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Do different diets affect oxidative stress biomarkers and metal bioaccumulation in two snake species?

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A B S T R A C T

In this study we examined possible differences in heavy metal accumulation and oxidative stress parameters in the liver and muscle of two semi-aquatic snakes: grass snake (*Natrix natrix*) and dice snake (*N. tessellata*), that inhabit the same environment but differ in prey diversity. The obtained results revealed some interspecies, inter-tissue, prey-snake and prey-prey differences in heavy metal concentrations. Grass snakes pray contained significantly higher concentrations of Al, Cr and Fe as compared to food of dice snakes. Both investigated snakes accumulated generally lower concentrations of metals than their prey, indicating that they are not at risk of contaminant biomagnification. A significant interspecies difference in accumulation was observed only for Cu and Mn concentrations. On the other hand, analysis of oxidative stress biomarkers showed clear differences between the investigated snake species and the two investigated tissues. The liver of grass snake had increased superoxide dismutase, glutathione reductase and glutathione-S-transferase activities in comparison to dice snake. In muscle, a reverse trend was observed for the activities of these three enzymes, as well as for glutathione peroxidase activity. The higher number of significant correlations observed between oxidative stress biomarkers and heavy metal concentrations in grass snake points to upregulation of the antioxidative system (AOS), which resulted in a lower TBARS concentration. Results show that while the investigated snake species did not differ significantly in the accumulated metals, their defense mechanisms were different. This reveals the complexity of the AOS and points to the cooperation of different AOS components in individuals from natural populations.

Keywords:

Natrix natrix

N. tessellata

Heavy metals

Bioaccumulation

Oxidative stress biomarkers

1. Introduction

Heavy metals cause serious health and environmental problems because they are toxic and cannot be degraded or modified like toxic organic compounds (Feng et al., 2015; Tokalioglu and Kartal, 2006). Elevated levels of various metals produce adverse biological effects on biochemical and metabolic reactions, growth, maturation, reproduction and survival of individuals (Łuszczek-Trojnar et al., 2014). One of these effects is the ability of metals to induce the production of increased levels of free radical and non-radical species (Valko et al., 2005). The consequences of increased generation of reactive oxygen species (ROS) is disruption of the prooxidant-antioxidant balance and damage to cellular macromolecules (Feng et al., 2013; Koch and Hill, 2017). The antioxidant defense system, which consists of enzymatic (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR), phase II biotransformation enzyme glutathione-S-transferase (GST)) and nonenzymatic compounds (glutathione (GSH), tocopherol, carotenoids) (Feng et al., 2014); Livingstone, 2001), has a crucial role in maintaining this balance. Therefore, assessment of the relationship between heavy metal pollution and oxidative stress indicators is of great interest for environmental and toxicological studies. Furthermore, measuring the changes in concentrations of thiobarbituric acid reactive substances (TBARS) as a standard marker for lipid peroxidation, and free sulfhydryl (–SH) groups as an indicator of increased cellular protein degradation may reveal the potential adverse effects of metals. According to Lionetto et al. (2013) measuring of cholinesterase (ChE) activity is widely recommended for monitoring not only organophosphate and carbamate pesticide exposure, but also many other contaminants, including heavy metals.

Although there has been considerable attention devoted to determining the adverse effects of heavy metals in other groups of vertebrates, mechanisms of contaminant exposure and metal bioaccumulation in snake are not fully established (Campbell and Campbell 2002). The close relationship with aquatic and surrounding terrestrial ecosystems, their comparative longevity, abundance, small home range, reasonable size and easy collection make semi-aquatic snakes a valuable model for environmental monitoring studies (Burger et al., 2006; Hopkins, 2000). Biomagnification through dietary exposure may represent the greatest risk for snakes as top-level carnivores (Burger et al., 2017). Hopkins et al. (2002) showed that feeding with contaminated prey was an important route of metal exposure for another semi-aquatic species *Nerodia fasciata*. Frog-eating individuals of *Natrix maura* were characterized by lower mean values of Hg concentration when compared to piscivorous snakes, suggesting strong differences in accumulation rates due to food peculiarities (Lemaire et al., 2018). According to Burger et al. (2017), differences in metal concentrations between snake species that inhabit the same area can point to bioaccumulation patterns and contaminant exposure. In addition to the diet, reptile species could also be exposed to contaminants via other sources, in particular through drinking water, dermal absorption and inhalation (Weir et al. 2014).

Some studies have shown that species-specific responses in antioxidative enzyme activities can be greater than spatial differences due to different physiological processes or species ecology (Fonseca et al., 2011). Our previous work conducted on frog species of the *Pelophylax esculentus* complex showed that certain differences in feeding and wintering habits could be responsible for the interspecific differences in accumulated heavy metals and levels of oxidative stress (Prokić et al., 2017). Two natricine species, *Natrix natrix* (grass snake) and *N. tessellata* (dice snake) are common throughout the Palearctic region (Šukalo et al., 2014).

Although the available data on prey composition show microgeographic variations in dietary preferences for both species (Filippi et al., 1996), it is well known that grass snakes feed mostly on amphibians, while fish are the main prey of dice snakes (Šukalo et al., 2014).

In order to study the effects of heavy metal pollution on wild snake in their relatively polluted natural habitats, we measured concentrations of 12 heavy metals (Al, As, Cd, Co, Cr, Cu, Fe, Mn, Mo, Ni, Pb and Zn), and examined a battery of oxidative stress biomarkers in the liver and skeletal muscle of *N. natrix* and *N. tessellata*. We also determined the concentrations of heavy metals in swallowed prey. Thus, this study provides evidence about heavy metals transferring to the succeeding trophic levels and possibility of their accumulation into snake tissues. The aim of our study was also to examine interspecies, inter-tissue and prey-prey differences in heavy metal concentrations, considering that the studied species of snakes have significantly different diets. Pearson correlation, Correspondence Analysis (CA) and Integrated Biomarker Response (IBR) were used with the purpose of linking metal concentrations with the antioxidant defense system parameters and to evidence an overall difference in biological responses between investigated snake species.

2. Materials and Methods

2.1. Site description and sample collection

Pančevački Rit (44°50'01.68'' N; 20°29'48.43'' E) is a wetland area in southern Vojvodina (Serbia), situated between the rivers Danube and Tamiš, close to the industrial zones of Belgrade and Pančevo (crude oil processing plant, production of plastic and fertilizers). The studied area was selected because it receives industrial and urban wastewater discharges.

Nine and six adult female specimens of *N. natrix* and N. *tessellata*, respectively, were captured by hand. The following mean values ± standard errors of snout-vent length (SVL) were recorded: 73.3±1.7 cm and 81.5±3.8 cm for *N. natrix* and *N. tessellata*, respectively. The snakes were sacrificed immediately after collection by severing the spinal cord. After dissection, liver and muscle tissue were carefully removed, frozen in liquid nitrogen and stored at -80 °C until analysis.

During capture and measuring procedures, *N. natrix* and *N. tessellata* would often regurgitate the ingested prey. Furthermore, we palpated some snakes that had obviously swallowed the prey a short time before capture to make them regurgitate the intact food from the upper portion of the digestive tract. *N. natrix* in Pančevački rit consumed mostly anurans: of 8 prey items, 6 were green frogs and 2 were lizards (*Podarcis muralis*). Prey of dice snake consisted 4 fish (without giving species-specific information) and 3 amphibians (*Lissotriton vulgaris*). The stomach contents of snakes were collected in plastic bags, stored at -20°C and used for heavy metal analyses.

Animal capture was approved by the Serbian Ministry of Energy, Development and Environmental Protection (Permission No: 353-01-77/2013-08). All animal procedures were in compliance with the Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes and was approved by the Ethical Committee for the Use of Laboratory Animals.

2.2. Determination of metal concentrations in water, snake tissues and their prey

The concentrations of Al, As, Cd, Co, Cr, Cu, Fe, Mn, Mo, Ni, Pb and Zn in water samples, prey, liver and muscle of two species of snakes, were examined using inductively coupled plasma optical emission spectrometry (ICP-OES) (Spectro Genesis EOP II, Spectro Analytical Instruments DmbH, Kleve, Germany). Water samples were collected from the surface (depth range<10 cm). The samples were then filtered and HNO_3 was added to avoid oxidation and preserved at 4°C prior to analysis. Analysis of the total element content in the water samples was performed according to the method provided in EPA (2007). The performance assessment of this method was done by examining a standard reference material: NIST-1643, RS stimulated fresh water trace elements. Analysis of each sample was performed in five sample replicates, and the concentrations were expressed in μg/L.

