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

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Abstract

We investigated the levels of five toxic 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 compartments of European brown bear populations in Croatia and Poland. Metal(loid) association with biomarkers of oxidative stress (superoxide dismutase, SOD; glutathione-peroxidase, GSH-Px; malondialdehyde, MDA) and of metal exposure (metallothionein, MT) was estimated to check for early signs of metal(loid)-related adverse health effects in this top predatory mammal. Lead was the most abundant of toxic metal(loid)s in both blood and hair, with 4/35 individuals having a blood level above the human threshold for haematological and cardiovascular effects in adults (100 µg/L). Positive Pb association with SOD activity in blood suggested possible impairment of antioxidative defence. Individuals under four years of age had certain blood and hair markers higher than adults. In the blood of females, SOD activity, Mn, Cd and Pb level were enhanced compared to males. In both of the sampled matrices, free-ranging bears had higher levels of metal(loid)s than captive bears. Hair showed a higher content of metal(loid)s when sampled in its growth phase and was not predictive of toxic metal(loid) blood levels. Established metal(loid) baseline values will enable future risk assessments in European brown bear populations.

Keywords: blood, hair, terrestrial mammal, oxidative stress, elements

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1. Introduction

The Dinarides and Carpathians are two of the few European habitats hosting three large carnivore species, of which the brown bear (*Ursus arctos*) is the most spread-out occurring in 22 European countries (Chapron et al. 2014). Brown bear population viability and health is of the highest interest for wildlife management and has been the target of substantial investments into conservation efforts in the last decades (Swenson et al. 2000, Kaczensky et al. 2012, Chapron et al. 2014). Concerning the long-range transport of metal-bearing particles, persistence and accumulation of inorganic pollutants along the food chain, the health of apex predatory mammals was recognized to be at an elevated risk due to their trophic position (Burger et al. 2007, Rodríguez-Jorquera et al. 2017). Toxic metal(oid)s (arsenic, cadmium, lead, and mercury) are among the health stressors (Tchounwou et al. 2012) recognized to adversely impact large terrestrial mammals (Reglero et al. 2009, Rodriguez-Estival et al. 2011, Berzas Nevado et al. 2012, Rodriguez-Estival et al. 2013), small mammals (Amuno et al. 2018) and birds (Koivula and Eeva 2010, Espin et al. 2014, Ortiz-Santaliestra et al. 2015), which might be reflected as perturbations in oxidative stress biomarkers. Oxidative stress-related toxic effects of metals are induced through the depletion of major antioxidants (e.g., glutathione, metallothionein), changes in activity of antioxidative enzymes (e.g., glutathione-peroxidase, superoxide-dismutase) and free radical levels which can damage biomolecules (DNA, proteins, membrane lipids) (Stohs and Bagchi 1995, Ercal et al. 2001). Except scavenging free radicals, small proteins metallothioneins (MTs) regulate level of essential metals (e.g., Zn, Cu) and detoxify non-essential metals (e.g., Cd, Hg) binding them to its sulfhydryl groups (Kägi 1991, Isani and Carpené 2014). If present in elevated levels, these MT binding metals can induce MTs (Kägi 1991, Klaassen et al. 1999), so MTs were proven to be suitable biomarkers of metal exposure (Gamberg and Scheuhammer 1994, M'kandawire et al. 2012, Ivanković et al. 2005, Durkalec et al. 2017). So far, MTs were regularly used in biomonitoring studies in aquatic ecosystems, while in terrestrial ecosystems their application involved metal exposure assessment in birds and large terrestrial mammals, but never in bear species. Adverse health effects of toxic metal(oid)s were also demonstrated through impairment of homeostasis of essential elements leading to their deficiency or excess state (Goyer 1997, Reglero et al. 2009, Durkalec et al. 2018, Kalisińska 2019).

Scarce data concerning environmental toxicant levels in the European brown bear populations were exclusively based on dead animal tissue levels of organic (Herceg Romanić et al. 2015) and inorganic contaminants (Medvedev 1999, Čelechovska et al. 2006, Flaten et al. 2008, Šprem et al. 2016, Lazarus et al. 2017, Lazarus et al. 2018a, Lazarus et al. 2018b). The Dinaric brown bear was recently found through invasive sampling to have the highest toxic metal(oid) liver and kidney levels among sympatric large mammals, with Pb and Cd in up to 5% of population above known threshold levels for mammals (Lazarus et al. 2017). Less invasive bioindicator tissues in European brown bears were not investigated for confirmation of health threats or possible adverse effects emerging in individuals bearing metal levels above the threshold limits. Concerning the strict protection status of brown bears under the Habitats Directive (92/43/EEC; EC/European Council 1992), non-lethal sampling for scientific purposes is of high conservational interest. Blood and hair represent a valuable biomonitoring data pool which excludes mortality, not to mention the possibility of longitudinal studies through resampling. Trace elements in blood are a marker of recent exposure, reflecting the net balance of element intake (mainly via food), deposition, and excretion. Also, elements in blood are bioavailable and thus relevant for toxicological risk assessment. Since there are still no bear-specific toxicity benchmarks for toxic metals in blood, the levels found in the literature were compared to those derived for humans. Consequently, the blood of the North American brown bear (grizzly, *Ursus arctos horribilis*), black bear (*Ursus americanus*), giant panda (*Ailuropoda melanoleuca*, Qinling subspecies) and polar bear (*Ursus maritimus*) was reported to have lead and mercury levels, respectively, ranging above the levels considered safe 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 because of the known interspecies differences in sensitivity to metal toxicity (Dietz et al. 2013), so relevant bear studies on metal-related exposure/effects and risk assessment should be a priority.

