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Metal(loid) exposure assessment and biomarker responses in captive and free-ranging European brown bear (*Ursus arctos*)

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ABSTRACT

We investigated the level of five non-essential metal(loid)s (As, Cd, Hg, Tl, Pb) and nine essential metals (Mg, Ca, Mn, Fe, Co, Cu, Zn, Se, Mo) in hair and blood components of captive and free-ranging European brown bear populations in Croatia and Poland. Metal(loid) associations with biomarkers of oxidative stress (superoxide dismutase, SOD; glutathione-peroxidase, GSH-Px; malondialdehyde, MDA) and metal exposure (metallothionein, MT) were estimated in this top predatory mammal. Lead was the most abundant non-essential metal(loid) in both blood and hair, with 4 of 35 individuals having blood levels over 100 μg/L. A positive association was found between Pb level and SOD activity in blood. Free-ranging bears had higher blood SOD activity, Mn, Zn and Cd levels, hair Co, Cd, Tl and Pb compared to captive individuals, while the opposite was true for Mg and hair Ca thereby reflecting habitat and diet differences. With increasing age, animals showed lower levels of SOD activity and certain essential metals. Females had higher SOD activity and blood levels of some essential metals than males. Hair showed a higher Fe and Co level when sampled during the growth phase and was not predictive of non-essential metal(loid) blood levels. The established metal(loid) baseline values will enable future risk assessment in both captive and wild European brown bear populations.

1. Introduction

Due to their trophic position, apex predators are known to face elevated health risks due to the long-range transport of metal-bearing particles, and the persistence and accumulation of inorganic pollutants along the food chain (Burger et al., 2007; Rodríguez-Jorquera et al., 2017, Rodríguez-Estival and Mateo, 2019). In particular, non-essential metal(oid)s (arsenic, cadmium, lead, and mercury) are health stressors known to adversely impact large terrestrial mammals (Reglero et al., 2009, Rodríguez-Estival et al., 2011; Berzas Nevado et al., 2012; Tchounwou et al., 2012, Rodríguez-Estival et al., 2013). These metal (oid)s have been demonstrated to impair homeostasis of essential metals, leading to their deficiency or surplus (Goyer, 1997; Reglero et al., 2009; Durkalec et al., 2018; Kalisińska, 2019), and their impacts can also be reflected as perturbations in oxidative stress biomarkers.

Metals induce oxidative stress causing toxic effects through the depletion of major antioxidants (e.g., glutathione, metallothionein), changes in the activity of antioxidative enzymes (e.g., glutathione-peroxidase, superoxide-dismutase) and free radical levels that can damage biomolecules (DNA, proteins, membrane lipids) (Stohs and Bagchi, 1995; Ercal et al., 2001). In addition to scavenging free radicals, small protein metallothionein (MT) regulate essential metal levels (e.g., Zn, Cu) and detoxify non-essential metals (e.g., Cd, Hg) by binding them to their sulfhydryl groups (Kägi, 1991, Isani and Carpenè, 2014).

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Table 1
The biometric data of European brown bears (*Ursus arctos*) sampled in this study.

Bear ID	Age	Sex[1]	Body mass	Status	Date	Country[3]	Sample[4]
1.	2	M	107	captive	May 2014	Cro	B(p,s,wb)
-resampled	4	M	150	captive	Nov 2016	Cro	B(p,s,wb) + H
2.	31	F	116	captive	Apr2015	Cro	B(p,s,wb) + H
3.	15	M	252	captive	Apr 2015	Cro	B(p,s,wb) + H
-resampled	15	M	244	captive	Sep 2015	Cro	B(s) + H
4.	19	M	209	captive	Jul 2016	Cro	B(p,s,wb)
-resampled	19	M	/	captive	Aug 2016	Cro	B(p,s,wb)
5.	5	M	170	free-ranging	May 2015	Cro	B(p,s,wb) + H
6.	4	M	186	free-ranging	May 2015	Cro	B(p,s,wb) + H
7.	5	M	178	free-ranging	May 2015	Cro	B(p,s,wb) + H
8.	2	M	100	free-ranging	Jun 2015	Cro	B(p,s,wb) + H
9.	2	M	73	free-ranging	Jun 2015	Cro	B(p,s,wb) + H
10.	2	F	80	free-ranging	Oct2015	Cro	B(s,wb) + H
11.	1	F	60	free-ranging	Oct 2015	Cro	B(p,s,wb)
12.	5	M	184	free-ranging	Oct 2015	Cro	B(p,s) + H
13.	6	M	109	free-ranging	Apr 2016	Cro	B(p,s,wb) + H
14.	1.5	M	39	free-ranging	May 2016	Cro	B(p,s,wb) + H
15.	8	M	176	free-ranging	May 2016	Cro	B(p,s,wb) + H
-resampled	8	M	176	free-ranging	May 2016	Cro	B(p,s,wb)
16.	9	F	101	free-ranging	May 2016	Cro	B(p,s,wb) + H
17.	0.7	F	30	free-ranging	Oct2016	Cro	B(s)
18.	0.7	F	30	free-ranging	Oct 2016	Cro	B(s)
19.	3	M	210	free-ranging	May 2017	Cro	B(p,s,wb) + H
20.	7	F	86	free-ranging	May 2017	Cro	B(p,s,wb) + H
21.	11	F	210	captive	Feb 2011	Pol	H
22.	16	M	340[2]	captive	Feb 2011	Pol	H
-resampled	21	M	350	captive	Apr 2016	Pol	B(s,wb) + H
23.	8	M	250[2]	captive	Feb 2011	Pol	H
24.	8	F	200[2]	captive	Feb 2011	Pol	H
-resampled	13	F	140	captive	Apr 2016	Pol	B(s,wb) + H
25.	36	F	112	captive	Feb 2011	Pol	H
26.	16	M	260	captive	Jun 2012	Pol	H
27.	24	M	210	captive	Dec 2014	Pol	H
28.	22	M	212	captive	Jun 2012	Pol	H
-resampled	26	M	250[2]	captive	Jan 2016	Pol	H
29.	22	F	145	captive	Apr 2016	Pol	B(s,wb) + H
30.	4	M	140	captive	Apr 2016	Pol	B(s,wb) + H
31.	10	F	100[2]	free-ranging	Oct 2010	Pol	H
32.	adult	M	/	free-ranging	Oct 2013	Pol	H
33.	11	M	215	free-ranging	Mar 2014	Pol	B(wb) + H
-resampled	12	M	168	free-ranging	May 2015	Pol	B(s,wb) + H
34.	adult	M	205	free-ranging	Apr 2014	Pol	B(wb) + H
35.	2	M	75	free-ranging	May 2014	Pol	H
36.	25	M	148	free-ranging	May 2014	Pol	B(s,wb) + H
-resampled	26	M	129	free-ranging	Jun 2015	Pol	B(s,wb)
37.	4	M	81	free-ranging	May 2014	Pol	B(s,wb)
38.	2	F	92	free-ranging	Oct 2014	Pol	B(s,wb) + H
39.	4	M	135	free-ranging	Nov 2014	Pol	B(s) + H
40.	17	F	190	free-ranging	Mar 2015	Pol	B(s) + H
41.	1	M	35	free-ranging	Mar 2015	Pol	H
42.	3	M	156	free-ranging	Mar 2015	Pol	B(s) + H
43.	3	M	270	free-ranging	Apr 2015	Pol	B(s,wb) + H
44.	5	M	120	free-ranging	May 2015	Pol	B(s) + H
45.	5	F	83	free-ranging	May 2015	Pol	B(s,wb) + H
46.	5	F	74	free-ranging	Oct 2015	Pol	B(wb) + H
47.	adult	M	250[2]	free-ranging	Mar 2016	Pol	H
48.	9	F	67	free-ranging	Apr 2016	Pol	B(wb) + H
49.	1	F	31	free-ranging	Apr 2016	Pol	H
50.	18	F	83	free-ranging	Apr 2016	Pol	B(wb) + H
51.	1	M	15	free-ranging	Apr 2016	Pol	B(wb) + H

[1] M-male, F-female.
[2] Estimated body mass.
[3] Cro-Croatia, Pol-Poland.
[4] B-blood, p-plasma, s-serum, wb-whole blood, H-hair.

When elevated, these MT binding metals can induce MTs (Kägi, 1991; Klaassen et al., 1999), and as such, MTs are suitable biomarkers of metal exposure (Gamberg and Scheuhammer, 1994; M'kandawire et al., 2012; Ivanković et al., 2005; Durkalec et al., 2017). Measurement of chemical exposure biomarkers and their effects on apex predators provide valuable information on population health, though such studies are scarce (Rodríguez-Estival and Mateo, 2019).

Brown bear (*Ursus arctos*) is found in 22 European countries (Chapron et al., 2014), including the Dinaric and Carpathian mountain ranges, as two of the few European habitats hosting three large carnivore species. Brown bear population viability and health is a major focus in wildlife management and has been the aim of conservation efforts in recent decades (Swenson et al., 2000; Kaczensky et al., 2012; Chapron et al., 2014). However, the scarce data available concerning

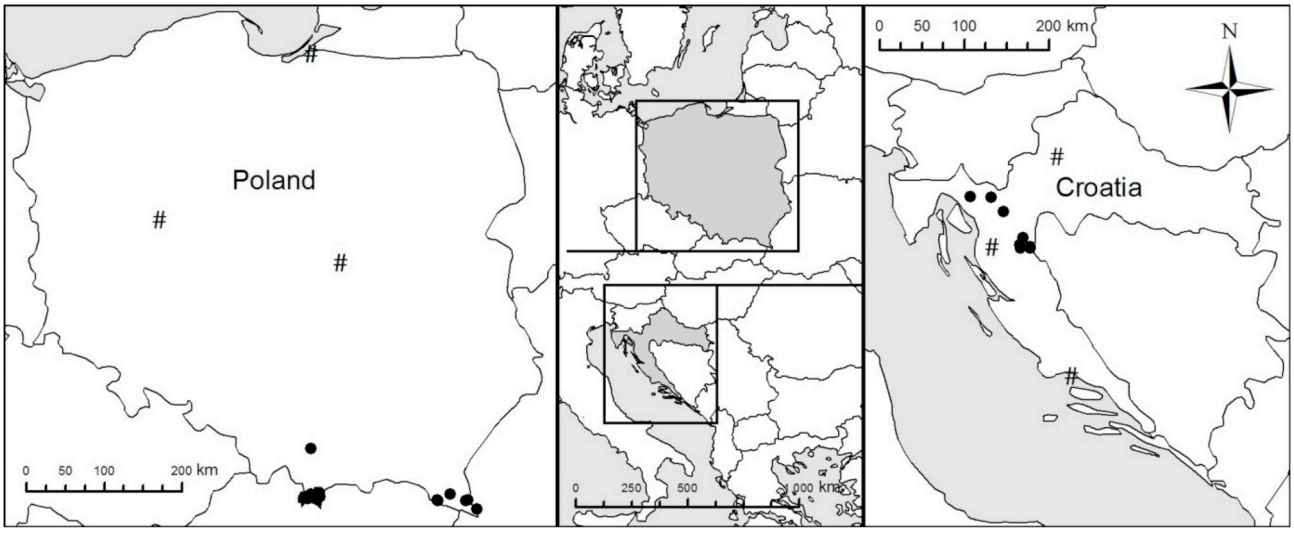

Fig. 1. European brown bear (*Ursus arctos*) sampling locations: dots indicate free-ranging animals and hash signs indicate captive animals.