For heavy metal analysis, the tissues were dried in a Freeze Dryers Rotational-Vacuum Concentrator (GAMMA 1-16 LSC, Germany). After drying, 6 mL ultrapure 65% HNO_3 and 4 mL 30% H_2O_2 were added to 0.2 g of each sample and transferred in a microwave digester (speedwave™ MWS-3+; Berghof Products Instruments GmbH, Eningen, Germany) at 100-170ºC. After cooling to room temperature, the samples were transferred to clean volumetric flasks and the volumes of each sample were adjusted to 25 mL using deionized water. The

quality of the analytical process was controlled using standard reference materials: SRM1577c (bovine liver) NIST. Metal concentrations in tissues were expressed in micrograms per gram dry weight (µg/g).

2.3. Tissue preparation for biochemical analyses

Liver and muscle tissue were minced and homogenized in Ultra-Turrax homogenizer (Janke & Kunkel, IKA-Werk, Staufen, Germany) (Rossi et al., 1983), in ice-cold 25 mM sucrose containing 10 mM Tris–HCl, pH 7.5 at a ratio of 1:5 (Lionetto et al., 2003). The subcellular structures were broken down with an ultrasonic homogenizer for 30 s at 40 kHz on ice. One part of the resulting sonicates was taken for determination of the total GSH concentration. The remaining sonicates were centrifuged at 10,000 × g for 90 min at 4°C (Takada et al., 1982). The supernatants were stored at -80°C and used for measuring the activities of antioxidant enzymes.

2.4. Biochemical analyses

The activity of SOD was assayed by the epinephrine method (Misra and Fridovich, 1972), which is based on the capacity of SOD to inhibit autoxidation of adrenaline to adrenochrome. CAT activity was evaluated by the rate of hydrogen peroxide (H_2O_2) composition following the method of Claiborne (1984). The activity of GSH-Px was determined using the method of Tamura et al. (1982), which measures the oxidation of NADPH, using t-butyl hydroperoxide as substrate. The activity of GR was detected following NADPH oxidation during reduction of oxidized glutathione (GSSG) (Glatzle et al., 1974). GST activity was measured at 340 nm according to Habig et al. (1974). This assay uses 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate, which conjugates with the thiol group of glutathione (GSH), causing an increase in

absorbance. All enzyme activities were expressed as the specific activity (U/mg protein). The protein content was estimated by the method of Lowry et al. (1951), using bovine serum albumin as standard.

The concentration of total GSH was detected according to the Griffith method (Griffith, 1980), and expressed as nmol/g of tissue. The concentration of –SH groups was determined according to the method of Ellman (Ellman, 1959) and expressed as μmol/g tissue.

ChE activity was determined according to Ellman's method (Ellman et al., 1961) by measuring the increase in absorbance of the sample at 405 nm in the presence of an alternative substrate, acetylthiocholine iodide and 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB). The reaction results in the production of 5-thio-2-nitrobenzoate (TNB) that has a yellow color due to the shift of electrons to the sulfur atom. ChE activity was expressed as μmol/min/g tissue.

Lipid peroxide concentrations (TBARS) were measured according to Ohkawa et al. (1979). The red color that was produced by the reaction of TBA with lipid peroxidation products (malondialdehyde) was measured at 532 nm. The results were expressed as nmol TBARS/mg tissue.

All measurements were performed in triplicate, using a Shimadzu UV-160 spectrophotometer with a temperature-controlled cuvette holder.

2.5. Integrated Biomarker Response

Activities of all investigated antioxidant defense enzymes (SOD, CAT, GSH-Px, GR and GST) as well as concentration of nonenzymatic compound GSH are combined into one IBR

comprehensive index. IBR value was calculated in R 3.4.1. using the script provided by Devin et al. (2014).

2.6. Statistical analyses

Reported metal concentrations and values of oxidative stress biomarkers are presented as the mean±SE (standard error). The Kolmogorov-Smirnov test was applied to examine the normal distribution, and when necessary, the data were log-transformed. We performed two-way analysis of variance (ANOVA) followed by the Tukey post hoc test to assess the differences in the examined oxidative stress biomarkers between tissues and species and differences in metal concentrations between species, investigated tissues and swallowed snake prey. Correlations between oxidative stress biomarkers and heavy metal concentrations were examined by Pearson's correlation test. These analyses were carried out using STATISTICA 8.0 software with a significance level determined at $p < 0.05$. Correspondence analysis was applied to obtain position of a species and tissue in relation to biomarkers and concentrations of heavy metals using XLSTAT software Version 2015.5.01.22537 and Microsoft Excel 2010.

3. Results

The concentrations of 12 heavy metals (Al, As, Cd, Co, Cr, Cu, Fe, Mn, Mo, Ni, Pb and Zn) in water samples from Pančevački Rit are presented in Table 1. The concentrations of accumulated metals in the liver, muscle and prey of both grass and dice snakes, expressed as μg/g dry weight, are presented in Table S1. Since the concentrations of some metals were below the limits of detection, we presented and compared the results for 8 metals detected in both

investigated tissues and prey in Fig. 2. The livers of both snakes accumulated markedly higher concentrations of Cu and Fe when compared to muscle ($p < 0.05$) (Fig. 2c, 2d). The same trend was observed for Mn in tissues of *N. tessellata* ($p < 0.05$) (Fig. 2e). As, Cd, Co and Mo were detected in the liver, while the concentrations of these metals were below the limits of detection in the muscles of both snake species (Table S1). Only Zn concentrations were significantly higher in muscle than in the liver of *N. tessellata* ($p < 0.05$) (Fig. 2h). The liver of dice snake contained significantly lower concentrations of Cu ($p < 0.05$) and significantly higher concentrations of Mn ($p < 0.05$) when compared to the liver of *N. natrix* (Fig. 2c, Fig. 2e). Dice snake muscle accumulated significantly lower Mn concentrations in comparison to grass snake muscle ($p < 0.05$) (Fig. 2e).

The prey of both investigated species contained significantly higher concentrations of Al, Cr, and Mn in comparison to both snake ($p < 0.05$) (Fig. 2a, 2b, 2e). In the liver of both snake species, the concentration of Fe was significantly higher when compared to their prey ($p < 0.05$) (Fig. 2d). Food of the grass snake had significantly higher concentrations of Pb when compared to the both investigated snake tissues ($p < 0.05$) (Fig. 2f), while food of the dice snake had significantly higher Zn concentrations than the liver ($p < 0.05$), and a significantly lower concentration than the muscle ($p < 0.05$) (Fig. 2h). The prey of *N. tessellata* had a markedly lower Cu concentration as compared to the liver ($p < 0.05$), whereas in the food of *N. natrix* the concentration of the same metal was significantly higher when compared to snake muscle ($p < 0.05$) (Fig. 2c). Although the highest concentrations of Ni were recorded in the food of the investigated snakes, statistical significance was obtained only in relation to the liver of *N. natrix* ($p < 0.05$) (Fig. 2f). The pray of *N. natrix* accumulated significantly higher concentrations of Al, Cr and Fe than the pray of *N. tessellata*) ($p < 0.05$) (Fig. 2a, 2b, 3d).

The activities of antioxidant enzymes, phase II bio-transformation enzyme GST and ChE, the concentrations of GSH and –SH groups, as well as the concentrations of TBARS in the liver and muscle of *N. natrix* and *N. tessellata* are presented in Fig. 3. The presented results show that the activities of SOD, GR and GST were considerably higher in the liver of *N. natrix* as compared to the liver of *N. tessellata* ($p < 0.05$) (Fig. 3a, 3d, 3e). At the same time, the activities of these three enzymes and of GSH-Px (Fig. 3c) were significantly lower in the muscle of *N. natrix* in comparison to the muscle of *N. tessellata* ($p < 0.05$). Grass snake liver had significantly reduced CAT activity than the dice snake ($p < 0.05$) (Fig. 3b). *N. natrix* had a significantly higher concentration of –SH groups and significantly lower TBARS concentrations than *N. tessellata* in both examined tissues ($p < 0.05$) (Fig. 3g, 3h). The concentration of GSH (Fig. 3f) and ChE activity (Fig. 3i) did not significantly differ between the investigated snakes ($p > 0.05$).