In contrast to blood, hair can reflect both recent and past exposure in time frames constrained by hair growth and moult. Only while growing, hair takes up elements from circulating blood and binds them to

abundant sulfhydryl groups in the cystine of the keratinized shaft, making elements inert for the host. This accumulative nature of toxic metals was amply used in hair pollution studies conducted on 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, Noël et al. 2015), mainly to track Hg biomagnification, but also in other predators, like the Iberian wolf (*Canis lupus signatus*; Hernandez-Moreno et al. 2013), golden jackal (*Canis aureus*; Malvandi et al. 2010), red fox (*Vulpes vulpes*; Dainowski et al. 2015), and arctic fox (*Vulpes lagopus*; Treu et al. 2018), but not the European brown bear. Characterization of hair-to-blood metal relation may help yield quality interpretations of toxic metal levels determined in hair, a minimally invasive sample, concerning toxicological risk for the individual and population. Although irrefutably connected, hair-to-blood metal levels showed a weak to strong relationship depending on the species, individual (age, foraging behaviour, weaning, growth and moulting status) and ecological factors (e.g., time of year) (Lieske et a. 2011, Peterson et al. 2016).

To the best of our knowledge, this study is the first to investigate hair and blood (plasma, serum, whole blood) levels of most relevant toxic metal(loid)s (As, Cd, Hg, Tl, Pb) and their hair-to-blood relation in European brown bears 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 check possible deficiencies/excess in both captive and free-ranging bears. In addition, we quantified MT as a biomarker of metal exposure, and the activity of some antioxidative enzymes (SOD, GSH-Px) and oxidative damage on lipids as biomarkers of metal-related toxic effects in the blood. This study aimed to set baseline values for given variables in European brown bears and explore the influence of various confounding individual and ecological factors.

2. Materials and methods

2.1. Animal sampling

We sampled hair from 46 and blood from 39 brown bears from Croatia and Poland in the period 2011-2017, of which 36 paired samples of hair and blood (at least one compartment: serum, plasma, whole blood) were collected (Table 1). Blood was resampled for six bears and hair was resampled for five bears. Altogether, four captive (one female, three males) and 16 free-ranging European brown bear individuals were sampled (six females, 10 males) in Croatia, and 10 captive (four females, six males) and 21 free-ranging bears (eight females, 13 males) in Poland. 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. Free-ranging bears in Poland (Carpathian population) were captured for telemetry research and management of problem bears in the Tatra and Bieszczady Mountains in Southern Poland, in the Western and Eastern part of Carpathians, respectively. Free-ranging bears in Croatia (Dinara-Pindos population) were captured in the Gorski kotar and Lika regions (Fig. 1). Complying with the Habitats Directive, the brown bear is strictly protected in both countries (Selva et al. 2011), but listed in Croatia also as a game species within the Directive's derogations provisions and managed through the Brown Bear Management Plan (Huber et al. 2008). Blood samples for this study were taken among samples designated for bear health investigations within frames of research and conservation projects at a time. Free-ranging bears were routinely aged using the premolar teeth cementum age determination method (Matson et al., 1993).

Captive bears were sampled in zoos and other captive institutions during regular veterinary checks, interventions or relocations. Before blood was collected from femoral vein in serum and whole blood (K_2EDTA coated) tubes, all bears were chemically immobilized and body measurements were taken. Serum and plasma were aliquoted into plastic tubes after the 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 a stainless steel scissors as close to the skin as possible (guard and undercoat hair together) and stored in a paper envelope at room temperature.

2.2. Element analyses

Hair was prewashed for 10 min on a vortex with 20 mL of ultrapure water (18 MΩ cm¸GenPure system, TKA, Germany) to eliminate soil particles as an external contamination source. The hair then

underwent the International Atomic Energy Agency (IAEA)-recommended washing procedure of 5 steps (acetone-water-water-water-acetone; acetone for gas chromatography MS SupraSolv®, Merck, Germany), each consisting of a 10 min vortex with 20 mL of solvent (Ryabukhin 1976). 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 a procedure described earlier (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 A.1 (hair) and Table A.2 (blood) together 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). The 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 of de-aerated supporting electrolyte (1 M NH_4Cl + 1 M NH_4OH, pH = 9.5, 6×10^{-4} M $[Co(NH_3)_6]Cl_3$) at constant temperature (20 °C). Serum was diluted twice with 0.9% NaCl (suprapur grade, Merck, Darmstadt, Germany), heated at 100 °C 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 by 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 (4.0x4.0 and 4.0x125.0 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 element for the purpose of statistical analysis. Blood samples collected from the same individual bear two times over the course of the study (bear 1, 3, 33 and 36, Table 1) were considered independent if the period between the resampling was more 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 (bear 4 and 15, Table 1) and 1 year for hair (bear 3) were averaged and taken as a single sample in analysis. We identified age, sex, status (captive vs. free ranging), country and date of sampling (only for hair) as factors potentially influencing the element levels in bear blood and hair. However, given the low sample number and taking into account all confounding factors, comprehensive linear regression analysis was not performed. Instead, a simpler model was used where each factor was tested separately by means of a *t* test for homogenous (Levene's test) and normally distributed (Shapiro-

