1 # Metal and metalloid exposure and oxidative status in free-
2 # 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 status 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 metals and metalloids (As, Ca, Cd, Co, Cu, Mn, Ni, Pb, Se, Zn) from individual fecal pellets. We measured enzymatic antioxidants (GP, CAT, SOD), total glutathione (tGSH) and ratio between reduced and oxidized glutathione (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.

Metal 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 (Regoli et al. 2011, Costantini et al. 2014). In bats, oxidative status 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 status are more scarce (Lilley et al. 2013). The combination of industrial disturbance, habitat destruction

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

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

114
2. Materials and methods
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2.1. Study species and study area

117 Bats were trapped during May-August 2014 and 2015 in the vicinity of a smelter in Harjavalta
118 (61°20′N, 22°10′E), and at an old water mill in Lieto (60°33'N, 22°27'E) with a combination
119 of harp traps and mist nets (2.5 m height; Ecotone, Poland) placed along flying corridors during
120 their emergence time from roosting sites in Harjavalta (n=32), and hand trapped into cloth bags

121 in Lieto (n=19). *Myotis daubentonii,* is an insectivorous trawling bat distributed across Europe
122 and Asia. The species roosts in tree cavities, but they also take human-made constructions i.e.
123 bird boxes or buildings (Joint Nature Conservation Committee 2007, Dietz et al. 2009). *Myotis*
124 *daubentonii* roosts close to water bodies, where it forages for insects, mainly Chironomidae
125 (Dietz et al. 2009, Encarnação et al. 2010, Vesterinen et al. 2016). Chironomids, or non-biting
126 flying midges spend a part of the life-cycle as filter-feeders within sediments of water bodies.
127 They are therefore prone to accumulate chemicals or toxicants discharged into the water bodies
128 and deposited over time in the water bottom (Lilley et al. 2012).

129 *Myotis daubentonii* normally breed in colonies, and they can form subgroups within a
130 colony due to their mobility thus not being loyal to a specific roosting site within a cave.
131 However, individuals do show area roost fidelity (Lucan and Hanak 2011, Ngamprasertwong
132 et al. 2014). Generally, *M. daubentonii* become sexually mature at their first year, being able
133 to reproduce in late summer (Encarnação et al. 2004). However, observations of male *M.*
134 *daubentonii* being sexually matured at their year of birth and consequently being able to
135 reproduce before their first hibernation period have been reported (Encarnação et al. 2006).

136 Here, we sampled a bat population in a forest patch close to an air metal emission point
137 source in Harjavalta, Western Finland. Harjavalta is an industrial town characterized for its
138 metal processing activities particularly the smelting of copper and nickel (Kiikkilä 2003).
139 Emissions also include arsenic, zinc, cadmium, mercury, lead and sulphur as the smelting
140 process by-products (Kiikkilä 2003). A river, Kokemäenjoki, runs through the town and is the
141 main feeding ground for the bats in our study. This river system has a large catchment basin
142 (27000 km^2) including 16% of agricultural land (Huttunen et al. 2016). In 2014, an accidental
143 metal discharge from the smelter in Harjavalta released 66 tons of nickel into the
144 Kokemäenjoki-river (KVVY ry. 2016). The second and less metal exposed bat group in our
145 study, roosts in an old water mill in Lieto. This bat population has been previously monitored
146 for some years (Laine et al. 2013, Vesterinen et al. 2016), but the metals are quantified for the
147 first time in this study. The water mill is located along the Aura-river in South-Western Finland
148 and has a catchment basin of 874 km^2 of which 37% is agricultural land (Huttunen et al. 2016).

149 ***2.2. Sampling and biometric measurements***

150 Caught bats were identified to species and banded. Weight was recorded to the nearest 0.1 g
151 with a Pesola spring balance and forearm length was recorded to the nearest 0.05 mm with a
152 sliding caliper. Sex was determined, and age was classified into adults and juveniles according

153 to the ossification state between phalanges (Brunet-Rossinni and Wilkinson 2009). Fur and
154 wing were inspected for ectoparasites. Bats often defecate when handled, thus fresh fecal
155 pellets were collected per individual and used for metal analysis. Blood was obtained (up to a
156 maximum of 65 μL) from the interfemoral vein into a heparinized capillary tube (Marienfield
157 80iu/ml) and immediately centrifuged at 4400 g for 5 minutes in a LW Scientific ZIPocrit
158 Hematocrit Centrifuge to separate the red blood cell fraction from plasma. The hematocrit
159 (proportion of red cells) was measured with a sliding caliper. The red blood cells and plasma
160 were placed separately into tubes, flash frozen in liquid nitrogen and stored at −80 ºC until the
161 oxidative marker analyses. The blood metal concentrations were not measured because there
162 was not enough blood material to quantify both the metals and oxidative status parameters. All
163 bats were released after sampling. Collection licences were approved by the Animal Ethics
164 Committee of the University of Turku (license number ESAVI/3221/04.10.07/2013) and
165 Centre for Economic Development, Transport and the Environment (license number
166 VARELY/948/2015).

167 ***2.3.Metal analysis***

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

181 The determination of metal element concentrations was conducted with inductively
182 coupled plasma mass spectrometer ICP-MS (Elan 6100 DRC+ from PerkinElmer-Sciex), by
183 using a quantitative standard mode. The detection limit for most of the metal elements was
184 around 1 ppt (ng/L) and below. The instrument was calibrated with a commercial multi-
185 standard from Ultra Scientific, IMS–102, ICP-MS calibration standard 2. Certified reference

186 materials from European Reference Material (mussel tissue ERM-CE278K-8G) were used for
187 method validation. In 2014, the mean recoveries (±SE) in nine reference samples were as
188 follows: Ca 98 ± 15.98%, Mn 98 ± 3.29%, Co 101 ± 1.52%, As 96 ± 1.79%, Pb 95 ± 3.25%,
189 Ni 120 ± 2.41%, Cu 100 ± 2.44%, Cd 91 ± 1.79%, Zn 87 ± 1.80%, Se 151 ± 26.20%. In 2015,
190 the mean recoveries (±SE) in six reference samples were as follows: Ca 113 ± 8.39%, Mn 112
191 ± 4.21%, Co 101 ± 2.25%, As 99 ± 1.24%, Pb 89 ± 2.05%, Ni 111 ± 4.85%, Cu 100 ± 2.43%,
192 Cd 92 ± 1.90%, Zn 93 ± 1.51%, Se 118 ± 5.44%. The results are expressed as µg/g on a dry
193 weight (d.w.) basis.

