Metal and metalloid exposure and oxidative status in free-living individuals of *Myotis daubentonii*

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Abstract

Metal elements, ubiquitous in the environment, can cause negative effects in long-lived organisms even after low but prolonged exposure. Insectivorous bats living near metal emission sources can be vulnerable to such contaminants. Although it is known that bats can bioaccumulate metals, little information exists on the effects of metal elements on their physiological status. For example, oxidative stress markers are known to vary after detoxification processes and immune reactions. Here, for two consecutive summers, we sampled individuals from a natural population of the insectivorous bat, *Myotis daubentonii*, inhabiting a site close to a metal emission source. We quantified essential metal elements (Ca, Co, Mn, Cu, Se, Zn), non-essential metal elements (Cd, Ni, Pb) and a non-essential metalloid (As, ~~Se~~) from individual fecal pellets. We measured antioxidant status (GP, CAT, SOD, tGSH, GSH:GSSG) from their red blood cells together with biometrics, hematocrit and parasite prevalence. In general, metal concentrations in feces of *M. daubentonii* reflected the exposure to ambient contamination. This was especially evident in the higher concentrations of Cd, Co, Cu and Ni close to a smelter compared to a site with less contaminant exposure. Annual differences were also observed for most elements quantified. Sex-specific differences were observed for calcium and zinc excretion. SOD and CAT enzymatic activities were associated with metal levels (principal components of six metal elements), suggesting early signs of chronic stress in bats. The study also shows promise for the use of non-invasive sampling to assess the metal exposure on an individual basis and metal contamination in the environment.

1. Introduction

Bats are vulnerable to the exposure of various environmental pollutants, including organic contaminants and heavy metals (Walker et al. 2007, Naidoo et al. 2013, Bayat et al. 2014, Zukal et al. 2015). The longevity (Salmon et al. 2009, Munshi-South and Wilkinson 2010) and high trophic position of bats increases the likelihood of bioaccumulating pollutants in their tissues (Senthilkumar et al. 2001, Wada et al. 2010, Zukal et al. 2015). Population-level adverse effects associated with sustained contaminant exposure have been found (Gerell and Lundberg 1993), while individual cases of metal and pesticide poisoning have been anecdotically reported (Zook et al. 1970, Sutton and Wilson 1983, Skerratt et al. 1998). Metal-related effects in bats can be genotoxic (Zocche et al. 2010, Karouna-Renier et al. 2014, Naidoo et al. 2015), neurologic (Nam et al. 2012) and immunological (Pilosof et al. 2014), all generally linked to a continued chronic exposure.

Metallic elements occur naturally in the environment (Tchounwou et al. 2012). However, anthropogenic activities including industrial (mining, smelting), agricultural (pesticide and fertilizer application), domestic (lead-based paint and leaded-gasoline exhaust) and technological applications have contributed to the increment and spread of metals in various terrestrial and aquatic ecosystems (Hoffman et al. 2003). Particularly, industrial activities emit a combination of metals into the atmosphere, which end up deposited into soil and living matters such as plants and soil-dwelling invertebrates. Thus, metals also enter the food chain, e.g. through invertebrate diet items consumed by higher-trophic positioned animals (Park et al. 2009, Lilley et al. 2012, Méndez-Rodríguez and Alvarez-Castañeda 2016).

Long-term toxicant exposure can cause immune system disturbances, antioxidant depletion and DNA damage (Zocche et al. 2010, Lilley et al. 2013, Stauffer et al. 2017). Heavy metals can modulate immunological responses, for example impairing phagocytic activity of the exposed individual (Boyd 2010). One of the proposed mechanisms of metal toxicity is via oxidative stress (Valko et al. 2005, Regoli et al. 2011), which is the imbalance between antioxidants and oxygen radicals. Oxidative stress as a response to metal related toxicity has been described for wildlife, including birds, mammals and fish (Regoli et al. 2011, Costantini et al. 2014). In bats, oxidative stress markers have been analysed in relation to immune challenge (Schneeberger et al. 2013), but studies investigating the effects of environmental pollutants in relation to oxidative stress are more scarce (Lilley et al. 2013). The combination

of industrial disturbance, habitat destruction and parasite presence can result in physiological stress (Gerell and Lundberg 1993, Kannan et al. 2010). One of the host responses to parasite infestation may be an excessive production of oxygen radicals by phagocytic cells, also referred to as oxidative burst (Costantini 2014).

Here, we measure oxidative status of free-living insectivorous bats exposed to industrial metal pollution. We studied Daubenton's bat (*Myotis daubentonii*) individuals from geographically separated natural populations, one of which roosted and forage close to a source of metal emissions i.e. a Copper (Cu) – Nickel (Ni) smelter and other individuals at a less contaminated site. In bats, studies linking toxicant challenge to physiological alterations unfortunately have mostly required destructive sampling, since internal organs have been used to determine metal concentrations. Here, we collected individual bat fresh fecal pellets to quantify the following elements: Arsenic (As), Calcium (Ca), Cadmium (Cd), Cobalt (Co), Cu, Manganese (Mn), Lead (Pb), Ni, Selenium (Se) and Zinc (Zn). In addition, we extracted a minimal amount of blood from the same individuals to measure markers of oxidative stress: the ratio between Reduced Glutathione (GSH) and Oxidized Glutathione (GSSG) i.e. GSH:GSSG ratio, Glutathione Peroxidase (GP), Catalase (CAT) and Superoxide Dismutase (SOD). Based on oxidative status alterations found in other small mammals exposed to toxic metals (Viegas-Crespo et al. 2003), we hypothesize that the metal-exposed bats develop oxidative stress in response to elevated toxic metals in the environment at contaminated sites compared to our less contaminated reference site. However, given the unique characteristics of insectivorous bats, i.e. use of torpor, longevity and high basal antioxidants compared to other mammals (Wilhelm Filho et al. 2007), it is possible that the antioxidant machinery in bats may counteract metal-related challenges. This is the first study reporting physiological oxidative status effects of metal contamination on non-captive bat individuals.

2. Materials and methods

2.1. Study species and study area

Bats were trapped during May-August 2014 and 2015 in the vicinity of a smelter in Harjavalta (61°20′N, 22°10′E), and at an old water mill in Lieto (60°33'N, 22°27'E) with a combination of harp traps and mist nets (2.5 m height; Ecotone, Poland) placed along flying corridors during their emergence time from roosting sites in Harjavalta (n=32), and hand trapped into cloth bags

in Lieto (n=19). *Myotis daubentonii,* is an insectivorous trawling bat distributed across Europe and Asia. The species roosts in tree cavities, bird boxes or buildings close to water bodies, where it forages for insects, mainly Chironomidae (Dietz et al. 2009, Encarnação et al. 2010, Vesterinen et al. 2016). Chironomids, or non-biting flying midges spend a part of the life-cycle as filter-feeders within sediments of water bodies. They are therefore prone to accumulate chemicals or toxicants discharged into the water bodies and deposited over time in the water bottom (Lilley et al. 2012).

Myotis daubentonii roost frequently in tree cavities, but they also take human-made constructions (Joint Nature Conservation Committee 2007, Dietz et al. 2009). They normally breed in colonies, and they can form subgroups within a colony due to their mobility thus not being loyal to a specific roosting site within a cave. However, individuals do show area roost fidelity (Lucan and Hanak 2011, Ngamprasertwong et al. 2014). Generally, *M. daubentonii* become sexually mature at their first year, being able to reproduce in late summer (Encarnação et al. 2004). However, observations of male *M. daubentonii* being sexually matured at their year of birth and consequently being able to reproduce before their first hibernation period have been reported (Encarnação et al. 2006). For summer roosts, they normally choose tree cavities, bird boxes or buildings.

