

## Trace Elements in Tissues of Wild Carnivores and Omnivores in Croatia

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**Abstract** The differences in metal exposure (As, Cd, Cu, Pb and Hg) in the muscle, liver and kidney tissues of brown bears (*Ursus arctos*), grey wolves (*Canis lupus*), Eurasian lynxes (*Lynx lynx*), Eurasian badgers (*Meles meles*) and pine martens (*Martes martes*) from Croatia were observed. The highest mean Cd levels were found in kidney and liver of Eurasian badger (3.05 and 0.537 mg/kg). The highest Cu concentrations (mg/kg) measured in liver tissue were obtained in order: Eurasian badger (15.2) > brown bear (12.1) > pine marten (10.3) > Eurasian lynx (8.43) > grey wolf (6.44). Result presented that Eurasian badger accumulated the highest levels of elements: As, Cu and Pb in

muscle; As, Cd, Cu and Pb in liver; Cd and Pb in kidney. Kidney of pine marten accumulated the highest concentrations of As, Cu and Hg. Omnivorous species observed present an important bioindicator for the accumulation of toxic elements indicating an enhanced vulnerability for response to ecological changes in forested terrain. Generally, element concentrations found in five species observed were lower in comparison to levels reported in previous studies and below levels related to toxicosis in mammals.

**Keywords** Trace elements · Tissues · Carnivorous · Omnivorous · Croatia

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Emissions from anthropogenic activities are the major cause of increased environmental concentrations of the toxic elements arsenic (As), cadmium (Cd), mercury (Hg) and lead (Pb). Chronic lower level intakes of these metals have damaging effects on humans and animals due to their potential toxicity and accumulation in various compartments (Millán et al. 2008). Copper is an essential metal required in the diet but may produce toxic effect such as liver cirrhosis, dermatitis and neurological disorders (Gamberg and Braune 1999).

Previous investigations have reported the risk of heavy metal exposure for carnivores in Spain (Millán et al. 2008; Reglero et al. 2008), also in American mink (*Mustela vison*) (Yates et al. 2005) and Florida panther (*Felis concolor coryi*) (Newman et al. 2004). Carnivorous mammals such as brown bear (*Ursus arctos*), grey wolf (*Canis lupus*) and Eurasian lynx (*Lynx lynx*) are at the top of the food web in the wild and therefore it is important to estimate any ongoing biomagnification processes.

Large terrestrial top carnivores, brown bear (*Ursus arctos*), grey wolf (*Canis lupus*) and Eurasian lynx (*Lynx*

*lynx*) live in the Gorski Kotar region in Croatia (Kusak et al. 2009). The wolf and Eurasian lynx are critically endangered species in most European countries, though are more widespread and abundant in Eastern Europe. It is estimated that only 160–200 lynx inhabit southern Spain (Millán et al. 2008). Habitat loss, depletion of prey and intensive hunting are reasons why the indigenous Eurasian lynx population is disappearing from the Dinaric Mountains of Croatia. It is estimated that only 130 Eurasian lynx inhabit an area of 9,374 km<sup>2</sup> in Croatia. In Europe, the Eurasian lynx largely preys on medium-sized ungulates (e.g. various deer species and domestic sheep), hares, foxes and larger birds (Kusak et al. 2009).

According to recent estimates, Croatia is inhabited by 181–253 grey wolves (*Canis lupus*) in 50 herds (State Institute for Nature Protection 2009). Wolves directly hunt and consume a variety of small and large mammals, such as red deer or wild boar, or scavenge carcasses and thus represent a valuable biomonitor among the free-living carnivores. The brown bear (*Ursus arctos*) is a widely distributed predator in Europe and its dietary habits have been widely studied. As omnivores, most brown bears feed on a variety of plant products, including berries, roots, and sprouts, and while they are not highly carnivorous, they will also feed on fish, insects, and small mammals (Nikolaos et al. 2010). The Eurasian badger (*Meles meles*) is territorial and lives in small groups that include one adult female of reproductive age. Rabbits are the main prey. The pine marten (*Martes martes*) is a medium-sized omnivore that prefers forest habitats, including deciduous, mixed, and coniferous forests and its diet is based on small mammals for most of the year (Sidorovich et al. 2010).