194 ***2.4. Oxidative status analysis***

195 Concentrations of antioxidants and enzymatic activities were measured from red blood cells in
196 triplicate using 96-well and 384-well microplates. Protein content was determined using the
197 Bradford method with bovine serum albumin as a standard and BioRad protein assay reagent
198 (Bradford 1976). The samples were diluted in phosphate buffer saline before being added to
199 the microplate. Inter-assay variation was normalized by using the same control samples of
200 known enzymatic activities. Measurements were obtained using EnSpire and Envision plate
201 readers (Perkin-Elmer).

202 **2.4.1. Glutathione**

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

214 **2.4.2. Glutathione Peroxidase**

215 Glutathione Peroxidase (GP) Cellular Activity Assay Kit was purchased from Sigma (Catalog
216 No CGP1). Glutathione peroxidase activity is determined indirectly, by first quantifying the

217 conversion of reduced glutathione (GSH) to oxidized glutathione (GSSG), followed by the
218 reduction of GSSG back to GSH, catalyzed by the enzyme glutathione reductase (GR) and
219 Nicotinamide Adenine Dinucleotide Phosphate Reduced (NADPH). Procedures were carried
220 out following the manufacturer instructions, except using 2 mM H_2O_2 as a substrate. The assay
221 was performed in a clear 384-well plate (Perkin Elmer). Briefly, to each well were added: 35
222 μL of assay buffer, 5 μL of NADPH assay reagent, 5 μL of 2 mM H_2O_2 and 5 μL of blood
223 sample to obtain a final volume of 50 μL. Five μL of assay buffer were used as blank. The
224 absorbance was measured at 340 nm (A340) for 60 seconds in a kinetic program using an
225 Envision microplate spectrophotometer. The activity of GP was calculated by dividing the
226 A340 by the extinction coefficient of NADPH (6.22) and it is expressed as pmol/min/mg.

227 **2.4.3. Catalase**

228 The activity of catalase (CAT), an enzyme which converts hydrogen peroxide into water and
229 oxygen, was quantified following the protocol instructions of the CAT-assay kit (Sigma
230 Catalog No CAT 100). To perform the assay in a 96-well microplate format, the volumes of
231 reagents and samples were reduced. Assay solutions (peroxide, peroxide-solution, assay buffer,
232 chromogen, Sodium Azide (NaN_3) - stop solution and enzyme dilution buffer) were prepared
233 according to the information in the Sigma kit technical bulletin. Briefly, 2 μL of sample
234 (1mg/mL) and 13 μL of assay buffer were mixed in a tube. The reaction was stopped with 180
235 μL of 15mM $NaN_{3.}$ The CAT activity expressed in μmol/min/mg was colorimetrically
236 quantified by adding 200 μL chromogen in each well to 2 μL aliquot of the stopped reaction
237 solution. Absorbance was measured at 520 nm.

238 **2.4.4. Superoxide Dismutase**

239 Superoxide dismutase (SOD) assay kit was purchased from Sigma-Aldrich (Catalog No
240 19160). The reaction determines the inhibition activity of SOD by a colorimetric method. The
241 water-soluble salt WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-
242 tetrazolium, monosodium salt) reacts with the superoxide anion to produce a formazan dye.
243 The reduction rate of reduced superoxide anion (i.e. O_2^-) is directly related to the enzyme
244 xanthine oxidase (XO) which is inhibited by SOD. In a 384-well plate, 45 μL of WST-solution
245 were added to 5 μL of sample (1mg/mL). Then 5 μL of xanthine oxidase (XO) enzyme were
246 added to each well and incubated at 37°C for 20 min. Absorbance was measured at 450 nm.
247 SOD activity is expressed as inhibition rate percentage.

248 ***2.5. Statistics***

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

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

266 For further modelling of the effects of pollution level on morphological and
267 physiological parameters we built principal components (PC) of six metal elements (As, Cd,
268 Co, Cu, Ni, Pb) to reduce the information of multiple inter-correlated variables (metal
269 concentrations) into smaller number of variables, or components, explaining most of the
270 variation in the data. The selection of these elements was based on their toxic degree
271 (Tchounwou et al. 2012), their strong correlation with each other and their consistent elevated
272 concentrations around the smelter source. The effects of metal exposure may negatively affect
273 vital physiological functions potentially leading to body mass loss (Eeva and Lehikoinen 1996,
274 Dauwe et al. 2006). We investigated this in a model with the first and second principal
275 components of metals (PC1, PC2) as an explanatory variable for body mass and with sex, age
276 and location as additional explanatory variables. Because some of the studied non-essential
277 metals capable of toxicity (e.g. Cd and Pb) can interfere with calcium metabolism and in turn,
278 calcium concentration in the body may affect bone development, we included calcium as a
279 predictor of forearm length. In this LM we included age, but not sex, because age represents
280 an important source of variation for forearm length in our data. Given the significant

281 correlations found between SOD and CAT with many metal elements known to cause toxic
282 effects (As, Co, Cu, Ni and Pb), we explored the effects of the first and second principal
283 components of metals (PC1, PC2) on these two oxidative status markers in a model including
284 also year as an explanatory variable.

285 All variables were modeled with Gaussian error distribution, except parasite prevalence,
286 which was built with binary error distribution. Geometric means of metal concentrations and
287 95% confidence intervals were calculated and back-transformed from models to express the
288 fold-level comparisons between explanatory variables. Similarly, estimates and standard errors
289 from the models are shown for biometrics.

290 ## ***3. Results***

291 ### *3.1. Biometrics*

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

299 ### *3.2. Parasite load*

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

310 ### *3.3. Metal levels*

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

315 Cobalt, copper and nickel were detected at higher concentrations in Harjavalta
316 compared to Lieto (Table 2, Figure 1). The concentrations of these elements also decreased
317 from 2014 to 2015 within Harjavalta (Table 2, Figure 1, Table S1). Cadmium was overall
318 markedly higher (i.e. 4.8-times) in Harjavalta compared to Lieto (Table 2, Figure 1, Table S1),
319 and 1.9-times higher in 2014 compared to the following year (Table 2, Figure 1, Table S1).
320 Selenium followed the same annual trends as cadmium. However, contrary to cadmium and
321 most other metals (except lead), selenium was significantly higher (i.e. 3.1-times) in Lieto than
322 in Harjavalta (Table 2, Table S1). Manganese only varied annually, being 2.0-times higher in
323 2014 than 2015 (Table 2, Table S1). Age had no effect in metal element levels (Table 2). Means
324 (±SE) for each element are given in supplementary Table S1.

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

328 Calcium was 1.8-times higher in 2014 compared to the following year, 2.0 times higher
329 in Lieto than in Harjavalta and 1.9-times higher in males than in females (Table 2, Figure 2,
330 Table S1). In contrast, zinc concentrations were 1.4-times higher in females than males (Table
331 2, Figure 2, Table S1). Same annual trends as in calcium were also observed for zinc i.e. higher
332 concentrations in the first year of sampling (Table 2, Table S1). Correlations between metals
333 are presented in supplementary Table S2.

