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A first record of the antioxidant defense and selected trace elements in *Salamandra salamandra* larvae on Mt. Avala and Mt. Vršački Breg (Serbia)

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Abstract: We investigated the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR) and the phase II biotransformation enzyme glutathione S-transferase (GST) in the whole body of fire salamander larvae (*Salamandra salamandra*) from two localities on Mt. Avala (AVS and ABP) and one locality on Mt. Vršački Breg (VSB), Serbia. We also determined the total glutathione (GSH) and sulfhydryl group (SH) contents, as well as the concentrations of manganese (Mn), copper (Cu), zinc (Zn), selenium (Se), arsenic (As), cadmium (Cd), lead (Pb) and uranium (U). The obtained results show that animals from VSB had significantly lower weights and lengths than animals from AVS and ABP. The activities of all investigated enzymes were significantly higher, while the SH content was significantly lower in animals from VSB compared to those from AVS and ABP. No correlations between trace-element concentrations in water and animal tissue were observed. We concluded that the obtained results were more likely a consequence of the combination of developmental differences and the effects of different habitat conditions, environmental and anthropogenic influences than of concentrations of trace elements in the water alone.

Keywords: antioxidant defense system; oxidative stress; trace elements; fire salamander; development

INTRODUCTION

The physiological cost of aerobic life is elevated damage of cellular components by highly reactive oxygen species (ROS) derived as byproducts of oxygen metabolism. Most of the oxygen used in respiration is completely reduced to water in the mitochondria, while 0.1-2.0% is constantly transformed to superoxide anion radicals ($O_2^{\cdot-}$) [1]. By spontaneous or enzymatic dismutation of $O_2^{\cdot-}$, hydrogen peroxide is formed (H_2O_2). Hydrogen peroxide can be reduced by some transition metal ions (Fe^{2+}) to produce the hydroxyl radical (HO•), which is the most reactive ROS. Hydrogen radicals attack and cause damage to proteins, lipids and DNA. When the production of ROS exceeds the capacity of cells to maintain redox balance through their antioxidant defense mechanisms, oxidative stress ensues [2]. However, in addition to their toxic effects, ROS have certain essential beneficial roles in the cell. When the concentrations of ROS in the cells are strictly controlled, they can act as intracellular signaling molecules that participate in critical functions in organisms, such as regulation of circulation, energy metabolism, reproduction and modulation of gene expression in cells [3]. To prevent the harmful effects of ROS, during evolution all aerobic organisms developed antioxidant defense, which is comprised of protective enzymes and nonenzymatic scavengers that prevent the uncontrolled formation of free radicals and activated oxygen species [4]. Antioxidant enzymes include superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GSH-Px, EC 1.11.1.9), glutathione reductase (GR, EC 1.6.4.2) [5] and the antioxidant and phase II biotransformation enzyme glutathione-S-transferase (GST, EC 2.5.1.18) [6]. Nonenzymatic components include some low molecular mass antioxidants, such as glutathione (GSH), sulfhydryl

groups (SH), the vitamins C and E, as well as other molecules [5]. The main cellular regulator of the expression of many antioxidant pathway genes is the transcription factor, nuclear factor-erythroid 2 (NF-E2)-related factor 2 (Nrf2). Nrf2 is the main inducer of genes encoding antioxidant enzymes and phase II detoxifying enzymes, which enhances the overall capacity of cells to detoxify and eliminate harmful substances [7].

Many environmental toxicants can induce oxidative stress in organisms. Environmental poisoning by metals has increased due to the extensive use of metals in agricultural, chemical and industrial processes [8]. Exposure to metals can increase ROS generation, leading to oxidative stress, as has been reported in many aquatic organisms after exposure to sublethal concentrations of some metals [9]. In addition to toxic effects, some metals such as Fe, Cu, Zn and Mn, are essential metals that play important roles in biological systems, whereas metals such as Hg, Pb and Cd are non-essential metals and are toxic even in trace amounts.

The fire salamander, *Salamandra salamandra*, belongs to the order *Caudata*, tailed amphibians. Amphibians with tails are one of the most endangered vertebrates on earth [10], and their larval stages are the most vulnerable in this regard. The fire salamander inhabits mostly central and southern parts of Europe in a variety of habitats from 100-250 m a.s.l. [11]. In the central Balkans, the fire salamander can be found in highland areas up to 1750 m a.s.l. [12]. *S. salamandra* inhabits more than 50% of Serbia in fragmented distributional ranges; however, due to insufficient data it is in potential danger [13]. In Serbia, it is protected by law and classified as a strictly protected species [14]. *S. salamandra* is a long-lived species with a complex life cycle comprising aquatic larvae and terrestrial adults. Mating takes place from March to September when females mate with different males and fertilization occurs in the oviduct. The reproductive process has an unusual course, as females do not lay eggs but retain them in their oviducts until the egg yolk is consumed, giving birth to well-developed aquatic larvae. Adult forms live in the floor of deciduous forests [15].

