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Concentrations of Selected Elements in Liver Tissue of Grey Wolves (*Canis lupus*) from Serbia

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Abstract The grey wolf (*Canis lupus*) is a large carnivore species and a top predator in the ecosystems that it inhabits. Considering its role in food webs, wolves may be exposed to high concentrations of potentially harmful elements. Therefore liver samples from 28 legally hunted wolves were analyzed for concentrations of 16 elements using inductively coupled plasma optical emission spectrometry. The Mann–Whitney *U* test showed a significant difference between the genders only for Li, and there were no differences between individuals caught in different years. The majority of statistically significant correlations between element levels were positive, except for three cases. Compliance with several criteria for suitable bioindicator organisms imply that wolves may serve for monitoring environmental contamination.

Keywords Ecotoxicology · Wolf · ICP-OES · Element concentration

Exposure to non-essential elements, such as arsenic (As) or cadmium (Cd), can cause different negative effects on health (mutagenic, carcinogenic or teratogenic effects, immune suppression, growth inhibition, impaired reproduction) (Gall et al. 2015). Moreover, these elements are toxic even in trace amounts (Yilmaz et al. 2010). Furthermore, some essential elements needed in biological systems, such as iron (Fe) or zinc (Zn), can show toxicity at high concentrations (Yilmaz et al. 2010).

The concentration of elements in soil depends primarily on the geological composition of parent material (Shan et al. 2013), although some elements are transported atmospherically and settled by wet or dry deposition (Rodriguez et al. 2012). Many plants are able to bioaccumulate elements and some species have the ability to hyperaccumulate various elements (Mann et al. 2011), and thus can become one of the first links of element transfer in the terrestrial food web (Gall et al. 2015).

The grey wolf (*Canis lupus* Linnaeus, 1758) is the most widespread large carnivore in Europe (Randi 2011), and represents one of the top predators in terrestrial ecosystems (Sergio et al. 2008). Conservation success, protective legislation, and supportive public opinion in the last few decades have led to the recovery of European populations (Chapron et al. 2014), so several areas have been recolonized (Stronen et al. 2013). In European Union countries, wolves are under strict legal protection (Kaczensky et al. 2012). In Serbia, this carnivore has a relatively continuous range, which encompasses most forested areas of western, southwestern, southern, southeastern and eastern parts of the country (Paunović et al. 2008). With the exception of a small isolated population in southern Banat, there is no protection of this species in Serbia, due to its stable population trend and a population size estimated at 700–800 individuals (Paunović et al. 2008; Djan et al. 2014).

The aims of this study are: (a) to evaluate the possibility of using the grey wolf, as the top predator, for monitoring of environmental contamination; (b) to determine the levels of toxic elements and trace metals in wolf liver; (c) to explore differences between the genders and correlations with regard to element concentrations; (d) to compare our findings with the results of other similar studies.

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Materials and Methods

Sjenica is located on the Pešter plateau, in the mountain area of south-western Serbia. The study area comprises 1056 km^2 of average altitude 1000 masl. Because of the surrounding mountains and poorly developed infrastructure, this region is scarcely populated. Vegetation is dominated by pastures and meadows, and the old conifer forests cover only about 20% of the investigated area. The primary commercial activity is still the traditional way of animal husbandry (Institute for nature conservation of Serbia 2013). Notable is the presence of the "Štavalj" coal mine inside the sampling area. The mine has a small production (70,000 t annually) of hard brown coal (Turković et al. 2012). With the exception of this mine, industrial production is not developed (Institute for nature conservation of Serbia 2013).

In co-operation with the local hunting association from Sjenica, the carcasses of legally hunted wolves were obtained during the winter season. Out of 28 animals, 16 were females (average length 112.95 ± 3.62 cm; average weight 28.30 ± 4.69 kg) and 12 were males (average length 119.75 ± 7.09 cm; average weight 34.38 ± 5.21 kg). Nine individuals were collected in 2011, 11 in 2012, 7 in 2013, and 1 in 2015. During post mortem analysis of these specimens, liver samples were taken, rinsed with distilled water and stored at −18°C prior to analysis by inductively coupled plasma optical emission spectrometry (ICP-OES). The liver was chosen as the target organ as it is the major detoxification site for contaminants, and it can accumulate inorganic substances (Becker 2000). Another reason for using liver tissue is to facilitate comparison of our findings with those from other toxicological studies performed on wolves.