Here, we sampled a bat population in a forest patch close to an air metal emission point source in Harjavalta, Western Finland. Harjavalta is an industrial town characterized for its metal processing activities particularly the smelting of copper and nickel (Kiikkilä 2003). Emissions also include arsenic, zinc, cadmium, mercury, lead and sulphur as the smelting process by-products (Kiikkilä 2003). A river, Kokemäenjoki, runs through the town and is the main feeding ground for the bats in our study. This river system has a large catchment basin (27000 km^2) including 16% of agricultural land (VARELY 2011). In 2014, an accidental metal discharge from the smelter in Harjavalta released 66 tons of nickel into the Kokemäenjoki-river (KVVY ry. 2016). The second and less metal exposed bat group in our study, roosts in an old water mill in Lieto. This bat population has been previously monitored for some years (Laine et al. 2013, Vesterinen et al. 2016), but the metals are quantified for the first time in this study. The water mill is located along the Aura-river in South-Western Finland and has a catchment basin of 874 km^2 of which 37% is agricultural land (Huttunen et al. 2016).

2.2. Sampling and biometric measurements

Caught bats were identified to species and banded. Weight was recorded to the nearest 0.1 g with a Pesola spring balance and forearm length was recorded to the nearest 0.05 mm with a sliding caliper. Sex was determined, and age was classified into adults and juveniles according to the ossification state between phalanges (Brunet-Rossinni and Wilkinson 2009). Fur and wing were inspected for ectoparasites. Bats often defecate when handled, thus fresh fecal pellets were collected per individual and used for metal analysis. Blood was obtained (up to a maximum of 65 µL) from the interfemoral vein into a heparinized capillary tube (Marienfield 80iu/ml) and immediately centrifuged at 4400 g for 5 minutes in a LW Scientific ZIPocrit Hematocrit Centrifuge to separate the red blood cell fraction from plasma. The hematocrit (proportion of red cells) was measured with a sliding caliper. The red blood cells and plasma were placed separately into tubes, flash frozen in liquid nitrogen and stored at −80 ºC until the oxidative marker analyses. The blood metal concentrations were not measured because there was not enough blood material to quantify both the metals and oxidative status parameters. All bats were released after sampling. Collection licences were approved by the Animal Ethics Committee of the University of Turku (license number ESAVI/3221/04.10.07/2013) and Centre for Economic Development, Transport and the Environment (license number VARELY/948/2015).

2.3.Metal analysis

Fecal pellets (one sample belonging to one individual) were dried separately at 50°C for 48 hours. Dried samples were weighted and dissolved in a mixture of Suprapure acids, 3 mL HNO_3 and 1 mL H_2O_2 with a microwave digestion system (Anton Paar Microwave Sample preparation System, Multiwave 3000). After that, samples were diluted to 50 µL per sample with de-ionized water. The elements chosen for quantification were: the essential elements (Ca, Co, Cu, Mn, Ni, Se and Zn), the non-essential metals (Cd, Pb) and the non-essential metalloid (As). Generally, most of these chosen elements have been referred to as “heavy metals”. However, no chemical consensus (e.g. atomic number, density, etc.) exists in the definition of heavy metals (Duffus 2002). The term“heavy metals” have been used to refer to a group of metals, metalloids and other elements or compounds which exert toxicity. Generally, “heavy metals” include Cd, Hg and Pb. In this manuscript, when referring to all the selected elements we quantified, we will address them as “metals” or “metal elements” since this arbitrary grouping includes essential and non-essential metals as well as metalloids.

The determination of metal element concentrations was conducted with inductively coupled plasma mass spectrometer ICP-MS (Elan 6100 DRC+ from PerkinElmer-Sciex), by using a quantitative standard mode. The detection limit for most of the metal elements was around 1 ppt (ng/L) and below. The instrument was calibrated with a commercial multi-standard from Ultra Scientific, IMS–102, ICP-MS calibration standard 2. Certified reference materials from European Reference Material (mussel tissue ERM-CE278K-8G) were used for method validation. In 2014, the mean recoveries (±SE) in nine reference samples were as follows: Ca 98 ± 15.98%, Mn 98 ± 3.29%, Co 101 ± 1.52%, As 96 ± 1.79%, Pb 95 ± 3.25%, Ni 120 ± 2.41%, Cu 100 ± 2.44%, Cd 91 ± 1.79%, Zn 87 ± 1.80%, Se 151 ± 26.20%. In 2015, the mean recoveries (±SE) in six reference samples were as follows: Ca 113 ± 8.39%, Mn 112 ± 4.21%, Co 101 ± 2.25%, As 99 ± 1.24%, Pb 89 ± 2.05%, Ni 111 ± 4.85%, Cu 100 ± 2.43%, Cd 92 ± 1.90%, Zn 93 ± 1.51%, Se 118 ± 5.44%. The results are expressed as μg/g on a dry weight (d.w.) basis.

2.4. Oxidative stress and oxidative damage analysis

Concentrations of antioxidants and enzymatic activities were measured from red blood cells in triplicate using 96-well and 384-well microplates. Protein content was determined using the Bradford method with bovine serum albumin as a standard and BioRad protein assay reagent (Bradford 1976). Inter-assay variation was normalized by using the same control samples of known enzymatic activities. Measurements were obtained using EnSpire plate reader (Perkin-Elmer).

2.4.1. ***Glutathione***

Glutathione, an important cellular antioxidant used as a substrate for the enzyme glutathione-S-transferase in Phase II detoxification of chemicals (Sies 1999) was quantified in its reduced (GSH) and oxidized form (GSSG) using a ThioStar glutathione detection reagent purchased from Arbor Assays. First, samples were pre-processed by removing proteins with a solution of 5% sulfosalicylic acid, then diluted to 1% SSA with sample dilution buffer. In a 384-well black microplate (Perkin Elmer), 6.5 μL of Thiostar reagent was added to 12.5 μL of standard, sample or blank, incubated in dark for 15 minutes and fluorescence emission measured at 510 nm, with excitation of 405 nm to determine the free GSH concentration. Then, 6.5 μL of reaction mixture (4 mM NADPH+8U/ml GR) were added, incubated for 15 minutes and fluorescence measured at same excitation and emission wavelengths to determine the total glutathione concentration (tGSH = GSH+GSSG). Unit.

2.4.2. Glutathione Peroxidase

Glutathione Peroxidase (GP) Cellular Activity Assay Kit was purchased from Sigma (Catalog No CGP1). Glutathione peroxidase activity is determined indirectly, by first quantifying the conversion of reduced glutathione (GSH) to oxidized glutathione (GSSG), followed by the reduction of GSSG back to GSH, catalyzed by the enzyme glutathione reductase (GR) and Nicotinamide Adenine Dinucleotide Phosphate Reduced (NADPH). Procedures were carried out following the manufacturer instructions, except using 2 mM H_2O_2 as a substrate. The assay was performed in a clear 384-well plate (Perkin Elmer). Briefly, to each well were added: 35 µL of assay buffer, 5 µL of NADPH assay reagent, 5 µL of 2 mM H_2O_2 and 5 µL of blood sample to obtain a final volume of 50 µL. Five µL of assay buffer were used as blank. The absorbance was measured at 340 nm (A340) for 60 seconds in a kinetic program using an Envision microplate spectrophotometer. The activity of GP expressed as pmol/min/mg was calculated by dividing the A340 by the extinction coefficient of NADPH (6.22).

2.4.3. Catalase

The activity of catalase (CAT), an enzyme which converts hydrogen peroxide into water and oxygen, was quantified following the protocol instructions of the CAT-assay kit (Sigma Catalog No CAT 100). To perform the assay in a 96-well microplate format, the volumes of reagents and samples were reduced. Assay solutions (peroxide, peroxide-solution, assay buffer, chromogen, Sodium Azide (NaN_3) - stop solution and enzyme dilution buffer) were prepared according to the information in the Sigma kit technical bulletin. Briefly, 2 µL of sample (1mg/mL) and 13 µL of assay buffer were mixed in a tube. The reaction was stopped with 180 µL of 15mM NaN_3. The CAT activity expressed in µmol/min/mg was colorimetrically quantified by adding 200 µL chromogen in each well to 2 µL aliquot of the stopped reaction solution. Absorbance was measured at 520 nm.