There are no studies of the activity of antioxidant defense enzymes, low-molecular mass antioxidants and trace-element concentrations in larvae of the fire salamander. Thus, the aim of our study was to provide reference data to support the potential inclusion of the fire salamander in different applications, from conservation biology, monitoring studies, effects of nutrition and in developmental studies. To that end, we investigated the activities of the antioxidant defense enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR), and the biotransformation phase II enzyme glutathione S-transferase (GST) in the whole body of the fire salamander (*Salamandra salamandra*) from the Avala and Vršački Breg mountains in Serbia. In the same samples, the total glutathione (GSH) and sulfhydryl group (SH) contents, and the concentrations of manganese (Mn), copper (Cu), zinc (Zn), selenium (Se), arsenic (As), cadmium (Cd), lead (Pb) and uranium (U), were determined.

MATERIALS AND METHODS

Study area and sampling

Samples of fire salamander larvae (Fig. S2) and water samples were collected in the spring of 2019 at two localities on Mt. Avala and one locality on Mt. Vršački Breg (Serbia). The Avala localities were the "Vranovac Stream" (AVS; latitude 44°69'46"N/longitude 20°51'92", 330 m a.s.l., with air temperature 11°C and water temperature 6.5°C on the day of sampling) (Fig. S3) and the village "Beli Potok" (ABP; latitude 44°70'13"N/longitude 20°51'79"E, 270 m a.s.l., air temperature: 11°C, water temperature: 9°C on the day of sampling) (Fig. S4). The locality on Mt. Vršački Breg was "Široko Bilo" (VSB; latitude 45°12'69"N/longitude 21.36'37"E, 220 m a.s.l., air temperature: 15°C, water temperature: 9°C on the day of sampling) (Fig. S5). AVS is in the northeastern part of Mt. Avala, in a deep, mixed deciduous forest. ABP is a remarkably similar site located in the western part of Mt. Vršački Breg near a mountain hut. In both cases, these are tourist sites without industrial or municipal pollution. The water trough is located within the ABP at the foothill of Mt. Avala. This is a rectangular masonry trough about 5 m long and 60 cm deep, surrounded by ruderal habitats and houses. The localities were chosen based on our earlier field studies of natural variations in the investigated parameters among three different populations of fire salamander larvae. The geographical positions of the sampling sites are given in Supplementary Fig. S1.

Sample processing

All animal procedures were performed in compliance with the ARRIVE guidelines which conform with the European Directive (2010/63/EU) on the protection of animals used for experimental and other scientific purposes. Animal capture was approved by the Serbian Ministry for Energy, Development and Environmental Protection (Permission No. 353-01-325/2019-04).

All animals used in the experiment were at the same stage of development. After collection, the lengths and weights of the animals were measured, and the animals were washed in ice-cold 0.6% NaCl and frozen in liquid nitrogen (-196°C) before storage at -80°C. The whole body of every animal was ground and homogenized in 5 volumes of 25 mmol/L sucrose containing 10 mmol/L Tris-HCl, pH 7.5 at 1500 rpm [16] using a Janke & Kunkel (Staufen, Germany) IKA-Werk Ultra-Turrax homogenizer at 4°C. The homogenates were sonicated for 30 s at 10 kHz on ice to release enzymes [17], and the sonicates were then centrifuged at 100000 × g for 90 min at 4°C. The resulting supernatants were used for further biochemical analyses.

Determination of antioxidant defense parameters

The protein concentrations of the supernatants were determined as described [18], using bovine serum albumin as a standard, and were expressed in mg/g wet mass. The activities of the antioxidant defense enzymes were measured simultaneously in triplicate for each sample using a Shimadzu UV-1800 spectrophotometer and a temperature-controlled cuvette holder.

SOD activity was measured by the epinephrine method [19]. One unit of SOD activity was defined as the amount of protein causing 50% inhibition of the autoxidation of adrenaline at 26°C and was expressed as specific activity (U/mg protein). The activity of CAT was evaluated by the rate of hydrogen peroxide (H_2O_2) decomposition [20]. The method is based on the degradation of H_2O_2 by CAT contained in the examined samples. According to this procedure, 30 mmol H_2O_2 as substrate is used. CAT activity was expressed as μmol H_2O_2/min/mg protein.

GSH-Px activity was assayed following the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) as a substrate with t-butyl hydroperoxide [21]. This reaction assesses the action of GSH-Px contained in the samples in the presence of *t*-butyl hydroperoxide (3 mmol) as a substrate in 0.5 M phosphate buffer, pH 7.0, at 37°C. The activity of GSH-Px was expressed as nmol NADPH/min/mg protein.