334 The principal component analysis (PCA) of metal elements including As, Cd, Co, Cu,
335 Ni and Pb revealed two principal components with eigenvalues larger than one. The first
336 principal component (PC1, eigenvalue = 3.05) represented 51% of the total variation, the main
337 loadings coming from Cd, Co, Cu and Ni. The second principal component (PC2, eigenvalue
338 = 1.60) represented 27% of the variation with main loadings from As and Pb and in a smaller
339 manner Ni.

340 *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 Status

Correlations between metals and oxidative status markers (tGSH, GSH:GSSG, SOD, CAT and GP), were observed for the most part in relation to SOD and CAT. In specific, CAT correlated negatively with the metals Cu (r=−0.43, p<0.01, n=46), Ni (r=−0.39, p<0.01, n=46) and Mn (r=-0.32, p=0.03, 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), As (r=0.36, p=0.02, n=45) and Ca (r=0.37, p=0.01, n=45). Total glutathione also showed a positive association with Ca (r=0.29, p<0.05, n=46), while GSH negatively correlated with Se (r=-0.35, p=0.02, n=46). We observed no significant relationships between biometrics and oxidative status.

We found that PC2 of metals (As, Cd, Co, Cu, Ni and Pb), and year predicted SOD activity (Table 3, Figure 4). SOD activity was higher in 2015 and positively related to PC2 (Table 3, Figure 4), probably due to elevated concentrations of Pb and As (main components of PC2) found around Lieto in 2015. Instead, PC1 showed a negative association with CAT (Table 3). Means (±SD) of oxidative status markers are presented in supplementary Table S3.

4. Discussion

Metal concentrations in the feces of *M. daubentonii* reflected the exposure to ambient contamination. 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.

Copper, cobalt and nickel were three of the metal elements found at elevated concentrations in the bats living around the smelter. This is consistent with studies on passerine

370 bird species around the smelter area, where same metals have been found at larger
371 concentrations when compared to clean site groups (Eeva and Lehikoinen 1996, Berglund et
372 al. 2011), explained by the historical atmospheric metal deposition of copper, nickel and other
373 smelting by-products of the facility (Kiikkilä 2003). When comparing the values of these
374 elements from our study with literature on bats (see Zukal et al. 2015), we observed that the
375 concentrations were in general comparable to what was reported for guano of other
376 insectivorous bats. For example, compared to the values reported in Zukal et al. 2015, the mean
377 values in our study were lower for copper (126.5 vs 205.7 µg/g d.w), similar for cobalt (1.3 vs.
378 2.0 µg/g d.w., minimum value), while ca. 3-times higher for nickel (12.3 vs. 4.5 µg/g d.w.).

379 The elevated bat fecal values observed, particularly of nickel, are possibly linked to the
380 metal spillage occurrence around the smelter during 2014, where 66 tons of nickel were
381 released into the river adjacent to the smelter, main feeding ground of the bats in study.
382 Furthermore, maximum fecal nickel values in our study corresponded to bats sampled during
383 the same year, suggesting that nickel in feces may reflect the water and sediment nickel
384 concentration. Similar findings have been reported for the frugivorous bat *Neoromicia nana*
385 and the diminutive serotine bat (*Eptesicus diminutus*) where nickel in internal organs correlated
386 to ambient nickel concentrations (Zocche et al. 2010, Naidoo et al. 2013). The extraction of
387 nickel is closely associated to cobalt presence, which may explain the elevated concentrations
388 of the latter in bat feces as well. Even though both nickel and cobalt are essential elements, at
389 high enough concentrations they can exert toxic effects by way of oxygen radical production
390 (Valko et al. 2005). Nickel may cause genotoxicity by overproduction of reactive oxygen
391 species (Costa 1996), whereas cobalt may lead to carcinogenic alterations related to the
392 respiratory system (Princivalle et al. 2017), possibly connected to the production of superoxide
393 radicals when cobalt reacts with hydrogen peroxide (Valko et al. 2005). Copper, also an
394 essential element under homeostatic regulation, forms part of active sites of antioxidant
395 enzymes namely catalase, superoxide dismutase and peroxidase (Nieminen and Lemasters
396 1996). Excess concentration of copper may trigger lipid peroxidation by excessive reactive
397 oxygen species production and depletion of glutathione (Nieminen and Lemasters 1996).

398 Accumulation of the non-essential cadmium in kidney and liver occurs with time
399 (Goyer 1997). Thus, the long-lived bats may be prone to the toxicity and prolonged exposure
400 of cadmium, even when this occurs at low concentrations. We expected cadmium
401 concentrations to differ between juveniles and adults, especially since the lifespan of *M.*
402 *daubentonii* in the wild can reach well over a couple of decades. 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). Nevertheless, cadmium exposure may exert negative effects due to its interaction with essential elements such as calcium and zinc, which stimulate the decalcification of bones (Scheuhammer 1987, Goyer 1997).

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; seeing that female bats require more calcium especially during lactation and gestation (Booher 2008), they may be more efficient at extracting calcium from the food items compared to males. But considering also that 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 our study females may reflect inadequate calcium in the body, and/or exhausted calcium storages during the breeding phase, as suggested by Studier et al. (1991). Understanding this would require measurements of calcium concentrations in internal organs, which were not part of this study. 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. Taking this into account, sex-differences may not only be related to absorption efficiencies between males and females, but also behavioral feeding patterns. For instance, calcium deficiency in female bats may be due to more opportunistic and less selective feeding during the reproductive period (Studier et al. 1991). Regardless of the reasons for the sex-differences in calcium, we observed negative associations between calcium and forearm length, being more relevant in juvenile bats compared to adults, possibly suggesting the vulnerable state of young's calcium metabolism, which may be compromised when exposed to metals known to interact with calcium, such as cadmium and lead (Ruiz et al. 2016).

Lead interferes with calcium absorption at the molecular level by competing for intestinal binding sites (Dauwe et al. 2006). We found unexpectedly high concentrations of lead, averaging 31 µg/g d.w., in feces of bats from the water mill in Lieto. Concentrations of 20.9 µg/g d.w. in 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

436 bats (Zook et al. 1970, Skerratt et al. 1998). Considering that in bats, fecal concentrations are
437 generally higher in feces compared to internal tissue concentrations (Zukal et al. 2015) and the
438 lack of symptoms characteristic of lead poisoning (Sutton and Wilson 1983), it is possible that
439 the fecal lead concentrations found in our study, although seemingly high, relate to internal
440 lead levels below concentrations to cause toxicity. However, it cannot be ruled out such levels
441 of lead exposure may have had negative consequences in other aspects of bat's health, such as
442 the aforementioned calcium disruption. Sources of lead exposure in urban areas originate from
443 industrial emissions (Hariono et al. 1993, Ruiz et al. 2016), lead-based paints in old buildings
444 and exhaust of vehicles running on leaded gasoline. However, the latter two have been banned
445 some decades ago (Clark 1979, US-EPA 1998). Therefore, it is possible that sources of lead
446 may come from a localized point of lead in the old building (water mill) in which the Lieto bats
447 roost. Though, this is only a conjecture and further studies to confirm this are needed.