Sample portions between 0.3 and 0.5 g (wet weight) were processed in a microwave digestor (ETHOS EASY ADVANCED MICROWAVE DIGESTION SYSTEM 230V/50 Hz, Mileston, Italy), using 9 mL of 65% HNO_3 (Suprapur®, Merck) and 1 mL of 30% H_2O_2 (Suprapur®, Merck) at a Fresh liver temperature program. Potential presence of the analyzed elements in the reagents was resolved by digestion of blank samples. After cooling to room temperature, the digested samples were diluted with distilled water to a total volume of 25 mL and the concentrations of 16 elements (Al, B, Ba, Cd, Co, Cr, Cu, Fe, Hg, Li, Mn, Mo, Ni, Pb, Sr, and Zn) determined by ICP-OES (Spectro Genesis EOP II, Spectro Analytical Instruments DmbH, Kleve, Germany). The following wavelength lines were used: Al 394.401 nm, B 249.773 nm, Ba 233.527 nm, Cd 228.802 nm, Co 228.616 nm, Cr 205.552 nm, Cu 324.754 nm, Fe 259.941 nm, Hg 184.950 nm, Li 460.289 nm, Mn 259.373 nm, Mo 202.095 nm, Ni 231.604 nm, Pb 220.353 nm, Sr 460.733 nm and Zn 206.1919 nm. The quality of the analytical process was controlled through analysis of BCR-185R reference materials of bovine liver, for the following elements: Cd, Cu, Mn, Pb, and Zn. IAEA-336 reference materials of lichen was used for the quality of the analytical process for the remaining elements, except for Li, Mo and Ni for which there were no reference materials. The concentrations found were within 90%–115% of the certified values for all measured elements. Concentrations of all elements were expressed as mg kg^{-1} wet weight (ww).

The Shapiro–Wilk test was used to infer if the data obtained showed normal distribution. Due to non-normal distribution, we applied the Mann–Whitney U test to determine the significance of differences in the levels of elements in liver tissue between the genders. The Kruskal–Wallis test for k independent samples was used to examine differences in levels of the elements in individuals between sampling years (2011, 2012, and 2013). Finally, correlation analysis (bivariate correlations with Spearman's correlation coefficients) was used to check for significant relationships between the length and weight of wolves and element content.

Results and Discussion

The levels of Al and B were below the detection limit, whereas the other elements were above the detection threshold in all samples. Mean values and ranges for element levels in females and males, as well as in all individuals, are presented in Table 1. The highest concentrations were detected for Fe and Zn.

Most published data on the subject of toxic element concentrations in large mammals concern animals important in the human diet, such as cattle, deer, sheep, wild boar (Gall et al. 2015). Several papers have focused on these problems in tissues from carnivorous species but only a few for the wolf (Gamberg and Braune 1999; Gnamuš et al. 2000; Shore et al. 2001; Flaten et al. 2008; Hoffmann et al. 2010; Bilandžić et al. 2012; Hernández-Moreno et al. 2013; Vihnanek Lazarus et al. 2013; Lazarus et al. 2017).

We found lower level of Cu and higher levels of Hg and Pb than reported for samples taken in neighbouring Croatia (Bilandžić et al. 2012), while Cd levels were similar. These authors also observed that liver content of Cd was higher than for Pb, which is not the case in our study. Due to the uneven sample size, comparison with the results of their paper should be kept under scrutiny. However, also in Croatia but with a larger sample, Vihnanek Lazarus et al. (2013) reported a higher content of Pb than Cd in liver, which is corroborated in our study. In a comprehensive ecotoxicology study on carnivores in Croatia, Lazarus et al. (2017) found higher contents of Co, Cu, and Zn, but lower contents of Hg, Mo, and Pb in wolf liver, than we obtained in our study. Levels of Cd, Fe, and Mn were similar. Our results for Pb were lower and for Hg (minimum and maximum values)

Table 1 Concentrations of Ba, Cd, Co, Cr, Cu, Fe, Hg, Li, Mn, Mo, Ni, Pb, Sr, and Zn in wolf liver samples (mg kg^{-1} ww) (mean value ± standard deviation; ranges are presented in brackets)