Wilk's test) variables, while Mann-Whitney *U* test was used for heterogeneous and/or non-normally distributed data. For testing age as a categorical variable, individual bears were assigned to two age classes: adult (≥4 years) and subadult bears (<4 years; Knott et al. 2014, Lazarus et al. 2018b). The status of bears from Croatia and Poland was either free-ranging or captive (bears kept in zoo or other captive institution). According to date of sampling, hair was categorized as being sampled in its growth (May-October) or quiescent (November-April) phase (corrected for southern populations from Cattet et al. 2018). Associations of element levels versus age, MT, MDA and antioxidant enzymes or correlations between hair and blood were explored with Spearman rank order correlations (r_S) and interpreted according to Hinkle et al. (2003) where $0.3<r_S\leq0.5$ indicated low correlation, $0.5<r_S\leq0.7$ indicated moderate correlation, $0.7<r_S\leq0.9$ indicated high correlation, and $r_S>0.9$ indicated very high correlation. 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

3.1. Blood

Brown bear blood as an indicator of recent exposure of the host pointed Pb as the toxic metal(loid) of the highest relevance among As and Hg, which were mostly below the limit of detection (68-94% and 74-84% of all samples, respectively), and Cd and Tl which had an order of magnitude lower values than Pb in all compartments (Table 2). Specifically, Ca, Co, Cu, Mo and Tl were compartmentalized primarily in the serum/plasma and Mn, Fe, Zn, Cd and Pb in whole blood. Presence of Fe and Pb (>1%, Matović et al. 2015) in the serum/plasma of some individuals indicated various grades of hemolysis, as both elements are characteristic for erythrocytes. Activity of GSH-Px in the serum of all bears was below detection limit of the method and thus unavailable. Increasing age was moderately associated with decreased activity of SOD and decreased levels of Fe (low association), Co and Mo in blood, so subadult bears (<4 years) had higher levels of mentioned parameters than adult (≥4 years) ones (Table A.3). Opposite to Cu levels, females had higher SOD activity and Mn, Cd and Pb levels than males. Free-ranging bears had higher Mn, Fe, Zn, Mo, but also Cd and Pb levels in their blood compared to captive bears (Table A.3). Data for serum and whole blood categorized by status of bears are presented in Table A.4; however, plasma was omitted from the table because of the low sample number. 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 A.3). Seasonal variation in toxic metal(loid)s (only Cd and Pb taken into account, as Hg and As were mostly below MDL) displayed in Fig. 2A and 2C indicated two seasonal peaks in whole blood Cd and Pb in April-May and October corresponding to emergence from den/extensive feeding compared to winter period and prior to denning/end of hyperphagic period, respectively. Toxic Pb in serum (N=26, r_S=0.40, p=0.04) and whole blood (N=33, r_S=0.52, p=0.002) was associated with activity of enzyme SOD in the whole blood (Fig. 3). Low to moderate correlations were observed between essential elements and biomarkers of exposure (MT) and oxidative stress in the serum. MT showed low negative association with Tl (N=31, r_S=-0.44, p=0.02). MDA as an indicator of lipid peroxidation was poorly correlated with Fe (N=35, r_S=0.50, p=0.004), Cu (N=35, r_S=0.36, p=0.04) and Se (N=35, r_S=0.42, p=0.02), while activity of SOD in serum was moderately correlated with Fe (N=37, r_S=0.50, p=0.003), Co (N=37, r_S=0.54, p=0.001) and Mo (N=37, r_S=0.59, p<0.001) levels. Correlation matrix between Pb and essential elements in blood compartments revealed low association 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 in hair was much more abundant than in the blood, while Se was present in the smallest amount among essential elements in hair, but it was the most abundant element (Ca and Fe in whole blood) in serum and plasma. In addition, majority of elements with blood values below MDL (mostly toxic ones) was quantified in a higher percent in hair samples. Negative low to moderate association implied that with aging bears have lower Mn, Fe, Co, Zn, but also As, Cd, Tl and Pb hair levels (Table A.3). Testing sex as a host factor revealed no significant influence on elements in bear

hair. Free-ranging Croatian and Polish bears had higher Mg, Fe, Co, As, Cd and Tl than captive ones. Possibly because of the larger sample set from Poland than from Croatia (N=34 *vs*. N=16), the tested differences between captive and free-ranging bears from Poland were also significant for Zn and Pb (higher in free-ranging animals), while Mg and Ca were higher in captive bears. All hair sampled in bears from Croatia had Fe, Co, Mo, Tl and Pb higher than in bears from Poland. In addition, hair captured in its growth phase (May-October) had higher Mn, Fe, Co, Tl, and toxic As, Cd and Pb compared to hair in the quiescent phase (November-April).

The correlation matrix of each element between two matrices (blood and hair) revealed a moderate relationship only for Co (serum *vs*. hair r_S=0.60, p<0.001; whole blood *vs*. hair r_S=0.55, p=0.002) and low relationship for Ca (whole blood *vs*. hair r_S=-0.38, p=0.04) and Mo (whole blood *vs*. hair r_S=0.42, p=0.02).