The objective of the present study was to conduct bio-monitoring of trace element levels as environmental contaminants (As, Cd, Cu, Pb and Hg) in tissues of brown bear, grey wolf, Eurasian lynx, pine marten and Eurasian badger living in mountainous and woodland areas of Croatia.

## Materials and Methods

During 2009 and 2010, muscle, liver and kidney tissues were collected from 15 specimens: two brown bear (*Ursus arctos*), four grey wolf (*Canis lupus*), three Eurasian lynx (*Lynx lynx*), three Eurasian badger (*Meles meles*) and four pine marten (*Martes martes*). Tissues were collected opportunistically, predominantly from animals that had been killed legally by hunters or had died from other causes, such as in road traffic. No animals were killed for the purpose of this study. All animals were collected in the wooded, mountainous area of Gorski Kotar in Primorje-Gorski Kotar County. All animals sampled were adults. Upon collection, all muscle, liver and kidney samples were

placed into labelled plastic bags and stored at –18°C to avoid tissue degradation prior to laboratory analyses. All tissue samples were homogenized prior to analysis.

All reagents were of analytical reagent grade, HNO<sub>3</sub>, H<sub>2</sub>O<sub>2</sub> and HCl (Analytical Grade, Kemika, Croatia). Double deionised water (Milli-Q Millipore, 18.2 MΩ/cm resistivity) was used for all dilutions. Plastic and glassware were cleaned by soaking in diluted HNO<sub>3</sub> (1/9, v/v) and rinsed with distilled water prior to use. Calibrations were prepared with element standard solutions of 1 g/L of each element supplied by Perkin Elmer. Stock solution was diluted in HNO<sub>3</sub> (0.2%). As matrix modifiers in each atomization for Pb and Cd, 0.005 mg Pd(NO<sub>3</sub>)<sub>2</sub> and 0.003 mg Mg(NO<sub>3</sub>)<sub>2</sub> (Perkin Elmer, USA) were used.

Sample tissues (2 g) were digested with 5 mL of HNO<sub>3</sub> (65% v/v), 1 mL of H<sub>2</sub>O<sub>2</sub> (30% v/v) with a microwave oven. A blank digest was carried out in the same way. The Multiwave 3000 microwave closed system (Anton Paar, Germany) was used for sample digestion. The digestion program began at a potency of 1,200 W and was then ramped for 10 min, after which samples were held at 1,200 W for 10 min. Digested samples were diluted to a final volume of 50 mL with double deionised water.

The analyses of As, Cd, Cu and Pb were conducted by graphite furnace using an AAnalyst 800 atomic absorption spectrometer (Perkin Elmer, USA) equipped with an AS 800 autosampler (Perkin Elmer, USA) set at 193.7, 228.8, 324.8 and 283.3 nm. For graphite furnace measurements, argon was used as the inert gas. Pyrolytic-coated graphite tubes with a platform were used. The atomic absorption signal was measured in peak area mode against a calibration curve. Mercury were quantified without acid digestion using the AMA-254 (Advanced Mercury Analyzer, Leco, Poland), set at 253.6 nm, which employs direct combustion of the tissue samples in an oxygen-rich atmosphere.

Randomly selected samples were analyzed in duplicate to estimate the precision of the results. The wet weight (w.w.) limits of detection for the metals (LODs, mg/kg) analyzed by GFAAS were: in kidney As 0.01, Cd 0.004, Cu 0.005 and Pb 0.005; in liver As 0.01, Cd 0.0004, Cu 0.0005 and Pb 0.0005; in muscle As 0.01, Cd 0.0004, Cu 0.0005 and Pb 0.005. For Hg analyzed by AMA, the detection limits were (mg/kg): Hg 0.00004 in kidney, Hg 0.0001 in liver and Hg 0.0005 in muscle. All specimens were run in batches that included blanks, a standard calibration curve, two spiked specimens, and one duplicate.