The activity of GR was measured as described by Glatzle et al. [22]. The method is based on the capability of GR to catalyze the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) using NADPH as a substrate in phosphate buffer (pH 7.4). GR activity was expressed as nmol NADPH/min/mg protein.

GST activity towards 1-chloro-2,4-dinitrobenzene (CDNB) was determined by the method of Habig et al. [23]. The method is based on the reaction of CDNB with the SH group of GSH, catalyzed by GST contained in the samples. The reaction proceeded in the presence of 1 mmol GSH in phosphate buffer (pH 6.5) at 37°C. GST activity was expressed as nmol GSH/min/mg protein.

The GSH content was measured according to the method of Griffith [24] based on the sequential oxidation of GSH by 5,5´-dithiobis 2-nitrobenzoicacid (DTNB) and reduction by NADPH in the presence of GR. The GSH content was expressed as μmol GSH/g tissue.

The concentration of SH groups was determined using DTNB according to the Ellman [25] method and expressed as nmol SH/g tissue. All chemicals were purchased from Sigma-Aldrich (Saint Louis, MO, USA) or Merck (Darmstadt, Germany).

Trace element analysis

The concentrations of eight elements (Mn, Cu, Zn, Se, As, Cd, Pb and U) were determined by inductively coupled plasma mass spectrometry, ICP-MS (ICAP Q_c; Thermo Scientific X series 2, Waltham, MA, USA). The entire system was controlled with Qtegra Instrument Control software.

Tissue samples were transferred into microwave cuvettes and decomposition was performed using the ETHOS 1 Microwave System (Milestone, Italy). Four mL of 65% nitric acid and 1 mL of 30% high-grade hydrogen peroxide (Merck, Darmstadt, Germany) were added to each cuvette and microwave digestion was performed as follows: warmup for 3 min to 85°C, 5 min to 135°C, and 15 min to 180°C. After cooling, the samples were

quantitatively transferred into a volumetric flask (25 mL) and diluted with ultrapure water (resistance: 18.2 MΩ; Milli Q plus system, Merck, Germany). The investigated water samples were diluted 10 times with 2% nitric acid.

Statistical analysis

The data are expressed as the mean±SE (standard error). Before testing, all data were checked for normality and homogeneity using Shapiro-Wilk and Lilliefors statistics to meet statistical demands. One-way analysis of variance (ANOVA) was performed to determine all interactive effects between the localities. When an interactive effect was observed, Tukey's HSD (honest significant difference) post-hoc test was used to obtain significant differences among the means. A minimum significance level of $P<0.05$ was accepted for all cases. Principal component analysis (PCA) was implemented to statistically determine the differences between the investigated groups based on all investigated antioxidant defense biomarkers, and to detect variables that significantly contributed to differences in the investigated parameters [26]. After examining the antioxidant defense parameters and trace-element concentrations in the bodies of larvae, three PCA models were constructed. Spearman's rank correlation was performed between the parameters of oxidative stress and trace-element concentrations, as well as between the parameters of oxidative stress and the weight and length of the larvae. Statistical analyses were performed using STATISTICA 10.0 software.