448 Zinc and selenium have protective roles against oxidative stress and the deficiency of
449 zinc can compromise the immune system (Valko et al. 2005, Rautio et al. 2010). In our study,
450 zinc in feces varied by sex, although this difference was significant only when considering the
451 year effect. In that sense, the annual differences between metal exposure may have influence
452 zinc values. We found positive associations between zinc with cadmium, copper and nickel.
453 Of these, zinc and cadmium interactions are better documented in literature. For example, a
454 deficiency of zinc contributes to cadmium absorption (Peraza et al. 1998, Reeves and Chaney
455 2004), while the presence of cadmium reduces zinc absorption, resulting in higher amounts of
456 zinc excreted in feces (Brzóska and Moniuszko-Jakoniuk 2001). The fact that the female bats
457 excreted more zinc than males, could be indicative of an adverse effect of elevated cadmium
458 concentration on females. However, sex-dependent differences in cadmium were not observed.
459 In the same way as explained for calcium, it is possible that sex-related differences in diet items
460 or feeding patterns may play more important roles in determining the observed sex differences
461 in fecal zinc. Selenium provides defense against copper toxicity (Valko et al. 2005). A
462 deficiency of selenium will impair reproduction in wild animals (Allen and Ullrey 2004). In
463 bats, the highest concentration of selenium in liver (8.96 µg/g d.w.) has been found in *Eptesicus*
464 *fuscus* in a study focused on a fungal disease, white-nose syndrome (Courtin et al. 2010). In
465 our study, selenium was below mean and maximum concentrations described for guano of
466 insectivorous bats (Zukal et al. 2015). In a similar manner as with cadmium, selenium
467 concentration varied annually and by location. Interestingly, only selenium and lead were
468 higher in Lieto than Harjavalta compared to the other elements analysed.

469 Some of the essential elements analysed in our study ameliorate the toxic effects of
470 non-essential metal elements when consumed in adequate amounts (e.g. zinc, calcium), while
471 others provide antioxidant protection (e.g. selenium). Antioxidants defend the organism from
472 the chemically reactive species formed after oxygen metabolism (Halliwell and Gutteridge
473 2007). At the same time, the production of such reactive oxygen species (ROS) can increase
474 due to immune reactions, pollution, reoxygenation after hypoxia during hibernation, food
475 scarcity and predation (Costantini 2014). Here, we observed marked differences in catalase
476 activities in our study groups. Catalase is an enzyme with antioxidant function which converts
477 hydrogen peroxide to water and oxygen in instances when hydrogen peroxide concentrations
478 are particularly elevated (Halliwell and Gutteridge 2007, Costantini 2014). The smelter bats
479 presented the lowest catalase activity during the year of accidental metal spillage. Catalase may
480 be inhibited by copper and other metal ions (Gaetke and Chow 2003). Our findings are in line
481 with the observed negative correlations between catalase and levels of non-essential metal
482 elements know for exerting toxicity (e.g. Cd). In addition, we also observed an effect of year
483 and the second principal component of metal elements (As, Cd, Co, Cu, Ni and Pb), which
484 main loadings belong to arsenic and lead, on superoxide dismutase activity. Superoxide
485 dismutase, which catalyzes the conversion of superoxide radicals into hydrogen peroxide and
486 oxygen (Halliwell and Gutteridge 2007) presented higher enzymatic activity during our second
487 and on average less polluted sampling year. It is possible that in a similar manner, as described
488 for the Algerian mice (*Mus spretus)* living in a polluted copper-mine area (Viegas-Crespo et
489 al. 2003), the exposure to elevated metal elements may decrease the superoxide dismutase
490 activity.

491 Immunotoxicity is described as the weakening of the immune system because of
492 sustained or elevated pollutant exposure (Propst et al. 1999), rendering the individual
493 vulnerable to parasite infestation, among other effects. At the same time, responses to immune
494 challenge (e.g. parasite infestation) can generate reactive oxygen species (Schneeberger et al.
495 2013, Lilley et al. 2014). In that sense, an immune response to pollutant challenge may activate
496 oxidative enzymes, while pollutants may on their own do the same by causing oxidative stress.
497 In this study, we observed that bats living close to the smelter in Harjavalta had higher parasite
498 infestation compared to the water mill bats from Lieto. In addition, variation between locations
499 in catalase activities was only observed in 2014, the same year in which higher concentrations
500 of cadmium, copper and nickel were detected around the smelter in Harjavalta. Although, the
501 positive correlations between cadmium and copper with parasites (in wing) were weak, we

502 speculate that the combination of pollutants and parasites may have contributed to a decrease
503 in the activity of catalase. Even though lower catalase activities in response to a metal pollutant
504 have already been described in other mammals (Ossola et al. 1997), immune marker tests
505 should accompany the current study to support the hypothesis of an additive effect of parasite
506 infestation and pollutant exposure on catalase activity in bats.

507 The limited alteration found in the other oxidative status markers examined (tGSH,
508 GSH:GSGG ratio, GP) may be explained by the resistance to oxidative stress characteristic of
509 bats, which by their life-history traits i.e. longevity, exposure to drastic oxygen fluctuations
510 (from entering torpor and hibernation) have possibly developed a stronger defense mechanism
511 against the generation of oxygen radicals (Brunet-Rossinni 2004, Wilhelm Filho et al. 2007,
512 Salmon et al. 2009). For example, compared to short-lived small mammals, bats release
513 hydrogen peroxide at lower rates (Brown et al. 2009).