2.4.4. Superoxide Dismutase

Superoxide dismutase (SOD) assay kit was purchased from Sigma-Aldrich (Catalog No 19160). The reaction determines the inhibition activity of SOD by a colorimetric method. The water-soluble salt WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) reacts with the superoxide anion to produce a formazan dye. The reduction rate of reduced superoxide anion (i.e. O_2^-) is directly related to the enzyme xanthine oxidase (XO) which is inhibited by SOD. In a 384-well plate, 45 µL of WST-solution were added to 5 µL of sample (1mg/mL). Then 5 µL of xanthine oxidase (XO) enzyme were

added to each well and incubated at 37°C for 20 min. Absorbance was measured at 450 nm. SOD activity is expressed as inhibition rate percentage. Unit.

2.3. Statistics

We used a dataset including Harjavalta and Lieto, observations for which metal data was available during 2014 and 2015 (n=51). To explain variation in biometric data (n=50), we built linear models (LMs) separately for body mass and forearm length (using the Glimmix procedure) in SAS 9.4. In these models, we included year, sex, age, location and the interaction between year and location as explanatory variables. For a more complete picture of bats' health condition, we also analyzed the effect of the same explanatory variables on hematocrit (ratio between red blood cells and whole blood volume; n=44) and parasite prevalence (in wings and fur; n=51).

We analyzed the correlations between metals with Pearson correlation analysis and investigated variation in metal concentrations (As, Ca, Cd, Co, Cu, Mn, Ni, Pb, Se, Zn) in individual bat feces (n=51) using the same explanatory variables used for biometrics i.e. year, sex, age location and the interaction between year and location. Terms were removed if not significant, one at a time starting with interactions. The metal concentrations were log-10 transformed before analysis to comply with normality requirements in the model. We also use Pearson correlation analysis to examine the associations of metals with parasite prevalence, biometric data and oxidative stress parameters (log-10-transformed SOD, CAT, tGSH, GSH:GSSG and GP).

For further modelling of the effects of pollution level on morphological and physiological parameters we built principal components of six metal elements (As, Cd, Co, Cu, Ni, Pb) to reduce the information of multiple inter-correlated variables (metal concentrations) into smaller number of variables, or components, explaining most of the variation in the data. The selection of these elements was based on their toxic degree (Tchounwou et al. 2012), their strong correlation with each other and their consistent elevated concentrations around the smelter source. The effects of metal exposure may negatively affect vital physiological functions potentially leading to body mass loss (Eeva and Lehikoinen 1996, Dauwe et al. 2006). We investigated this in a model with the first and second principal components of metals (PC1, PC2) as an explanatory variable for body mass and with sex, age and location as additional explanatory variables. Because some of the studied non-essential metals capable of toxicity (e.g. Cd and Pb) can interfere with calcium metabolism and in turn, calcium concentration in

the body may affect bone development, we included calcium as a predictor of forearm length. In this LM we included age, but not sex, because age represents an important source of variation for forearm length in our data. Given the significant correlations found between SOD and CAT with many metal elements known to cause toxic effects (As, Co, Cu, Ni and Pb), we explored the effects of PC1 and PC2 on these two oxidative stress markers in a model including year as an explanatory variable.

All variables were modeled with Gaussian error distribution, except parasite prevalence, which was built with binary error distribution. Geometric means of metal concentrations and 95% confidence intervals were calculated and back-transformed from models to express the fold-level comparisons between explanatory variables. Similarly, estimates and standard errors from the models are shown for biometrics.

3. Results

3.1. Biometrics

Adult bats weighed more than juveniles ($F_{df}=44.59_{1,47}$, $p<0.01$, Table 1) and bats in Harjavalta were heavier than in Lieto ($F_{df}=14.71_{1,47}$, $p<0.01$, Table 1). Body mass did not vary between years, nor did we observe significant sex-related differences in body mass (Table 1). The forearm length of adults was significantly larger than juveniles ($F_{df}=40.83_{1,47}$, $p<0.01$, Table 1). Females had larger forearms (Estimate ± SE: 37.44 ± 0.25 mm, n=20, Table 1) compared to males (Estimate ± SE: 36.70 ± 0.28 mm, n=40, Table 1). Hematocrit did not vary by sex, age, year or location (Table 1).

3.2. Parasite load

Parasite prevalence on wings, defined as the presence of one or more ectoparasites in the wing membrane (mites, Spinturnicidae) was significantly different between years and locations: bats from Harjavalta showed higher mite prevalence on their wings compared to Lieto ones ($F_{df}=12.33_{1,48}$, $p<0.01$, Table 1), and wing parasite prevalence was greater during 2015 ($F_{df}=4.56_{1,48}$, $p=0.04$, Table 1). Parasite prevalence in fur, defined as the presence of bat flies (Nycteribidae) also varied by location but this effect was different in two years (Year*Location: $F_{df}=6.07_{1,47}$, $p=0.02$, Table 1). We observed positive significant correlations between wing mites with cadmium ($r=0.35$, $p=0.01$, $n=51$) and copper ($r=0.29$, $p=0.04$, $n=51$), and a negative association with lead ($r=-0.30$, $p=0.03$, $n=51$). Bat flies in fur were negatively correlated with arsenic ($r=-0.30$, $p=0.03$, $n=51$) and cobalt ($r=-0.34$, $p=0.02$, $n=51$).

3.3. Metal levels

Overall, elevated concentrations of cobalt, copper, cadmium and nickel were found around the Harjavalta smelter area compared to the water-mill Lieto bats, particularly during the first year of the study. However, surprisingly elevated levels of lead were observed around the water-mill during the second study year.

Cobalt, copper and nickel were detected at higher concentrations in Harjavalta compared to Lieto (Table 2). The concentrations of these elements also decreased from 2014 to 2015 within Harjavalta (Table 2, Figure 1, Table S1). Cadmium was overall markedly higher (i.e. 4.8-times) in Harjavalta compared to Lieto (Table 2, Figure 1, Table S1), and 1.9-times higher in 2014 compared to the next year (Table 2, Figure 1, Table S1). Selenium followed the same annual trends as cadmium. However, contrary to cadmium and most other metals (except lead), selenium was significantly higher (i.e. 3.1-times) in Lieto than in Harjavalta (Table 2, Table S1). Manganese only varied annually, being 2.0-times higher in 2014 than 2015 (Table 2, Table S1). Age had no effect in metal element levels (Table 2). Geometric means for each element are given in Table S1.

For reasons unknown, lead was on average 8.9-times higher in the water-mill bats in Lieto than around the smelter in Harjavalta during 2015 (Table 2, Table S1). Arsenic concentration was not significantly different among years or locations (Table 2).

Calcium was 1.8-times higher in 2014 compared to the following year, 2.0 times higher in Lieto than in Harjavalta and 1.9-times higher in males than in females (Table 2, Figure 2, Table S1). In contrast, zinc concentrations were 1.4-times higher in females than males (Table 2, Figure 2, Table S1). Same annual trends as in calcium were also observed for zinc i.e. higher concentrations in the first year of sampling (Table S1). Correlations between metals are in Table S2.

The principal component analysis (PCA) including As, Cd, Co, Cu, Ni and Pb revealed two principal components with eigenvalues larger than one. The first principal component (PC1, eigenvalue = 3.05) represented 51% of the total variation, the main loadings coming from Cd, Co, Cu and Ni. The second principal component (PC2, eigenvalue = 1.60) represented 27% of the variation with main loadings from As and Pb and in a smaller manner Ni.

3.4. Metals and biometrics

The second, but not first, principal component of metals (PC2) had a significant negative association to body mass, when age was considered as an additional explanatory variable in the same model (PC2: $F_{df}=5.20_{1,47}$, p=0.0271; Age: $F_{df}=31.21_{1,47,}$ p<0.0001), adult bats being heavier the smaller the metal load was. Forearm length showed an age-related negative association to calcium concentration (Ca: $F_{df}=24.6_{1,46}$, p<0.0001; Age: $F_{df}=5.05_{1,46,}$ p=0.0295; Ca*Age: $F_{df}=8.31_{1,46}$; p=0.0060, Figure 3), potentially connected to intercorrelation between Ca and Pb levels.

3.5. Oxidative Stress

Correlations between metals and oxidative stress markers (tGSH, GSH/GSSG, SOD, CAT and GP), were observed in relation to SOD and CAT. In specific, CAT correlated negatively with the metals Cu (r=−0.43, p<0.01, n=46), and Ni (r=−0.39, p<0.01, n=46). SOD correlated negatively with Cu (r=−0.34, p=0.02, n=45) and Co (r=−0.33, p=0.03, n=45), but positively with Pb (r=0.32, p=0.03, n=45) and As (r=0.36, p=0.02, n=45). We observed no significant relationships between biometrics and oxidative stress.

We found that PC2 of metals (As, Cd, Co, Cu, Ni and Pb), and year predicted SOD activity Table 3). SOD activity was higher in 2015 and positively related to PC2 (Figure 4), probably due to elevated concentrations of Pb and As (main components of PC2) found around Lieto in 2015. Instead, PC1 was negatively associated to CAT (Table 3).

4. Discussion

Metal concentrations in the feces of *M. daubentonii* reflected the exposure to ambient contamination. This was especially evident in the higher concentrations of cadmium, nickel, cobalt and copper close to a smelter source compared to a site with less contaminant exposure. Annual variations were also observed for most elements quantified. Calcium and zinc levels differed between males and females. Superoxide dismutase and catalase varied with the exposure to a combination of metals. Additionally, parasite prevalence was higher close to the pollution source.

We found lower fecal calcium concentrations in females compared to males, in line with previous findings in an insectivorous bat (Studier et al. 1991). It is possible that the fecal calcium concentrations reflect the sex-dependent absorption efficiencies, being that female bats require larger calcium amounts for maintenance especially during lactation and gestation (Booher 2008), they may be more efficient at extracting calcium from the food items compared to males. But seeing also that in this study variation of calcium levels in feces within adult females is also lowest among groups, it is not possible to rule out that these low fecal calcium concentrations in females in our study may reflect inadequate calcium in the body, and/or exhausted calcium storages during the breeding phase, as suggested by Studier et al. (1991), at least not without measurements of calcium concentrations in internal organs. Furthermore, it has been shown that insectivorous bats may also suffer from seasonal deficiencies of calcium (Studier et al. 1994). There is no doubt that the calcium composition in diet, particularly recently consumed items, will account for much of calcium detected in feces. In that sense, sex-differences can not only be related to absorption efficiencies between males and females, but also behavioral feeding patterns. For instance, the opportunistic and less selective feeding of female bats, which are generally found at calcium deficit, may be a reflection of “atypical” pellet appearance and its relation to calcium composition (Studier et al. 1991). Calcium deficiency is also more prevalent in insectivorous bats compared to frugivorous or piscivorous ones (Studier et al. 1994).

We found unexpectedly high concentrations of lead, averaging 31 µg/g d.w., in feces of bats from the water-mill. Earlier, concentrations of 20.9 µg/g d.w. in cave guano of *Myotis griscenses* have been described (Ryan et al. 1992), but maximum concentrations of lead (370 µg/g in kidney and 2000 µg/g d.w. in liver) attributed to lead-based paint ingestion with evidence of lead poisoning have been reported in frugivorous bats (Zook et al. 1970, Skerratt et al. 1998). It has been found that fecal concentrations of most metal elements (except selenium, Berglund et al. 2011) correlate with and are higher than internal tissue concentrations, e.g. in liver and kidney (Zukal et al. 2015). Based on the above and on the lack of symptoms characteristic of lead poisoning (Sutton and Wilson 1983), it is possible that the fecal lead concentrations found in our study, although seemingly high, relate to internal lead levels below concentrations to cause toxicity. However, it cannot be ruled out that lowest observable adverse effects level (LOAEL) has been reached. Sources of lead exposure in urban areas originate from industrial emissions (Hariono et al. 1993, Ruiz et al. 2016), lead-based paints in old buildings and exhaust of vehicles running on leaded gasoline. However, it is worth

mentioning that the use of the latter two have been banned some decades ago (Clark 1979, US-EPA 1998). Therefore, it is possible that sources of lead may come from a localized point of lead in the old building (water-mill) in which the Lieto bats roost. However, this is only a conjecture and further studies to confirm this are needed.

The non-essential cadmium interacts with essential elements such as calcium and zinc, stimulating the decalcification of bones (Scheuhammer 1987, Goyer 1997). Accumulation of cadmium in kidney and liver occurs with time (Goyer 1997). Thus, the long-lived bats may be prone to the toxicity and prolonged exposure of cadmium, even when this occurs at low concentrations. We expected cadmium concentrations to differ between juveniles and adults, especially since the lifespan of *M. daubentonii* in the wild can reach well over a couple of decades, and the oldest recorded individual from the *Myotis* genus was 40 years (Podlutsky et al. 2005). However, we did not observe age-dependence in concentrations and it is likely that those would only be observable in internal tissues e.g. kidney, and not necessarily in feces (Berglund et al. 2011). Sex was not a determinant factor in explaining differences in cadmium concentration. However, the highest cadmium concentrations 26.11 and 18.5 µg/g d.w., which represent 4.0 and 2.9-fold of the arithmetic mean in that group respectively, belonged to two adult females sampled from Harjavalta during 2014. Cadmium accumulation in the tissues of certain mammals can be influenced by sex, some studies reporting higher accumulation of cadmium in females (Komarnicki 2000), while in others, sex effects are not observed (Rautio et al. 2010). In our study, the mean arithmetic cadmium concentration was 4.09 µg/g d.w. in all places combined. In literature, fecal cadmium concentrations of 8.5 µg/g d.w have been described in *M. grisescens* (Clark and Shore 2001) roosting in caves with no described metal pollution source. In a recent review on metal elements in bats, Zukal et al. (2015) reported mean cadmium concentrations of 4.13 µg/g d.w. in guano of insectivorous bat species.

Nickel was ca. 3-times higher in feces of *M. daubentonii* than the mean in guano from insectivorous bats (Zukal et al. 2015). Maximum concentrations in our study were 44.4 µg/g d.w., whereas Zukal et al. 2015 report <16.0 µg/g d.w. as a maximum nickel concentration. Naidoo (2013) found mean and maximum nickel concentrations of 3.3 and 19.7 µg/g d.w. in kidneys of the frugivorous bat *Neoromicia nana* around wastewater treatment plants, which correlated well with nickel concentrations in water. Given that the accidental spillage of 66 tons of nickel into the river adjacent to the smelter and the highest mean and maximum values of nickel in bat feces in our study occurred in the bats sampled during the same year suggests that nickel in feces may reflect the water or sediment nickel concentration. Additionally,

Zocche et al. 2010 already postulated that insectivorous bats may not only ingest contaminants via food, but contaminated water may also be an important source of pollutants. In this case, mean nickel concentrations of 8.6 µg/g d.w. and other metals in liver of the diminutive serotine bat (*Eptesicus diminutus*) around a coal mine were linked to DNA damage (Zocche et al. 2010). Although nickel is an essential element, at high enough concentrations it may cause genotoxicity by overproduction of reactive oxygen species, and it has been linked to development of cancer in the respiratory system (Costa 1996). Furthermore, the extraction of nickel is closely associated to the presence of cobalt, a naturally occurring dietary essential element, part of vitamin B_{12} or cobalamin (Valko et al. 2005). Exposure to cobalt may lead to carcinogenic alterations related to the respiratory system (Princivalle et al. 2017), possibly connected to the production of superoxide radicals when cobalt reacts with hydrogen peroxide (Valko et al. 2005). The minimum cobalt concentration reported in guano of insectivorous bats was 2.0 µg/g d.w. (Zukal et al. 2015). In our study, we found a mean of 1.3 µg/g d.w. and a maximum of 4.8 µg/g d.w., the latter corresponding again to the year of the nickel spillage. Thus, high correlations between cobalt-nickel in our study would indicate that the elevated presence of cobalt is probably linked to the excessive nickel in 2014.

Copper was the third metal element found at elevated concentrations around the smelter during 2014. Copper, an essential element under homeostatic regulation, forms part of active sites of enzymes namely catalase, superoxide dismutase and peroxidase (Nieminen and Lemasters 1996). Excess concentration of copper may trigger lipid peroxidation by excessive reactive oxygen species production and depletion of glutathione (Nieminen and Lemasters 1996). Copper concentration in bats has mostly been reported for liver, being ca. 40 µg/g d.w. on average (Méndez and Alvarez-Castañeda 2000, Hoenerhoff and Williams 2004, Courtin et al. 2010, Williams et al. 2010, Zocche et al. 2010). In our study, mean concentrations of copper were ca. 40% lower compared to mean concentrations reported in guano of insectivorous bats in a study by Zukal et al. 2015 (i.e. 126.5 vs 205.7 µg/g d.w.). Similarly, maximum copper concentrations in our study were well below the one reported in guano of other insectivorous bats (Zukal et al. 2015).

In other studies around the smelter study area, these metals (i.e. Cd, Cu, Ni and Pb) have also been found at elevated concentrations in feces and internal organs of birds inhabiting near the smelter in our polluted site (Eeva and Lehikoinen 1996, Berglund et al. 2010, Berglund et al. 2011). Berglund et al. (2011) also concluded that metal concentrations in feces are not necessarily correlated with internal tissue concentrations, at least not in the studied passerine

birds inhabiting close to our polluted study area, but comparative studies of metal concentrations in internal organs and feces are lacking for bats, which make toxicity assessment difficult in this study. It is known that concentrations of non-essential elements (As, Cd, Pb) in bat tissues such as bone and fur may reflect long-term exposure, whereas softer tissues including brain, muscle and blood would represent recent exposure (Hariono et al. 1993). In a similar manner, metal concentrations in feces will most likely reflect recent exposure mostly via diet, water (Naidoo et al. 2016) and transfer to feces via biliary excretion (Gregus and Klaassen 1986). Insectivorous bats defecate several times a day, and *M. daubentonii* has excretion times ranging from 15 to 90 minutes after food ingestion (Webb et al. 1993), thus likely making the metal turnover fast.

Zinc and selenium have protective roles against oxidative stress and the deficiency of zinc can compromise the immune system (Valko et al. 2005, Rautio et al. 2010). The toxicity of zinc in bats has only been experimentally tested exposing little brown bats to zinc phosphide (Hurley and Fenton 1980), where mortalities were explained by elevated zinc concentrations and the confined space of the exposure experiment. In our study, zinc in feces varied between males and females. Few sex-related studies on zinc accumulation or excretion in wild mammals are found in literature. For example, a study in the red fox (*Vulpes vulpes*) found no sex-dependence in the accumulation of zinc, lead and cadmium (Pérez-López et al. 2016). Zinc and cadmium interactions, on the other hand, are well documented. Zinc deficiency contributes to cadmium gastrointestinal absorption, hence cadmium accumulation in internal organs (Peraza et al. 1998, Reeves and Chaney 2004). Furthermore, the presence of cadmium reduces zinc absorption, resulting in higher amounts of zinc excreted in feces (Brzóska and Moniuszko-Jakoniuk 2001). In our study, females excreted more zinc than males. This could be indicative of an adverse effect of elevated cadmium concentration on females. However other factors including sex-related differences in diet could contribute to the sex-related differences in concentrations observed in both zinc and calcium.

Selenium provides defense against copper-induced toxicity (Valko et al. 2005). A deficiency of selenium will impair reproduction in wild animals (Allen and Ullrey 2004). In mammals, however, the range to cause selenium deficiency and toxicosis is narrow, and the former is more commonly observed (Ohlendorf 2003). However, selenium toxicosis seems not to cause embryo malformations in mammals, as it does in birds (Clark 1987, Mora et al. 2002), selenium-exposed mammals may suffer from lower fertility (Santolo 2009). In bats, the highest concentration of selenium in liver (8.96 µg/g d.w.) has been found in *Eptesicus fuscus* in a

study focused on a fungal disease, white-nose syndrome. However, no correlation between the syndrome and selenium was found (Courtin et al. 2010). In our study, selenium was below mean and maximum concentrations described for guano of insectivorous bats and similarly to cadmium, selenium concentration was influenced by year and location. Interestingly, only selenium and lead were higher in Lieto than Harjavalta compared to the other elements analyzed.

In our study, manganese levels were about half the mean concentration reported in other insectivorous bats (Zukal et al. 2015). Guidelines for toxicity of manganese are inexistent, as it is considered an essential micronutrient (Tchounwou et al. 2012), however it has been shown that manganese exposure can provoke adverse neurobehavioral responses (Burger and Gochfeld 1995). It is likely that no case for excess amounts of manganese exposure are present neither from the smelter, nor around the water-mill, although the latter one is located around agricultural areas, known potential sources of manganese in the environment from fertilizers and pesticides in the agricultural runoffs (Martinez-Finley et al. 2012). The low average amounts of manganese (also selenium) found in the bats around the smelter study site, could be responses from the organism to diminish excretion of vital nutrients. However, the effect of interactions between essential nutrients and non-essential metals that exert toxicity cannot be neglected (Goyer 1997, Peraza et al. 1998). For example, manganese and aluminum in high concentrations combined with a low calcium-magnesium diet have been linked to neurological disorders in humans (Goyer 1997). Based on our available data, it is not possible to conclude whether a deficiency of manganese is present around the smelter or an excess occurs around the water-mill, but it is likely that elevated concentrations of non-essential metals capable of toxicity around the smelter, specifically cadmium, known to interact with the nutrients zinc, calcium, selenium and copper, may negatively affect their homeostasis (Goyer 1997).

Some of the essential elements analyzed in our study diminish the toxic effects of non-essential metal elements when consumed in adequate amounts (e.g. zinc, calcium), while others provide antioxidant protection (e.g. selenium). Antioxidants defend the organism from the chemically reactive species formed after oxygen metabolism (Halliwell and Gutteridge 2007). At the same time, the production of such reactive oxygen species (ROS) can increase due to immune reactions, pollution, reoxygenation after hypoxia during hibernation, food scarcity and predation (Costantini 2014). Here, we observed marked differences in catalase activities in our study groups. Catalase is an enzyme with antioxidant function which converts hydrogen peroxide to water and oxygen in instances when hydrogen peroxide concentrations are

particularly elevated (Halliwell and Gutteridge 2007, Costantini 2014). The smelter group presented the lowest CAT activity during the year of accidental metal spillage. Catalase may be inhibited by copper and other metal ions (Gaetke and Chow 2003). Our findings are in line with this in that we observed negative correlations between CAT and levels of non-essential metal elements capable of toxic effects. We also observed an effect of year and the second principal component of metals (As, Cd, Co, Cu, Ni and Pb), which main loadings belong to arsenic and lead, on SOD activity. Superoxide dismutase catalyzes the conversion of superoxide radicals into hydrogen peroxide and oxygen (Halliwell and Gutteridge 2007). Superoxide dismutase activity was higher during our second and on average less polluted sampling year. In line with that, the activity of SOD decreased in Algerian mice (*Mus spretus*) from a copper-mine area polluted with elevated concentrations of selenium, manganese and iron (Viegas-Crespo et al. 2003).

Longevity and drastic oxygen fluctuations, a consequence of entering torpor and hibernation are correlated with the resistance to oxidative stress (Salmon et al. 2009) and low oxygen radical generation in bats (Brunet-Rossinni 2004, Wilhelm Filho et al. 2007). For example, compared to short-lived small mammals, bats release hydrogen peroxide at a lower rate (Brown et al. 2009). These studies partly support the limited alteration in the antioxidant machinery observed in our study.

Immunotoxicity is described as the weakening of the immune system because of sustained or elevated pollutant exposure (Propst et al. 1999), rendering the individual vulnerable to parasite infestation, among other effects. At the same time, responses to immune challenge (e.g. parasite infestation) can generate reactive oxygen species (Schneeberger et al. 2013, Lilley et al. 2014). In that sense, an immune response to pollutant challenge may activate oxidative enzymes, while pollutants may on their own do the same by causing oxidative stress. In this study, we observed that bats living close to the smelter in Harjavalta had higher parasite infestation compared to the water-mill bats from Lieto. In addition, variation in catalase activities was only observed in 2014, the year in which higher concentrations of cadmium, copper and nickel were detected around the smelter in Harjavalta. And although, the positive correlations between cadmium and copper with parasites (in wing) were weak. We speculate that the combination of pollutants and parasites may have led to a depression in the activity of catalase. However, immune marker tests should accompany the study to support this statement and build a more robust case. Nevertheless, other studies have also described depressed catalase

activities (but also glutathione peroxidase) in other small mammals when exposed to a metal pollutant (Ossola et al. 1997).

~~Here, the bats around the smelter site presented higher ectoparasite (Nycteribidae, Spinturnicidae) prevalence, which may be related to the specific roosts used by the two bat populations. The presence of parasites generates an immune reaction accompanied by production of oxygen radicals (Costantini 2014, Lilley et al. 2014). Because significant differences in catalase activity were only observed during 2014, we speculate that the combination of parasite infestation with toxicant challenge, (i.e. higher than average concentrations of copper, nickel, cadmium around the smelter), may have led to a depression of catalase activity. However, to support this, catalase tests should be accompanied with immune marker tests. Similarly, in another study on a small mammal, low catalase and glutathione peroxidase activities were observed in rats after exposure to copper sulphate (Ossola et al. 1997). However, this may instead be related to the specific roosts used by the two bat populations and not necessarily to the pollution levels around the smelter as weak correlations between parasite load and metals showed.~~

Other factors, such as timing of sampling in relation to entering or leaving hibernation are also important to consider. For example, *M. daubentonii* are lighter in body mass after arousal from hibernation (April) because they have depleted their fat-reserves during northern-hemisphere winter. This can affect release of toxicants accumulated in fat into the bloodstream. However, such factor is more relevant to lipophilic contaminants such as polyaromatic hydrocarbons, which tend to accumulate in adipose tissue (Bayat et al. 2014). Metals, unless found in their organometallic form (e.g. methylmercury, tetraethyl lead) behave chemically different and generally tend not to accumulate in fat (Yates et al. 2014).

5. Conclusions

Our study makes use of a minimally invasive and understudied format (i.e. fecal pellets) to evaluate exposure to metal contaminants in free-ranging bats. The elevated concentrations of metal elements (cadmium, copper, nickel) commonly found in other vertebrate species around the smelter study site (Eeva and Lehikoinen 1996, Eeva et al. 2009) and the correlations between an incidental metal discharge around our polluted study site (smelter) indicate that

fresh fecal pellets can be a suitable material to assess metal exposure on an individual basis and show promise for use in biomonitoring studies. Furthermore, significant differences in catalase and superoxide dismutase between our study sites may suggest the onset of physiological stress, possibly caused by excessive non-essential metal concentrations capable of toxicity in the environment, although contribution from parasite prevalence cannot be ruled out. To our best knowledge, this is the first study in which individual fecal pellets of non-captive bats have been used to assess the metal exposure and correlations with biomarkers of contaminant effect. Further studies making use of fresh fecal pellets of bats around contaminated places are encouraged to if similar patterns can be observed.

Acknowledgments/ Permits

Paul Ek and Sten Lindholm (Åbo Akademi) are acknowledged for the heavy metal analysis. We thank Ville Vasko for help during field sampling and Janina Stauffer for advice on oxidative stress protocols. Silvia Espín and Pablo Sánchez-Virosta are acknowledged for assistance in laboratory. Approval was granted by the Animal Ethics Committee of the University of Turku (license number ESAVI/3221/04.10.07/2013) and Centre for Economic Development, Transport and the Environment collection permit (license number VARELY/948/2015). This study was financed by Academy of Finland (project: 265859) to Tapio Eeva and the University of Turku Foundation and the Department of Biology to Sandra Ruiz.

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Table 1. The effect of year, sex, age and location on biometrics (body mass and forearm length), hematocrit and parasite prevalence (in wings and fur).

		Year		Sex		Age		Location		Year*Location	
	n	F_{df}	p	F_{df}	p	F_{df}	p	F_{df}	p	F_{df}	p
Body mass[a]	50	0.00(1,46)	0.97	0.17(1,44)	0.68	44.59(1,47)	<0.01	14.71(1,47)	<0.01	2.74(1,45)	0.10
Forearm length[a]	50	0.00(1,45)	0.99	4.92(1,47)	0.03	40.83(1,47)	<0.01	1.82(1,46)	0.18	1.56(1,44)	0.22
Hematocrit[a]	44	0.49(1,41)	0.49	0.06(1,39)	0.82	0.49(1,40)	0.49	1.49(1,42)	0.23	0.68(1,38)	0.41
Parasite Wing[b]	51	4.56(1,48)	0.04	2.25(1,47)	0.14	0.21(1,46)	0.65	12.33(1,48)	<0.01	0.00(1,45)	0.98
Parasite Fur[b]	51	0.00(1,47)	0.98	2.18(1,46)	0.15	0.00(1,45)	0.98	0.09(1,47)	0.76	6.07(1,47)	0.02

	n	female	n	male	n	adult	n	juvenile
Body mass (grams)[c]	30	8.56 ± 0.22	20	8.06 ± 0.24	40	9.25 ± 0.16	10	7.37 ± 0.31
Forearm length (millimeters)[c]	30	37.44 ± 0.25	20	36.70 ± 0.28	40	38.38 ± 0.19	10	35.77 ± 0.36

[a]Linear model (LM) with Gaussian distribution.

[b]Generalized linear model (GLM) with Binomial distribution. Final terms in models are bolded. Significance set at $p<0.05$.

[c]Estimates ±SE calculated using LMs with sex and age as explanatory variables. N is the number of individuals.

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Table 2. The effect of year, location (Lieto and Harjavalta), sex and age on the fecal metal concentrations of Myotis daubentonii (n=51).

	Year		Sex		Age		Location		Year*Location	
	F_{df}	p	F_{df}	p	F_{df}	p	F_{df}	p	F_{df}	p
Arsenic	0.50(1,47)	0.48	0.13(1,46)	0.72	1.51(1,49)	0.23	0.82(1,48)	0.37	3.08(1,45)	0.09
Calcium	18.74(1,47)	<0.01	7.16(1,47)	0.01	3.17(1,46)	0.08	8.16(1,47)	<0.01	0.04(1,45)	0.84
Cadmium	4.96 (1,48)	0.03	0.08(1,46)	0.78	0.30(1,47)	0.59	29.80(1,48)	<0.01	2.18(1,45)	0.15
Cobalt	14.65(1,47)	<0.01	0.34(1,46)	0.56	0.51(1,45)	0.48	7.16(1,47)	0.01	11.27(1,47)	<0.01
Copper	22.25(1,47)	<0.01	0.58(1,45)	0.45	0.62(1,46)	0.43	28.91(1,47)	<0.01	4.11(1,47)	<0.05
Lead	15.29(1,47)	<0.01	0.24(1,45)	0.63	0.51(1,46)	0.48	37.71(1,47)	<0.01	17.33(1,47)	<0.01
Manganese	9.36(1,49)	<0.01	2.06(1,48)	0.16	2.20(1,47)	0.14	0.19(1,46)	0.66	1.57(1,45)	0.22
Nickel	17.58(1,47)	<0.01	0.32(1,46)	0.57	0.50(1,45)	0.48	8.88(1,47)	<0.01	5.11(1,47)	0.03
Selenium	4.07(1,48)	<0.05	0.01(1,45)	0.94	0.49(1,46)	0.49	9.28(1,48)	<0.01	2.23(1,47)	0.14
Zinc	4.89(1,48)	0.03	4.65(1,48)	0.04	1.04(1,45)	0.31	1.08(1,47)	0.30	2.05(1,46)	0.16

GLM with Gaussian distribution. Final terms in models are bolded. Significance set at p<0.05

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Table 3. Effects of metal load (first and second principal components of As, Cd, Co, Cu, Ni and Pb) and year on the enzymatic activities of superoxide dismutase (SOD) and catalase (CAT).

	PC1		PC2		Year		PC1*Year		PC2*Year	
	Fdf	p	Fdf	p	Fdf	p	Fdf	p	Fdf	p
SOD	0.05(1,41)	0.8837	6.25(1,42)	0.0164	21.38(1,42)	<0.0001	1.52(1,39)	0.2253	0.92(1,39)	0.3443
CAT	8.07(1,44)	0.0068	0.04(1,42)	0.8433	0.99(1,43)	0.3248	2.83(1,40)	0.1006	0.41(1,40)	0.5243

PC1 and PC2 are first and second principal components of metals. Final terms in model are bolded. Significance set at p<0.05

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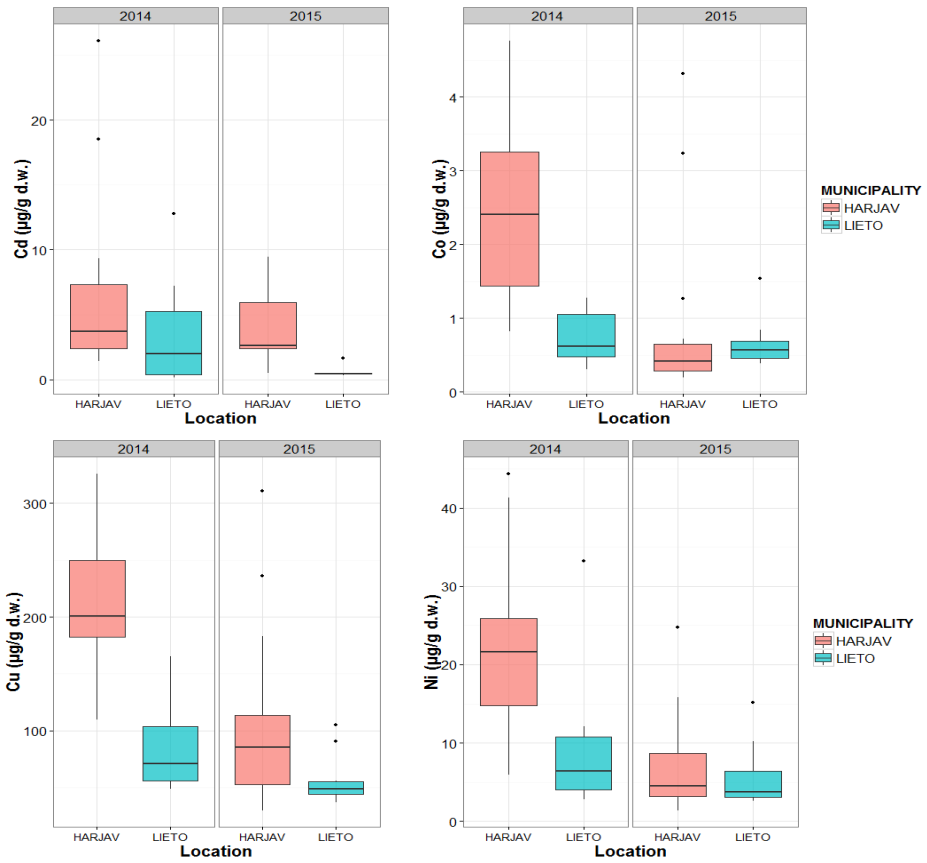

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632 Figure 1. Concentrations (µg/g dry weight) of cadmium (Cd), cobalt (Co), copper (Cu) and nickel
633 (Ni) in feces of Myotis daubentonii collected during the years 2014 (Harjavalta: n=17; Lieto: n=9)
634 and 2015 (Harjavalta: n=15; Lieto: n=10).

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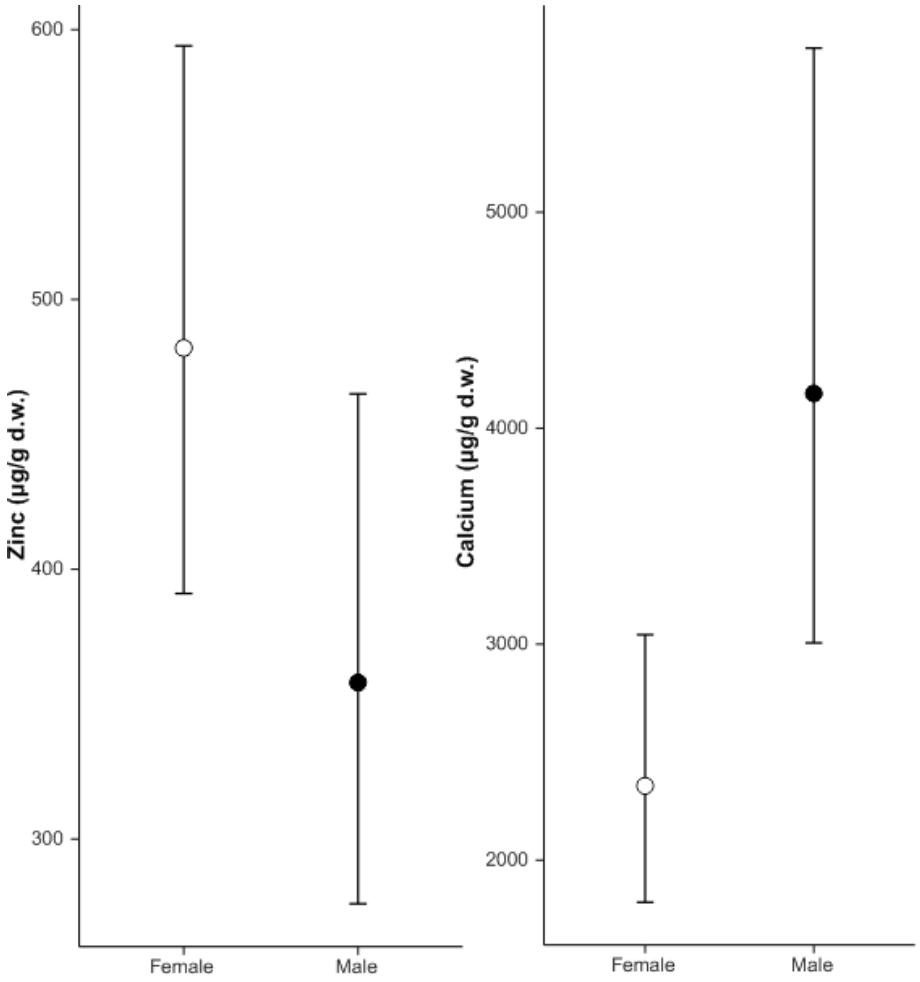

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640 Figure 2. Mean (±95%CI) zinc and calcium concentrations in feces of female (n=31) and male
641 (n=20) *Myotis daubentonii*.

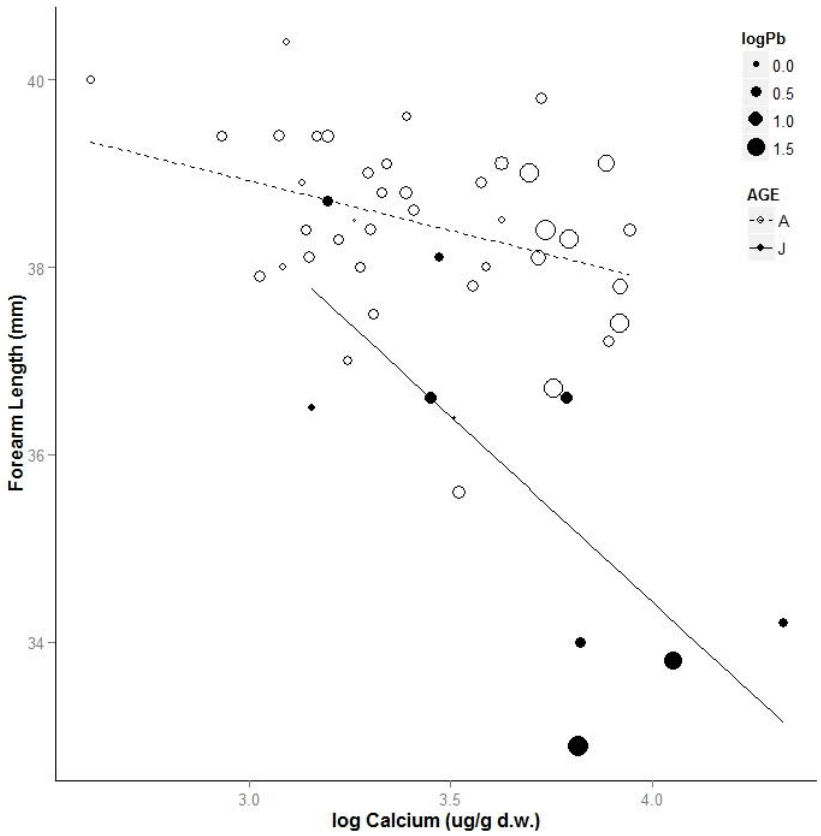
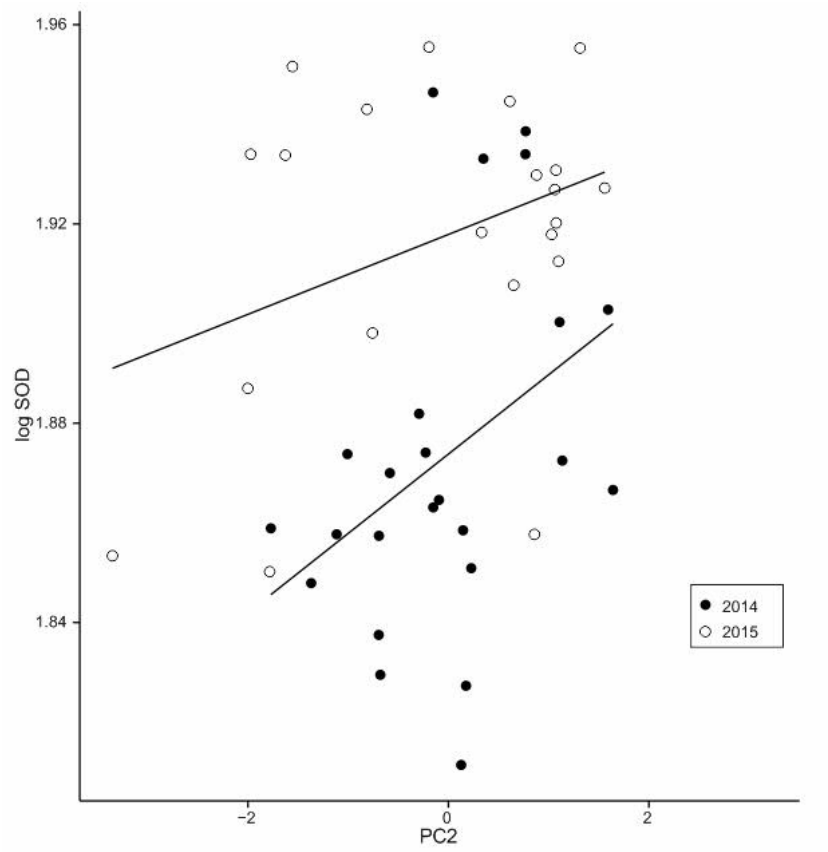


Figure 3. Relationship between fecal calcium concentration (µg/g d.w.) and forearm length (mm). Empty and filled circles denote adults (A) and juveniles (J), respectively; regression lines correspond to adults (dashed) and juveniles (solid); size of the circles denotes the fecal concentrations of lead. Calcium and lead concentrations are expressed in µg/g of dry weight.. (GLM: Ca: $F_{1,46}=24.6$, $p<0.0001$; Age: $F_{1,46}=5.05$, $p=0.03$; Ca*Age: $F_{1,46}=8.31$, $p<0.01$)



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652 Figure 4. Relationship between second principal component of metals (As, Cd, Co, Cu, Ni and
653 Pb) and superoxide enzymatic activity. Filled and empty circles are individuals trapped
654 during 2014 and 2015 respectively.

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Table S1. Arithmetic means (± SE) of metal concentrations (µg/g d.w.) in feces of Myotis daubentonii per year and location.

	2014				2015			
	Harjavalta (n=17)		Lieto (n=9)		Harjavalta (n=15)		Lieto (n=10)	
	mean	± SE	mean	± SE	mean	± SE	mean	± SE
As	5.10	1.86	1.76	0.35	9.45	5.96	7.22	2.25
Ca	1911	277	4019	866	4398	1280	6658	748
Cd	6.46	1.61	3.60	1.43	4.05	0.65	0.54	0.12
Co	2.47	0.31	0.75	0.12	0.90	0.31	0.66	0.11
Cu	210.2	13.0	82.7	12.5	104.4	20.6	56.8	7.1
Mn	199.1	28.1	152.3	38.6	101.3	24.6	94.0	19.3
Ni	22.15	2.70	9.37	3.16	7.24	1.60	5.67	1.29
Pb	3.81	0.43	5.32	0.82	5.25	2.17	30.91	4.95
Se	2.21	0.29	3.91	0.60	1.91	0.51	5.32	0.83
Zn	654.0	59.0	422.7	105.2	469.7	81.2	373.2	34.5

Table S2. Correlations between metallic elements in feces of Myotis daubentonii.

	logCa	logCd	logCo	logCu	logMn	logNi	logPb	logSe	logZn
logAs	0,18	-0,09	0,19	0,07	0,23	0,18	0,40	0,03	0,01
	0,211	0,544	0,179	0,642	0,103	0,205	0,003	0,859	0,971
logCa	1	-0,53	-0,39	-0,48	-0,15	-0,38	0,52	0,14	-0,27
		<0,0001	0,005	<0,001	0,291	0,006	<0,0001	0,336	0,053
logCd			0,47	0,66	0,49	0,46	-0,56	-0,30	0,50
			0,001	<0,0001	<0,001	0,001	<0,0001	0,034	0,0002
logCo				0,77	0,57	0,72	-0,09	0,01	0,62
				<0,0001	<0,0001	<0,0001	0,520	0,947	<0,0001
logCu					0,68	0,75	-0,36	-0,19	0,72
					<0,0001	<0,0001	0,009	0,192	<0,0001
logMn						0,49	-0,05	-0,17	0,77
						<0,001	0,723	0,242	<0,0001
logNi							0,01	0,15	0,52
							0,942	0,302	<0,001
logPb								0,34	-0,20
								0,015	0,168
logSe									-0,25
									0,073

N=51, Pearson correlation coefficient (above), p-value (below).
Bolded values are significant correlations. Significance at p<0.05

Table S3. Oxidative stress mean (±SD) in red blood cells of Myotis daubentonii per Year and Location. Below are GLM for the effects of Year, Location and their interaction of the oxidative stress markers.

Year	Location		GSH:GSSG ratio		tGSH (µmol/mg)		GP (pmol/min/mg)		SOD (% Inhibition)		CAT (µmol/min/mg)	
2014	Harjavalta	Mean ± SD	n=16	14.29 ± 12.19	n=16	22.64 ± 9.17	n=16	0.13616 ± 0.03127	n=16	74.40 ± 6.12	n=16	70.69 ± 21.29
	Lieto	Mean ± SD	n=8	12.98 ± 4.63	n=8	30.04 ± 7.07	n=8	0.14797 ± 0.03307	n=8	76.03 ± 7.20	n=8	96.42 ± 11.38
2015	Harjavalta	Mean ± SD	n=14	26.82 ± 16.05	n=14	33.25 ± 8.30	n=14	0.18169 ± 0.06142	n=14	83.17 ± 6.51	n=14	95.66 ± 12.37
	Lieto	Mean ± SD	n=8	20.55 ± 19.61	n=8	24.07 ± 6.37	n=8	0.20766 ± 0.10649	n=7	82.05 ± 4.60	n=8	97.68 ± 10.47
			n=46		n=46		n=46		n=45		n=46	
GLM	Year	F_{df}; p		$6.68_{(1,44)}$;0.01		$1.03_{(1,42)}$; 0.32		$0.48_{(1,44)}$; 0.49		$18.31_{(1,43)}$; <0.0001		$5.85_{(1,44)}$;0.02
	Location	F_{df}; p		$0.53_{(1,44)}$; 0.47		$0.01_{(1,42)}$; 0.93		$0.13_{(1,44)}$;0.72		$0.04_{(1,43)}$; 0.85		$3.66_{(1,44)}$; 0.06
	Year*Location	F_{df}; p		$0.32_{(1,42)}$; 0.58		$10.79_{(1,42)}$; <0.01		$0.63_{(1,42)}$; 0.43		$0.42_{(1,41)}$; 0.52		$2.95_{(1,42)}$; 0.09

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