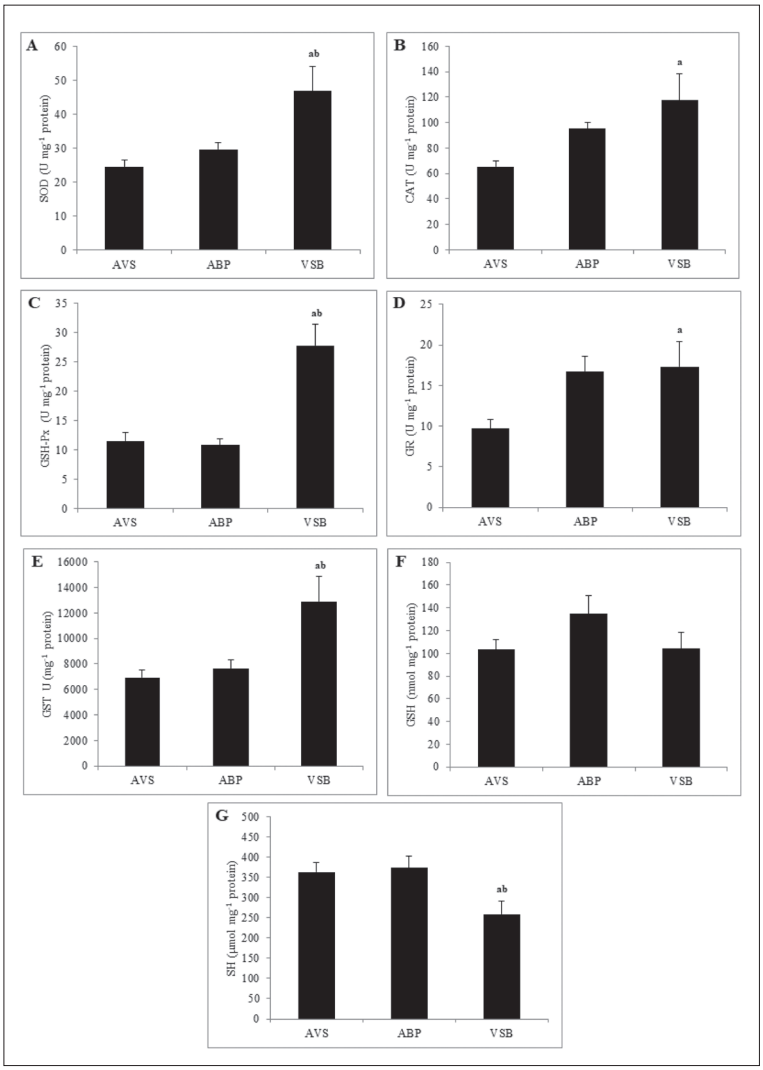

Fig. 1. The activities of antioxidant enzymes and the concentrations of total glutathione and sulfhydryl groups in fire salamander larvae. **A** – superoxide dismutase (SOD), **B** – catalase (CAT), **C** – glutathione peroxidase (GSH-Px), **D** – glutathione reductase (GR), **E** – glutathione S-transferase (GST); the concentrations of **F** – total glutathione (GSH) and **G** – sulfhydryl. The data are expressed as the mean±SE. One-way ANOVA post hoc Tukey's HSD for unequal N, with $P<0.05$ as the level of significance. Significant differences between groups: [a]AVS vs VSB; [b]ABP vs VSB.

RESULTS

The obtained results show significantly lower body weight (AVS: 0.32±0.002g; ABP: 0.40±0.18 and VSB: 0.20±0.03) and body length (AVS: 3.25±0.002 cm; ABP: 3.13±0.08 cm and VSB: 2.54±0.16 cm) of animals from the VSB group with respect to the animals from both AVS ($P<0.05$) and ABP ($P<0.05$) groups. The condition factor (CF) of animals from the AVS locality (AVS: 0.98±0.03; ABP: 1.36±0.09 and VSB: 1.06±0.03) was lower in comparison to animals from ABP ($P<0.05$). At the same time, the CF of animals from VSB was significantly lower than in animals from ABP ($P<0.05$).

The activities of antioxidant enzymes and the concentrations of GSH and SH are presented in Fig. 1;

in Table 1 statistics are provided (one-way ANOVA, F, *P*-values and standard error of estimate; raw data, post hoc Tukey's HSD for unequal N with *P*). The obtained data show that the activity of SOD (Fig. 1A) was significantly higher in animals from VSB compared to animals from AVS ($P<0.05$) and ABP ($P<0.05$). The same trend was observed for GSH-Px (Fig 1C, $P<0.05$) and GST (Fig. 1E, $P<0.05$) activities. The activities of CAT (Fig. 1B) and GR (Fig. 1D) were markedly higher in animals from VSB only in comparison those from AVS ($P<0.05$). The concentration of the SH group (Fig. 1G) was significantly lower in animals from VSB compared to animals from both AVS ($P<0.05$) and ABP ($P<0.05$). There was no change in total GSH concentration among the investigated groups of animals (Fig. 1F).

Spot measurements of investigated trace-element concentrations (mg/L) are presented in Table 2. Values that differ significantly are marked in bold. As can be seen, higher concentrations of Se were obtained at sites ABP and VSB, of As at site AVS and of U at sites AVS and ABP.

The concentrations of selected trace elements in the tissue of fire salamander larvae are expressed in ng/g tissue and are presented in Table 3. We detected only a few significant differences, for Cd, Pb and U. The concentration of Cd was markedly lower in tissues of animals from locality VSB than in tissues of animals from AVS ($P<0.05$) and ABP ($P<0.05$). The concentration of Pb was significantly higher in animals from ABP ($P<0.05$) and VSB ($P<0.05$) with respect to animals from AVS, as well as in larvae from VSB in comparison to those from ABP ($P<0.05$). The concentration of U in the bodies of fire salamander from VSB was markedly higher relative to the animals from AVS ($P<0.05$) and ABP ($P<0.05$).