environmental toxicant levels in European brown bear populations are based exclusively on dead animal tissue levels of organic (Herceg Romanić et al., 2015) and inorganic contaminants (Medvedev, 1999; Čelechovská et al., 2006; Flaten et al., 2008; Šprem et al., 2016; Lazarus et al. 2017, 2018a, 2018b). Less invasive bioindicator tissues in European brown bears have not been investigated to determine health threats or possible adverse effects. Since brown bears enjoy strict protection status under the Habitats Directive (EC/European Council Directive 92/43/EEC, 1992), non-lethal sampling for scientific purposes has high conservation interest. Blood and hair can be considered a valuable biomonitoring data pool that excludes mortality, and enables longitudinal studies through resampling. Metal(loid)s in blood are a marker of recent exposure, reflecting the net balance of metal(loid) intake (mainly via food), deposition, and excretion. Also, metal(loid)s in blood are bioavailable and thus relevant for toxicological risk assessment. Since there are no bear-specific toxicity benchmarks for non-essential metals in blood, other authors have compared non-European ursid levels with those derived for humans (Rogers et al., 2012; Dietz et al., 2013; Chen et al., 2018). However, human guidelines are highly conservative and of questionable relevance for bears given the known interspecies differences for metal toxicity sensitivity (Dietz et al., 2013), so relevant bear studies on metal-related exposure/effects and risk assessment should be a priority.

Unlike blood, hair can reflect both recent and past exposure in time frames constrained by hair growth and moult. Only during growth, hair takes up elements from circulating blood and binds them to abundant sulfhydryl groups in the cystine of the keratinized shaft, making the elements inert for the host. This accumulative nature of non-essential metals has often been used in hair pollution studies on different non-ursid predators (Malvandi et al., 2010; Hernandez-Moreno et al., 2013; Dainowski et al., 2015; Treu et al., 2018), and polar bears (Dietz et al., 2013; Cardona-Marek et al., 2009; Bechshoft et al., 2016) and North American brown bears (Felicetti et al., 2004; Noël et al. 2014, 2015), but there are no data available for the European brown bear. Characterization of hair-to-blood metal relation may help yield useful interpretations of non-essential metal levels in hair, as a minimally invasive sample, to determine the toxicological risk for the individual and population. Although irrefutably connected, the relationships hair-to-blood metal levels is highly dependent on species, individual factors (age, foraging behaviour, weaning, growth and moulting status) and ecological factors (e.g., time of year) (Lieske et al., 2011; Peterson et al., 2016).

The aim of this study was to investigate hair and blood (plasma, serum, whole blood) levels of most relevant non-essential metal(loid)s (As, Cd, Hg, Tl, Pb) and their hair-to-blood relation in captive and free-ranging European brown bears in order to estimate the possible toxicological risk due to exposure to environmental contaminants, and to assess essential element levels (Mg, Ca, Mn, Fe, Co, Cu, Zn, Se, Mo) to examine for possible deficiencies or surpluses. Furthermore, we tested the associations between levels of blood metal(loid)s and biomarkers: biomarker of metal exposure (MT) and biomarkers of effect (antioxidative enzyme activity (SOD, GSH-Px) and oxidative damage to lipids). This study also aimed to set baseline values for the given metal (loid)s in European brown bears and to explore the influence of various confounding individual and ecological factors.

2. Materials and methods

2.1. Animal sampling

We sampled 51 brown bears from Croatia and Poland in the period 2010–2017, and paired samples of hair and blood (at least one blood component: serum, plasma, whole blood) were collected from 36 individuals (Table 1). Blood was resampled for six bears and hair was resampled for five bears. Altogether, 14 captive (five female, nine males) and 37 free-ranging European brown bear individuals were sampled (14 females, 23 males). In Poland, free-ranging bears were captured for telemetry research and management of problem bears in the Tatra and Bieszczady Mountains in Southern Poland, in the Western and Eastern Carpathians, respectively. Free-ranging bears in Croatia (Dinara-Pindos population) were captured in the Gorski kotar and Lika regions (Fig. 1). Bears are permanently present in both countries, and all sampling areas can be considered pristine without point contamination sources. In compliance with the Habitats Directive, the brown bear is strictly protected in both countries (Selva et al., 2011), though in Croatia it is also listed as a game species within the Directive's derogation provisions, and managed under the Brown Bear Management Plan (Huber et al., 2008). Blood, hair and tooth samples for this study were taken within the framework of research and conservation projects in effect at the time. Captive bears were sampled in zoos and other institutions during regular veterinary checks, interventions or relocations. All bears were chemically immobilized and body measurements were taken (Huber et al., 1996; Kaczensky et al., 2002), and blood was collected from femoral vein in serum and whole blood (K_2EDTA coated) tubes). Body mass was measured by suspending the bear from a spring-loaded weigh scale. Serum and plasma were aliquoted into plastic tubes after centrifugation at 3000 rpm for 10 min and stored with aliquoted whole blood samples at −20 °C until

analysis. Neck or shoulder hair was cut with stainless steel scissors as close to the skin as possible (guard and undercoat hair together) and stored in a paper envelope at room temperature. A premolar tooth was extracted from every free-ranging bear to determine age using the counts of cementum annuli (Matson's Lab, Milltown, Montana, USA; Matson et al., 1993).

2.2. Element analyses

Hair was prewashed for 10 min on a vortex with 20 mL ultrapure water (18 MΩ cm, GenPure system, TKA, Germany) to eliminate soil particles as an external contamination source. The International Atomic Energy Agency (IAEA) recommended 5-step washing procedure was implemented (acetone-water-water-water-acetone; acetone for gas chromatography MS SupraSolv®, Merck, Germany), each consisting of a 10 min vortex with 20 mL solvent (Ryabukhin, 1976). The hair was then dried for 24 h at 40 °C, weighted and digested in an UltraCLAVE IV (Milestone, Italy) microwave digestion system. Elements (Mg, Ca, Mn, Fe, Co, Cu, Zn, As, Se, Mo, Cd, Hg, Tl, Pb) in hair were then quantified by inductively coupled plasma mass spectrometry (ICP-MS; Agilent 7500cx, Germany) together with diluted blood (serum, plasma and whole blood) samples according to accepted procedure (Vihnanek Lazarus et al., 2013; Živković et al., 2014). Ultrapure water and purified (duoPUR, Milestone, Italy) nitric acid (p.a. 65%, Merck, Germany) were used for sample preparation and dilution. The reference material Human hair IAEA-086 (Vienna, Austria), certified reference material (CRM) No.13 Human hair (National Institute for Environmental Studies, Japan), Seronorm™ Trace Elements Whole blood L-1, L-2 and L-3, Serum L-1 and L-2 (Sero AS, Billingstad, Norway) were processed in duplicate with hair and blood samples to control for the quality of the analytical method. Results of reference material analyses are presented in Table A1 (hair) and Table A2 (blood), with respective method detection limits (MDL) as a supplementary material. Blood element results are expressed in mg or µg per L and hair results in mg or µg per kg of dry hair mass.

2.3. Biomarker analyses in serum and whole blood

The activity of total superoxide-dismutase (SOD; EC 1.15.1.1) and glutathione-peroxidase (GSH-Px; EC 1.11.1.9) was determined by commercial Ransod and Ransel kits (Randox Laboratories, Crumlin, UK), respectively, on a SABA 18 biochemistry analyser (AMS, Rome, Italy). SOD and GSH-Px activity were expressed as units per L of serum/whole blood.

Metallothionein (MT) was quantified in heat-treated samples by differential pulse voltammetry following a modified Brdička procedure (Raspor et al., 2001) on 797 VA Computrace (Metrohm, Herisau, Switzerland) with a three-electrode system (hanging mercury drop electrode (HMDE), an Ag/AgCl/sat, KCl reference electrode and a Pt counter electrode). Measurements were performed in 10 mL de-aerated supporting electrolyte (1 M NH4Cl + 1 M NH4OH, pH = 9.5, $6 \times 10-4$ M [Co(NH3)6]Cl3) at constant temperature (20 °C). Serum was diluted twice with 0.9% NaCl (suprapur grade, Merck, Darmstadt, Germany), heated at 100 °C for 15 min in Techne Dri-Block (Bibby Scientific Limited, Staffordshire, UK), and then cooled on ice for 30 min prior to centrifugation for 30 min at 10,000 g and 4 °C. Metallothionein was quantified in the resulting supernatant using the calibration straight line gained by commercially available ≥95% - pure MT-2 from rabbit liver (Enzo Life Sciences, Inc., NY, USA) dissolved in 0.25 M NaCl, and expressed in mg per mL of serum.

The malondialdehyde (MDA) as an index of lipid peroxidation was measured using a high-performance liquid chromatography based (HPLC; Shimadzu Corporation, Kyoto, Japan) thiobarbituric acid (TBA) assay (Drury et al., 1997). Within the HPLC apparatus, degasser, isocratic pump, column oven, Shimadzu UV detector set at 532 nm, C-18 reverse-phase (LiChrospher, Merck, Darmstadt, Germany) guard column and analytical column with 5 µm particles (4.0 × 4.0 and 4.0 × 125.0 mm, respectively) were used. Malondialdehyde content was reported in µmol per L of serum.