4. Discussion

The brown bear is a long-living predatory mammal. Therefore, it was recently proposed as a good indicator species for environmental exposure to toxic metal(loid)s, relevant for both the bear population and ecosystem health assessment due to its trophic position, with defined influences of host and ecological factors on levels of metal(loid)s in target organs (liver, kidney, bone) during low lifetime exposure (Lazarus et al. 2017, Lazarus et al. 2018a, Lazarus et al. 2018b). This study examined blood as a non-lethal indicator of recent exposure and hair as blood-related tissue, covering exposure history somewhere between blood and liver or kidney. The obtained baseline data will enable future assessment of toxicological risk related to element toxicity or deficiency in the Dinara-Pindos and Carpathian population, or any of 10 European brown bear populations (Chapron et al. 2014).

4.1. Blood

Lead was the most abundant toxic metalmeasured in brown bear blood (Table 2), which is known as a critical target of Pb toxicity (Matović et al. 2015). 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, which makes up from 45-75% in adults up to 90% of PbB levels in children (Barbosa et al. 2005). These proportions are unknown for bear and other wildlife, but surely differ between humans and bears due to physiological differences, e.g., black bears supressed bone remodelling during hibernation (McGee-Lawrence et al. 2015). Mean (median) PbB levels in free-living (61.2 (64.0) µg/L) and captive (49.2 (29.6) µg/L) brown bears from Croatia and Poland (Table A.4) were similar to those in North American brown bears (median, 44 µg/L), but higher than those 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 the concentration in µg/g with blood density of 1060 g/L, Chen et al. 2018). Although very rarely reported in the literature, a few reports of wildlife mammalian PbB in Iberian ibex (*Capra pyrenaica*, detectable data were around 10.6 µg/L, recalculated from Ráez-Bravo et al. 2016), Australian Tasmanian devil (*Sarcophilus harrisii*, mean, 26.9 µg/L, recalculated by multiplying the concentration in µmol/g with Pb molar mass of 207 µg/µmol, Hivert et al. 2018) and Matschie's tree kangaroos (*Dendrolagus matschiei*) from Papua New Guinea (mean, 1.3 and 4.6 µg/L in wild and captive animals, respectively, Travis et al. 2012) showed lower levels than those found in bears from this study. However, PbB in captive giant pandas, tree kangaroos and Tasmanian devils were higher than in respective wild animals, which is opposite to the majority of Croatian and Polish captive bears. They have a two times lower median PbB than free-ranging brown bears (Table A.4). As for urbanized zoo locations, higher exposure to Pb in captive animals was expected as argued by Travis et al. (2012), but for brown bears it seems that food 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), thus high Pb is not expected, and the diet is fairly uniform throughout the year. On the contrary, free-ranging bears have seasonal variation in available food and especially in spring, when the majority were sampled in the current study, 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 rise with higher altitudes (ATSDR 2019), so this might have also influenced the higher Pb level in free-ranging bears inhabiting mountainous areas of Croatia and Poland. Another possible source of Pb for wildlife scavengers are bullet fragments left in carcasses of hunted animals (Martin et al. 2019). Although this source of Pb was confirmed in e.g., toxicosis in cougar (*Puma concolor*, Burco et al. 2012) and terrestrial birds of prey (Espin et al. 2014), 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 reported as part of a bear's diet (Cicnjak et al. 1987, Kusak and Huber 1998, Ważna et al. 2017, Bojarska 2015). Lacking a relevant effect threshold PbB levels for each wildlife species, other authors assessed possibility of adverse effects in wildlife regarding human (100 µg/L for haematological and cardiovascular effects in adults, ATSDR 2019; 50 µg/L, CDC reference value for children, Ettinger et al. 2019), domestic animal data (high PbB in cattle: 318-424 µg/L, horse: 318-636 µg/L, sheep: 742-954 µg/L, recalculated from Puls 1994) or threshold derived from various mammalian toxicity studies (180 µg/L, Buekers et al. 2009). Fifteen individuals (43%, N=35) from this study had a PbB above 60 µg/L, considered as a background level for cattle (Ma, 2011). Similar background Pb-B (mean 70 µg/L, median 50 µg/L) was reported for control mammals in dose-response studies reviewed by Buekers et al. (2009). 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 in background Pb-B 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 PbB ranging 60-350 µg/L. Also in brown bears from this study some indices of Pb-induced impairment of antioxidative defence (significant association of Pb with SOD in whole blood, Fig. 3), confirmed before in many epidemiological and animal studies (Flora et al. 2012, Matović et al. 2015), were seen, although 57% of individuals had PbB within the background range (<60 µg/L, Ma 2011). Increased activity of SOD found in erythrocytes of bear from this study with elevated serum and PbB levels can be a consequence of higher production of reactive oxygen species (Flora et al. 2012). However, the highest PbB levels measured in one male captive bear (168 µg/L) was still below the currently considered threshold for infertility and foetal abnormalities in mammals (>200 µg/L, Ma 2011). Median PbB levels in captive brown bears (29.6 µg/L) were similar to those recently reported for a general human population of men in Croatia (30.3 µg/L, range 3.2-142 µg/L, Kljaković-Gašpić et al. 2016), but levels in free-ranging bears were two times higher (64.0 µg/L, Table A.4). 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 is the second highest toxic metal in the blood of brown bears (Table 2), known to bind erythrocyte membranes, protein albumin and MT (Matović et al. 2015) upon absorption from the intestines. Croatian and Polish brown bears showed generally 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 East Greenland polar bears (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 come from the difference in diet, i.e., exposure rate or interspecies differences in Cd metabolism. Whatever influenced such a variation between species in blood as a marker of recent assimilation of Cd (ATSDR 2012), the result was a similar range of Cd 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), indicating that long-term exposure was similar. As in brown bears, Cd in whole blood of other wildlife species was reported low and under the limit of method detection (tree kangaroos, <1 µg/L, Travis et al. 2012; Iberian ibex, <10.6 µg/L, Ráez-Bravo et al. 2016) and near the general human population mean value (0.315 µg/L, ATSDR 2012). The highest measured whole blood value (1.21 µg/L) in brown bears was well below toxic thresholds for cattle (>42 µg/L, Puls 1994) and sheep (106-212 µg/L, Puls 1994). In addition, we found no association between Cd in blood and biomarkers of exposure (MT) or oxidative stress (SOD, MDA) in the studied individuals. Lack of Cd-MT relation could be due to an overall low Cd level in bear blood being unable to induce MT synthesis, as reported for marine