Data quality was checked by analysis of the recovery rate using certified reference materials: bovine liver (BCR 185R, IRMM, Belgium) and muscle tissue (ERM-CE278, IRMM, Belgium). A reference sample of bovine liver (BCR 185R, IRMM, Belgium) was analyzed (n = 5) and the recovery (mean % recovery ± SE) was 93.9% ± 5.7% for As, 98.3% ± 2.5% for Cd, 98.2% ± 3.6% for Cu, and

97.1%  $\pm$  4.2% for Pb (no Hg certified value is available). A reference sample of muscle tissue (ERM-CE278, IRMM, Belgium) was analyzed ( $n = 5$ ) and the recovery was 96.6%  $\pm$  6.3% for As, 97.9%  $\pm$  5.5% for Cd, 99.3%  $\pm$  4.6% for Cu, 96.4%  $\pm$  3.6% for Hg and 97.5%  $\pm$  4.1% for Pb. All concentrations are given in wet weight.

Statistical analysis was performed using the Statistica® 6.1 software package (StatSoft® Inc., Tulsa, USA). Data were grouped according to species. One-way analysis of variance was used to test for differences in tissue metal concentrations. Data were log-transformed to improve normality prior to analysis to meet the underlying assumptions of the analysis of variance; the values given are therefore geometric means. Student's  $t$  test was applied to test for differences in element concentrations between species and between tissues. Statistical significance was set at  $p < 0.05$ .

## Results and Discussion

This is the first study, to the extent of our knowledge, to determine the accumulation of toxic metals and copper in brown bear (*Ursus arctos*), grey wolf (*Canis lupus*), Eurasian lynx (*Lynx lynx*), Eurasian badger (*Meles meles*) and pine marten (*Martes martes*) in Croatia. The concentrations of five elements in muscle, liver and kidney tissues of five examined species are shown in Tables 1, 2 and 3. Statistical analysis showed a significant difference in As ( $p < 0.001$ ), Cd ( $p < 0.01$ ), Cu ( $p < 0.01$ ) and Pb ( $p < 0.001$ ) levels among the species. There were no significant differences in mercury levels among the species.

The highest mean Cd levels were found in kidney and liver of Eurasian badger (3.05 and 0.537 mg/kg). In a previous study carried out in Urbino-Pesaro Province in Italy (Alleva et al. 2006), the highest Cd levels were found in three species of omnivorous mammals: badger (*Meles meles*), stone marten (*Martes foina*), and fox (*Vulpes vulpes*). Mean Cd concentrations in liver reported in various carnivores ranged from 0.10 to 0.30 mg/kg (Hoekstra et al. 2003; Millán et al. 2008). The presented liver levels were more than 30-fold higher than concentrations found in badger in Andalusia, Spain (0.017 mg/kg, Millán et al. 2008), but similar to levels detected in badger liver from Italy (0.67 mg/kg; Alleva et al. 2006). However, Cd levels found in the liver of pine marten were more than 8-fold lower than those found in stone marten from Italy (0.96 mg/kg, Alleva et al. 2006). Muscle and liver Cd observed in Eurasian lynx in this survey were similar to muscle levels and 3- to 5-fold higher than those in liver found in Iberian lynx from Andalusia, Spain (0.005 mg/kg, 0.057 to 0.122 mg/kg; Millán et al. 2008). On the other hand, mean muscle and liver Cd levels found in brown bear

were 1.3- and 10-fold lower than concentrations reported in polar bears from Alaska (0.1 and 0.47 mg/kg; Woshner et al. 2001). Kidney and liver Cd levels measured in wolves in this study were 25- and 18-fold lower than those reported in wolves from Yukon Territory, Canada (6.3 and 1.5 mg/kg; Gamberg and Braune 1999). However, Cd liver levels detected were similar to those reported in wolverine from the Canadian Arctic (0.1 mg/kg; Hoekstra et al. 2003). Cadmium kidney concentrations detected in all five species were much lower than the suggested injury thresholds of Cd in the renal cortex (200 mg/kg) related to chronic intoxication (Millán et al. 2008).