Pearson's correlation coefficients between metal concentrations and investigated oxidative stress biomarkers are presented in Table 2. The concentration of Al significantly correlated with GSH concentration ($r = 0.89$, $p < 0.05$), while the concentrations of Cr ($r = –0.92$, $p < 0.05$), Pb ($r = –0.90$, $p < 0.05$) and Ni ($r = –0.98$, $p < 0.05$) negatively correlated with GST activity in the liver of *N. natrix*. In dice snake liver, the only significant correlation was found between the concentration of Ni and GR activity ($r = 0.98$, $p < 0.05$). The most significant correlations were observed in the muscle of *N. natrix* as follows: Cr negatively correlated with GSH concentration ($r = –0.98$, $p < 0.05$); Fe positively correlated with the GR activity and GSH concentration ($r = 0.94$, $p < 0.05$; $r = 0.90$, $p < 0.05$); Ni positively correlated with GST activity ($r = 0.95$, $p < 0.05$); Pb positively correlated with GR activity ($r = 0.96$, $p < 0.05$), and Zn positively correlated with SOD activity ($r = 0.94$, $p < 0.05$). In the muscle of *N. tessellata* Al displayed a strong negative correlation with GST activity ($r = –0.96$, $p < 0.05$), and a strong

positive correlation with GSH concentration (r = 0.90, p < 0.05). At the same time, a positive correlation was found between Cu concentration and CAT activity (r = 0.91, p < 0.05), while Ni had a negative effect on GSH-Px activity (r = –0.90, p < 0.05).

The calculated scores for IBR among liver and muscle of both investigated snake species was presented in the same star plot (Fig. 4). The greatest response of antioxidant defense system parameters (5.517) was observed in the liver of *N. natrix*, followed by liver of *N. tessellata* with IBR values of 2.905. The lower response was in muscle with IBR of 0 and 0.188 for *N. natrix* and *N. tessellata* respectively.

Correspondence analysis showed that the first two dimensions explain 99, 51% of total inertia (Fig. 5). Tissues of both species were clearly separated. The biomarkers which contributed to differentiation of liver were GST, GR, SOD, GSH-Px in relation to heavy metals: Cu, Cd, As, Fe. The biomarkers which contributed to differentiation of muscles were GSH together in relation to heavy metals: Pb, Cr, Zn, Al, Ni.

4. Discussion

Our understanding of the toxicokinetics of metals in reptiles is limited as most research involving heavy metal concentrations in wildlife has focused on other vertebrate groups (fish, birds and mammals). This data gap for reptile ecotoxicology represents a challenge for ecological risks assessment of pollutants to this group of animals (Hopkins, 2000; Drewett et al., 2013). Laboratory and field studies have suggested that metals have the potential to pass in the food chain to higher trophic levels, which inevitably leads to an increase in their concentration in internal organs and tissues of reptiles (Campbell and Campbell, 2001; Márquez-Ferrando et al.,

2009). It was confirmed that heavy metals cause histological, morphological and endocrine malformations in this group of vertebrates (Crain and Guillette, 1998; Gribbins, 2011). Hopkins et al. (1999) demonstrated that long-term exposure to some trace elements (As, Cd and Se) provokes an increase in the standard metabolic rate of banded water snake (*Nerodia fasciata*), which means that less energy is available for growth and reproduction. Increased Pb concentrations near high-traffic routes determine the longevity and health of snakes (Kaur, 1988).

Concentrations of some very toxic metals such as As, Cd, Co and Mo were below the detection limits in the muscle of both snakes, whereas Cu and Mn concentrations were significantly lower in the muscle than in the liver of *N. tessellata*. The livers of both species also had significantly higher concentrations of Fe than the muscles. Such results are consistent with the specific function of the liver and the presence of metallothioneins, cysteine-rich, low molecular weight proteins that have a role in the detoxification of heavy metals and in the maintenance of essential metal ion homeostasis (Ruttkay-Nedecky et al et al., 2013). The metal concentrations in muscle, as the main tissue in whole snake bodies, provide useful information about potential risks for consumers in the food chain. We found that only the concentration of Zn was significantly higher in muscle than in the liver of dice snake.

The mean Cd concentrations in the livers of grass and dice snake were lower than those found for banded water snakes from coal ash-polluted site (0.4-0.6 ppm), but higher than in snakes from a reference site (0.12 ppm) (Hopkins et al., 1999). It was shown that Cd rapidly accumulated in the gut of European lizards by binding to the epithelial surface, but its movement across the basal membrane into the bloodstream is a much slower process (Mann et al., 2006). According to our results, among the investigated snake species there were significant differences

in the concentrations of bioaccumulated Cu and Mn, whereas the prey of *N. natrix* contained significantly higher concentrations of Al, Cr and Fe than the prey of *N. tessellata*. Although As was not detected in prey nor in the water, both species accumulated similar concentrations of this metal in the livers, which pointed to some other exposure route or inability of elimination. According to Mogren et al. (2013), irrigation, lead-arsenate insecticides and atmospheric deposition are some of the main sources of As in the environment. Toxic As^{3+} that can be found in many pesticides, herbicides and insecticides binds to –SH groups of enzymes and inhibits their activity (Squibb and Fowler, 1983).

Our results demonstrate that snakes are not significantly affected by the biomagnification process of some metals (Al, Cr, Mn and Pb), as the accumulated concentrations were significantly lower when compared to organisms that snakes feed on. It was shown that sea snakes, *Emydocephalus annulatus,* bind heavy metals to melanin in their skin, thus preventing metal entry into internal organs (Goiran et al., 2017). Other studies have also confirmed that shed skins might be a route of elimination of some metals, such as Pb, Hg and Cr (Burger, 1992). Brown house snakes (*Lamprophis fuliginosus*) exposed to excessive concentrations of dietary Se transferred this metal into eggs at concentrations that exceeded the proposed reproductive toxicity thresholds for birds and fish (Hopkins et al., 2004). Although some snakes have been used to determine the amount of heavy metals in the environment (Campbell et al., 2005; Campbell and Campbell, 2002), it was assumed that they were not representative for comparing the metal levels in different localities. This was concluded based on the results of studies that showed that differences in bioaccumulated concentrations of some very toxic metals in snakes collected from polluted sites were less apparent in comparison to control sites (Burger, 1992). Our findings are in agreement with the results of Albrecht et al. (2007) who investigated metal

concentrations in ribbon snake tissue and anuran larvae and demonstrated that they were not biomagnified through upper trophic levels.

Previous studies were mostly focused on the species-dependent bioaccumulation patterns of heavy metals and their tissue distributions in snakes (Burger, 1992; Wylie et al., 2009). Therefore, it is necessary to examine not only the accumulated concentrations of metals and other pollutants, but also their effects and mechanisms of toxicity. Oxidative stress biomarkers have been recognized as early warning signals of toxicant exposure and are integrated in environmental monitoring programs (Pavlović et al., 2010). As a consequence of the exposure to toxins, 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 (Carvalho et al., 2012). Although the concentrations of most investigated metals did not differ significantly between the examined species (with the exception of Cu and Mn), we observed different responses of the antioxidant defense enzymes.

SOD protects the cell by transforming the superoxide anion radical to H_2O_2, which is removed from the system. According to Stoliar and Luschchak (2012), decreased SOD activity in tissues of some fish species occurs at low pollution exposure levels over long time periods and points to a limitation of the antioxidative defenses. Our results demonstrated that *N. tessellata* has a markedly lower liver SOD activity and increased CAT activity in the same tissue. CAT directly catalyzes the decomposition of H_2O_2 to water and molecular oxygen, while GSH-Px uses H_2O_2 to oxidize GSH. Elevated CAT activity can occur unrelated to SOD (Falfushynska and Stoliar, 2009), and often indicates the presence of certain prooxidative compounds that can lead to oxidative stress in organisms from chronically polluted industrial sites (Karadag et al., 2014).