2.4. Statistical analyses

Data below the method detection limit were assigned half of the value of the MDL for the respective metal(loid) for the purpose of statistical analysis. Blood samples collected from the same individual bear twice during the study (i.e. bears 1, 3, 33 and 36, Table 1) were considered independent if the period between resampling was longer than 3 months (Rogers et al., 2012). The same period for hair resampling was set at one year which is the time needed for complete hair exchange in bears (Felicetti et al., 2004). Data for bears resampled less than 3 months apart for blood (i.e. bears 4 and 15, Table 1) and 1 year for hair (bear 3) were averaged and used as a single sample in the analysis. The status of bears from Croatia and Poland was either free-ranging or captive (bears kept in zoos or other captive institutions). According to date of sampling, hair was categorized as sampled during growth (May–October) or quiescence (November–April; corrected for southern populations from Cattet et al., 2018). First, each predictor was tested separately by means of a *t*-test for homogenous (Levene's test) and normally distributed (Shapiro-Wilk's test) variables, while the Mann-Whitney *U* test was used for heterogeneous and/or non-normally distributed data in blood and hair (Table A3). Spearman rank order correlations (r_S) were used to analyse univariate associations between age and other continuous variables, and interpreted according to Hinkle et al. (2003), where $0.3 < r_S \leq 0.5$ indicated low correlation, $0.5 < r_S \leq 0.7$ moderate correlation, $0.7 < r_S \leq 0.9$ high correlation, and $r_S > 0.9$ very high correlation. Multivariate linear regression analyses (*b*, p-value, R^2) were then performed to test differences in variables between captive and free-ranging bears taking into account the predictors of age, sex, country and date of sampling (i.e. hair growth phase) in hair. However, given the lower sample number for serum and whole blood variables, multiple analyses of blood included only individual predictors of age and sex while testing if status (captive *vs.* free-ranging) influenced element and biomarker levels. Furthermore, significant predictors from the preceding multivariate model were used in the following model to test for an association between non-essential metals (Cd, Tl and Pb) and biomarkers (MT, MDA, SOD) in blood. Also, blood-hair associations were explored for non-essential metal(loid)s. Residual analyses were performed to check for normality and homoscedasticity, and in cases when those criteria were not met, data were transformed (log_{10}, inversion, square root; Table A4). The level of significance was set at $\alpha = 0.05$. Statistica for Windows software, version 13.0 (StatSoft, Inc., Tulsa, USA) was used in all statistical analyses.

3. Results

Bears aged between 0.7 and 31 years weighted between 15 and 350 kg, with captive bears being older (mean 17 years *vs.* 7 years old, $t(52) = 4.68$, $p < 0.001$) and heavier than free-ranging bears (mean 205 kg *vs.* 121 kg, $t(56) = 4.50$, $p < 0.001$), on average.

3.1. Blood

Brown bear blood as an indicator of recent exposure of the host showed that Pb is the most relevant non-essential metal(loid), while As and Hg were mostly below the method detection limit (68–94% and 74–84% of all samples, respectively), and Cd and Tl had values an order of magnitude lower than Pb in all compartments (Table 2). Specifically, Ca, Co, Cu, Mo and Tl were compartmentalized primarily in serum/plasma and Mn, Fe, Zn, Cd and Pb in whole blood. Activity of GSH-Px in the serum of all bears was below the method detection limit and thus unavailable. For certain variables, the results of multivariate modelling differed from univariate testing (Table A3 *vs.* Table A4). When bear

Table 2
Levels of metallothionein, oxidative stress biomarkers and metal(loid)s in European brown bear (*Ursus arctos*) blood components.

	Serum		Plasma		Whole blood	
	N	mean ± SD (range) median	N	mean ± SD (range) median	N	mean ± SD (range) median
MT (mg/mL)	31	1.64 ± 0.73 (0.497–3.67) 1.76				
MDA (μmol/L)	35	3.86 ± 2.92 (0.846–11.7) 2.83				
SOD (U/mL)	37	1.01 ± 0.55 (0.022–2.33) 0.980			35	548 ± 197 (264–961) 477
Mg (mg/L)	37	19.3 ± 2.06 (15.5–24.0) 19.2	18	19.1 ± 1.57 (15.8–22.1) 19.6	35	21.2 ± 3.11 (16.2–28.5) 21.1
Ca (mg/L)	37	91.6 ± 8.0 (69.3–110) 92.4	18	110 ± 6 (101–124) 107	35	31.5 ± 6.9 (18.8–48.6) 31.2
Mn (μg/L)	37	3.47 ± 1.33 (1.69–7.66) 3.26	18	2.64 ± 0.59 (1.63–3.64) 2.73	35	22.0 ± 7.4 (9.22–34.2) 23.4
Fe (mg/L)	37	6.55 ± 3.59 (0.594–17.6) 6.06	18	6.05 ± 3.28 (0.910–12.2) 6.33	35	354 ± 55 (195–449) 356
Co (μg/L)	37	0.771 ± 0.574 (0.130–2.55) 0.670	18	0.733 ± 0.343 (0.284–1.43) 0.652	35	0.322 ± 0.271 (0.074–1.11) 0.247
Cu (mg/L)	37	0.793 ± 0.487 (0.326–2.34) 0.669	18	0.852 ± 0.433 (0.331–1.78) 0.709	35	0.473 ± 0.189 (0.225–1.04) 0.390
Zn (mg/L)	37	1.30 ± 0.27 (0.719–1.84) 1.27	18	1.26 ± 0.28 (0.703–1.75) 1.23	35	2.00 ± 0.28 (1.48–2.66) 1.94
As (μg/L)	37	(<0.678–2.11)[1]	18	(<0.678–0.881)[2]	35	(<2.37–2.49)[3]
Se (μg/L)	37	114 ± 25 (73–207) 110	18	81.1 ± 16 (51.0–109) 81.7	35	141 ± 30 (80–204) 139
Mo (μg/L)	37	50.7 ± 10.8 (22.6–71.2) 48.4	18	49.8 ± 11.9 (22.2–68.5) 48.2	35	13.4 ± 4.5 (3.38–23.3) 13.7
Cd (μg/L)	37	0.105 ± 0.048 (<0.070–0.248) 0.101[4]	18	0.106 ± 0.038 (<0.070–0.203) 0.102[5]	35	0.251 ± 0.234 (<0.247–1.21) 0.123[6]
Hg (μg/L)	37	(<0.375–1.11)[7]	18	0.658 ± 1.847 (<0.375–8.47)[8]	35	(<1.31–6.32)[9]
Tl (μg/L)	37	0.049 ± 0.039 (<0.009–0.158) 0.041[10]	18	0.144 ± 0.105 (0.024–0.378) 0.117	35	0.063 ± 0.028 (0.032–0.141) 0.053
Pb (μg/L)	37	0.944 ± 0.688 (0.147–3.14) 0.691	18	3.90 ± 2.91 (0.285–12.0) 4.51	35	58.0 ± 34.7 (5.08–168) 49.6

MT-metallothionein, MDA-malondialdehyde, SOD-superoxide-dismutase, MDL-method detection limit. Elements are listed in order of increasing atomic mass.
[1] 25/37 < As MDL (0.678 μg/L serum).
[2] 13/18 <As MDL (0.678 μg/L plasma).
[3] 33/35 <As MDL (2.37 μg/L blood).
[4] 5/37 < Cd MDL (0.070 μg/L serum).
[5] 2/18 < Cd MDL (0.070 μg/L plasma).
[6] 22/35 < Cd MDL (0.247 μg/L blood).
[7] 31/37 < Hg MDL (0.375 μg/L serum).
[8] 15/18 < Hg MDL (0.375 μg/L plasma).
[9] 26/35 < Hg MDL (1.31 μg/L blood).
[10] 3/37 < Tl MDL (0.009 μg/L serum).

status was analysed while controlling for age and sex, free-ranging animals showed higher activity of SOD in whole blood (b = 0.40, p = 0.04, R^2 = 0.18), serum and whole blood Mn (b = 0.54, p = 0.01, R^2 = 0.35 and b = 0.44, p = 0.01, R^2 = 0.35, respectively), Zn (b = 0.46, p = 0.05, R^2 = 0.13) and Cd (b = 0.54, p = 0.02, R^2 = 0.24) than captive animals, while the opposite was true for serum Mg (b = −0.49, p = 0.02, R^2 = 0.26; Table A4). However, relatively small biomarker/element variation in the blood compartments (13–35%; Table A4) could be attributed to the influence of age, sex and captivity status of bears. Older animals had lower SOD in serum (b = −0.45, p = 0.01), Co (b = −0.48, p = 0.03 and b = −0.49, p = 0.009) and Mo (b = −0.50, p = 0.02 and b = −0.65, p < 0.001) in serum and whole blood, respectively (Table A4). Unlike whole blood Cu levels (b = −0.37, p = 0.03), females had higher serum SOD activity (b = 0.35, p = 0.02), Mg (b = 0.38, p = 0.03) and Mn (b = 0.32, p = 0.04) and whole blood Mn (b = 0.38, p = 0.02) and Se (b = 0.34, p = 0.05) levels than males (Table A4). When the association between non-essential metals and biomarkers was explored controlling for significant predictors, serum Pb revealed a significant correlation with SOD activity in whole blood (b = 0.47, p = 0.04, R^2 = 0.19; Table A5, Fig. 3). Data for serum and whole blood categorized by captivity status of bears are presented in Table A6; however, plasma was omitted from the table due to the low sample number. According to univariate analyses, bears sampled in Croatia showed higher SOD activity, levels of Co, Cu and Mo compared to bears from Poland, while the opposite was evident for Mn and Zn in the blood (Table A3). Seasonal variation in non-essential metal(loid)s (only Cd and Pb analysed, as Hg and As were mostly below the MDL) displayed in Fig. 2A and C indicated two seasonal peaks in whole blood Cd and Pb in April–May and October corresponding to emergence from the den/extensive feeding compared to winter period, and prior to denning/end of hyperphagic period, respectively. The correlation matrix between Pb, as the most abundant non-essential metal, and essential metals in blood compartments revealed a low association of Pb with Mn (N = 35, r_S = 0.39, p = 0.02), Zn (N = 35, r_S = 0.36, p = 0.03) and Mo (N = 35, r_S = −0.35, p = 0.04).

3.2. Hair

In general, essential elements in hair had a similar distribution as in the blood compartments, except for Zn and Se (Table 3). Zn was much more abundant in hair than in blood, while Se was the lowest of all essential elements in hair, but the most abundant element (Ca and Fe in whole blood) in serum and plasma. In addition, the majority of elements with blood values below the MDL (mostly non-essential ones) were quantified in a higher percentage in hair samples. Results of multivariate linear regression revealed captivity status as an important predictor for Mg (b = −0.53, p = 0.008, R^2 = 0.17), Ca (b = −0.87, p < 0.001, R^2 = 0.56), Co (b = 0.33, p = 0.007, R^2 = 0.69), Cd (b = 0.53, p < 0.001, R^2 = 0.59), Tl (b = 0.34, p = 0.01, R^2 = 0.66) and Pb levels (b = 0.39, p = 0.02, R^2 = 0.45) explaining up to 69% of element variation, while controlling for age, sex, country and hair growth phase. Free-ranging bears had higher Co, Cd, Tl and Pb and lower Mg and Ca levels than captive ones. The other four predictors were not significant in this model, except age for Mn (b = −0.35, p = 0.02), Fe (b = −0.37, p = 0.006) and Co (b = −0.25, p = 0.04), country for Co (b = −0.21, p = 0.04), Mo (b = −0.41, p = 0.01) and Tl (b = −0.48, p < 0.001), and hair growth phase for Fe (b = −0.32, p = 0.01) and Co (b = −0.25, p = 0.02; Table A.4). Regression analyses of blood-to-hair non-essential metal levels with status and/or country as confounding factors revealed no significant relationship between the two matrices (Table A7).