mammals with even higher Cd levels than in brown bears (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 order to be able to handle higher challenges of toxic metal exposure, oxidative stress and maintenance of essential element homeostasis, as primary functions of MT (Kägi 1991, Isani and Carpené 2014). In this study, MT was quantified in the brown bear (or any bear) species for the first time and MT levels were somewhat higher compared to a healthy population of Croatian men (0.230-0.687 mg/mL, Tariba et al. 2015) using the same method and apparatus. An age-related increase in human Cd blood levels and sex differences (higher Cd in females) in the reproductive period (Lee and Kim 2014) were not observed in brown bears. However, distinction in food 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) of free-living bears (Table A.4) compared to captive ones (0.073, 0.076 and 0.123 µg/L, respectively).

Other toxic metal(loid)s, like As and Hg were detected in 6-32% and 16-26% of brown bear samples (Table 2), respectively, and in 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) with the highest Hg levels in blood up to 739 µg/L (reviewed in Dietz et al. 2013). The polar bear is a part of the marine food web known of higher Hg bioaccumulation and biomagnification (Scheuhammer et al. 2015) compared to brown bears and Florida black bears which are exclusively part of the terrestrial food web. Thus, the Hg difference between our bears and Florida black bears point to geographical differences, because the diet of these populations is very similar (Murphy et al. 2017, Cicnjak et al. 1987, Kusak and Huber 1998). All of the brown bears had blood Hg within normal range established for cattle, sheep (<106 µg/L, Puls 1994) and humans (<20 µg/L, Klaassen et al. 2013). Thallium was present in blood of brown bears in much lower levels than the mentioned toxic metals like Cd, Pb, or Hg, but its toxicity was 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), while lacking any wildlife data.

According to mammalian and human thresholds and guidelines for toxic metal(loid)s, only the level of Pb in the brown bear blood compartments raised concern regarding possible adverse health effects, taking into account blood as a marker of recent exposure. Interaction of Pb with essential elements (Na, Mg, Ca, Fe, Cu, Zn) due to chemical and physical resemblance can cause their deficiency, or affect enzyme activity and function of cells and organs (Telišman et al. 1995, Goyer 1997, Flora et al. 2012). Associations of Pb with Mn, and Zn found in brown bear might point to the replacement of Pb with essential elements in δ-ALAD and SOD or shared binding sites on transporters in intestinal cells (Telišman et al. 1995, Goyer 1997, Peraza et al. 1998). For adverse relations between Pb and Mo, authors have no apparent explanation, but it might be connected to well-defined Cu-Mo interaction, as Pb was reported to reduce Cu storage in sheep (Puls 1994).

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 toxic metal in the hair of brown bears, followed by Hg, As Cd and Tl. All toxic metal(loid)s except for 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 as more important storage site than the hair, taking into account the exposure timeframe that hair reflects (medium exposure window).

Bearing in mind 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 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. North American brown bear females from Yellowstone had comparable Hg with brown bears in this study, while males had higher Hg in hair (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 toxic 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).

Hair reflected higher toxic metal(loid) levels in subadult bears (<4 years) compared to adults in contrast to the observed lack of age differences noted in blood of studied bears. This discrepancy between blood and hair is expected as hair is a long(er)-term storage site for elements compared to blood. Absence of age-related differences in hair Hg was in line with the report of Cardona-Marek et al. (2009) for Southern Beaufort sea polar bear and Icelandic arctic fox (Treu et al. 2018), but differed from findings of higher Hg in adult individuals' hair in Western Hudson bay polar bear (Bechshoft et al. 2016) and Alaskan reindeer (Duffy et al. 2005). 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. A similarly opposite direction of age-related renal Pb accumulation was reported between Croatian grey wolf and brown bears (Lazarus et al. 2017). Also the essential elements (Mn, Fe, Co, Zn) were higher in hair of subadult bears from this study than of adults. This may reflect the known enhanced demand for essential elements in young/growing organism which is then followed by enhanced absorption from the gastrointestinal tract and consequently higher deposition in the hair. Cardona-Marek et al. (2009) reported higher Hg in hair of female polar bears, while elements in bears from this study failed to differentiate between the sexes, as seen also for the Iberian wolf (Hernandez-Moreno et al. 2013) and arctic fox (Treu et al. 2018). Free-ranging brown bears had enhanced hair toxic metal(loid)s level with more pronounced fluctuations compared to captive bears. The same as for blood, our assumption was that lower exposure of captive bears through human-intended food triggered the aforementioned difference. Also, metal content in hair plotted against sampled months reflected the uniformity of captive bear diet throughout the year, especially in 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, except for seasonal changes in the diet of free-ranging bears, seasonal cycle of hair complicates the interpretation of element levels found in bear hair. In general, brown bear hair in 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 are different from free-living bears, because of the shorter hibernation and higher food availability (Cattet et al. 2018). Thus, hair physiology might also make impact on elements in captive *vs*. free-living populations. That may have influenced significant differences in metal(loid)s measured in hair sampled in May-October *vs*. hair sampled in November-April (Table A.3), which disappeared once animals were categorized according to captivity status (captive/free-living; data not shown). However, we believe that the peaks observed in hair element levels sampled in May and October mostly reflected enhanced dietary exposure in spring and hyperphagia in fall, as a similar pattern was also confirmed in the blood of bears (Fig. 2). Nonetheless, more firm conclusions can be drawn once a larger sample is investigated in the future.