In the present study, differences in Pb levels were observed among species. The Eurasian badger had the highest Pb muscle and liver concentrations among the studied species. However, observed liver Pb levels in badger were more than 10-fold lower than those reported in Italy and Spain (0.4 mg/kg, Alleva et al. 2006; 0.46 mg/kg, Millán et al. 2008). Mean liver Pb levels observed in pine marten were more than 40-fold lower than those found in stone marten from Italy (0.33 mg/kg; Alleva et al. 2006) and more than 5-fold lower than levels found in Egyptian mongoose and Eurasian badger (0.054 and 0.046 mg/kg; Millán et al. 2008). Lead muscle and liver levels observed in Eurasian lynx were 10- to 62-fold lower than those found in Iberian lynx from Spain (0.005 and 0.043 to 0.248 mg/kg; Millán et al. 2008). Generally, Pb concentrations found in wolves in this study were lower than reported in previous studies. Mean muscle and liver Pb levels in brown bear were 7-fold lower than levels reported in polar bear from Alaska (0.02 and 0.08 mg/kg, Woshner et al. 2001). Lead liver and kidney concentrations in all five species observed were below levels related to Pb toxicosis in mammals (10–25 mg/kg; Ma 1996).

In the present study, the highest Cu concentrations were measured in liver tissue in the order: Eurasian badger > brown bear > pine marten > Eurasian lynx > grey wolf. In average, Cu levels in liver were 4- to 8.7-fold higher than muscle levels and 2- to 3.2-fold higher than in kidney. Copper liver levels vary widely among species: 18.2 and 35.4 mg/kg in Iberian lynx, 16.9 mg/kg in Egyptian mongoose, 89.5 mg/kg in Eurasian badger (Millán et al. 2008), 47–69 mg/kg in wolf (Gamberg and Braune 1999), 30 mg/kg in polar bear (Woshner et al. 2001), and 47.3–55.9 mg/kg in red deer (Reglero et al. 2008). Mean Cu muscle and liver levels found in badger were 2.7- and 6-fold lower than Cu levels reported in badger from Spain (8.15 and 89.5 mg/kg; Millán et al. 2008). Mean muscle and liver Cu levels measured in pine marten were 2- to 5-fold lower than concentrations reported in Iberian lynx, Egyptian mongoose, red fox and Eurasian badger from Spain (Millán et al. 2008). Muscle and liver Cu measured in Eurasian lynx in this survey were similar to muscle

**Table 1** Trace element concentrations (geometric mean and range; mg/kg, w.w.) in muscle of wild carnivores and omnivores from Croatia

Region	N	Statistics	mg/kg				
			As	Cd	Cu	Hg	Pb
Brown bear ( <i>Ursus arctos</i> )	2	Geometric mean	0.006 <sup>b</sup>	0.004	2.87 <sup>b</sup>	0.005	0.003 <sup>b</sup>
		Minimum	0.005	0.003	2.62	0.004	0.002
		Maximum	0.007	0.005	3.14	0.006	0.004
Eurasian lynx ( <i>Lynx lynx</i> )	2	Geometric mean	0.009 <sup>a</sup>	0.003	1.51	0.008	0.005 <sup>a</sup>
		Minimum	0.008	0.002	1.06	0.007	0.004
		Maximum	0.011	0.004	2.16	0.009	0.006
Pine marten ( <i>Martes martes</i> )	4	Geometric mean	0.009 <sup>a</sup>	0.005	1.59	0.017	0.004 <sup>b</sup>
		Minimum	0.004	0.001	0.684	0.005	0.002
		Maximum	0.02	0.02	3.36	0.048	0.009
Eurasian badger ( <i>Meles meles</i> )	3	Geometric mean	0.034 <sup>a,b</sup>	0.009	2.99 <sup>a</sup>	0.005	0.077 <sup>a,b</sup>
		Minimum	0.02	0.006	1.56	0.002	0.037
		Maximum	0.047	0.02	7.73	0.016	0.133
Grey wolf ( <i>Canis lupus</i> )	4	Geometric mean	0.008 <sup>a</sup>	0.01	0.74 <sup>a,b</sup>	0.006	0.021
		Minimum	0.005	0.003	0.530	0.001	0.005
		Maximum	0.02	0.142	1.36	0.027	0.084

Vertically, letters show statistically significant differences among species: <sup>a</sup>  $p < 0.05$ ; <sup>b</sup>  $p < 0.01$

**Table 2** Trace element concentrations (geometric mean and range; mg/kg, w.w.) in liver of wild carnivores and omnivores from Croatia

Region	N	Statistics	mg/kg				
			As	Cd	Cu	Hg	Pb
Brown bear ( <i>Ursus arctos</i> )	2	Geometric mean	0.006	0.347	12.1	0.029	0.012 <sup>b</sup>
		Minimum	0.001	0.286	8.65	0.027	0.011
		Maximum	0.01	0.421	16.8	0.032	0.012
Eurasian lynx ( <i>Lynx lynx</i> )	2	Geometric mean	0.014	0.325	8.43	0.041	0.004 <sup>a,c</sup>
		Minimum	0.01	0.251	7.81	0.036	0.003
		Maximum	0.02	0.422	9.11	0.047	0.005
Pine marten ( <i>Martes martes</i> )	4	Geometric mean	0.007	0.11	10.3	0.027	0.008 <sup>b</sup>
		Minimum	0.001	0.004	7.45	0.019	0.002
		Maximum	0.02	0.819	23.0	0.056	0.037
Eurasian badger ( <i>Meles meles</i> )	3	Geometric mean	0.033	0.537 <sup>b</sup>	15.2	0.037	0.106 <sup>b,c</sup>
		Minimum	0.013	0.307	7.31	0.019	0.078
		Maximum	0.14	1.04	23.65	0.072	1.19
Grey wolf ( <i>Canis lupus</i> )	4	Geometric mean	0.008	0.085 <sup>b</sup>	6.44	0.017	0.068 <sup>a,b</sup>
		Minimum	0.005	0.042	1.94	0.007	0.029
		Maximum	0.033	0.208	20.4	0.099	0.355

Vertically, letters show statistically significant differences among species: <sup>a</sup>  $p < 0.05$ ; <sup>b</sup>  $p < 0.01$ ; <sup>c</sup>  $p < 0.001$

levels and 2- to 4-fold lower than those found in the liver of Iberian lynx (0.9–1.07 and 18.2–35.4 mg/kg; Millán et al. 2008). Kidney and liver Cu levels measured in wolves in the present study were 2- to 8-fold lower than concentrations found in wolves (15 and 49 mg/kg; Gamberg and Braune 1999) and liver tissues in wolverines from Canada (32 mg/kg; Hoekstra et al. 2003). The presented muscle and liver Cu levels found in brown bear were similar to muscle but 2.5- and 9-fold lower than those found in liver

of polar bear from Alaska and the Canadian Arctic (2.97 and 30 mg/kg, Woshner et al. 2001). Concentrations measured in the present study were below the concentrations indicative of potential toxic effects, i.e. 20 mg/kg in liver and 400–3,000 mg/kg in kidney (Puls 1994).

In this survey, the highest mean Hg levels were found in the kidney of pine marten (0.106 mg/kg) followed by levels in Eurasian badger of 0.084 mg/kg. A study conducted in Italy (Alleva et al. 2006) shown the highest Hg levels

**Table 3** Trace element concentrations (geometric mean and range; mg/kg, w.w.) in kidney of wild carnivores and omnivores from Croatia (elements is not detected in kidney of brown bear and Eurasian lynx)

Region	N	Statistics	mg/kg				
			As	Cd	Cu	Hg	Pb
Pine marten ( <i>Martes martes</i> )	4	Geometric mean	0.013 <sup>a</sup>	1.06	4.94	0.106	0.009 <sup>a</sup>
		Minimum	0.01	0.285	2.59	0.073	0.001
		Maximum	0.02	4.83	7.16	0.162	0.047
Eurasian badger ( <i>Meles meles</i> )	3	Geometric mean	0.012 <sup>a</sup>	3.05	4.69	0.084	0.156 <sup>a</sup>
		Minimum	0.01	1.75	2.67	0.04	0.073
		Maximum	0.02	7.61	7.93	0.168	0.426
Grey wolf ( <i>Canis lupus</i> )	4	Geometric mean	0.009 <sup>a</sup>	0.249	3.33	0.027	0.055
		Minimum	0.005	0.09	1.18	0.008	0.032
		Maximum	0.015	1.21	10.11	0.309	0.143

Vertically, letter show statistically significant differences among species: <sup>a</sup>  $p < 0.05$

in three species of omnivorous mammals, badger (*Meles meles*), stone marten (*Martes foina*), and fox (*Vulpes vulpes*). In the present study, liver Hg levels in pine marten were more than 6-fold lower than concentrations found in stone marten (0.11 mg/kg; Alleva et al. 2006). Badger Hg liver concentrations were 36- and 18-fold lower than those reported in badger (*Meles meles*) from Italy and Spain (0.18 mg/kg, Alleva et al. 2006; 0.09 mg/kg, Millán et al. 2008). Also, muscle Hg concentration detected in badger were 13-fold lower than those found in Italy (0.069 mg/kg, Millán et al. 2008). Muscle and liver Hg levels found in Eurasian lynx in this survey were 2-fold lower in muscle and 3- to 6-fold lower in liver than those reported in Iberian lynx from Spain (0.017–0.024 mg/kg, 0.135–0.233 mg/kg; Millán et al. 2008). However, kidney Hg levels found in wolves from the Yukon (0.76 mg/kg; Gamberg and Braune 1999) were more than 28-fold higher than our findings. Mean Hg liver levels in wolves were 7-fold lower (0.12 mg/kg) than those reported in wolves from the Canadian Arctic (Hoekstra et al. 2003) and 85-fold lower (1.45 mg/kg) than levels found in wolves from Canada (Gamberg and Braune 1999). Mercury levels measured in brown bear were similar to muscle levels but more than 450-fold lower than levels (14.22 mg/kg) found in polar bears from Alaska (Woshner et al. 2001). Very high Hg liver levels were registered in polar bears Javan mongooses (*Herpestes javanicus*) from Japan (1.75–55.5 mg/kg; Horai et al. 2006). As previously concluded, Hg accumulation is related to local contamination and natural background variability, age of the animal and duration of exposure (Hoekstra et al. 2003; Millán et al. 2008). It has been suggested that low levels of mercury recorded in recent years may be due a reduction in the use of fungicides containing mercury, which have since been banned (Hoekstra et al. 2003; Millán et al. 2008).

In the present study, the highest As levels (0.034 mg/kg) were measured in muscle tissue of Eurasian badger.

Related to muscle As concentrations observed in carnivores in Spain, the measured muscle levels in badger were higher than in Spanish badgers, but more than 4- to 12-fold lower than those in Iberian lynx, Egyptian mongoose and common genet (Millán et al. 2008). Muscle levels of Eurasian lynx in this study were more than 20-fold lower in comparison to Iberian lynx. However, observed hepatic As levels in bear and wolf were generally lower than in other reported carnivores: 0.041 in Iberian lynx and 0.047 mg/kg in red fox (Millán et al. 2008), 0.09 mg/kg in polar bears (Woshner et al. 2001) and 0.21 mg/kg in wolves (Gamberg and Braune 1999).

Generally, element concentrations found in the five investigated species were lower than levels reported in previous studies and below levels related to toxicosis in mammals.

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