4. Discussion

This study examined blood as a non-lethal indicator of recent exposure and hair as a blood-related tissue, depicting medium exposure history. All three blood components were investigated to enable future

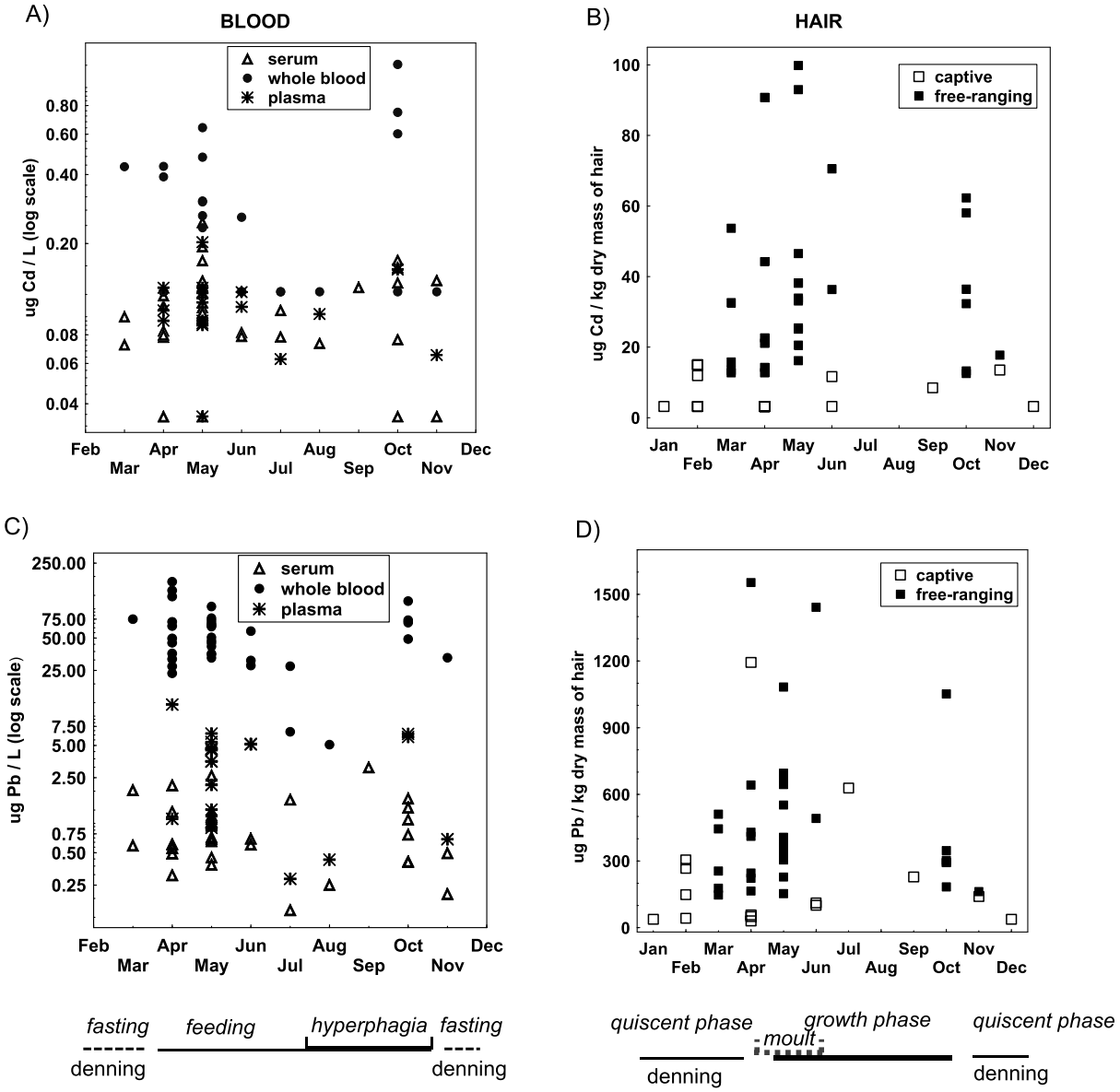

Fig. 2. Cadmium (**A** and **B**) and lead (**C** and **D**) levels in blood compartments on a log scale and hair of captive *vs*. free-ranging European brown bear (*Ursus arctos*, individual values) by sampling month.

between-studies comparisons and correlation of measured metal(loid) levels with biomarkers of exposure and effect. The obtained baseline data will enable future assessment of toxicological risk related to metal (loid) toxicity or deficiency (defined in whole blood or plasma/serum, depending on the element) in the Dinara-Pindos and Carpathian populations, or in any of the 10 European brown bear populations (Chapron et al. 2014).

4.1. Blood

Differential metal(loid) compartmentalization in the blood addresses the need for both whole blood and serum/plasma sampling in monitoring studies. As serum and plasma metal(loid) levels were similar for most elements, future studies should focus on plasma sampling to rationalize multiple tube handling and the amount of drawn blood and avoid coagulation-related changes in measured biomarkers of lipid peroxidation.

Lead was the most abundant non-essential metal measured in brown bear blood (Table 2), known as a critical target of Pb toxicity (Matović et al. 2015). The presence of Fe and Pb (>1%, Matović et al. 2015) in the serum/plasma of some individuals indicated various grades of haemolysis, as both elements are characteristic for erythrocytes. Erythrocytes are a dominant blood subcompartment repository for Pb, although more slowly exchangeable than plasma. Except recent exposure, Pb in human whole blood (PbB) also reflects past exposure due to mobilization of Pb from bone during remodelling (Barbosa et al. 2005). Mean (median) PbB levels in free-ranging (61.2 (64.0) μg/L) and captive (49.2 (29.6) μg/L) brown bears from Croatia and Poland (Table A6) were similar to those in North American brown bears (median, 44 μg/L), but higher than in black bears (median, 16 μg/L) from the greater Yellowstone ecosystem, USA (Rogers et al. 2012) and captive giant panda from Longuantai, China (mean 181 μg/L, recalculated by multiplying 0.171 μg/g with blood density of 1060 g/L, Chen et al. 2018). Blood Pb in captive giant pandas was higher than in free-ranging animals (Chen et al. 2018), which was contrary to the results in Croatian and Polish captive bears which had a median PbB half that of free-ranging brown bears (Table A6), although multivariate analysis declined significance of status for PbB levels (Table A4). Higher exposure to Pb in captive animals was expected given the urbanised zoo locations (Travis et al. 2012); however, it appears that for brown bears, food

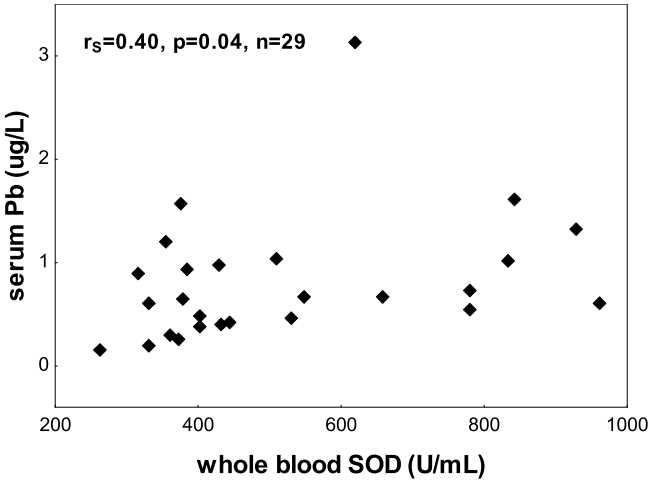

Fig. 3. Relationship between whole blood superoxide-dismutase (SOD) activity (x-axis) and lead level (y-axis) in the serum of European brown bear (*Ursus arctos*). Spearman's correlation coefficient (r_S) with the level of significance indicates the degree of relation between the variables.

origin plays a crucial role in deriving differences. Food given to bears in captive institutions across Croatia and Poland is primarily produced and intended for human consumption (e.g., bread, corn, fruits, vegetables) and the diet is fairly uniform year round, and therefore high Pb is not expected. On the contrary, free-ranging bears have seasonal variation in food availability, especially in spring, when the majority of animals were sampled in this study and when they rely on leafy plants (Cicnjak et al. 1987; Kusak and Huber 1998) which are most vulnerable to airborne Pb atmospheric deposition (ATSDR 2019). Atmospheric Pb deposition is known to increase with altitude (ATSDR 2019), and therefore, this might have also influenced the higher Pb level in free-ranging bears inhabiting the mountainous areas of Croatia and Poland. Another possible source of Pb for wildlife scavengers are bullet fragments left in the carcasses of hunted animals (Taggart et al. 2011; Martin et al. 2019). Although this source of Pb was confirmed in cougar (*Puma concolor*, Burco et al. 2012), but not for bears (Rogers et al. 2012), we suspect brown bears might be exposed to Pb from ammunition while scavenging on hunted wild ungulates as a minor component of the bear diet (Cicnjak et al. 1987; Kusak and Huber 1998; Ważna et al. 2017; Bojarska 2015). Given the lack of a relevant effect threshold PbB levels for wildlife species, other authors have assessed the possibility of estimating the adverse effects in wildlife using data based on humans (ATSDR 2019; Ettinger et al. 2019), domestic animals (Puls 1994) or thresholds derived from various mammalian toxicity studies (Buekers et al. 2009). Fifteen individuals (43%, N = 35) in this study had PbB above 60 μg/L, considered the background level for cattle (Ma, 2011). Rodriguez-Estival et al. (2012 and references cited therein) found decreased activity of an enzyme involved in haeme biosynthesis, δ-aminolevulinic acid dehydratase (δ-ALAD), in cattle even at the background PbB level range (60 μg/L), while oxidative stress, an imbalance in essential elements and interferences of Pb with metabolism of vitamin D were reported in PbBs ranging from 60 to 350 μg/L. In this study, a positive relationship was found between serum Pb and SOD activity in whole blood (Fig. 3), although 57% of individuals had PbB within the background range (<60 μg/L, Ma 2011). This elevation in enzyme activity could be an adaptive mechanism of the enzyme to low Pb exposure (hormesis) as a consequence of higher production of reactive oxygen species (Flora et al. 2012). In addition, the highest PbB level (168 μg/L) measured in a male captive bear, was still below the currently considered threshold for infertility and foetal abnormalities in mammals (>200 μg/L, Ma 2011). Three adult and one yearling (1 year old) brown bear surpassed the adult human threshold for PbB, above which a decreased activity of several haeme biosynthesis enzymes and elevated blood pressure was reported (100 μg/L, ATSDR 2019).