While investigating element associations between two matrices (blood and hair) of brown bear, we noted that toxic metal(loids) in one matrix were not predictive of levels in the other matrix. 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), diet exposure had seasonal variations and some bears could have been impacted by a fasting period marked with element mobilization into blood without subsequent transfer into hair (as hair was in the quiescent phase). So, our study scheme aiming at annual monitoring, was not able to address the blood-to-hair correlation very well. In order to do so, newly grown hair after moulting should be sampled.

5. Conclusion

The here reported first quantification of toxic metal(loid)s in blood and hair of European brown bear revealed Pb as the most abundant metal, possibly toxicologically relevant for bears. In 11 % of individuals, blood Pb crossed human threshold levels. Association with a biomarker of oxidative stress (activity of enzyme SOD) and some essential elements (Mn, Zn and Mo) pointed at possible Pb-related adverse health effects needing confirmation in future, higher sample scale studies. All of the tested confounding factors (age, sex, status, country, hair growth phase) showed significant influence on metal(loid)s level and even some oxidative stress biomarkers, with the exception of sex factor in hair parameters analysis. Hair sampled throughout the year was not proven to reflect toxic metal(loid) levels in blood of bears. Taking into account also Pb, brown bears from this study had toxic 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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The authors have no competing interests to declare.

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Figure caption

Fig. 1 European brown bear (*Ursus arctos*) sampling locations are marked with dots (free-ranging animals) and hash marks (captive animals)

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) according to sampling month

Fig. 3 Relationship between superoxide-dismutase (SOD) activity (x-axis) and lead level (y-axis) in the whole blood of 33 European brown bear (*Ursus arctos*). Spearman's correlation coefficient (r_S) with the level of significance indicates the degree of relation between the variables

Table1

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

Table2

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

	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 were listed according to 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)

Table3

Table 3. Level of metal(loid)s in European brown bear (*Ursus arctos*) hair

	All (N=50)	Croatia			Poland		
	mean±SD (range) median	Free-ranging (N=13)	Captive (N=3)	*p*	Free-ranging (N=21)	Captive (N=13)	*p*
Mg (mg/kg)	121±72 (24.6-317) 116	136±64 (24.6-213) 150[a]	85.9±38.0 (31.4-116) 98.1	0.243	83.9±43.0 (28.4-176) 67.2[a]	178±86 (39.8-317) 180	**<0.001**
Ca (mg/kg)	940±733 (224-3757) 694	769±347 (402-1764) 717[a]	1068±450 (397-1355) 1260	0.248	488±304 (224-1656) 412[b]	1801±846 (615-3757) 1821	**<0.001**
Mn (mg/kg)	10.3±8.9 (0.487-38.2) 7.86	17.4±10.4 (5.66-38.2) 16.5[a]	3.61±3.76 (0.487-8.58) 2.68	**0.011**	10.2±7.7 (2.42-35.7) 7.95[b]	4.82±3.41 (1.30-10.3) 3.82	**0.003**
Fe (mg/kg)	69.3±64.7 (7.90-289) 57.1	128±60 (62.4-270) 115[a]	27.9±20.4 (12.2-56.1) 21.6	**<0.001**	67.2±45.6 (13.9-180) 60.7[b]	40.0±75.4 (7.90-289) 17.5	**0.003**
Co (µg/kg)	67.3±70.9 (<8.14-300) 39.8[1]	150±80 (38.8-300) 130[a]	24.9±24.5 (<8.14-51.4) 22.0	**0.008**	56.3±42.7 (<8.14-165) 42.1[b]	15.5±15.1 (<8.14-51.4) 9.23	**<0.001**
Cu (mg/kg)	10.0±2.1 (6.30-15.5) 9.77	10.6±2.3 (7.24-15.5) 10.8	8.62±1.88 (6.52-15.5) 8.81	0.127	9.77±2.40 (6.30-15.0) 9.50	10.1±1.44 (8.12-12.9) 9.90	0.608
Zn (mg/kg)	140±13 (112-177) 140	144±13 (126-177) 142	144±17 (128-161) 143	0.959	142±12 (124-173) 142	130±11 (112-152) 128	**0.011**
As (µg/kg)	95.3±102.8 (<22.4-404) 60.7[2]	164±123 (48.2-404) 102[a]	25.7±17.2 (<22.4-44.7) 23.5	**<0.001**	91.0±101.3 (32.9-403) 55.1[b]	54.4±55.0 (<22.4-187) 28.8	**0.020**
Se (mg/kg)	0.489±0.167 (0.238-1.00) 0.446	0.516±0.167 (0.284-0.809) 0.516	0.522±0.096 (0.390-0.622) 0.538	0.798	0.450±0.167 (0.238-1.00) 0.421	0.516±0.188 (0.312-1.00) 0.446	0.232
Mo (µg/kg)	71.8±45.0 (<12.0-258) 61.7[3]	103±59 (36.7-258) 97.4[a]	76.2±37.8 (43.8-128) 66.4	0.429	52.3±29.6 (<12.0-108) 47.0[b]	70.3±35.5 (20.3-140) 67.2	0.157
Cd (µg/kg)	26.8±24.6 (<6.37-99.8) 16.9[4]	46.0±28.0 (16.2-99.8) 35.1	7.09±4.96 (<6.37-13.5) 5.83	**0.002**	31.3±20.6 (12.5-93.0) 27.6	6.59±5.12 (<6.37-15.1) 3.19	**<0.001**
Hg (µg/kg)	128±128 (<43.0-562) 91.8[5]	188±184 (<43.0 -562) 94.6	59.4±75.8 (<43.0-173) 21.5	0.172	140±118 (<43.0-486) 104	78.8±66.0 (<43.0-192) 60.2	0.060
Tl (µg/kg)	3.99±2.80 (<0.6-12.5) 3.72[6]	7.66±2.55 (4.04-12.5) 6.59[a]	3.31±1.19 (2.24-4.52) 3.24	**0.003**	3.70±1.97 (0.852-10.2) 3.59[b]	1.84±1.55 (<0.6-5.35) 1.24	**0.002**
Pb (µg/kg)	401±354 (30.3-1553) 302	542±367 (153-1442) 382	521±584 (140-1194) 228	0.538	439±339 (147-1553) 347	151±176 (30.3-628) 79.2	**<0.001**