Element	Detection limits (mg L^{-1})	Females	Males	Total
Ba	0.000531	0.26 ± 0.62 (0.01–2.40)	0.32 ± 0.63 (0.01–2.14)	0.29 ± 0.61 (0.01–2.40)
Cd	0.000132	0.11 ± 0.08 (0.004–0.36)	0.07 ± 0.04 (0.003–0.11)	0.09 ± 0.07 (0.003–0.36)
Co	0.00024	0.02 ± 0.02 (0.01–0.06)	0.04 ± 0.04 (0.005–0.10)	0.03 ± 0.03 (0.005–0.10)
Cr	0.000366	0.15 ± 0.10 (0.08–0.49)	0.14 ± 0.03 (0.09–0.20)	0.15 ± 0.08 (0.08–0.49)
Cu	0.000588	5.26 ± 2.48 (2.52–11.37)	5.82 ± 4.90 (2.50–19.41)	5.50 ± 3.65 (2.50–19.41)
Fe	0.000562	143.77 ± 60.65 (49.92–292.31)	168.19 ± 62.25 (69.62–276.84)	154.24 ± 62.69 (49.92–292.31)
Hg	0.00553	0.16 ± 0.08 (0.08–0.38)	0.13 ± 0.07 (0.02–0.28)	0.15 ± 0.07 (0.02–0.38)
Li	0.042	1.00 ± 0.33* (0.50–1.45)	0.67 ± 0.26* (0.42–1.09)	0.86 ± 0.34 (0.42–1.45)
Mn	0.000403	3.94 ± 0.98 (2.82–6.24)	3.35 ± 0.64 (2.32–4.54)	3.69 ± 0.88 (2.32–6.24)
Mo	0.000784	0.85 ± 0.36 (0.41–1.52)	0.71 ± 0.22 (0.38–1.08)	0.79 ± 0.31 (0.38–1.52)
Ni	0.00114	0.04 ± 0.07 (0.004–0.31)	0.02 ± 0.01 (0.003–0.04)	0.03 ± 0.06 (0.003–0.31)
Pb	0.00343	0.49 ± 0.50 (0.16–2.18)	0.88 ± 0.99 (0.08–3.47)	0.66 ± 0.76 (0.08–3.47)
Sr	0.00138	0.50 ± 0.29 (0.19–1.07)	0.47 ± 0.21 (0.23–0.89)	0.49 ± 0.26 (0.18–1.07)
Zn	0.000391	24.01 ± 7.77 (13.22–39.28)	24.24 ± 6.52 (18.09–38.53)	24.11 ± 7.13 (13.22–39.28)

The values in the same row are significantly different (Mann–Whitney *U* test, $p < 0.05$)

were higher than Shore et al. (2001) found in wolf livers from Russia.

The study of Hernández-Moreno et al. (2013) showed higher contents of Cd and Pb in liver of the Iberian wolf (*C. lupus signatus*) than we found for the grey wolf, while Zn levels were similar. Higher concentrations of Cd, Co and Zn were reported in arctic wolves from Canada by Gamberg and Braune (1999). They also found lower concentration of Pb but similar concentrations of Hg when compared with our results. In a survey of wolves from Norway, Flaten et al. (2008) obtained higher levels of Cd, Cu, Mn, and Zn than we observed in our paper. They found lower levels of Ba, Hg, Mo, Pb, and Sr, while Co and Ni levels were similar to ours. For comparisons with these studies, we converted the results expressed, published in dry weight, to wet weight using 3.33 as the conversion factor, as the water content in wolf liver is about 70% (Gamberg and Braune 1999).

The Mann–Whitney test revealed a significant difference between the genders, only for Li concentration (females had a higher level). According to the Kruskal–Wallis test, there were no statistically significant differences between individuals sampled in different years.

Currently, there are few papers concerning differences in element concentration between the genders in wolves. Hernández-Moreno et al. (2013) also observed no significant differences in liver element content between the genders but there was a difference in Cd concentration in kidney tissue. Element analysis in wolf kidneys, from the USA and Canada, showed no disparity between the genders, except for Fe concentration (Hoffmann et al. 2010). Lastly, Lazarus et al. (2017) found differences between the genders for concentrations of Cu, Fe, Mn and Tl in muscle tissue, for As, Ca and Tl concentration in liver tissue, and for Tl in kidney tissue. Other carnivores also exhibited no difference between genders, with regard to element concentration, which is shown by Millán et al. (2008) and Pérez-López et al. (2016), in their studies on red foxes (*Vulpes vulpes*).

The majority of statistically significant correlations between length, weight and element levels in liver were positive. The only statistically significant negative correlations were between length and Ni concentration ($r = -0.454$, $p < 0.05$), between weight and Ni concentration ($r = -0.389$, $p < 0.05$) and between the concentrations of Co and Mo ($r = -0.449$, $p < 0.05$).

A good bioindicator organism should meet several criteria such as: (1) abundance and wide distribution to enable continual sampling and comparison with other research; (2) a long lifespan in order to compare accumulation at various life stages; (3) a suitable target tissue for analysis; (4) easy sampling; (5) an important position in the food chain (Zhou et al. 2008). When considering these criteria, the wolf is a favorable model organism for monitoring element contamination in its home range. It is relatively abundant in Serbia (Djan et al. 2014), it is widespread in Europe (Randi 2011), has a life span of around 13 years (Mech 1988; Holyan et al. 2005), and it is one of the top predators in the food chain (Sergio et al. 2008). Moreover, samples of target tissues are easily obtainable during annual wolf hunts.

This is a first study of element concentration in tissues of the grey wolf and only the second (Ćirović et al. 2015) for a canid species from Serbia. Our results are meaningful due to an adequate sample size from a relatively small area, and the number of detected elements. Also this is the first study that considers correlations between element levels in wolf tissues. As a species, the wolf, could potentially be used for environmental monitoring in Serbia, but more work is needed, especially to determine the levels of toxic and trace elements in the wolf's prey, as well as in other carnivore species.

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