Cadmium was the second highest non-essential metal measured in the blood of brown bears (Table 2). Absorbed from the intestines, cadmium is known to bind erythrocyte membranes, protein albumin and MT in the blood (Matović et al. 2015). Croatian and Polish brown bears showed the lowest blood Cd levels among ursids. Other studies reported a tenfold higher Cd mean (2.54 μg/L, recalculated from Chen et al. 2018) in whole blood of captive giant panda and a 100-fold higher level in polar bears in East Greenland (plasma mean, 16.4 μg/L; range, 0.034–181 μg/L, recalculated using the density of plasma of 1025 g/L; Uba 2013) than in bears from this study (mean plasma, 0.106 μg/L; whole blood, 0.251 μg/L). The cause for such a difference is not clear and may ensue from dietary differences, i.e., exposure rates or interspecies differences in Cd metabolism. Whatever influenced such

Table 3
Level of metal(loid)s in hair of free-ranging and captive European brown bear (*Ursus arctos*).

	All (N = 50)	Free-ranging (N = 34)	Captive (N = 16)
	mean ± SD (range) median		
Mg (mg/kg)	121 ± 72 (24.6–317) 116	104 ± 57 (24.6–213) 104	156 ± 86 (31.4–317) 145
Ca (mg/kg)	940 ± 733 (224–3757) 694	595 ± 345 (224–1764) 524	1629 ± 823 (397–3757) 1548
Mn (mg/kg)	10.3 ± 8.9 (0.487–38.2) 7.86	13.0 ± 9.4 (2.42–38.2) 9.70	4.52 ± 3.42 (0.487–10.3) 3.71
Fe (mg/kg)	69.3 ± 64.7 (7.90–289) 57.1	86.9 ± 57.6 (13.9–270) 74.0	37.1 ± 66.1 (7.90–289) 17.5
Co (μg/kg)	67.3 ± 70.9 (<8.14–300) 39.8[1]	92.1 ± 74.7 (<8.14–300) 70.8	17.7 ± 17.3 (<8.14–51.4) 9.23
Cu (mg/kg)	10.0 ± 2.1 (6.30–15.5) 9.77	10.1 ± 2.36 (6.30–15.5) 9.71	9.79 ± 1.63 (6.52–12.9) 9.90
Zn (mg/kg)	140 ± 13 (112–177) 140	143 ± 12 (124–177) 142	134 ± 14 (112–161) 130
As (μg/kg)	95.3 ± 102.8 (<22.4–404) 60.7[2]	119 ± 114 (32.9–404) 70.9	47.7 ± 49.8 (<22.4–187) 28.8
Se (mg/kg)	0.489 ± 0.167 (0.238–1.00) 0.446	0.476 ± 0.167 (0.238–1.00) 0.432	0.517 ± 0.168 (0.312–1.00) 0.461
Mo (μg/kg)	71.8 ± 45.0 (<12.0–258) 61.7[3]	71.7 ± 49.3 (<12.0–258) 59.4	71.9 ± 34.8 (20.3–140) 67.2
Cd (μg/kg)	26.8 ± 24.6 (<6.37–99.8) 16.9[4]	36.8 ± 24.3 (12.5–99.8) 32.8	6.71 ± 4.92 (<6.37–15.0) 3.18
Hg (μg/kg)	128 ± 128 (<43.0–562) 91.8[5]	156 ± 143 (<43.0–562) 102	74.2 ± 66.5 (<43.0–192) 21.5
Tl (μg/kg)	3.99 ± 2.80 (<0.6–12.5) 3.72[6]	4.98 ± 2.84 (0.852–12.5) 4.15	2.18 ± 1.58 (<0.6–5.35) 1.86
Pb (μg/kg)	401 ± 354 (30.3–1553) 302	479 ± 348 (147–1553) 374	225 ± 311 (30.3–1194) 112

Elements are listed in order of increasing atomic mass
[1] 9/50 < Co MDL (8.14 μg/kg).
[2] 6/50 < As MDL (22.4 μg/kg).
[3] 1/50 < Mo MDL (12.0 μg/kg).
[4] 10/50 < Cd MDL (6.37 μg/kg).
[5] 14/50 < Hg MDL (43.0 μg/kg).
[6] 1/50 < Tl MDL (0.6 μg/kg).

variation between species in blood as a marker of recent assimilation of Cd (ATSDR 2012), the resulting accumulation in target organs (kidney and liver, 20.3 and 1.77 μg/g wet weight, respectively in polar bear, Uba 2013 *vs*. 19.4 and 1.26 μg/g wet weight, respectively in brown bear, Lazarus et al. 2017) indicate a similar long-term exposure. The highest measured whole blood value (1.21 μg/L) in brown bears was well below toxic thresholds for large domestic animals (Puls 1994), used for comparison due to the lack of appropriate wildlife thresholds. Furthermore, no association was found between Cd in blood and biomarkers of exposure (MT) or oxidative stress (SOD, MDA) in the studied individuals (Table A5). The lack of a Cd-MT relation could be due to low Cd levels in bear blood that are too low to induce MT synthesis, as reported for marine mammals (Polizzi et al. 2017). Adam et al. (2007) found that the MT level depended on trophic levels of the species, thus carnivorous animals had higher MT levels in blood than herbivores. In this study, MT was quantified for the first time in some ursid species. Dietary differences and consequently Cd exposure resulted in higher serum (mean, 0.120 μg/L), plasma (0.122 μg/L) and whole blood Cd levels (0.298 μg/L) in free-living bears (Table A6) compared to captive ones (0.073, 0.076 and 0.123 μg/L, respectively), though the difference was statistically significant only for serum (multivariate analyses, Table A4). Cd-rich viscera of domestic and especially wild ungulate animals (Lazarus et al. 2008; Taggart et al. 2011) left in the woods can increase the intake of Cd for free-living bears compared to captive ones. However, it should be noted that food of animal origin accounts for only a minor part of the bear's diet, with preference on plant-based foods (Cicnjak et al. 1987; Kusak and Huber 1998; Ważna et al. 2017; Bojarska 2015). Other non-essential metal(loid)s, like As and Hg were detected in 6–32% and 16–26% of brown bear samples (Table 2), respectively, and at an order of magnitude lower than reported for Florida black bears (*Ursus americanus floridanus*, whole blood Hg mean, 53 μg/L, recalculated from Julian and Cunningham 2013), captive giant pandas (whole blood mean, As 25.5 μg/L, recalculated from Chen et al. 2018) or polar bears (plasma mean, As 37.2 μg/L and Hg 57.5 μg/L, Uba 2013; whole blood mean, Hg 74.2 μg/L, Cardona-Marek et al. 2009). The highest Hg levels measured in blood were 739 μg/L in polar bear (reviewed in Dietz et al. 2013), which is known to be affected by higher Hg bioaccumulation and biomagnification given its marine diet (Scheuhammer et al. 2015), as opposed to the brown bears and Florida black bears which are exclusively part of the terrestrial food web. Thus, the Hg difference in bears from this study and in Florida black bears suggest geographical differences, as their diets are very similar (Murphy et al. 2017; Cicnjak et al. 1987; Kusak and Huber 1998). In this study, all brown bears had blood Hg within the normal range established for cattle, sheep (< 106 μg/L, Puls 1994) and humans (< 20 μg/L, Klaassen et al. 2013), as no wildlife threshold is available. Thallium was present in the blood of brown bears at much lower levels than non-essential metals such as Cd, Pb, or Hg, though its toxicity is much higher (Peter and Viraraghavan 2005). The blood Tl (Table 2) reported here can be considered normal according to CDC human guidelines (< 2 μg/L), given the lack of wildlife data.

According to mammalian and human thresholds and guidelines for non-essential metal(loid)s, only the Pb levels in brown bear blood compartments raised concern regarding possible adverse health effects. Due to the chemical and physical resemblance, the interaction of Pb with essential elements (Na, Mg, Ca, Fe, Cu, Zn) can cause their deficiency, or affect enzyme activity and function of cells and organs (Telišman 1995; Goyer 1997; Flora et al. 2012). Associations of Pb with Mn and Zn found in brown bear might indicate a replacement of Pb with essential elements in δ-ALAD and SOD or shared binding sites on transporters in intestinal cells (Telišman 1995; Goyer 1997; Peraza et al. 1998). The adverse relations between Pb and Mo might be connected to the well-defined Cu–Mo interaction, as Pb was reported to reduce Cu storage in sheep (Puls 1994). However, essential elements in brown bear fitted the adequate ranges defined for domestic animals (Puls 1994), as relevant data for wild large mammals does not exist in the literature, and therefore, we can propose that no excess or deficiency is expected in our bears.

4.2. Hair

For this study, guard and underfur hair was sampled to assess the baseline element levels over a larger period of time and to promote the use of snagged hair in future research as the most easily available hair sample. Lead was the most abundant non-essential metal in the hair of brown bears, followed by Hg, As Cd and Tl. All non-essential metal (loid)s except As were much lower in hair than in liver and kidney of the Croatian bear population (recalculated to dry mass from Lazarus et al. 2017), confirming those two organs are more important storage sites than hair, taking into account the medium exposure window reflected by hair.

Bearing in mind the differences in age and sex distribution, other ursids had higher Hg (North American brown bear, Noël et al. 2014; Florida black bear, Julian and Cunningham 2013; polar bear, Uba 2013, Dietz et al., 2013) and Cd (Uba 2013), similar Se and Zn (Uba 2013), but lower Pb and As (Uba 2013) hair levels than the bears in our study. Hair Hg levels were similar in female but higher in male North American brown bears from Yellowstone than in brown bears in this study (Felicetti et al. 2004). The mean level of Hg detected in brown bears from Croatia and Poland (128 μg/kg) was 40 times lower than the neurotoxic threshold suggested for Hg in polar bear (5400 μg/kg dry mass, Dietz et al. 2013) so adverse health effects are not expected. Other hair non-essential metal(loid)s were in the range considered normal for domestic animals (Puls 1994). Also, due to a lack of data for wild large mammals, essential elements in bear hair were compared and seen to fit the ranges regarded as adequate for domestic mammals (Puls 1994).

The absence of age-related differences in brown bear hair Hg was in line with the report of Cardona-Marek et al. (2009) for Southern Beaufort Sea polar bear, but less than in Western Hudson Bay polar bear (Bechshoft et al. 2016). In contrast to brown bears in this study, Hernandez-Moreno et al. (2013) reported higher hair Pb in adult Iberian wolves than in young individuals. Cardona-Marek et al. (2009) reported higher Hg in the hair of female polar bears, while metal(loid)s in bears from this study failed to differentiate between the sexes, as seen also for the Iberian wolf (Hernandez-Moreno et al. 2013). Free-ranging brown bears had enhanced hair non-essential metal(loid)s level with more pronounced fluctuations compared to captive bears (Tables 3, A4). As for blood, the lower exposure of captive bears through human-intended food can be assumed to cause this difference. Also, metal content in hair plotted against sampled months reflected the uniformity of the captive bear diet throughout the year, especially for Cd levels (Fig. 2). Noël et al. (2014, 2015) confirmed hair Hg, Cu and Zn, but not Pb, Cd and Fe as good indicators of grizzly bear dietary changes. However, in addition to seasonal changes in the diet of free-ranging bears, the seasonal cycle of hair also complicates interpretation of element levels found in bear hair. In general, brown bear hair in the two studied populations grows (and takes up elements from the blood) from May to October (Cattet et al. 2018, corrected for southern populations), i.e., in the period of non-hibernation and good food availability. Moulting occurs between April and June, so hair sampled after June and before hibernation will contain only elements taken up to newly grown hair in that period. Also, hair cycle phases in captive bears differ from free-ranging bears, because of the shorter hibernation and higher food availability (Cattet et al. 2018). Thus, hair physiology might also have an effect on metal(loid)s in captive *vs*. free-ranging populations, though no differences were seen in non-essential metal(loid)s between the two periods (Table A4). However, the peaks observed in hair Cd and Pb levels sampled in May and October can be considered to mostly reflect enhanced dietary exposure in spring and hyperphagia in fall, as a similar pattern was also confirmed in blood (Fig. 2). Nonetheless, for firmer conclusions, future studies should include a larger sample.

While investigating non-essential metal associations between two matrices (blood and hair) of brown bear, it was found that metals in one matrix were not predictive of levels in the other (Table A7). One of the reasons could be the sampling timing. Hair reflects blood levels best when: i) new hair is sampled; ii) hair is in its growth phase (after moulting); iii) exposure is relatively constant (Peterson et al. 2016). The bears in this study were sampled opportunistically throughout the year (except hibernation), and diet exposure had seasonal variations, and accordingly some bears may have been impacted by a fasting period marked with element mobilization into the blood without subsequent transfer into hair (as hair was in the quiescent phase). Therefore, this study design was not able to address the blood-to-hair correlation. To do so, newly grown hair after moulting should be sampled.

5. Conclusion

This first report of the quantification of non-essential metal(loid)s in blood and hair of European brown bear revealed Pb as the most abundant metal that is possibly toxicologically relevant for bears. In 11% of individuals, blood Pb levels surpassed human threshold levels applied due to the lack of wildlife threshold data. The association between Pb and a biomarker of oxidative stress (activity of enzyme SOD) and some essential elements (Mn, Zn and Mo) indicated the need for larger sample scale studies to explore possible Pb-related adverse health effects in brown bear. Higher non-essential (Cd, Tl and Pb) and essential metal levels (Mn, Zn, Co) in free-ranging compared to captive bears confirmed habitat and diet differences. Significant influence of confounding factors (age, sex, status, country, hair growth phase) on metal (loid)s level and even some oxidative stress biomarkers, imply the importance of the inclusion of these elements in future biomonitoring studies of apex terrestrial mammals. Hair sampled throughout the year did not reflect non-essential metal(loid) levels in bear blood. Brown bears from this study had non-essential and essential metal(loid)s in the range of currently available ursid and wildlife data, under the toxic thresholds and within the normal levels for essential elements published for domestic animals and wild mammals.

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Sample collection in Croatia was approved by the Ministry of Agriculture of the Republic of Croatia, Directorate for Forestry, Hunting and Wood Industry (annual permit no. UP/1-323-03/15-01/107) and Ministry of Environment and Energy (permit no. UP/I-612-07/15–48/47). Sample collection in Poland was approved by the General Directorate for Environmental Protection (permit nos. DOP-OZ.6401.08.2.2013.ls.1 and DOP-WG.6401.08.4.2014.JRO,kka) and the appropriate Ethics Committee (Local Ethics Committee in Krakow, permit no. 21/2013).

CRediT authorship contribution statement

Maja Lazarus: Conceptualization, Formal analysis, Writing - original draft, Visualization. **Tatjana Orct:** Methodology, Formal analysis. **Agnieszka Sergiel:** Investigation, Resources, Data curation, Writing - review & editing, Project administration, Funding acquisition. **Lana Vranković:** Formal analysis, Investigation, Resources. **Vlatka Filipović Marijić:** Methodology, Formal analysis, Investigation, Writing - review & editing. **Dubravka Rašić:** Methodology, Formal analysis. **Slaven Reljić:** Resources, Writing - review & editing. **Jasna Aladrović:** Validation, Formal analysis, Resources. **Tomasz Zwijacz-Kozica:** Investigation, Resources, Writing - review & editing. **Filip Zięba:** Investigation, Resources. **Jasna Jurasović:** Resources, Funding acquisition. **Marijana Erk:** Supervision, Writing - review & editing. **Robert Maślak:** Resources. **Nuria Selva:** Resources, Writing - review & editing, Funding acquisition. **Đuro Huber:** Resources, Writing - review & editing, Supervision.

Declaration of competing interest

The authors have no competing interests to declare.

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Appendix A. Supplementary data

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Supplementary material

Journal: Environmental Research

Title: Metal(loid) exposure assessment and biomarker responses in captive and free-ranging European brown bear (*Ursus arctos*)

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Supplementary material contains: 7 Tables

Table A.1 Method detection limit (MDL)[1] for metal(loid) quantification in brown bear hair and results of the analyses of standard/certified reference materials used for quality control

Element		MDL[a]	**IAEA-086 Human hair**		**NIES CRM No.13 Human hair**		**NIST SRM 1577a Bovine liver**	
		hair	Recommended (mean (95% CI))	Measured (mean±SD)	Certified (mean (range))	Measured (mean±SD)	Certified (mean (range))	Measured (mean±SD)
Mg	mg/kg	5.51	177 (156-197)[2]	174±6	160[2]	157 (155-160)	600 (585-615)	591±12
Ca	mg/kg	18.5	1120 (1000-1240)[2]	1114±26	820[2]	819 (809-830)	120 (113-127)	118±6
Mn	mg/kg	0.037	9.6 (8.9-10.3)[2]	9.9±0.4	3.9[2]	4.0 (3.9-4.1)	9.9 (9.1-10.7)	9.9±0.2
Fe	mg/kg	2.81	123 (110-136)	123±3	140[2]	137 (136-138)	194 (174-214)	182±4
Co	µg/kg	8.14	-	-	70[2]	71 (68-73)	210 (160-260)	221±6
Cu	mg/kg	0.152	17.6 (16.6-18.5)[2]	18.1±0.6	15.3 (14.0-16.6)	14.9 (14.5-15.3)	158 (151-165)	155±6
Zn	mg/kg	1.12	167 (159-174)	166±6	172 (161-183)	173 (166-179)	123 (115-131)	129±6
As	µg/kg	22.4	-	-	100[2]	101 (96-106)	47 (41-53)	51±2
Se	µg/kg	35.6	1000 (800-1200)[2]	980±49	1790 (1620-1960)	1755 (1717-1793)	710 (640-780)	701±39
Mo	µg/kg	12.0	-	-	-	-	3500 (3000-4000)	3485±75
Cd	µg/kg	6.37	-	-	230 (200-260)	219 (211-227)	440 (380-500)	415±22
Hg	µg/kg	43.0	573 (534-612)	577 (547-607)	4420 (4220-4620)	4436 (4404-4468)	-	-
Tl	µg/kg	0.600	-	-	-	-	-	-
Pb	µg/kg	16.8	-	-	4600 (4200-5000)	4597 (4494-4700)	135 (120-150)	125±12

[1]Method detection limit (MDL) is calculated as the mean plus three times the standard deviation of a set of method blanks multiplied by the dilution factor
[2]Non-certified (information) values

Table A.2 Method detection limit (MDL)[1] for metal(loid) quantification in brown bear blood compartments and results of the analyses of standard/certified reference materials used for quality control

Element		MDL[a]	**Seronorm Whole blood L-1**		**Seronorm Whole blood L-2**		**Seronorm Whole blood L-3**	
		whole blood	Certified (mean (95% CI))	Measured (mean±SD)	Certified (mean (95% CI))	Measured (mean±SD)	Certified (mean (95% CI))	Measured (mean±SD)
Mg	mg/L	0.0027	14.7[2]	14.2 (13.8-14.6)	14.4[2]	14.0 (13.8-14.1)	17.2[2]	15.5 (15.2-15.9)
Ca	mg/L	0.208	15.0[2]	14.9 (14.7-15.2)	15.0[2]	14.4 (14.2-14.6)	14.2[2]	13.2 (12.2-14.3)
Mn	µg/L	0.750	18.4 (14.7-22.1)	18.5 (18.4-18.6)	31.4 (25.1-37.7)	31.8 (31.4-31.7)	47.3 (37.8-56.8)	47.5 (47.1-47.9)
Fe	mg/L	0.730	334[2]	333 (320-347)	332[2]	330 (317-344)	383[2]	386 (373-399)
Co	µg/L	0.149	0.20 (0.12-0.28)	0.22 (0.20-0.24)	5.18 (4.13-6.22)	5.12 (5.06-5.19)	11.4 (10.2-12.6)	11.5 (11.3-11.7)
Cu	mg/L	0.022	0.64 (0.51-0.76)	0.65 (0.64-0.65)	1.34 (1.07-1.60)	1.32 (1.31-1.34)	2.47 (2.22-2.72)	2.44 (2.36-2.53)
Zn	mg/L	0.025	4.3 (3.4-5.2)	4.41 (4.39-4.43)	7.1 (5.7-8.5)	7.4 (7.3-7.4)	9.11 (7.28-10.9)	9.01 (8.97-9.06)
As	µg/L	2.37	4.6 (3.7-5.5)	2.1 (1.9-2.3)	14.1 (11.3-17.0)	13.8 (13.2-14.5)	30.4 (23.1-37.7)	29.7 (28.8-30.6)
Se	µg/L	1.21	60 (48-72)	62 (60-64)	161 (128-193)	164 (163-165)	272 (217-327)	273 (267-279)
Mo	µg/L	0.456	0.51 (0.41-0.61)	0.67 (0.65-0.70)	5.31 (4.24-6.37)	5.3 (5.1-5.5)	7.5 (6.0-9.0)	7.5 (7.3-7.7)
Cd	µg/L	0.247	0.28 (0.17-0.40)	0.270 (0.26-0.28)	5.01 (4.00-6.20)	5.2 (5.1-5.3)	12.1 (10.8-13.4)	11.9 (11.7-12.2)
Hg	µg/L	1.31	1.48 (1.18-1.77)	1.48 (1.48-1.49)	17.0 (13.6-20.4)	18.5 (18.4-18.6)	37.1 (29.6-44.6)	37.7 (37.5-37.9)
Tl	µg/L	0.031	0.007 (0.003-0.011)	<MDL	10.2 (8.1-12.2)	10.8 (10.1-11.5)	34.1 (27.2-41.0)	34.9 (34.3-35.5)
Pb	µg/L	0.240	9.9 (7.9-11.9)	10.9 (10.8-10.9)	337 (269-405)	340 (336-344)	447 (401-493)	443 (440-445)

Element		MDL	**Seronorm Serum L-1**		**Seronorm Serum L-2**	
		serum	Certified (mean (95% CI))	Measured (mean±SD)	Certified (mean (95% CI))	Measured (mean±SD)
Mg	mg/L	0.0008	16.8 (13.4-20.1)	16.5 (16.2-16.8)	33.9 (27.1-40.7)[2]	32.6 (32.3-32.8)
Ca	mg/L	0.060	86 (69-104)	89 (88-90)	119 (95-143)	122 (121-123)
Mn	µg/L	0.214	9.9 (7.9-11.9)	9.9 (9.8-10.0)	14.5 (11.6-17.4)[2]	14.9 (14.7-15.1)
Fe	mg/L	0.208	1.47 (1.17-1.77)	1.43 (1.4-1.5)	2.15 (1.72-2.58)[2]	2.0 (2.0-2.1)
Co	µg/L	0.043	1.12 (0.67-1.57)	1.10 (1.08-1.11)	3.05 (2.13-3.97)[2]	2.9 (2.8-3.0)
Cu	mg/L	0.0063	1.09 (0.999-1.18)	1.06 (1.03-1.09)	1.85 (1.7-2.0)[2]	1.86 (1.85-1.87)
Zn	mg/L	0.0072	1.1 (0.952-1.24)	1.1 (1.09-1.13)	1.62 (0.952-1.24)	1.61 (1.57-1.64)
As	µg/L	0.678	0.40[2]	0.43 (0.41-0.44)	0.38[2]	0.53 (0.50-0.57)
Se	µg/L	0.347	87 (76-99)	89 (87-90)	138 (120-157)	138 (136-140)
Mo	µg/L	0.130	0.76[2]	0.74 (0.72-0.75)	1.21[2]	1.13 (1.12-1.14)
Cd	µg/L	0.070	0.13[2]	0.14 (0.13-0.14)	0.14[2]	0.15 (0.14-0.15)
Hg	µg/L	0.375	1.07 (0.53-1.60)	1.07 (0.99-1.14)	2.07 (1.44-2.67)	1.95 (1.93-1.98)
Tl	µg/L	0.0089	0.090[2]	0.096 (0.089-0.103)	0.108[2]	0.109 (0.102-0.116)
Pb	µg/L	0.068	0.40[2]	0.45 (0.44-0.46)	0.66[2]	0.63 (0.63-0.64)

[1]Method detection limit (MDL) is calculated as the mean plus three times the standard deviation of a set of method blanks multiplied by the dilution factor
[2]Non-certified (information) values

Table A.3 Results of univariate testing influence of single confounding factor on biomarker and metal(loid) levels in blood and hair of European brown bear (*Ursus arctos*)

Variable	Compartment	Transformation	Age	Sex male/female	Status captive/free-ranging	Country Croatia/Poland	Hair growth phase growth/quiescent
MT	serum	none	–	–	–	–	na
MDA	serum	$\log_{10}(x)$	–	–	–	–	na
SOD	serum	none	r_S=-0.54, p<0.001	t(35)=-2.21, p=0.03	–	t(35)=2.35, p=0.02	na
	whole blood	$\log_{10}(x)$	–	–	t(33)=-2.46, p=0.02	–	na
Mg	serum	none	–	–	t(35)=2.17, p=0.04	–	na
	plasma	none	–	–	–	na	na
	whole blood	none	–	–	–	–	na
	hair	$\sqrt{x}$	–	–	t(48)=2.41, p=0.02	–	–
Ca	serum	none	–	–	–	–	na
	plasma	none	–	–	–	na	na
	whole blood	none	–	–	–	–	na
	hair	$\log_{10}(x)$	r_S=0.33, p=0.03	–	t(48)=6.68, p<0.001	–	–
Mn	serum	$\log_{10}(x)$	–	t(35)=-2.09, p=0.04	t(35)=-3.08, p=0.004	–	na
	plasma	none	–	t(16)=-3.17, p=0.006	–	na	na
	whole blood	none	–	U=78, p=0.04	t(33)=-2.13, p=0.04	U=85, p=0.03	na
	hair	$\log_{10}(x)$	r_S=-0.53, p<0.001	–	t(48)=-4.98, p<0.001	–	t(48)=3.23, p=0.002
Fe	serum	none	r_S=-0.37, p=0.03	–	t(35)=-2.97, p=0.006	–	na
	plasma	none	–	–	t(16)=-3.55, p=0.002	na	na
	whole blood	none	–	–	–	–	na
	hair	$\log_{10}(x)$	r_S=-0.69, p<0.001	–	t(48)=-4.91, p<0.001	t(48)=2.33, p=0.02	t(48)=3.89, p<0.001
Co	serum	$\log_{10}(x)$	r_S=-0.55, p<0.001	–	–	t(35)=4.39, p<0.001	na
	plasma	none	–	–	–	na	na
	whole blood	$\sqrt{x}$	r_S=-0.57, p<0.001	–	–	t(33)=2.45, p=0.02	na
	hair	$\log_{10}(x)$	r_S=-0.64, p<0.001	–	t(48)=-5.42, p<0.001	t(48)=3.27, p=0.002	U=142, p<0.001
Cu	serum	$\log_{10}(x)$	–	–	–	–	na
	plasma	none	–	U=10, p=0.04	–	na	na
	whole blood	$\log_{10}(x)$	–	–	–	t(33)=2.24, p=0.03	na
	hair	none	–	–	–	–	–
Zn	serum	none	–	–	–	–	na
	plasma	none	–	–	t(16)=-2.71, p=0.01	na	na
	whole blood	none	–	–	–	t(33)=-2.12, p=0.04	na
	hair	none	r_S=-0.50, p<0.001	–	t(48)=-2.27, p=0.03	–	–
Se	serum	$\log_{10}(x)$	–	–	–	–	na
	plasma	none	–	–	–	na	na
	whole blood	none	–	–	–	–	na
	hair	$\log_{10}(x)$	–	–	–	–	–
As	hair	$\log_{10}(x)$	r_S=-0.30, p=0.05	–	t(48)=-4.34, p<0.001	–	t(48)=2.07, p=0.04
Mo	serum	none	r_S=-0.43, p=0.008	–	–	–	na

	plasma	none	r_S=-0.56, p=0.01	–	t(16)=-2.92, p=0.009	na	na
	whole blood	none	r_S=-0.56, p<0.001	–	–	t(33)=3.05, p=0.004	na
	hair	$\log_{10}(x)$	–	–	–	t(48)=2.91, p=0.006	–
Cd	serum	none	–	–	t(35)=-2.96, p=0.006	–	na
	plasma	none	–	–	t(16)=-2.93, p=0.01	na	na
	hair	$\sqrt{x}$	r_S=-0.61, p<0.001	–	U=17, p<0.001	–	t(48)=3.48, p=0.001
Hg	hair	$\sqrt{x}$	–	–	t(48)=-2.47, p=0.02	–	–
Tl	serum	$\log_{10}(x)$	–	–	–	–	na
	plasma	$\log_{10}(x)$	–	–	–	na	na
	whole blood	1/x	–	–	–	–	na
	hair	$\sqrt{x}$	r_S=-0.57, p<0.001	–	t(48)=-4.25, p<0.001	t(48)=4.47, p<0.001	t(48)=2.68, p=0.01
Pb	serum	$\log_{10}(x)$	–	–	–	–	na
	plasma	$\sqrt{x}$	–	t(16)=-2.50, p=0.02	–	na	na
	whole blood	$\sqrt{x}$	–	–	U=67, p=0.02	–	na
	hair	$\log_{10}(x)$	r_S=-0.40, p=0.008	–	U=89, p<0.001	t(48)=2.38, p=0.02	U=174, p=0.01

MT-metallothionein, MDA- malondialdehyde, SOD- superoxide-dismutase, "–" non significant, na- non applicable, r_S-Spearman correlation coefficient. Differences were tested by the Student t-test (t(df), p) or Mann-Whitney U-test (U, p). Elements are listed in order of ascending atomic mass. Variables with more than 60% of samples lower than the method detection limit were not analysed or presented in the table. Number of MT data is 31(N=31), MDA N=35, serum SOD N=37, whole blood SOD N=35, metal(loid) data for serum N=37, plasma N=18, whole blood N=35, hair N=50. Sex was coded as 1 for males and 2 for females; status was coded as 1 for captive and 2 for free-ranging bears; country was coded as 1 for Croatia and 2 for Poland; hair growth phase was coded as 1 for growth and 2 for quiescent

Table A.4 Results of linear regression modelling revealing associations of biomarkers and metal(loid) levels with confounding factors in blood and hair of European brown bear (*Ursus arctos*)