p-level of significance for differences in elements between free-ranging and captive bears of the same country tested by the Student t-test or Mann-Whitney U-test. Elements were listed according to 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)

[ab]Different superscript letters point to significantly different (p<0.05) element values for free-ranging bears between countries. None of the elements tested in captive bears showed significant differences between countries. Differences were tested by the Student t-test or Mann-Whitney *U*-test

Figure1

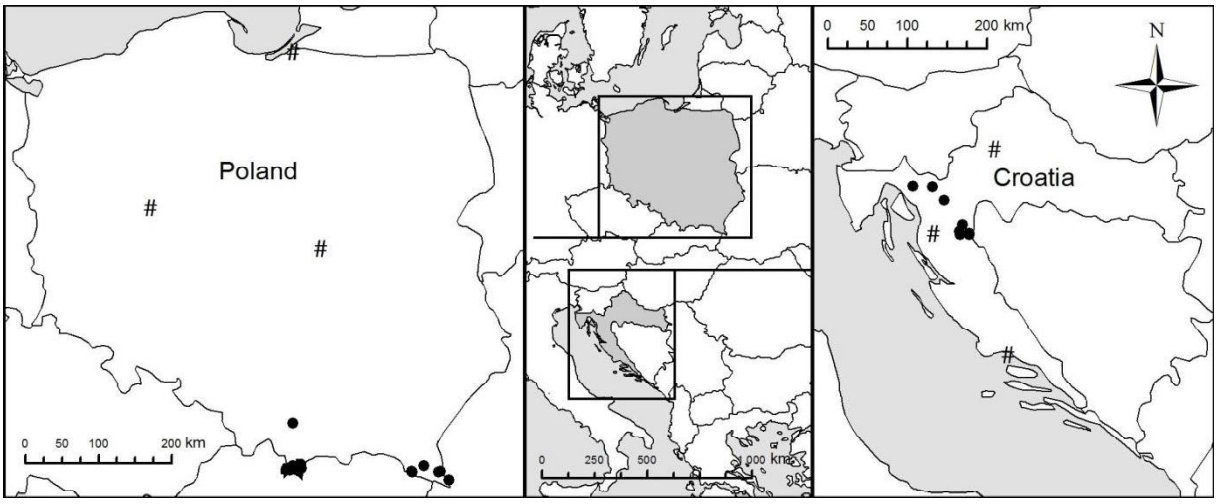

Fig 1.