Although, CAT and GSH-Px have a similar physiological function, the level of H_2O_2 is mainly determined by GSH-Px, except during increased production of this ROS when CAT is more prominent in decomposing H_2O_2 (Reddy et al., 2015). Interspecies differences observed in GSH-Px suggest that peroxide metabolism may affect glutathione homoeostasis differently in these species. The significantly lower GR activity in the liver of *N. tessellata* suggests that under conditions of excessive GSH oxidation, this species may experience greater accumulation of oxidized glutathione disulfide (GSSG) than *N. natrix*. The studied snake species had similar GSH concentrations, but changes in GR activity can promote specific differences in the GSH/GSSG ratio (Zhang et al., 2017). GST activity can be significantly increased or significantly reduced depending on the type of metal or exposure conditions (Mani et al., 2014). It was shown that after an initial increase in GST activity due to ROS overproduction, its enzymatic activity progressively decreases (Fonseca et al., 2011). According to correspondence analysis GST, SOD and GR contributed to differentiation of *N. natrix* liver and this is consistent with the significantly higher activity of these enzymes in the liver of grass snake compared to the dice snake liver. Although, heavy metals can cause ChE inhibition (Bocquené et al., 1995), there was no significant difference in activity of this enzyme between species, and no significant correlations with metals. According to Espín et al. (2014), the increase in total GSH content and the induction of some antioxidant enzymes can be interpreted as a protective response against enhanced lipid peroxidation. Increased heavy metal concentrations were associated with increased concentrations of GSH and –SH groups (Prokić et al., 2016). *N. natrix* had a significantly lower TBARS level and higher concentration of free –SH groups in both investigated tissues and thus appeared to possess more effective protection against oxidation in

comparison to *N. tessellata*. These results are in agreement with greatest IBR value observed in the liver of *N. natrix*.

Metals with the highest number of significant correlations with oxidative stress parameters were Al and Ni. We previously observed that heavy metals can modulate the antioxidative defense parameters in the liver of dice snake during pre-hibernation and post-hibernation periods (Gavrić et al., 2017). A greater number of correlations were found in tissues of *N. natrix* compared to *N. tessellata*. The results showed that the concentration of Al positively correlated with the concentration of GSH in the liver of *N. natrix* as well as in the muscle of *N. tessellata*. Al induced oxidative stress in lymphocytes of common carp, which consequently caused higher levels of lipid peroxidation and oxidized proteins (García-Medina et al., 2010). Zn concentration positively correlated with SOD activity, which is expected considering that Zn ions are responsible for stabilizing the globular structure of the CuZn SOD isoform (Tarhan et al., 2007). Results of Pearson's correlations obtained between some metal concentrations and investigated parameters (GST and GR activities as well as GSH concentrations) indicate that these biomarkers could be efficiently used in monitoring studies.

5. Conclusions

This multi-biomarker approach contributes to a better understanding of the diverse effects of heavy metal exposure on dice and grass snakes. Since dice and grass snakes generally accumulate lower concentrations of metal than their prey, with the exception of Fe, it can be concluded that snakes are not at risk of contaminant biomagnification. Among the many factors that can affect the activities of antioxidative enzymes, for dice and grass snakes species-specific responses could be crucial in determining the patterns of antioxidative defense enzymes rather

than differences in their feeding habits. The increased TBARS levels together with decreased concentrations of free –SH groups that were observed in the liver and muscle of dice snake suggested that this species may be less tolerant to pollution exposure.

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References

Albrecht, J., Abalos, M., Rice, T.M., 2007. Heavy metal levels in ribbon snakes (*Thamnophis sauritus*) and anuran larvae from the Mobile-Tensaw River Delta, Alabama, USA. Arch. Environ. Contam. Toxicol. 53, 647–654.

Bocquené, G., Bellanger, C., Cadiou, Y., Galgani, F., 1995. Joint action of combinations of pollutants on the acetylcholinesterase activity of several marine species. Ecotoxicology 4, 266–279.

Burger, J., 1992. Trace element levels in pine snake hatchlings: Tissue and temporal differences. Arch. Environ. Contam. Toxicol. 22, 209–213.

Burger, J., Murray, S., Gaines, K.F., Novak, J.M., Punshon, T., Dixon, C., Gochfeld, M., 2006. Element levels in snakes in South Carolina: difference between a control site and exposed site on the Savannah River site. Environ. Monit. Assess. 112, 35–52.

Burger, J., Gochfeld, M., Jeitner, C., Zappalorti, R., Pittfield, T., DeVito, E., 2017. Arsenic, cadmium, chromium, lead, mercury and selenium concentrations in pine snakes

(*Pituophis melanoleucus*) from the New Jersey Pine Barrens. Arch. Environ. Contam. Toxicol. 72, 586–595.

Campbell, K.R., Campbell, T.S., 2001. The accumulation and effects of environmental contaminants on snakes: a review. Environ. Monit. Assess. 70, 253–301.

Campbell, K.R., Campbell, T.S., 2002. A logical starting point for developing priorities for lizard and snake ecotoxicology: a review of available data. Environ. Toxicol. Chem. 21, 894–898.

Campbell, K.R., Campbell, T.S., Burger, J., 2005. Heavy metal concentrations in northern water snakes (*Nerodia sipedon*) from East Fork Poplar Creek and the Little River, East Tennessee, USA. Arch. Environ. Contam. Toxicol. 49, 239–248.

Carvalho, C.S., Bernusso, V.A., Araújo, H.S.S., Espíndola, E.L.G., Fernandes, M.N., 2012. Biomarker responses as indication of contaminant effects in *Oreochromis niloticus*. Chemosphere 89, 60–69.

Claiborne, A., 1984. Catalase activity. In: Greenwald, R.A. (Ed.), Handbook of Methods for Oxygen Radical Research. CRC Press Inc., Boca Raton, pp. 283–284.

Crain, D.A., Guillette, L.J.Jr., 1998. Reptiles as models of contaminant-induced endocrine disruption. Anim. Reprod. Sci. 53, 77–86.

Devin, S., Burgeot T., Giambérini, L., Minguez, L., Pain-Devin, S., 2014. The integrated biomarker response revisited: optimization to avoid misuse. Environ. Sci. Pollut. Res. 21, 2448–2454.

Drewett, D.V., Willson, J.D., Cristol, D.A., Chin, S.Y., Hopkins,W.A., 2013. Inter- and intraspecific variation in mercury bioaccumulation by snakes inhabiting a contaminated river floodplain. Environ. Toxicol. Chem. 32, 1178–1186.

Ellman, G.L., 1959. Tissue sulfhydryl groups. Arch. Biochem. Biophys. 82, 70–77.

Ellman, G.L., Courtney, K.D., Andres, V.J.R., Featherstone, R.M., 1961. Determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95.

Espín, S., Martínez-López, E., Jiménez, P., María-Mojica, P., García-Fernández, A.J., 2014. Effects of heavy metals on biomarkers for oxidative stress in Griffon vulture (*Gyps fulvus*). Environ. Res. 129, 59–68.

Falfushynska, H.I., Stoliar, O.B., 2009. Function of metallothioneins in Carp *Cyprinus carpio* from two field sites in Western Ukraine. Ecotox. Environ. Saf. 72, 1425–1432.

Feng, M., He, Q., Meng, L., Zhang, X., Sun, P., Wang Z., 2015. Evaluation of single and joint toxicity of perfluorooctane sulfonate, perfluorooctanoic acid, and copper to Carassius auratus using oxidative stress biomarkers. Aquat Toxicol. 161, 108–116.

Feng, M.B., Qu, R.J., Li, Y., Wei, Z.B., Wang, Z.Y., 2014. Biochemical biomarkers in liver and gill tissues of freshwater fish Carassius auratus following in vivo exposure to hexabromobenzene. Environ. Toxicol. 29, 1460–1470.

Feng, M.B., Qu, R.J., Wang, C., Wang, L.S., Wang, Z.Y., 2013. Comparative antioxidant status in freshwater fish *Carassius auratus* exposed to six current-use brominated flame retardants: a combined experimental and theoretical study. Aquat. Toxicol. 140–141, 314–323.

Filippi, E., Capula, M., Luselli, L., Agrimi, U., 1996. The prey spectrum of *Natrix natrix* (Linnaeus, 1758) and *Natrix tessellata* (Laurenti, 1768) in sympatric populations (Squamata: Serpentes: Colubridae). Herpetozoa 8, 155–164.

Fonseca, V.F., França, S., Serafim, A., Company, R., Lopes, B., Bebianno, M.J., Cabral, H.N., 2011. Multi-biomarker responses to estuarine habitat contamination in three fish species: *Dicentrarchus labrax*, *Solea senegalensis* and *Pomatoschistus microps*. Aquat. Toxicol. 102, 216–227.

García-Medina, S., Razo-Estrada, A.C., Gomez-Olivan, L.M., Amaya-Chavez, A., Madrigal - Bujaidar, E., Galar-Martinez, M., 2010. Aluminum-induced oxidative stress in lymphocytes of common carp (*Cyprinus carpio*). Fish Physiol. Biochem. 36, 875–882.

Gavrić, J.P, Anđelković, M.Z., Tomović, Lj.M., Prokić, M.D., Despotović, S.G., Gavrilović, B.R., Radovanović, T.B., Borkovic-Mitić, S.S., Pavlović, S.Z., Saicić, Z.S., 2017. Oxidative stress biomarkers, cholinesterase activity and biotransformation enzymes in the liver of dice snake (*Natrix tessellata* Laurenti) during pre-hibernation and post-hibernation: A possible correlation with heavy metals in the environment. Ecotoxicol. Environ. Saf. 138, 154–162.

Glatzle, D., Vuilleumier, J.P., Weber, F., Decker, K., 1974. Glutathione reductase test with whole blood, a convenient procedure for the assessment of the riboflavin status in humans. Experientia 30, 665–667.

Goiran, C., Bustamante, P., Shine, R., 2017. Industrial melanism in the seasnake *Emydocephalus annulatus*. Curr. Biol. 27, 2510–2513.

Gribbins, K.M, 2011. Reptilian spermatogenesis: A histological and ultrastructural perspective. Spermatogenesis 1, 250–269.

Griffith, O.W., 1980. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal. Biochem. 106, 207–212.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.

Hopkins, W.A., 2000. Reptile toxicology: challenges and opportunities on the last frontier of vertebrate ecotoxicology. Environ. Toxicol. Chem. 19, 2391–2393.

Hopkins, W.A., Rowe, C.L., Congdon, J.D., 1999. Elevated trace element concentrations and standard metabolic rate in banded water snakes, *Nerodia fasciata*, exposed to coal combustion wastes. Environ. Toxicol. Chem. 18, 1258–1263.

Hopkins, W.A., Roe, J.H., Snodgrass, J.W., Staub, B.P., Jackson, B.P., Congdon, J.D., 2002. Effects of chronic dietary exposure to trace elements on banded water snakes (*Nerodia fasciata*). Environ. Toxicol. Chem. 21, 906–913.

Hopkins, W.A., Staub, B.P., Baionno, J.A., Jackson, B.P., Roe, J.H., Ford, N.B., 2004. Trophic and maternal transfer of selenium in brown house snakes (*Lamprophis fuliginosus*) Ecotoxicol. Environ. Saf. 58, 285–293.

Karadag, H., Fırat, Ö., Fırat, Ö., 2014. Use of oxidative stress biomarkers in *Cyprinus carpio* L. for the evaluation of water pollution in Ataturk Dam Lake (Adiyaman, Turkey). Bull. Environ. Contam. Toxicol. 92, 289–293.

Kaur, S., 1988. Lead in the scales and cobras and wall snakes from rural and urban areas of Punjab, India. Sci. Total. Environ. 77, 289–290.

Koch, R.E., Hill, G.E., 2017. An assessment of techniques to manipulate oxidative stress in animals. Funct. Ecol. 31, 9–21.

Lemaire, J., Bustamante, P., Olivier, A., Lourdais, O., Michaud, B., Boissinot, A., Galán, P., Brischoux, F., 2018. Determinants of mercury contamination in viperine snakes, *Natrix maura*, in Western Europe. Sci. Total. Environ. 635, 20–25.

Lionetto, M.G., Caricato, R., Giordano, M.E., Pascariello, M.F., Marinosci, L., Schettino, T., 2003. Integrated use of biomarkers (acetylcholinesterase and antioxidant enzymeactivities) in *Mytilus galloprovincialis* and *Mullus barbatus* in an Italian coastal marine area. Mar. Poll. Bull. 46, 324–330.

Lionetto, M.G., Caricato, R., Calisi, A., Giordano, M.E., Schettino, T., 2013. Acetylcholinesterase as a biomarker in environmental and occupational medicine: new insights and future perspectives. Biomed. Res. Int., Article ID 321213

doi: 10.1155/2013/321213

Livingstone, D.R., 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar. Pollut. Bull. 42, 656–666.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Łuszczek-Trojnar, E., Drag-Kozak, E., Szczerbik, P., Socha, M., Popek, W., 2014. Effect of long-term dietary lead exposure on some maturation and reproductive parameters of a female Prussian carp (*Carassius gibelio* B.). Environ. Sci. Pollut. Res. 21, 2465–2478.

Mani, R., Meena, B., Valivittan, K., Suresh, A., 2014. Glutathione-S-transferase and catalase activity in different tissues of marine catfish *Arius arius* on exposure to cadmium. Int. J. Pharm. Pharm. Sci. 6, 326–332.

Mann, R.M., Serra, E.A., Soares, A.M.V.M., 2006. Assimilation of cadmium in a European lacertid lizard: is trophic transfer important? Environ. Toxicol. Chem. 25, 3199–3203

Márquez-Ferrando, R., Santos, X., Pleguezuelos, J.M., Ontiveros, D., 2009. Bioaccumulation of heavy metals in the lizard *Psammodromus algirus* after a tailing-dam collapse in Aznalcóllar (Southwest Spain). Arch. Environ. Contam.Toxicol. 56, 276–285.

Misra, H.P., Fridovich, I., 1972. The role of superoxide anion in the autoxidation of epinephrine and simple assay or superoxide dismutase. J. Biol. Chem. 247, 3170–3175.

Mogren, C.L., Walton, W.E., Parker, D.R., Trumble, J.T., 2013. Trophic transfer of arsenic from an aquatic insect to terrestrial insect predators. PLoS ONE 8:e67817. doi: 10.1371/journal.pone.0067817

Ohkawa, H., Okishi, N., Yogi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95, 351–358.

Pavlović, S.Z., Borković Mitić, S.S., Radovanović,T.B., Perendija, B.R., Despotović, S.G., Gavrić, J.P., Saičić, Z.S., 2010. Seasonal variations of the activity of antioxidant defense enzymes in the Red Mullet (*Mullus barbatus* l.) from the Adriatic Sea. Mar Drugs. 8, 413–428.

Prokić, M.D., Borković-Mitić, S.S., Krizmanić, I.I., Mutić, J.J., Vukojević, V., Nasia, M., Gavrić, J.P., Despotović, S.G., Gavrilović, B.R., Radovanović, T.B., Pavlović, S.Z., Saičić, S.S., 2016. Antioxidative responses of the tissues of two wild populations of *Pelophylax* kl. *esculentus* frogs to heavy metal pollution. Ecotoxicol. Environ. Saf. 128, 21–29.

Prokić, M.D., Borković-Mitić, S.S., Krizmanić, I.I., Mutić, J.J., Gavrić, J.P., Despotović, S.G., Gavrilović, B.R., Radovanović, T.B., Pavlović, S.Z., Saičić, S.Z., 2017. Oxidative stress parameters in two *Pelophylax esculentus* complex frogs during pre- and post-hibernation: Arousal vs heavy metals. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 202, 19–25.

Reddy, U.A., Prabhakar, P.V., Rao, G.S., Rao, P.R., Sandeep, K., Rahman, M.F., Kumari, S.I., Grover, P., Khan, H.A., Mahboob, M., 2015. Biomarkers of oxidative stress in rat for assessing toxicological effects of heavy metal pollution in river water. Environ. Sci. Pollut. Res. 22, 13453–13463.

Rossi, M.A., Cecchini, G., Dianzani, M.M., 1983. Glutathione peroxidase, glutathione reductase and glutathione transferase in two different hepatomas and in normal liver. IRCS Med. Sci. Biochem. 11, 805.

Ruttkay-Nedecky, B., Nejdl, L., Gumulec, J., Zitka, O., Masarik, M., Eckschlager, T., Stiborova, M., Adam, V., Kizek, R., 2013. The role of metallothionein in oxidative stress Int. J. Mol. Sci. 14, 6044–6066.

Squibb, K.S., Fowler, B.A., 1983. The toxicity of arsenic and its compounds. In: Fowler, B.A. (Ed.), Biological and Environmental Effects of Arsenic, Elsevier, Amsterdam, pp. 233–269.

Stoliar, O.B., Lushchak, V.I., 2012. Environmental pollution and oxidative stress in fish. In: Lushchak, V.I. (Ed), Oxidative Stress - Environmental Induction and Dietary Antioxidants, InTech, pp. 132–166.

Šukalo, G., Đorđević, S., Gvozdenović, S., Simović, A., Anđelković, M., Blagojević, V., Tomović, LJ., 2014. Intra- and inter-population variability of food preferences of two *Natrix* species on the Balkan Peninsula. Herpetol. Conserv. Biol. 9, 123–136.

Takada, Y., Noguchit, T., Kayiyama, M., 1982. Superoxide dismutase in various tissues from rabbits bearing the Vx-2 carcinoma in the maxillary sinus. Cancer Res. 42, 4233–4235.

Tamura, M., Oshino, N., Chance, B., 1982. Some characteristics of hydrogen-andalkylhydroperoxides metabolizing systems in cardiac tissue. J. Biochem. 92, 1019–1031.

Tarhan, C., Pekmez, M., Karaer, S., Arda, N., Sarikaya., A.T., 2007. The effect of superoxide dismutase deficiency on zinc toxicity in *Schizosaccharomyces pombe*. J. Basic Microb. 47, 506–512.

Tokalioglu, S., Kartal, S., 2006. Multivariate analysis of the data and speciation of heavy metals in street dust samples from the organized industrial district in Kayseri (Turkey). Atmos. Environ. 40, 2797–2805.

Valko, M., Morris, H., Cronin, M.T.D., 2005. Metals, toxicity an oxidative stress. Curr. Med. Chem. 12, 1161–1208.

Weir, S.M., Talent, L.G., Anderson, T.A, Salice, C.J., 2014. Unraveling the relative importance of oral and dermal contaminant exposure in reptiles: insights from studies using the Western fence lizard (*Sceloporus occidentalis*). PLoS One 9:e99666.

Wylie, G.D., Hothem, R.L., Bergen, D.R., Martin, L.L., Taylor, R.J., Brussee, B.E., 2009. Metals and trace elements in Giant Garter Snakes (*Thamnophis gigas*) from the Sacramento Valley, California, USA. Arch. Environ. Contam. Toxicol. 56, 577–587.

Zhang, W., Niu, C., Liu, Y., Chen, B., 2017. Glutathione redox balance in hibernating Chinese soft-shelled turtle Pelodiscus sinensis hatchlings. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 207, 9–14.

Table 1

Water temperature, pH and concentrations of metals dissolved in water (μg/L) at the Pančevački Rit locality. Data are given as the mean values from five measurements±SE; nd indicates values below the detection limit.

Parameter		
Water temperature (°C)	14 ± 0.15	
pH	8.4 ± 0.02	
Metal		Detection limit
Al	47.37 ± 9.96	3.091
As	nd	6.666
Cd	0.39 ± 0.03	0.238
Co	nd	0.829
Cr	nd	0.490
Cu	nd	10.458
Fe	619.23 ± 15.14	13.234
Mn	1730.50 ± 2.65	7.305
Mo	nd	6.573
Ni	nd	11.352
Pb	nd	2.996
Zn	nd	0.362

Table 2

Statistically significant correlations between metal concentrations and investigated parameters in the liver and muscle of grass snake (*Natrix natrix*) and dice snake (*Natrix tessellata*).

Liver					
N. natrix			*N. tessellata*		
Investigated parameters	Metal	*r*	Investigated parameters	Metal	*r*
GSH	Al	0.89	GR	Ni	0.98
GST	Cr	-0.92			
GST	Pb	-0.90			
GST	Ni	-0.98			
Muscle					
N. natrix			*N. tessellata*		
Investigated parameters	Metal	*r*	Investigated parameters	Metal	*r*
GSH	Cr	-0.98	GST	Al	-0.96
GR	Fe	0.94	GSH	Al	0.90
GSH	Fe	0.90	CAT	Cu	0.91
GST	Ni	0.95	GSH-Px	Ni	-0.90
GR	Pb	0.96			
SOD	Zn	0.94			

r - Pearson's correlation coefficient, $p < 0.05$.

FIGURE CAPTIONS

Fig. 1. The geographical position of the investigated locality, Pančevački Rit, Serbia.

Fig. 2. Ratio of heavy metal concentrations in the liver and muscle of *Natrix natrix* and *Natrix tessellata* and their prey. Significant differences at the level of $p < 0.05$ are marked with the letters a and b, which include differences with respect to liver (a) and muscle (b) for each snake species. Interspecies differences for liver, muscle and pray are marked with *.

Fig. 3. The activities of (A) superoxide dismutase (SOD), (B) catalase (CAT), (C) glutathione peroxidase (GSH-Px), (D) glutathione reductase (GR), (E) phase II biotransformation enzyme glutathione-S-transferase (GST), (F), concentrations of total glutathione (GSH), (G) sulfhydryl (–SH) groups, (H) lipid peroxide (TBARS) and (I) cholinesterase activity (ChE) in the the liver and muscle of grass (*Natrix natrix*) and dice snake (*Natrix tessellata*). The data are expressed as the mean ± SE. * $p < 0.05$ represents a minimal significant level.

Fig. 4. Integrated Biomarker Response (IBR) star plots for the liver and muscle of *Natrix natrix* and *Natrix tessellata* using all investigated oxidative stress parameters.

Fig. 5. Correspondence analysis ordination plot and position of the four analyzed groups: two species and two tissue relative to the analyzed parameters (NNL - *Natrix natrix* liver, NTL- *Natrix tessellata* liver, NNM- *Natrix natrix* muscle and NTM- *Natrix tessellata* muscle).

Graphical abstract

Highlights

- Examined snakes accumulated generally lower concentrations of metals than their prey.
- Oxidative stress biomarkers showed clear differences between the investigated snakes.
- Species-specific responses determined the patterns of antioxidative defense enzymes.
- Dice snake may be less tolerant to pollution exposure.

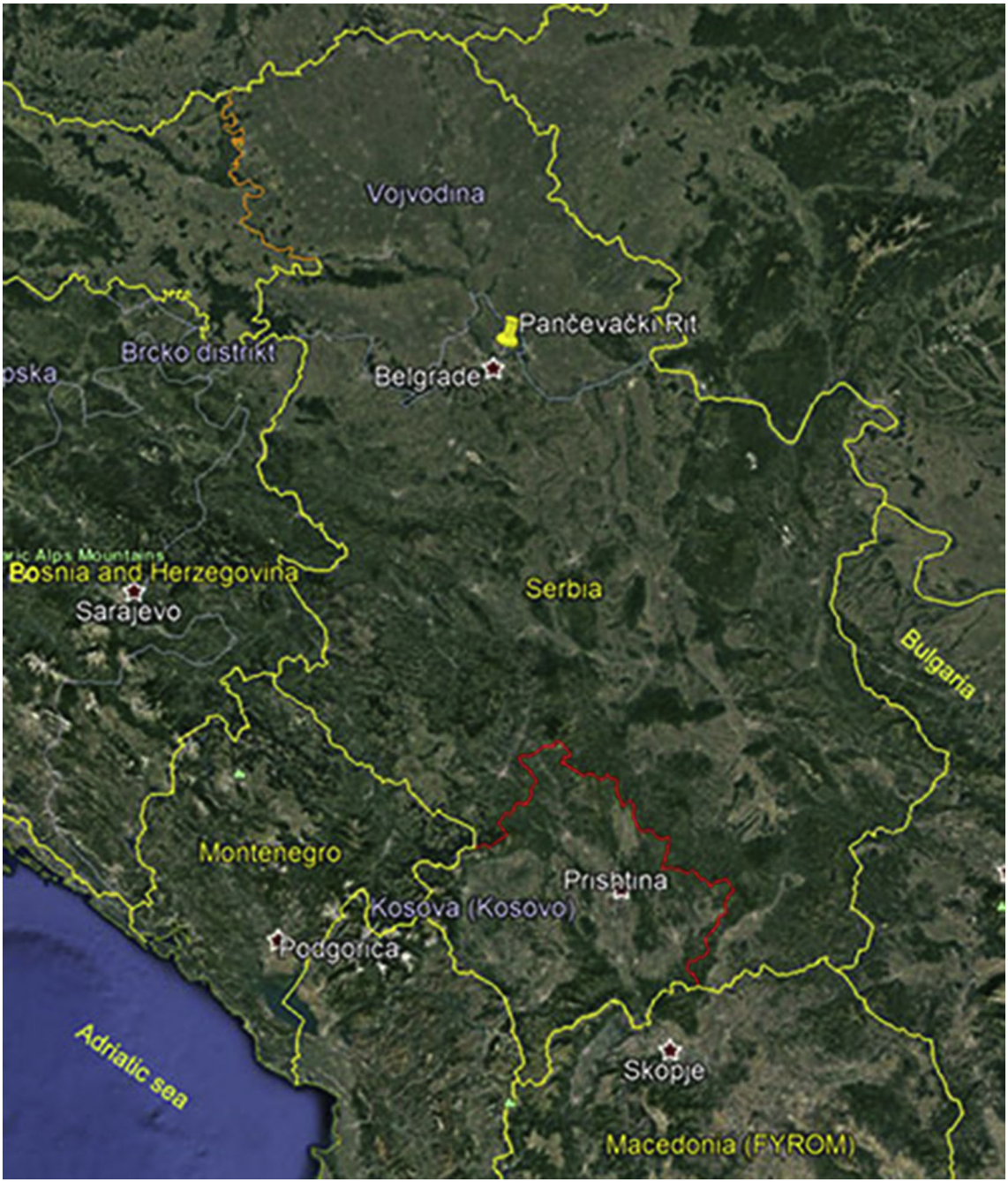
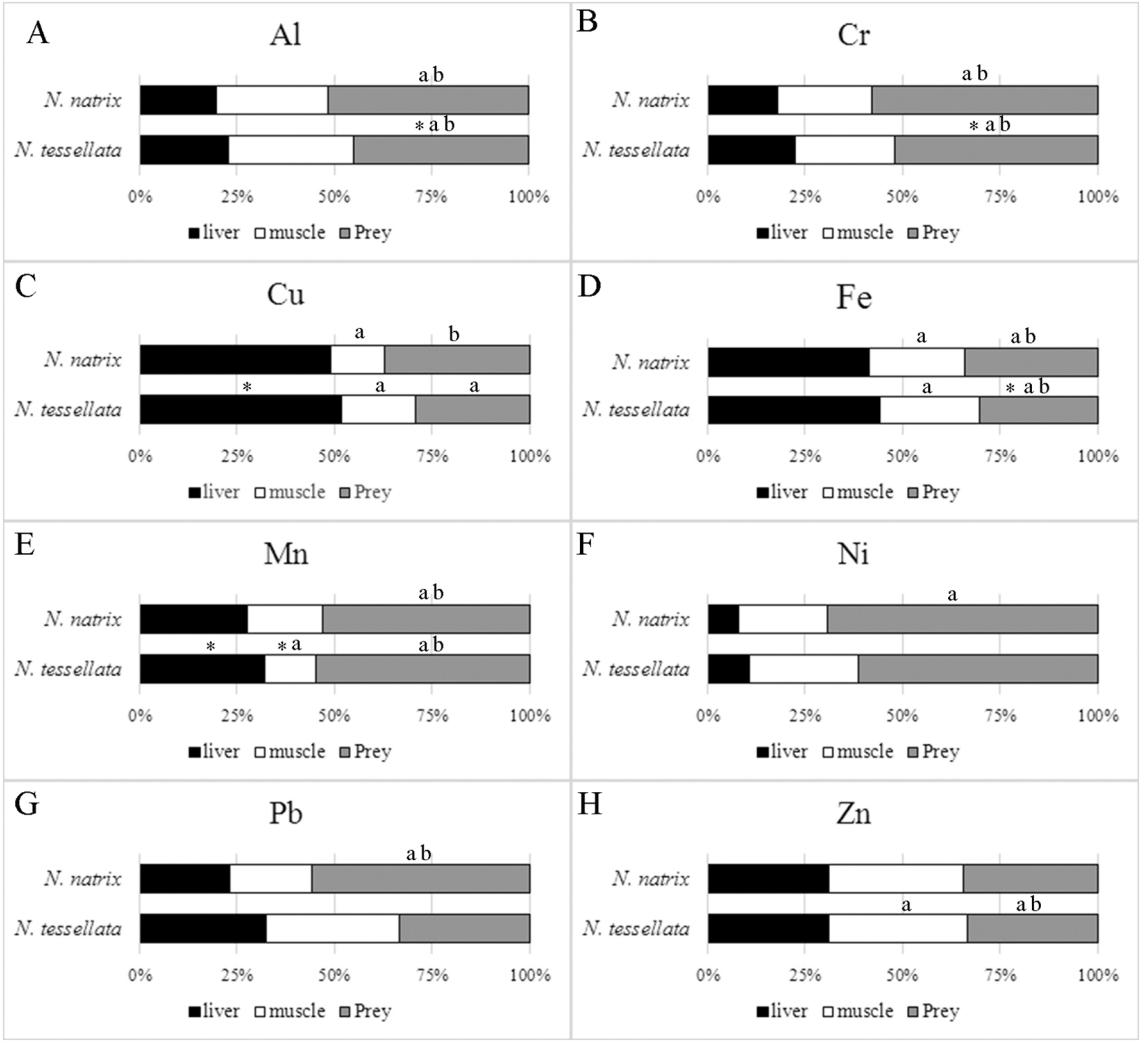

Vojvodina
Pančevački Rit
Brcko distrikt
Belgrade
Bosnia and Herzegovina
Sarajevo
Serbia
Bulgaria
Montenegro
Prishtina
Kosova (Kosovo)
Podgorica
Adriatic sea
Skopje
Macedonia (FYROM)

Figure 1


A
Al
a b
N. natrix
* a b
N. tessellata
0%
25%
50%
75%
100%
liver
muscle
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B
Cr
a b
N. natrix
* a b
N. tessellata
0%
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75%
100%
liver
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Prey
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b
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N. tessellata
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Figure 2

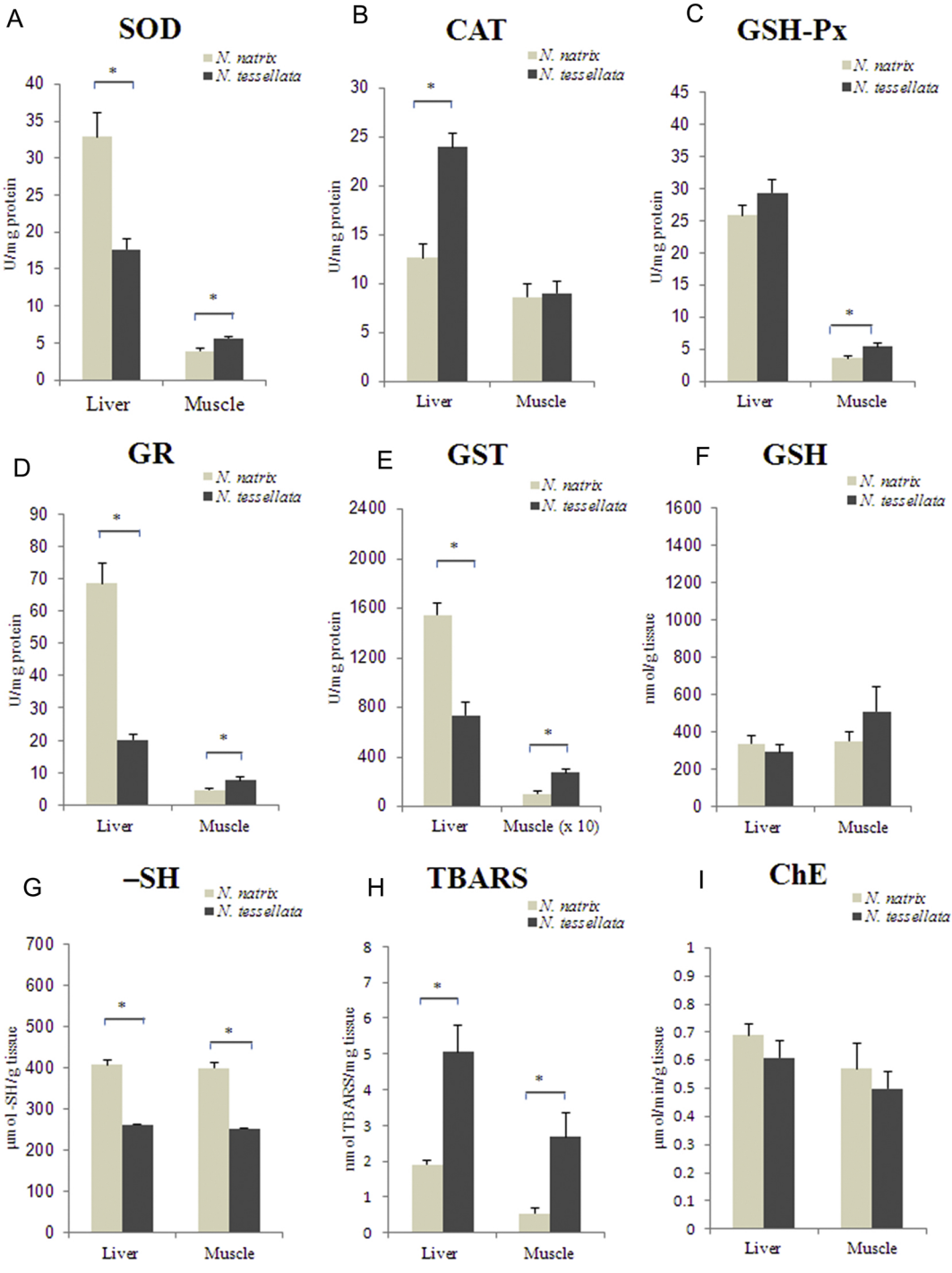

A
SOD
N. natrix
N. tessellata
U/mg protein
Liver
Muscle
B
CAT
C
GSH-Px
D
GR
E
GST
Muscle (x 10)
F
GSH
nmol/g tissue
G
–SH
μmol -SH/g tissue
H
TBARS
nmol TBARS/mg tissue
I
ChE
μmol/min/g tissue

Figure 3

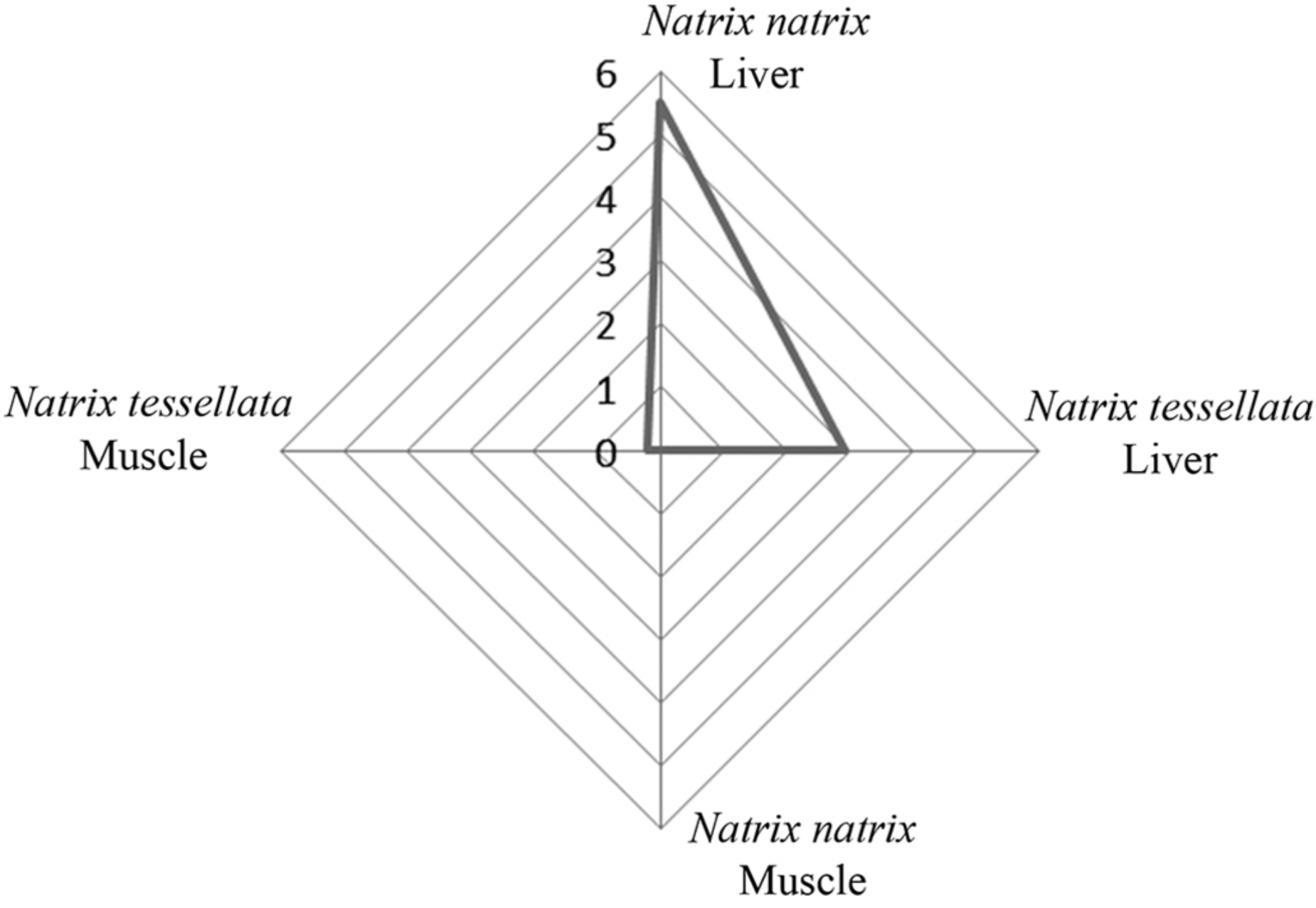
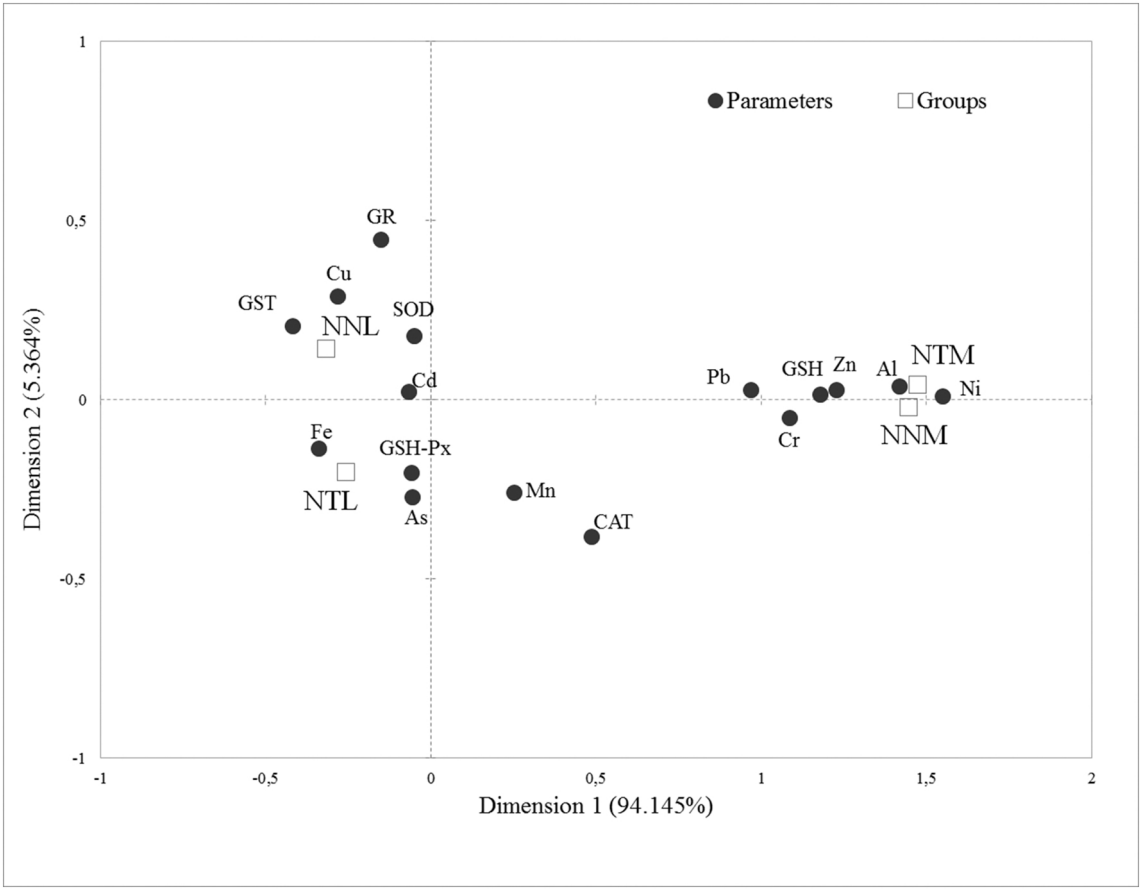

Natrix natrix
Liver
6
5
4
3
2
1
0
Natrix tessellata
Muscle
Natrix tessellata
Liver
Natrix natrix
Muscle

Figure 4


Parameters
Groups
GR
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GST
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GSH-Px
NTL
As
Mn
CAT
Pb
GSH
Zn
Al
NTM
Ni
Cr
NNM
1
0,5
0
-0,5
-1
-1
-0,5
0
0,5
1
1,5
2
Dimension 2 (5.364%)
Dimension 1 (94.145%)

Figure 5