Variable	Compartment	Transformation	Age	Sex male/female	Status captive/free-ranging	Country Croatia/Poland	Hair growth phase growth/quiescent	
			b					*R-squared*
MT	serum	none	-0.04	0.33	0.06	na	na	0.12
MDA	serum	$\log_{10}(x)$	0.25	-0.14	0.34	na	na	0.09
SOD	serum	none	**-0.45***	**0.35***	0.03	na	na	0.34
	whole blood	$\log_{10}(x)$	-0.04	-0.16	**0.40***	na	na	0.18
Mg	serum	none	-0.18	**0.38***	**-0.49***	na	na	0.26
	whole blood	none	-0.31	0.02	-0.006	na	na	0.09
	hair	$\sqrt{x}$	-0.23	0.01	**-0.53****	-0.05	-0.10	0.17
Ca	serum	none	-0.27	0.002	-0.17	na	na	0.04
	whole blood	none	-0.20	-0.22	-0.23	na	na	0.11
	hair	$\log_{10}(x)$	-0.17	0.02	**-0.87*****	0.14	-0.07	0.56
Mn	serum	$\log_{10}(x)$	0.10	**0.32***	**0.54***	na	na	0.35
	whole blood	none	0.30	**0.38***	**0.44***	na	na	0.35
	hair	$\log_{10}(x)$	**-0.35***	-0.13	0.26	0.02	-0.24	0.52
Fe	serum	none	-0.15	0.07	0.35	na	na	0.22
	whole blood	none	-0.09	0.22	0.25	na	na	0.14
	hair	$\log_{10}(x)$	**-0.37****	-0.06	0.22	-0.13	**-0.32***	0.64
Co	serum	$\log_{10}(x)$	**-0.48***	0.12	-0.003	na	na	0.23
	whole blood	$\sqrt{x}$	**-0.49****	-0.001	0.02	na	na	0.25
	hair	$\log_{10}(x)$	**-0.25***	-0.18	**0.33****	**-0.21***	**-0.25***	0.69
Cu	serum	$\log_{10}(x)$	-0.03	-0.28	0.11	na	na	0.09
	whole blood	$\log_{10}(x)$	0.16	**-0.37***	0.28	na	na	0.19
	hair	none	-0.31	0.20	-0.12	-0.10	0.08	0.09
Zn	serum	none	0.25	-0.13	**0.46***	na	na	0.13
	whole blood	none						na
	hair	none	-0.33	0.08	0.16	-0.09	0.06	0.20
Se	serum	$\log_{10}(x)$	-0.26	0.12	-0.19	na	na	0.04
	whole blood	none	-0.32	**0.34***	-0.09	na	na	0.18
	hair	$\log_{10}(x)$	0.16	-0.21	-0.02	**-0.33***	0.33	0.22
As	hair	$\log_{10}(x)$						na
Mo	serum	none	**-0.50***	0.22	-0.08	na	na	0.23
	whole blood	none	**-0.65*****	-0.07	-0.22	na	na	0.36
	hair	$\log_{10}(x)$	0.06	-0.27	-0.03	**-0.41***	0.14	0.25
Cd	serum	none	0.12	0.11	**0.54***	na	na	0.24
	hair	$\sqrt{x}$	-0.15	0.01	**0.53*****	-0.08	-0.18	0.59
Hg	hair	$\sqrt{x}$	-0.04	-0.21	0.38	-0.04	0.16	0.17
Tl	serum	$\log_{10}(x)$	-0.03	-0.18	-0.06	na	na	0.04
	whole blood	1/x	-0.07	0.21	-0.22	na	na	0.08

	hair	$\sqrt{x}$	-0.23	0.04	**0.34***	**-0.48*****	-0.06	0.66
Pb	serum	$\log_{10}(x)$	0.18	0.13	0.20	na	na	0.05
	whole blood	$\sqrt{x}$	0.20	0.26	0.34	na	na	0.18
	hair	$\log_{10}(x)$	-0.10	-0.06	**0.39***	-0.16	-0.21	0.45

MT-metallothionein, MDA- malondialdehyde, SOD- superoxide-dismutase, *b*-standardized multiple regression coefficient, *p<0.05, **p<0.01, ***p<0.001, na- non applicable (factor not included in the model or conditions for regression analyses not fulfilled: Zn in whole blood, As in hair). Number of MT data is 31(N=31), MDA N=35, serum SOD N=37, whole blood SOD N=35, metal(loid) data for serum N=37, plasma N=18, whole blood N=35, hair N=50. Sex was coded as 1 for males and 2 for females; status was coded as 1 for captive and 2 for free-ranging bears; country was coded as 1 for Croatia and 2 for Poland; hair growth phase was coded as 1 for growth and 2 for quiescent

Table A.5 Results of linear regression modelling revealing associations between biomarkers and metal(loid) levels in blood of European brown bear (*Ursus arctos*), based only on significant confounding factors from Table A.4

Dependent variable	Independent variable	N	*b*	*p-value*	*R-squared*
Cd serum	MT serum	29			na
	status				
	MDA	34			na
	status				
	SOD serum	35	-0.21	0.28	0.25
	age		0.08	0.75	
	sex		0.11	0.55	
	status		**0.59**	0.03	
	SOD whole blood	29	-0.38	0.05	0.33
	status		**0.58**	0.004	
Tl serum	SOD serum	35	0.13	0.54	0.07
	age		0.02	0.90	
	sex		-0.28	0.16	
	SOD whole blood	29	-0.19	0.38	0.04
	status		-0.004	0.98	
Tl whole blood	SOD serum	29	-0.04	0.89	0.07
	age		0.03	0.89	
	sex		0.26	0.24	
	SOD whole blood	35	0.04	0.86	0.03
	status		-0.18	0.36	
Pb serum	SOD serum	35	0.30	0.17	0.13
	age		0.19	0.35	
	sex		0.12	0.54	
	SOD whole blood	29	**0.47**	0.04	0.19
	status		-0.06	0.78	
Pb whole blood	SOD serum	29	-0.01	0.97	0.13
	age		0.11	0.64	
	sex		0.34	0.12	
	SOD whole blood	35	0.28	0.13	0.23
	status		0.29	0.12	

MT-metallothionein, MDA- malondialdehyde, SOD- superoxide-dismutase, *b*-standardized multiple regression coefficient, na- non applicable (conditions for regression analyses not fulfilled). Sex was coded as 1 for males and 2 for females; status was coded as 1 for captive and 2 for free-ranging bears. Some variables are transformed: SOD whole blood ($\log_{10}(x)$), Tl serum ($\log_{10}(x)$), Tl whole blood ($1/x$), Pb serum ($\log_{10}(x)$) and Pb whole blood ($\sqrt{x}$)

Table A.6 Serum and whole blood (mean±SD (range) median) metal(loid) levels of European brown bears (*Ursus arctos*) categorized by status

	Serum		Whole blood	
	Free-ranging (N=27)	Captive (N=10)	Free-ranging (N=26)	Captive (N=9)
Mg (mg/L)	18.8±2.0 (15.5-23.1) 18.9	20.3±1.7 (17.7-24.0) 20.3	21.5±3.1 (17.0-28.5) 21.2	20.6±3.1 (16.2-25.4) 19.8
Ca (mg/L)	91.6±8.0 (69.3-104) 92.7	91.6±8.5 (81.9-110) 92.1	30.9±4.9 (18.8-44.1) 31.2	33.0±10.7 (22.5-48.6) 28.9
Mn (µg/L)	3.80±1.37 (1.91-7.66) 3.60	2.56±0.62 (1.68-3.97) 2.42	23.6±6.7 (9.35-34.2) 23.4	18.0±7.8 (9.22-26.4) 18.1
Fe (mg/L)	7.57±3.49 (2.26-17.5) 6.57	3.98±2.41 (0.594-9.29) 4.37	363±50 (239-449) 356	328±65 (195-422) 350
Co (µg/L)	0.862±0.597 (0.151-2.55) 0.689	0.565±0.482 (0.130-1.46) 0.286	0.348±0.270 (0.074-1.10) 0.269	0.253±0.275 (0.074-0.812) 0.0744
Cu (mg/L)	0.789±0.401 (0.351-2.05) 0.680	0.803±0.667 (0.326-2.34) 0.499	0.480±0.158 (0.312-0.901) 0.406	0.452±0.272 (0.225-1.04) 0.344
Zn (mg/L)	1.35±0.28 (0.949-1.84) 1.31	1.18±0.19 (0.719-1.42) 1.18	2.06±0.28 (1.48-2.66) 2.03	1.86±0.27 (1.63-2.56) 1.78
As (µg/L)	(<0.678-1.50)[1]	0.684±0.570 (<0.678-2.11) <0.678[2]	<2.37-2.49[3]	<2.37[4]
Se (µg/L)	114±27 (72.7-207) 110	113±18 (92.7-156) 107	142±28 (80.2-195) 142	137±36 (94.2-204) 123
Mo (µg/L)	52.5±9.8 (38.4-71.2) 49.6	46.6±12.2 (22.6-65.1) 45.1	13.5±4.7 (3.38-23.3)14.1	13.0±4.2 (8.58-19.7) 11.5
Cd (µg/L)	0.120±0.046 (<0.070-0.248) 0.111[5]	0.073±0.035 (<0.070-0.129) 0.078[6]	0.298±0.260 (<0.247-1.21) <0.247[7]	<0.247[8]
Hg (µg/L)	(<0.375-1.11)[9]	<0.375[10]	1.41±1.49 (<1.31-6.32) <1.31[11]	<1.31[12]
Tl (µg/L)	0.050±0.043 (<0.009-0.158) 0.035[13]	0.048±0.027 (<0.009-0.087) 0.049[14]	0.067±0.031 (0.032-0.141) 0.053	0.052±0.013 (0.036-0.073) 0.054
Pb (µg/L)	0.893±0.538 (0.389-2.63) 0.691	1.07±0.99 (0.147-3.14) 0.858	61.2±25.5 (27.7-139) 64.0	49.2±53.0 (5.08-168) 29.6

[1]20/27 < As MDL (0.678 µg/L serum); [2]6/10 < As MDL (0.678 µg/L serum); [3]24/26 <As MDL (2.37 µg/L blood); [4]9/9 <As MDL (2.37 µg/L blood)
[5]1/27 < Cd MDL (0.070 µg/L serum); [6]4/10 < Cd MDL (0.070 µg/L serum); [7]14/26 < Cd MDL (0.247 µg/L blood); [8]9/9 < Cd MDL (0.247 µg/L blood)
[9]22/27 < Hg MDL (0.375 µg/L serum); [10]10/10 < Hg MDL (0.375 µg/L serum); [11]18/26 < Hg MDL (1.31 µg/L blood); [12]9/9 < Hg MDL (1.31 µg/L blood)
[13]2/27 < Tl MDL (0.009 µg/L serum); [14]1/10 < Tl MDL (0.009 µg/L serum)

Table A.7 Results of linear regression modelling revealing associations of non-essential metal levels between blood and hair of European brown bear (*Ursus arctos*), based only on significant confounding factors from Table A.4

Dependent variable	Independent variable	N	*b*	*p-value*	*R-squared*
Cd serum	Cd hair	28	-0.01	0.97	0.13
	status		0.37	0.18	
Tl serum	Tl hair	28	0.39	0.17	0.09
	status		-0.21	0.38	
	country		0.15	0.56	
Tl whole blood	Tl hair	30	0.17	0.52	0.10
	status		-0.22	0.33	
	country		0.33	0.19	
Pb serum	Pb hair	28	0.30	0.18	0.09
	status		-0.24	0.27	
Pb whole blood	Pb hair	30	0.07	0.76	0.09
	status		0.27	0.21	

b-standardized multiple regression coefficient. Status was coded as 1 for captive and 2 for free-ranging bears; country was coded as 1 for Croatia and 2 for Poland. Some variables are transformed: Cd hair ($\sqrt{x}$), Tl serum ($\log_{10}(x)$), Tl whole blood (1/x), Tl hair ($\sqrt{x}$), Pb serum and hair ($\log_{10}(x)$), Pb whole blood ($\sqrt{x}$)