Considering that individuals from VSB had significantly lower weight and shorter length than individuals from AVS and ABP, we calculated Spearman's rank correlations between the lengths and weights, as well as between the enzymatic and nonenzymatic components of the antioxidant defense system. The calculation results of Spearman's rank correlation coefficients are presented in Table 4. We obtained a significant negative correlation between the weight and GSH-Px activity (-0.519107, $P<0.05$) and a positive correlation between the weight and GR activity (0.483439, $P<0.05$). We also obtained negative correlations between the length and GSH-Px activity (-0.567547, $P<0.05$), as well as between the length and GST activity (-0.602260, $P<0.05$).

Table 1. Results of one-way ANOVA of the comparison between different localities.

Variable	*F*	*P*	SE of estimate	*N*
SOD	6.0437	0.007486	3.113147	27
CAT	4.6045	0.021972	7.56316	24
GSH-Px	17.2779	0.000022	2.006004	27
GR	4.1522	0.031028	1.329486	24
GST	6.8225	0.004509	859.709	27
GSH	1.7225	0.199152	8.23595	28
SH	5.4784	0.011729	20.22086	25

SOD – superoxide dismutase; CAT – catalase; GSH-Px – glutathione peroxidase; GST – glutathione S-transferase; GR – glutathione reductase; GSH – glutathione; SH – SH groups; N – number of individuals.

Table 2. Concentrations of trace elements (mg/L) in water from the investigated localities.

mg/L	MAC/MCL	AVS	ABP	VSB
Manganese (Mn)	0.10*	N.D.	N.D.	N.D.
Copper (Cu)	2.00*	0.85	0.77	0.28
Zinc (Zn)	5.00*	0.14	0.22	0.20
Selenium (Se)	0.05**	0.04	**0.09**	**0.23**
Arsenic (As)	1.00*	**16.35**	0.41	0.03
Cadmium (Cd)	0.10*	0.02	N.D.	N.D.
Lead (Pb)	1.00*	N.D.	N.D.	N.D.
Uranium (U)	0.03***	**0.83**	**0.19**	N.D.

* MAC-EQS – maximum allowable concentration (Environmental Quality Standards) according to TNMN standards (ICPDR, 2006)
**MCL – maximum contaminant level according to Water Quality Association (www.wqa.org)
***MCL – maximum contaminant level according to US EPA (United States Environmental Protection Agency)

Table 3. Concentrations of trace elements (ng/g) in fire salamander tissue.

ng/g	AVS		ABP		VSB	
	Mean	SE	Mean	SE	Mean	SE
Manganese (Mn)	0.53	0.02	0.42	0.03	1.12	0.31
Copper (Cu)	43.01	1.00	34.5	2.62	86.86	29.51
Zinc (Zn)	59.05	2.00	46.9	3.56	117.94	40.07
Selenium (Se)	25.80	2.00	19.07	0.84	51.92	22.45
Arsenic (As)	44.37	1.72	68.00	5.85	28.66	0.91
Cadmium (Cd)	34.20	5.22	195.18[a]	27.23	9.47[c]	5.25
Lead (Pb)	0.56	0.01	20.21[a]	6.12	68.23[bc]	3.50
Uranium (U)	0.07	0.004	1.07	0.26	7.95[bc]	2.10

The data are presented as the mean±SE. One-way ANOVA post hoc Tukey's HSD for unequal N, with $P<0.05$ as the level of significance. Significant differences between groups: [a]AVS vs ABP; [b]AVS vs VSB; [c]ABP vs VSB.

Table 4. Calculation results of Spearman's rank correlation coefficients between oxidative stress parameters and weight, length and trace-element concentrations in fire salamander larvae.

Investigated parameter	Weight r	Length r	Element r	
CAT			Pb	**0.64**
GR			Se	**-0.71**
GR			Pb	**0.76**
SH			Mn	**-0.62**
GSH-Px	**-0.52**	**-0.57**		
GST		-0.60		

A significant relationship assumed at $P<0.05$

Spearman's rank correlations between the enzymatic and nonenzymatic components of the antioxidant defense system and the concentrations of the investigated trace elements were performed (Table 4). Negative correlations were obtained between the content of SH groups and Mn concentration (-0.622378, $P<0.05$), as well as between GR activity and Se concentration (-0.709091, $P<0.05$). Positive correlations were obtained between CAT activity and Pb concentration (0.645455, $P<0.05$), as well as between GR activity and Pb concentration (0.757576, $P<0.05$).

PCA was used to detect possible separation of all three examined groups of animals based on all investigated parameters. PCA was performed in three ways as follows: projection of the relative contribution of every antioxidant component in the factor plane (Fig. 2A), projection of localities based on antioxidant defense parameters (Fig. 2B) and projection of localities based on trace-element concentrations (Fig. 2C).