Figure2

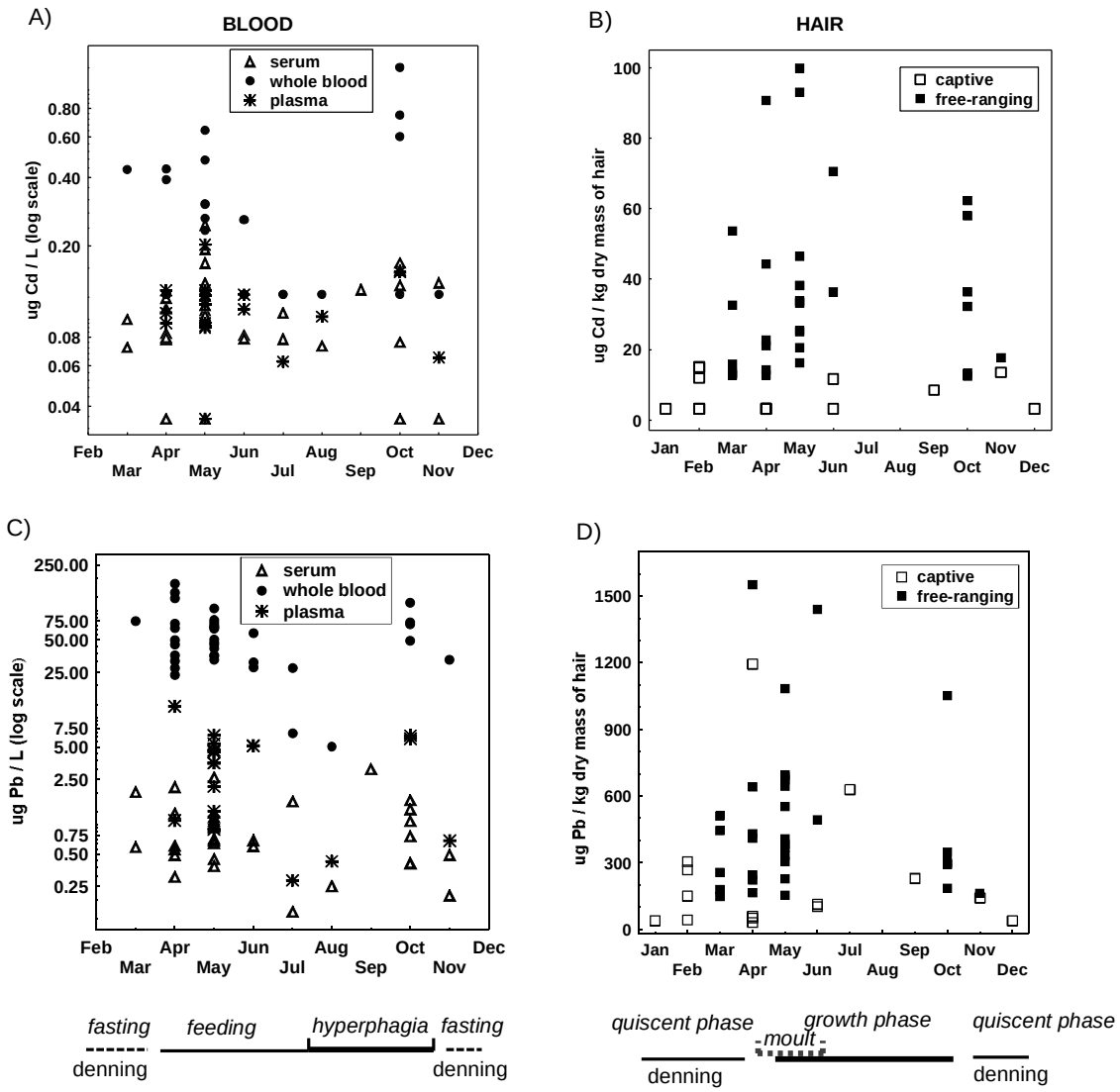

Fig. 2

Figure3

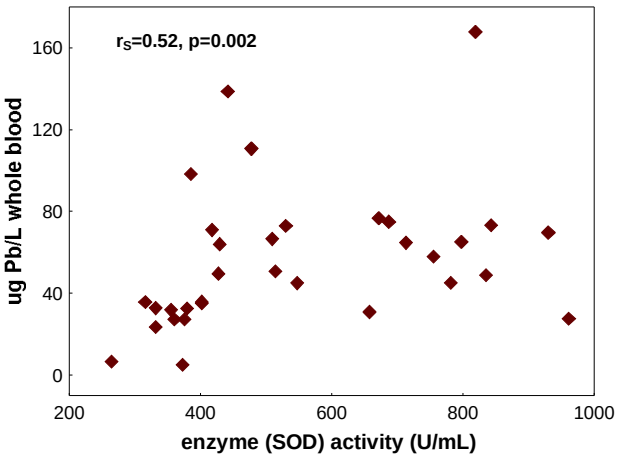

Fig 3.

Supplementary Material

Click here to download Supplementary Material: Appendix A.docx

CRediT author statement

M.L.: Conceptualization, Formal Analysis, Writing-Original Draft, Visualization. **T.O.:** Methodology, Formal Analysis. **A.S.:** Investigation, Resources, Data Curation, Writing – Review & Editing, Project Administration, Funding Acquisition. **L.V.:** Formal Analysis, Investigation, Resources. **V.F.M.:** Methodology, Formal Analysis, Investigation, Writing - Review & Editing. **D.R.:** Methodology, Formal Analysis. **S.R.:** Resources, Writing - Review & Editing. **J.A.:** Validation, Formal Analysis, Resourses. **T.Z.K.:** Investigation, Resources, Writing-Review & Editing. **F.Z.:** Investigation, Resources. **J.J.:** Resources, Funding Acquisition. **M.E.:** Supervision, Writing - Review & Editing. **R.M.:** Resources. **N.S.:** Resources, Writing-Review & Editing, Funding Acquisition. **Đ.H.:** Resources, Writing-Review & Editing, Supervision.

Conflict of Interest form

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: