between localites were detected during the prehibernation period. During the prehibernation period, the activity of CAT (Fig. 2B) was significantly higher in the blood of the snakes collected from the PR as compared to the snakes from OB ($p<0.05$). Significant differences between the localities were not observed during the posthibernation period. At the same time, the activity of CAT in the snakes from the PR during the posthibernation period was significantly lower than during the prehibernation period ($p<0.05$). The activity of GSH-Px (Fig. 2C) during the prehibernation period was markedly lower at PR when compared to OB ($p<0.05$).

In the snakes from OB, GR activity (Fig. 2D) was significantly lower during the posthibernation period when compared to the prehibernation period ($p<0.05$). However, the activity of GR from snakes caught in the PR was significantly decreased during the prehibernation period in comparison to the posthibernation period ($p<0.05$).

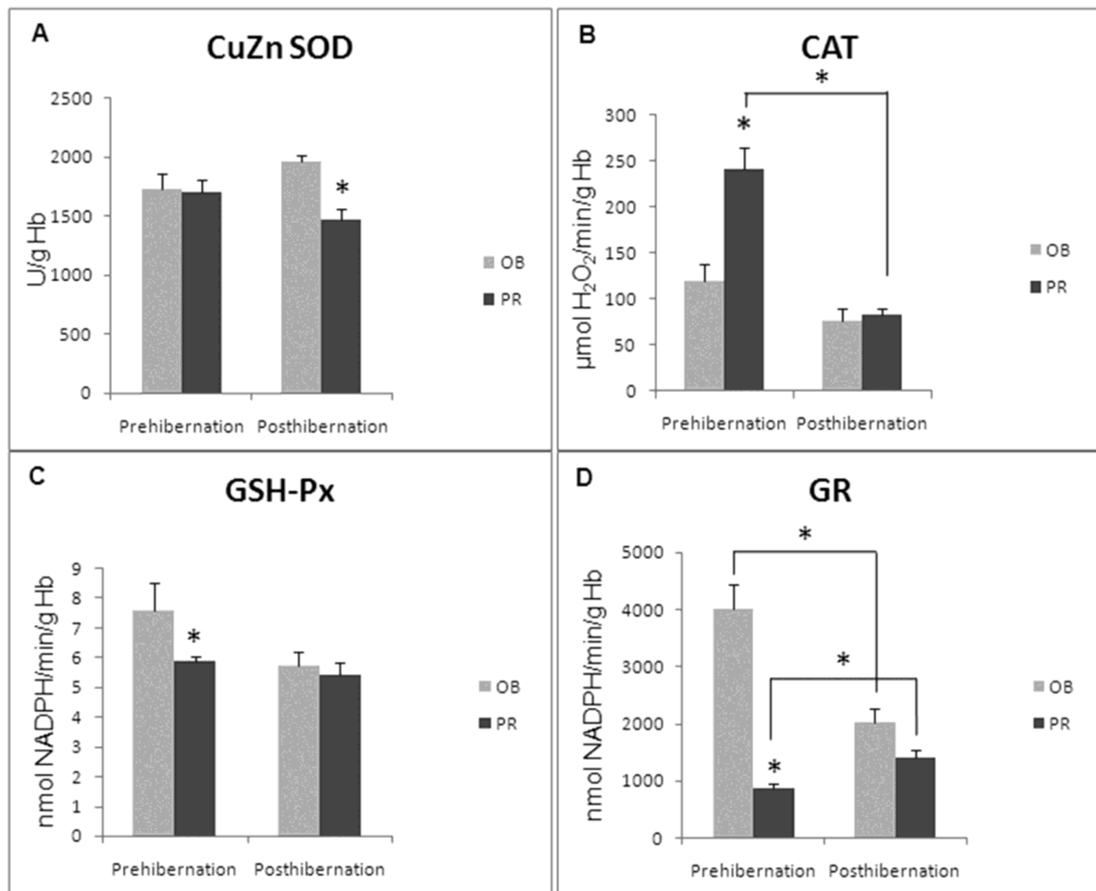

Figure 2 - The activities of: A) copper zinc containing superoxide dismutase (CuZn SOD); B) catalase (CAT); C) glutathione peroxidase (GSH-Px); D) glutathione reductase (GR) in red blood cells (RBCs) of the grass snake (*Natrix natrix*) from Obedska Bara (OB) and Pančevački Rit (PR) during the prehibernation and posthibernation periods. Specific enzyme activities are expressed as U/g hemoglobin (Hb). Significant differences between the means were established by the non-parametric Mann-Whitney U-test. * $p<0.05$ represents a minimal significant level.

Before hibernation, GR activity was markedly elevated in the snakes from OB as compared to snakes from PR ($p<0.05$). The activity of GST (Fig. 3A) during posthiberanation was higher in the snakes from the PR than during the prehibernation period ($p<0.05$). Before hibernation, GST activity was significantly decreased in the snakes from the PR as compared

to the snakes from OB ($p<0.05$). After hibernation, GST was increased in the snakes from the PR as compared to the snakes from OB ($p<0.05$).

The concentration of GSH (Fig. 3B) in the plasma was significantly increased in the snakes from the PR during the posthibernation period than during the prehibernation period ($p<0.05$). GSH was markedly increased in the snakes from the PR when compared to the snakes from OB during both the periods ($p<0.05$). At both the localities, the concentration of lipid peroxides, expressed as the TBARS blood concentration (Fig. 3C), was significantly decreased after hibernation ($p<0.05$). At the same time, during the prehibernation period, TBARS was significantly decreased in the snakes from the PR when compared those from OB ($p<0.05$). Plasma AChE activity in the snakes from OB (Fig. 3D) was lower in the snakes during posthibernation ($p<0.05$). Before hibernation, the activity of AChE was significantly decreased in the snakes from the PR in comparison to snakes from OB. After hibernation the opposite result was obtained.

Figure 4 shows PCA of all of the investigated parameters during the prehibernation and posthibernation periods in snakes from both sites. The data showed separation of the two investigated sampling localities during prehibernation and posthibernation (Factor 1:46.18% and Factor 2:34.57%).

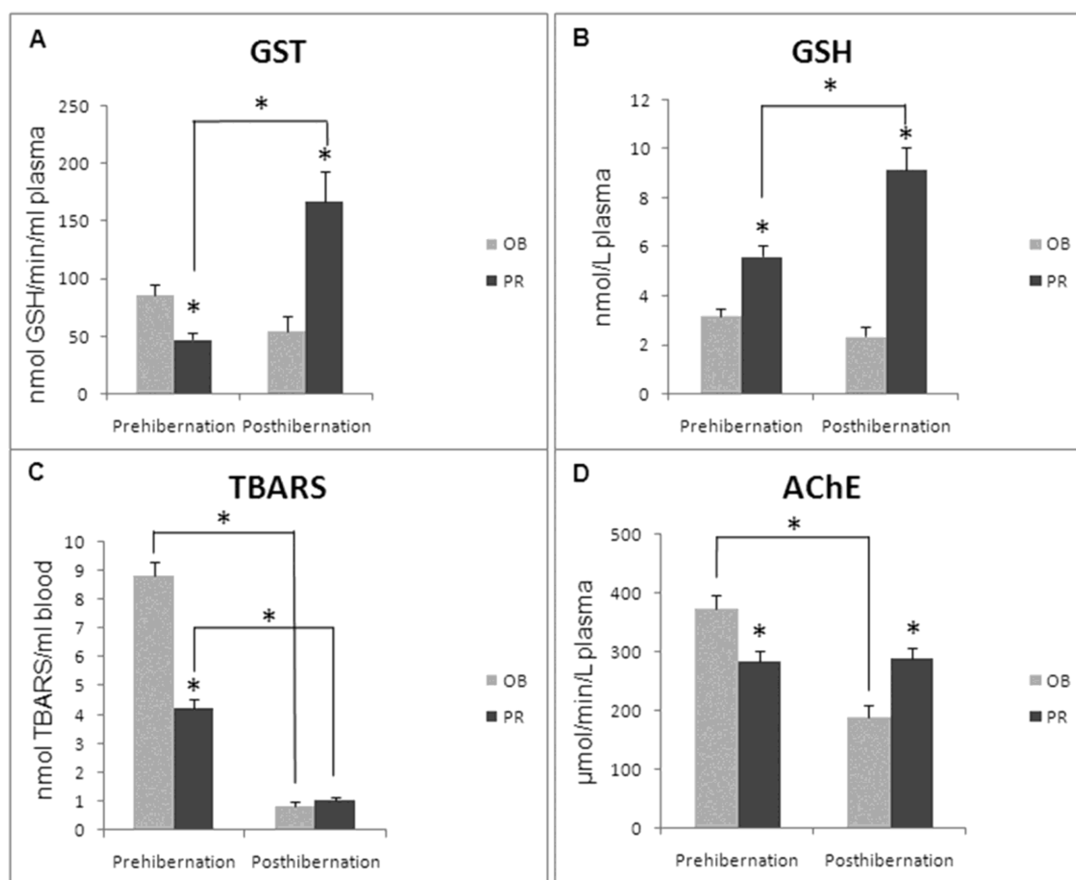

Figure 3 - Activity of A) plasma phase II biotransformation enzyme glutathione-S-transferase (GST; U/mL plasma); B) plasma concentration of total glutathione (GSH; nmol/L plasma); C) blood concentration of lipid peroxides (TBARS; nmol/L blood); D) plasma acetylcholinesterase activity (AChE; U/L plasma) in the grass snake *N. natrix* from Obedska Bara (OB) and Pančevački Rit (PR) during the prehibernation and posthibernation periods. Significant differences between the means were established by the non-parametric Mann-Whitney U-test. * $p<0.05$ represents a minimal significant level.

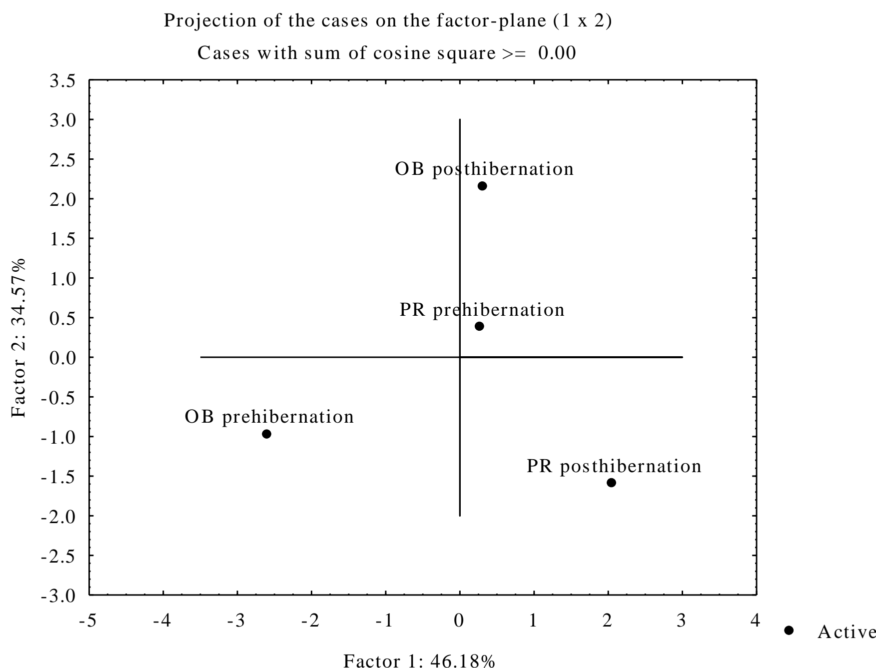

Figure 4 - Principal Component Analysis (PCA) of all of the investigated parameters: CuZn SOD, CAT, GSH-Px, GR, GST and AChE activities and GSH and TBARS concentrations in the blood of the grass snake *N. natrix* from the sites, The localities Obedska Bara (OB) and Pančevački Rit (PR) during the prehibernation and poshibernation periods are on the factor plane.

DISCUSSION

Hibernation is a prominent feature of the annual cycle in many reptiles, hence, prehibernation, hibernation and arousal from hibernation could be considered as the phases with specific, different demands for physiological and biochemical activities (El-Deib 2005). Since animals recovering from the hypometabolic state are exposed to increasing oxidative stress, they have developed mechanisms to prevent irreversible structural damage and maintain normal cell functions. Reperfusion in the animals adapted to anoxia/hypoxia or freezing is a physiological process that is an integral part of their natural life cycle (Orr et al. 2009). Pratihar et al. (2010) suggested that the high activity of antioxidant enzymes during hibernationwas an adaptive mechanism for preventing increased lipid peroxidation.

Data on antioxidant defence enzymes in the blood of the snakes during prehibernation and posthibernation are scarce, and comparisons between different animal groups need to be undertaken. Study on the changes of the antioxidant systems in *Rana ridibunda* during recovery from winter hibernation showed that the activity of the primary antioxidant defence enzymes, SOD and CAT, increased when the frogs were transferred to higher temperature (Bagnyukova et al. 2003). Because many reptile species could survive certain periods without O_2, Hermes-Lima et al. (1993) hypothesized that reptile species contained a powerful antioxidant defence system capable of protecting these organisms from post-anoxic generation of excess amounts of ROS. Hermes-Lima and Zenteno-Savín (2002) observed significant increases in the total-SOD in the muscle and liver of anoxia-exposed garter snakes, while these conditions generally did not have any effect on CAT activity. In this study, no significant difference in SOD activities before and after hibernation was observed at both the localities where the snakes were caught, but in posthibernating period, decrease of SOD activity at the PR in respect to OB was observed. Apparently the activity of SOD remained at a level capable of catalyzing the

dismutation of the superoxide anion into oxygen and H_2O_2, and therefore, opposed the adverse effects of this free radical. According to the present results, CAT activity was higher at both the sites during the period before hibernation; however, statistical significance was only observed at the PR. There was a significant increase of CAT activity ($p<0.05$) at the PR in comparison to OB in prehibernating period. Salway et al. (2010) suggested that a relatively rapid up-regulation of MnSOD, GSH-Px and GR occurred early during arousal from the estivation of the Giant African snail (*Achatina fulica*). Gabryelak et al. (1983) who studied the enzymes involved in peroxide metabolism (SOD, CAT and GSH-Px) in the erythrocytes of freshwater fish species, detected higher activities in spring than in autumn.

The change in GSH-Px activity observed during different hibernating periods was not statistically significant. GR activity during the posthibernation period was significantly decreased in the snakes from OB, whereas the snakes from the PR displayed an opposite pattern. According to Hayes and McLellan (1999), GR is one of the most important components of cellular protection against oxidative stress due to its ability to reduce oxidized glutathione using NADPH as a reductant. The level of GSH was significantly lower in the snakes from OB during both the periods, and was also significantly increased during the posthibernation period in the snakes from the PR. The elevation in GSH concentration probably protects from the oxidative stress that occurs during arousal (Avci et al. 2014). There was an increased concentration of GSH in the blood of grass snakes from the PR, a locality that was exposed to high anthropogenic pressure. GSH binds metals and prevents them from reacting with other biomolecules, and animal cells respond to contaminants by increasing the concentrations of this most abundant intracellular thiol (Olakolu et al. 2012). Metals such as Cu(II), Co(II), Mn(II), Fe(II) and Cr(VI) can react with GSH, bringing about its oxidation (Christie and Costa 1984).

According to Kaisarevic et al. (2009), polychlorinated biphenyls (PCBs) and their methylated and alkylated derivatives are the major toxicants in the sediments of wastewater canals in the Pančevo industrial zone and surrounding area that is a well-known hot-spot of contamination. The phase II biotransformation enzyme GST catalyzes the binding of GSH with electrophilic substrates and is involved in the detoxification of a wide variety of chemicals (Eaton and Bammler 1999). Also, the secondary substrates for GST are toxic products generated by tissue damage (e.g., compounds resulting from lipid peroxidation of membranes). Several studies have shown that GST activity is increased in the brain of *Rana pipiens* (Hermes-Lima and Storey 1998) during anoxic exposure, and lowered in the kidneys and heart of *R. sylvatica* during freezing exposure (Joanisse and Storey 1996).

Lipid peroxidation often serves as an indicator of ROS-induced damage and it represents a very useful and sensitive biomarker (Abu Youssef et al. 2014). Willmore and Storey (1997) reported a decrease in TBARS level in the muscle of the freshwater turtle, *Trachemys scripta elegans*, after anoxic submersion. The present results showed a significant reduction of lipid peroxidation during the posthibernation period in the blood of the snakes from both the localities. Lower TBARS levels suggested that upon awakening, there was no oxidative damage of lipid molecules. Increased CAT and GSH-Px activities before hibernation could represent an adaptive response and a mechanism that prepared the organism for hibernation and increased ROS generation that occurred during arousal when the metabolic rate rapidly increased. Findings obtained from the studies of mammals have shown that the level of lipid peroxidation declined during late hibernation (Pratihar et al. 2010; Avci et al. 2014).

AChE is inhibited when organisms are exposed to organophosphate and carbamate pesticides (Srain and Rudolph 2010). Organophosphorus insecticides, carbamates, and organochlorine pesticides, pyrethroids, are potent AChE inhibitors (Robillard et al. 2003), and exposure to these chemicals elicits toxic effects that are usually sublethal (Amaral et al. 2012). In accordance with the fact that other chemicals, such as polycyclic aromatic hydrocarbons (Payne et al. 1996) and heavy metals (Bocquené et al. 1995) can also inhibit AChE, Lionetto et al. (2005) suggested that AChE was a very useful biomarker of the biological effect of a mixture of neurotoxic pollutants in the aquatic environment. There was inhibition of AChE activity in the blood of the snakes collected from OB in the spring (posthibernation period). In the snakes from the PR, AChE activity was almost the same during the investigated period. Several studies (Abdel-Halim et al. 2006; Chitmanat et al. 2008) have described

the seasonal changes in AChE activity throughout the year, apparently linked with water temperature and the amount of precipitation.

Figure 4 shows the summary results of PCA for both the sampling localities. These showed that Factor 1 and Factor 2 explained over 80% of total variance. Regarding the position of the sites, Factor 1 (46.18%) discriminated between OB-prehibernation and OB-posthibernation/PR-prehibernation/PR-posthibernation. Factor 2 (34.57%) discriminated between OB-prehibernation/PR-posthibernation and OB-posthibernation/PR-prehibernation. Factor 1 showed clear separation between prehibernation and posthibernation periods at OB, but no separation at the PR, suggesting different results of the investigated parameters at polluted locality. The results also showed clear separation of the analyzed parameters between prehibernation and posthibernation periods at both the localities (Factor 2).

CONCLUSIONS

In contrast to the great interest in mammalian hibernation, little is known about the role of the markers of the oxidative stress in reptile species, particularly in the snakes during preparation for hibernation and after arousal. In conclusion, the observed seasonal differences in the oxidative stress and neurotoxicity markers between prehibernation and posthibernation periods represented an adaptive mechanism to the oxidative stress that was induced by tissue reoxygenation during arousal from hibernation. Results also showed that external factors modulated both the biomarkers of oxidative stress and of neurotoxicity in the blood of the grass snake during the prehibernation and posthibernation periods.

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