The PCA of all investigated antioxidant defense parameters is presented in Fig. 2A. The PCA referring to the relative contribution of every antioxidant component showed that PC1 and PC2 can explain about 78% of the total variance in the data matrix. PC1 explains 55.26% of the total variance with SOD and GST as the parameters that contributed most to the separation. PC2 explains 22.86% of the total variance with GSH and SH as the parameters that contributed most to the separation (Table 5).

A summary of the results of PCA for all three investigated groups of animals considering parameters of oxidative stress (Fig. 2B) indicates that PC1 and PC2 can explain 100% of the total variance. PC1 (69.04%)

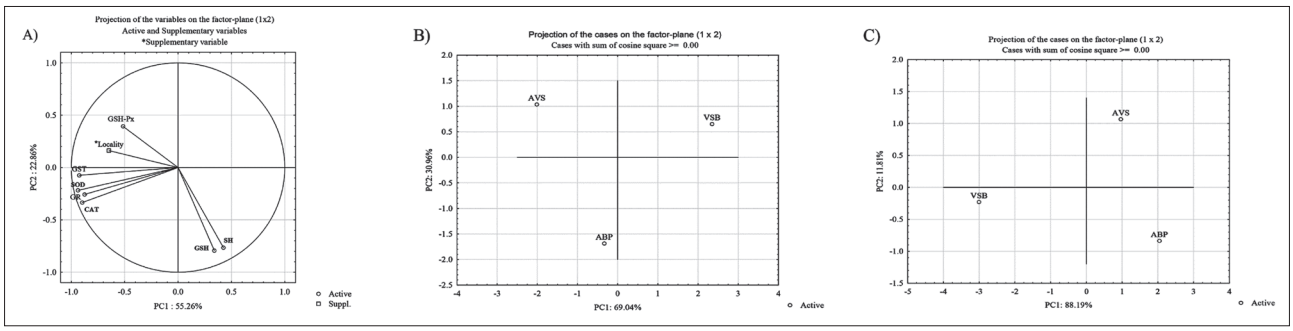

Fig. 2. Principal component analysis (PCA) of oxidative stress biomarkers of fire salamander larvae on the factor plane. **A** – projection of the relative contribution of every antioxidant component; **B** – projection of groups based on antioxidant defense parameters; **C** – projection of groups based on trace element concentrations.

Table 5. Loadings of variables onto the principal components (PC). Parameters that contributed most to the separation are marked in bold.

	PC1	PC2	PC3	PC4	PC5	PC6	PC7
SOD	-0.936356	-0.217993	0.096654	0.128494	0.003723	0.111084	-0.193677
CAT	-0.897851	-0.334664	0.033899	-0.159759	0.068418	0.169933	0.147083
GSH-Px	-0.515166	0.393474	-0.747470	0.052499	-0.132861	0.024764	0.007021
GR	-0.875191	-0.256611	0.221059	-0.045862	-0.312834	-0.135439	0.031815
GST	-0.925915	-0.074117	-0.113040	0.109880	0.286952	-0.172233	0.018163
GSH	0.340039	-0.793168	-0.327693	-0.375530	0.021700	-0.045402	-0.065733
SH	0.425297	-0.765100	-0.150251	0.454313	-0.044686	0.015933	0.050172
Eigenvalues	3.868078	1.600179	0.760805	0.406390	0.205021	0.092153	0.067374
% of each axis	55.26 %	22.86 %	10.87 %	5.81 %	2.93 %	1.32 %	0.95 %

clearly discriminates animals from the Mt. Avala sites (AVS and ABP), from animals from Mt. Vršački Breg (VSB). PC2 (30.96%) distinctly differentiates AVS and VSB animals from ABP animals. As regards trace-element concentrations (Fig. 2C), PC1 (88.19%) clearly separates VSB from AVS and ABP specimens, while PC2 (11.81%) discriminates AVS from VSB and ABP animals.

DISCUSSION

Amphibian populations are declining worldwide, primarily because of habitat loss, but also due to the influence of anthropogenic contaminants. According to a report of the International Union for Conservation of Nature (IUCN) of 6285 amphibian species, 1895 are at risk of extinction, making them the most endangered group of vertebrates [27]. Amphibians represent a significant part of aquatic ecosystems and are valuable indicators of environmental influences. They interact with the aquatic environment as larvae and with both water and soil as adults. In both aquatic and terrestrial environments, amphibians are exposed to different stressors throughout their life cycle [28].

Our results show that the average body weight and length of animals from Mt. Vršački Breg were significantly lower when compared to animals from Mt. Avala. At the same time, the animals from ABP had a higher CF when compared to animals from AVS and VSB. In the literature there are some potential explanations for this phenomenon. In some populations of amphibians, certain characteristics such as egg size or size at birth are recognized as highly variable. Many of these variabilities represent adaptations to environmental influences. Climatic conditions and food availability can contribute to increased variability in size at birth. Studies have shown that larvae developing in ponds had some adaptations, such as greater larval weight at birth, the ability to grow on lower food sources and earlier metamorphosis onset when food is limited [29]. Egg size is influenced by temperature and food availability. Variance in the size of larvae can be explained by their individual size at birth; larvae with a larger size at birth tend to have a shorter duration of development [30]. In addition, ectotherms in cold climates mature slowly and attain maturity later, thus predicting a smaller size at a given age in colder climates, which is in accordance with the results obtained in our study. In high-density populations, fire salamander larvae also increase their body length in response to these conditions [31]. The body size of larvae at birth depends on the body size of females. One important non-destructive technique used in amphibian field studies is to assess the body condition factor. The CF is related to the movements and survival of amphibians, as well as to population stressors; it is also a reflection of the status of the overall energy stores, the fats and proteins in the organism [32].

Amphibian development involves increased growth, absorption of larva-specific organs, the appearance of adult-type organs, many physiological and biochemical changes and increased oxidative metabolism. The requirements of increased cellular activity during metabolic processes can lead to the elevated production of ROS as a byproduct of metabolism [33]. The relationship between growth and ROS production depends on the developmental stage of the organism and the metabolic rate. During these processes, different defense mechanisms against elevated ROS production have developed [34]. Gomez-Mestre et al. [35] showed that during early developmental stages, tadpoles cope with the ROS production induced by growth and external environmental factors. During the earliest stages of anuran development, the produced ROS are removed by the activity of SOD and CAT, while the GSH system is activated for the first time after exposure to environmental stressors [36].

In addition to the physiological overproduction of ROS during development, many pollutants can also cause oxidative stress. For these reasons, many defense mechanisms have developed during evolution [37]. SOD, CAT and GSH-Px play important roles in protection against ROS in cells. SOD removes toxic superoxide anion radicals, while CAT and GSH-Px are the main antioxidant components involved in H_2O_2 elimination. The Michaelis-Menten constant (K_m) for H_2O_2 in CAT is significantly higher than in GSH-Px [38]. Thus, GSH-Px is efficient in H_2O_2 elimination when its concentration is near the physiological level [37] and CAT is efficient during H_2O_2 overproduction. GST activity can be significantly increased or significantly reduced depending on the type of toxic substance or exposure conditions. It was shown that after an initial increase in GST activity due to ROS

overproduction, its enzymatic activity progressively decreases [39]. Induction of antioxidant defenses is usually interpreted as the adaptation of an organism to environmental disturbances, whereas inhibition reflects the toxic effect of pollutants that points to cell damage [40]. Measuring the activities of antioxidant enzymes serves as an indicator of an organism's antioxidant status and has been used as a biomarker of oxidative stress.

Amphibian species can be important indicators of ecosystem health. Terrestrial salamanders can be sensitive to many anthropogenic disturbances [41]. Amphibians have a high potential for contaminant uptake because of their thin and permeable skin, which is important for respiration and osmoregulation. These characteristics make them susceptible to chemical contaminants. During early developmental stages, the larvae are the most sensitive to contaminants. Experiments with perfluoroalkyl substances show that larval amphibians rapidly bioaccumulate these compounds. Amphibians serve as prey for diverse aquatic and terrestrial species with different food-web positions, and thus could enhance the biomagnification potential of toxic substances [42].

Investigations of the bioaccumulation and influence of metals on fire salamander are scant. Heavy metals are toxic and cause environmental problems and affect the health of animals and humans. They cannot be degraded or modified like toxic organic compounds and are mainly deposited in tissues. Increased levels of metals in an organism produce many harmful effects on biochemical and metabolic processes, growth, maturation, reproduction and the survival of individuals [43]. Many metals generate oxidative stress either through direct ROS generation or by scavenging thiols (glutathione and cysteine) that act as important nonenzymatic antioxidants. Metals induce the production of increased levels of free radicals and non-radical species. The consequences of increased generation of ROS can lead to oxidative damage of cellular macromolecules causing oxidative stress [44]. The redox active metals, Fe, Cu, Cr and Co can produce ROS (such as superoxide anion radicals and hydroxyl radicals), mainly via the Fenton reaction. ROS generation is considered as the main mechanism of metal toxicity [45]. On the other hand, redox inactive metals (Cd, As and Pb) inhibit the antioxidant system (AOS) by covalently binding protein SH groups and depleting glutathione (GSH) [46]. Therefore, the AOS is directly involved in protection against the harmful effects of heavy metals and in homeostasis maintenance. The phase II biotransformation enzyme GST, in addition to its other roles [6], prevents lipid peroxidation and assists in heavy metal detoxification [47]. Antioxidative defense enzymes are early warning signals of toxicant exposure and are integrated into environmental monitoring programs [48]. As a result of exposure to metals, antioxidant enzyme activities can be enhanced or reduced, depending on the intensity and duration of chemical stress, as well as the sensitivity of the investigated species [49]. Therefore, assessment of the relationship between heavy metal pollution and oxidative stress indicators is of great interest to environmental and toxicological studies.

Cu, Pb, Cd, As and Hg are the most toxic heavy metals to humans, animals and the environment. Cu is an essential metal that participates in enzymatic reactions and is found in many tissues at relatively high concentrations. Cu toxicity is the consequence of the generation of ROS by Cu ions via the Fenton or Haber-Weiss reactions. It has been suggested that the Cu ion displays a high affinity for protein thiol and amino groups [50]. Cd is highly toxic and one of the most investigated heavy metal. The molecular mechanisms underlying Cd-induced cellular toxicity include interference with antioxidant enzymes, alterations in thiol proteins, inhibition of energy metabolism, alterations of DNA structure, altered membrane structure/function and induction of the expression of a wide variety of stress genes. Cd also plays an important role in disturbing enzyme activities and inducing lipid peroxidation [51]. Mn, Zn and Se at low concentrations are essential microelements for the activity of many enzymes, such as SOD or GSH-Px; however, at high concentrations they are toxic [5]. One of the most deleterious metal pollutants in water is Pb, which can cause morphophysiological and metabolic changes in amphibians [52]. Pb exposure has an inhibitory effect on SOD activity. It can cause alterations in the mitochondria and facilitate the release of $O_2^{\bullet-}$ that can inhibit CAT activity. Increasing Pb concentration lowers SOD and CAT activities. In fish that were exposed to As, CAT activity was not affected [53], further supporting the efficacy of increased GSH in preventing oxidative perturbations. This is in contrast

with the observation that in *Clarias batrachus* CAT activity increased after exposure to As [54]. Uranium can cause both chemical and radiological toxicity. The chemical toxicity of U is mainly caused by uranyl ions (UO_2^{2+}), which are dissolved in the soil and water. U increases ROS production in cells and increases SOD expression, indicating that SOD is the first line of defense against U toxicity [55]. Increased ROS in cells induce lipid peroxidation and cause a decrease in oxidative stress markers such as CAT and GSH and disturb sulfhydryl homeostasis. U toxicity depends on both its concentration and the length of exposure. Experiments have shown that U toxicity in zebrafish (*Danio rerio*) positively correlated with the time of exposure. After entering the body, U first accumulates in organs, affecting their functioning; thus, the activity of antioxidant enzymes in *D. rerio* was seriously affected and the gills were the most affected organs. U inhibited egg production and caused DNA damage in embryonic cells, thereby altering the reproductive capacity of *D. rerio* and destroying the genetic integrity of embryos. Acute exposure to U can also be toxic to kidneys [56].

The results of the PCA analysis point to two important conclusions: that there were clear differences between animals from Mt. Avala and animals from Mt. Vršački Breg, and that there were differences between animals from the two localities on Mt. Avala (AVS and ABP). The parameters that contributed the most were SOD and GST (PC1), and GSH and SH (PC2).

There are some limitations in the elucidation interpretation of antioxidant responses because in some cases environmental, toxicological and ecological factors may complicate the interpretation of the changes in the investigated parameters. Many other factors can impact the antioxidative defense system and interfere with biomarker responses. Such factors are the health status, sex, age, nutritional status, metabolic activity, migratory behavior, reproductive and developmental status, population density, as well as factors such as the season, ambient temperature, heterogeneity of the environmental pollution, etc. For example, in the gills of the freshwater catfish, elevated water temperature caused changes in antioxidant defense by increasing SOD activity and decreasing GSH level and GSH-Px activity, while food deprivation also affected GST activity in the liver of rainbow trout; another limitation is that most antioxidant responses are not specific for individual compounds. Thus, in order to avoid these limitations, it is important to carefully define the experimental conditions and pay special attention during sampling and handling of samples [6].

CONCLUSIONS

While the antioxidative defense system and trace-element concentrations have not been systematically investigated in fire salamander larvae, the presented results provide a contribution to the general knowledge of antioxidant defense parameters in these organisms. It can be concluded that the obtained results are more likely a consequence of the combination of developmental differences and the effects of different habitat conditions, environmental and anthropogenic influences than of concentrations of trace elements in the water. The study of the antioxidant defense system and its possible effects on different salamander populations is an important step in understanding the physiology of these organisms with the aim of better conservation and protection of this species.

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Supplementary Material

The Supplementary Material is available at: http://serbiosoc.org.rs/NewUploads/Uploads/Pavlovic%20et%20al_5753_Supplementary%20Material.pdf