514 Other factors, such as timing of bat sampling in relation to entering or leaving
515 hibernation (sampling month) are also important to consider. For instance, *M. daubentonii* are
516 lighter in body mass after arousal from hibernation (April) because they have depleted their
517 fat-reserves during the boreal winter. This can affect release of toxicants accumulated in fat
518 into the bloodstream. However, such factor is more relevant when evaluating lipophilic
519 contaminants such as polyaromatic hydrocarbons, which tend to accumulate in adipose tissue
520 (Bayat et al. 2014). Metals, unless found in their organometallic form (e.g. methylmercury,
521 tetraethyl lead) behave chemically different and generally tend not to accumulate in fat (Yates
522 et al. 2014). Another factor to contemplate in the interpretation of fecal metal concentrations is
523 how well they correlate to internal (i.e. organ) concentrations, because the latter are the ones
524 usually representative of potential toxic or adverse effects in the organism. Comparative studies
525 of metal concentrations among different tissues in passerine birds concluded that metal
526 concentrations in feces are not necessarily correlated with internal tissue concentrations
527 (Berglund et al. 2011). In bats, concentrations of non-essential elements (As, Cd, Pb) in tissues
528 such as bone and fur may reflect long-term exposure, whereas softer tissues including brain,
529 muscle and blood would represent recent exposure (Hariono et al. 1993). In a similar manner,
530 metal concentrations in feces will most likely reflect recent exposure mostly via diet, water
531 (Studier et al. 1994, Naidoo et al. 2016) and transfer to feces via biliary excretion (Gregus and
532 Klaassen 1986). However, comparative studies of metal concentrations in internal tissues and
533 feces at the individual level from the same study are to our best knowledge lacking for bats,
534 because obtaining internal tissues may require sacrifice of bats. Still, the lack of these

535 comparative reference values makes the assessment of toxic effects difficult in this study.
536 Lastly, excretion rates of insectivorous bats which range from 15 to 90 minutes after food
537 ingestion in *M. daubentonii* (Webb et al. 1993), likely make the metal turnover fast. Thus,
538 possibly affecting the metal values observed in feces.

539 # 5. Conclusions

540 Our study makes use of a minimally invasive and understudied format (i.e. fecal pellets) to
541 evaluate exposure to metal contaminants in free-ranging bats. The elevated concentrations of
542 metal elements (cadmium, copper, nickel) commonly found in other vertebrate species around
543 the smelter study site (Eeva and Lehikoinen 1996, Eeva et al. 2009) and the correlations
544 between an incidental metal discharge around our polluted study site (smelter) indicate that
545 fresh fecal pellets can be a suitable material to assess metal exposure on an individual basis
546 and show promise for use in biomonitoring studies. However, careful consideration on how
547 representative the fecal metal values are of the internal metal body burden should be taken into
548 account. Significant differences in catalase and superoxide dismutase between our study sites
549 may suggest the onset of physiological stress, possibly caused by excessive non-essential toxic
550 metal concentrations in the environment, although contribution from parasite prevalence
551 cannot be ruled out. To our best knowledge, this is the first study in which metal exposure in
552 relation to oxidative status is reported on an individual basis in non-captive bats. Further
553 studies, adding the evaluation of immune status markers would be valuable in understanding
554 the effect and relation of metal pollutant exposure with oxidative status.

555 # Acknowledgments/ Permits

556 Paul Ek and Sten Lindholm (Åbo Akademi) are acknowledged for the heavy metal analysis.
557 We thank Ville Vasko for help during field sampling and Janina Stauffer for advice on
558 oxidative status protocols. Silvia Espín and Pablo Sánchez-Virosta are acknowledged for
559 assistance in laboratory. We thank three anonymous reviewers for their comments on earlier
560 versions of this manuscript. Approval was granted by the Animal Ethics Committee of the
561 University of Turku (license number ESAVI/3221/04.10.07/2013) and Centre for Economic
562 Development, Transport and the Environment collection permit (license number
563 VARELY/948/2015). This study was financed by Academy of Finland (T.E., project: 265859),

564 the University of Turku Foundation and the Department of Biology (S.R.R.) and the H2020
565 Marie Sklodowska-Curie Actions (T.M.L., 706196).

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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 (g)[c]	30	8.56 ± 0.22	20	8.06 ± 0.24	40	9.25 ± 0.16	10	7.37 ± 0.31
Forearm length (mm)[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]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

LM 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 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	0.02(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 (As, Cd, Co, Cu, Ni and Pb). LM with Gaussian distribution. Final terms in model are bolded. Significance set at p<0.05

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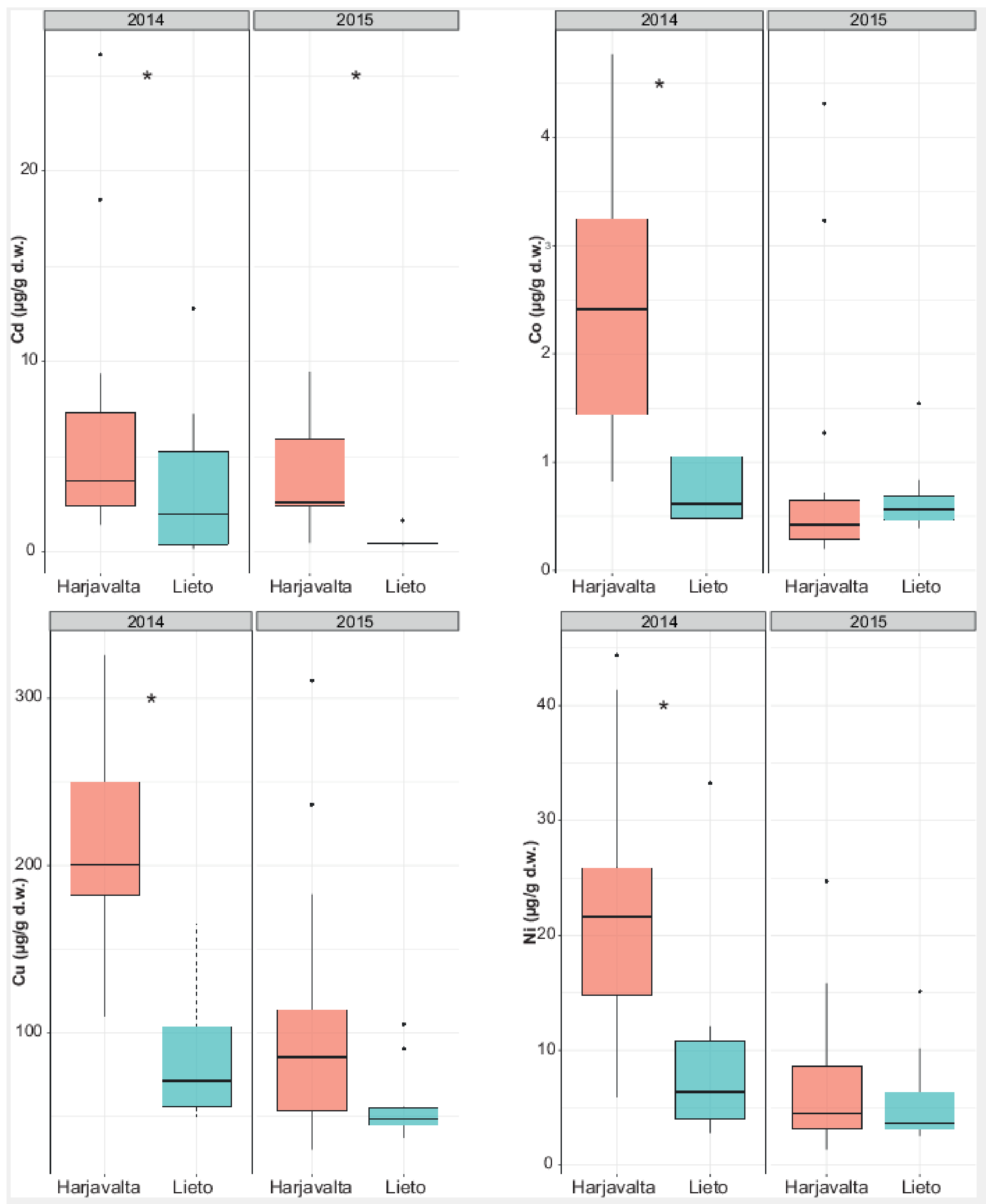

574 **Figure 1.** Concentrations (µg/g dry weight) of cadmium (Cd), cobalt (Co), copper (Cu) and
575 nickel (Ni) in feces of *Myotis daubentonii* collected during the years 2014 (Harjavalta: n=17;
576 Lieto: n=9) and 2015 (Harjavalta: n=15; Lieto: n=10). Asterisks denote significant differences
577 between locations within a year.

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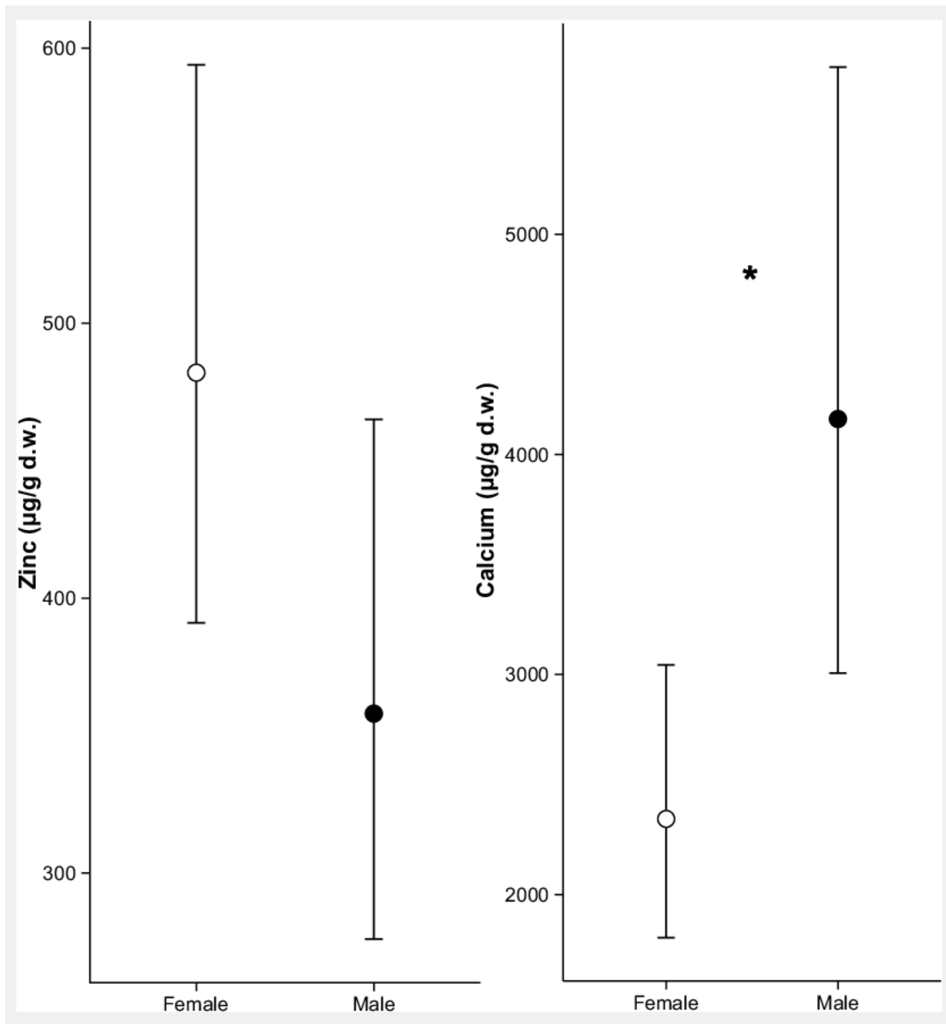

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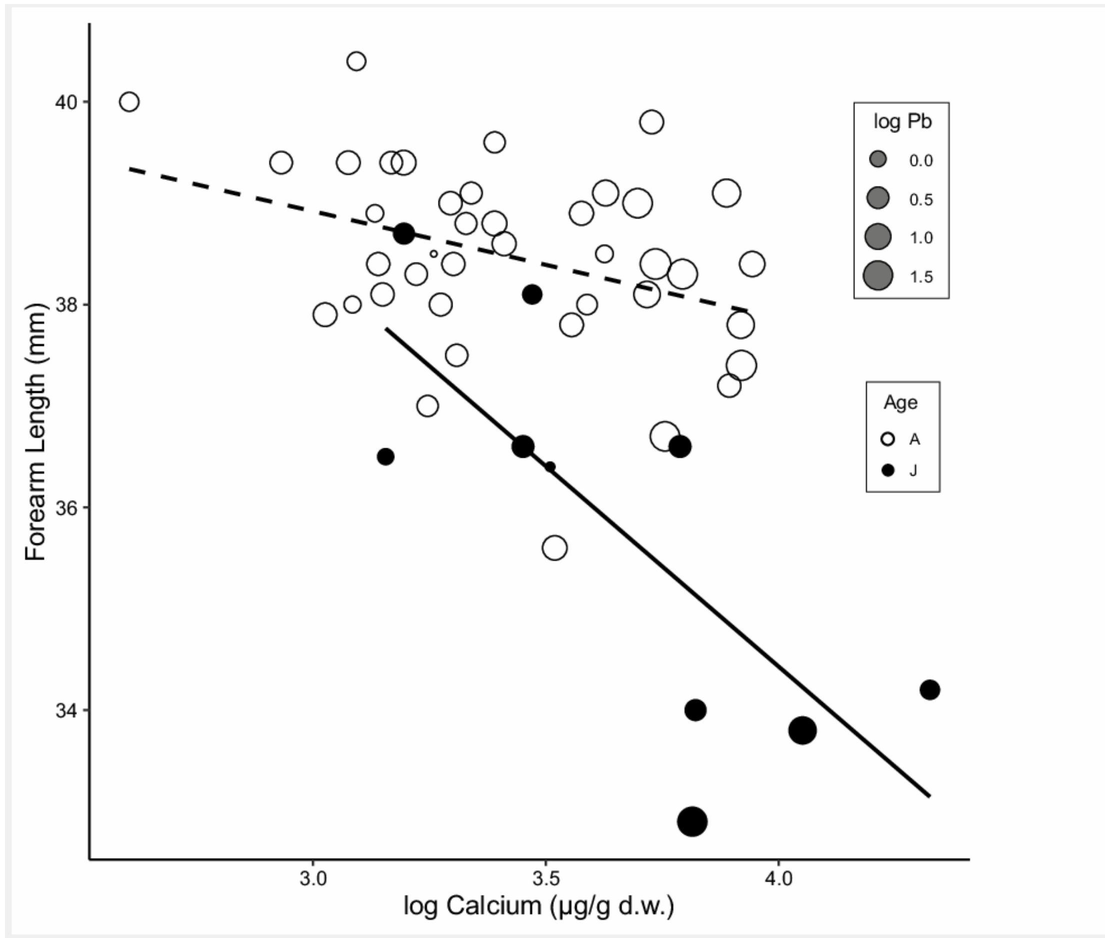
583 **Figure 2.** Mean (±95%CI) Zinc and Calcium concentrations (µg/g dry weight) in feces of female
584 (n=31) and male (n=20) *Myotis daubentonii*. LM(Zinc): Sex: $F_{df}=3.19_{(1,49)}$, p=0.08. LM(Calcium):
585 Sex: $F_{df}=7.65_{(1,49)}$, p<0.01. Asterisk denotes significant difference between females and males.



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587 **Figure 3.** Relationship between fecal calcium concentration (µg/g dry weight) and forearm
588 length (mm). Empty and filled circles denote adults (A) and juveniles (J), respectively;
589 regression lines correspond to adults (dashed) and juveniles (solid); size of the circles denotes
590 the fecal concentrations of lead. LM (Forearm Length): Calcium: $F_{df}=24.6_{(1,46)}$, $p<0.0001$; Age:
591 $F_{df}=5.05_{(1,46)}$, $p=0.03$; Calcium*Age: $F_{df}=8.31_{(1,46)}$, $p<0.01$).

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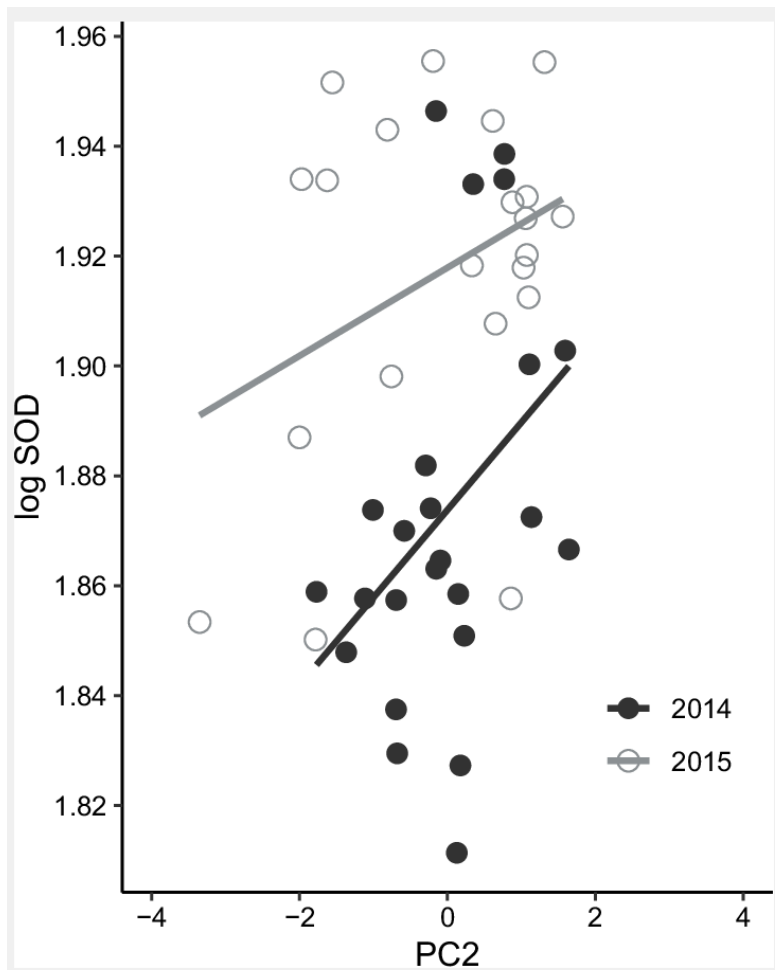

595 **Figure 4.** Relationship between second principal component (PC2) of metal elements (As, Cd,
596 Co, Cu, Ni and Pb) and superoxide dismutase (SOD) enzymatic activity. Filled and empty circles
597 are individuals trapped during 2014 and 2015 respectively. LM for SOD are shown in Table 3.

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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

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Table S2. Correlations between metal 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 $p<0.05$

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Table S3. Oxidative status mean (±SD) in red blood cells of *Myotis daubentonii* per Year and Location.

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

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614 # References

615 Allen M, Ullrey D (2004) Relationships among nutrition and reproduction and relevance for wild
616 animals. Zoo Biol 23:475-487

617 Bayat S, Geiser F, Kristiansen P, Wilson SC (2014) Organic contaminants in bats: Trends and new issues.
618 Environ Int 63:40-52

619 Berglund ÅMM, Koivula MJ, Eeva T (2011) Species- and age-related variation in metal exposure and
620 accumulation of two passerine bird species. Environmental Pollution 159:2368-2374

621 Booher CM (2008) Effects of calcium availability on reproductive output of big brown bats. J Zool
622 274:38-43

623 Boyd RS (2010) Heavy Metal Pollutants and Chemical Ecology: Exploring New Frontiers. J Chem Ecol
624 36:46-58

625 Bradford MM (1976) Rapid and Sensitive Method for Quantitation of Microgram Quantities of Protein
626 Utilizing Principle of Protein-Dye Binding. Anal Biochem 72:248-254

627 Brown JCL, McClelland GB, Faure PA, Klaiman JM, Staples JF (2009) Examining the mechanisms
628 responsible for lower ROS release rates in liver mitochondria from the long-lived house sparrow
629 (*Passer domesticus*) and big brown bat (*Eptesicus fuscus*) compared to the short-lived mouse (*Mus*
630 *musculus*). Mech Ageing Dev 130:467-476

631 Brunet-Rossinni AK, Wilkinson GS (2009) Methods for age estimation and the study of senescence in
632 bats. In: Kunz TH, Parsons S (eds) Ecological and Behavioral Methods for the Study of Bats. Johns
633 Hopkins University Press, pp 315-325

634 Brunet-Rossinni AK (2004) Reduced free-radical production and extreme longevity in the little brown
635 bat (*Myotis lucifugus*) versus two non-flying mammals. Mech Ageing Dev 125:11-20

636 Brzóska M, Moniuszko-Jakoniuk J (2001) Interactions between cadmium and zinc in the organism.
637 Food and Chemical Toxicology 39:967-980

638 Clark D (1979) Lead Concentrations - Bats Vs Terrestrial Small Mammals Collected Near a Major
639 Highway. Environ Sci Technol 13:338-341

640 Costa M (1996) Mechanisms of nickel toxicity and carcinogenicity. In: Chang LW, Magos L, Suzuki T
641 (eds) Toxicology of metals. Lewis Publishers, USA, pp 245-251

642 Costantini D (2014) Oxidative sress and hormesis in evolutionary ecology and physiology. A marriage
643 between mechanistic and evolutionary approaches. Springer, Heidelberg, Germany

644 Costantini D, Meillere A, Carravieri A, Lecomte V, Sorci G, Faivre B, Weimerskirch H, Bustamante P,
645 Labadie P, Budzinski H, Chastel O (2014) Oxidative stress in relation to reproduction, contaminants,
646 gender and age in a long-lived seabird. Oecologia 175:1107-1116

647 Courtin F, Stone WB, Risatti G, Gilbert K, Van Kruiningen HJ (2010) Pathologic Findings and Liver
648 Elements in Hibernating Bats With White-Nose Syndrome. Vet Pathol 47:214-219

649 Dauwe T, Snoeijs T, Bervoets L, Blust R, Eens M (2006) Calcium availability influences lead
650 accumulation in a passerine bird. Animal Biology 56:289-298

651 Dietz C, Nill D, von Helversen O (2009) Bats of Britain, Europe and Northwest Africa. A & C Black,
652 London

653 Duffus J (2002) "Heavy metals" - A meaningless term? (IUPAC technical report). Pure and Applied
654 Chemistry 74:793-807

655 Eeva T, Ahola M, Lehikoinen E (2009) Breeding performance of blue tits (*Cyanistes caeruleus*) and great
656 tits (*Parus major*) in a heavy metal polluted area. Environmental Pollution 157:3126-3131

657 Eeva T, Lehikoinen E (1996) Growth and mortality of nestling great tits (*Parus major*) and pied
658 flycatchers (*Ficedula hypoleuca*) in a heavy metal pollution gradient. Oecologia 108:631-639

659 Encarnação JA, Becker NI, Ekschmitt K (2010) When do Daubenton's bats *(Myotis daubentonii*) fly far
660 for dinner?. Canadian Journal of Zoology-Revue Canadienne De Zoologie 88:1192-1201

661 Encarnação JA, Kierdorf U, Ekschmitt K, Wolters V (2006) Age-related variation in physical and
662 reproductive condition of male Daubenton's bats (*Myotis daubentonii*). J Mammal 87:93-96

663 Encarnação JA, Dietz M, Kierdorf U (2004) Reproductive condition and activity pattern of male
664 Daubenton's bats (*Myotis daubentonii*) in the summer habitat. Mammalian Biology 69:163-172

665 Gaetke L, Chow C (2003) Copper toxicity, oxidative stress, and antioxidant nutrients. Toxicology
666 189:147-163

667 Gerell R, Lundberg KG (1993) Decline of a Bat *Pipistrellus pipistrellus* Population in an Industrialized
668 Area in South Sweden. Biol Conserv 65:153-157

669 Goyer R (1997) Toxic and essential metal interactions. Annu Rev Nutr 17:37-50

670 Gregus Z, Klaassen C (1986) Disposition of Metals in Rats - a Comparative-Study of Fecal, Urinary, and
671 Biliary-Excretion and Tissue Distribution of 18 Metals. Toxicol Appl Pharmacol 85:24-38

672 Halliwell and Gutteridge (2007) Free radicals in biology and medicine. Oxford University Press, New
673 York

674 Hariono B, Ng J, Sutton RH (1993) Lead Concentrations in Tissues of Fruit Bats (*Pteropus* sp) in Urban
675 and Nonurban Locations. Wildl Res 20:315-320

676 Hoffman DJ, Rattner BA, Burton GA, Cairns J (2003) Handbook of Ecotoxicology. Lewis Publishers, USA

677 Huttunen I, Huttunen M, Piirainen V, Korppoo M, Lepistö A, Räike A, Tattari S, Vehviläinen B (2016) A
678 National-Scale Nutrient Loading Model for Finnish Watersheds-VEMALA. Environmental Modeling &
679 Assessment 21:83-109

680 Joint Nature Conservation Committee (2007) Conservation status assessment for Species: S1314 - 681 *Myotis daubentonii* - Daubenton's bat. Second Report by the UK under Article 17 on the 682 implementation of the Habitats Directive from January 2001 to December 2006. Peterborough: JNCC.

683 Kannan K, Yun SH, Rudd RJ, Behr M (2010) High concentrations of persistent organic pollutants 684 including PCBs, DDT, PBDEs and PFOS in little brown bats with white-nose syndrome in New York, USA. 685 Chemosphere 80:613-618

686 Karouna-Renier NK, White C, Perkins CR, Schmerfeld JJ, Yates D (2014) Assessment of mitochondrial 687 DNA damage in little brown bats (*Myotis lucifugus*) collected near a mercury-contaminated river. 688 Ecotoxicology 23:1419-1429

689 Kiikkilä O (2003) Heavy-metal pollution and remediation of forest soil around the Harjavalta Cu-Ni 690 smelter, in SW Finland. Silva Fenn 37:399-415

691 KVVY ry. (2016) Norilsk Nickel Harjavalta Oy. 5.-6.7.2014 tapahtuneen nikkelipäästön vaikutusten 692 selvittäminen. Loppuraportti 2015. Tampere: KVVY ry.

693 Laine VN, Lilley TM, Norrdahl K, Primmer CR (2013) Population genetics of Daubenton's bat (*Myotis* 694 *daubentonii*) in the Archipelago Sea, SW Finland. Ann Zool Fenn 50:303-315

695 Lilley TM, Stauffer J, Kanerva M, Eeva T (2014) Interspecific variation in redox status regulation and 696 immune defence in five bat species: the role of ectoparasites. Oecologia 175:811-823

697 Lilley TM, Ruokolainen L, Meierjohann A, Kanerva M, Stauffer J, Laine VN, Atosuo J, Lilius E-, Nikinmaa 698 M (2013) Resistance to oxidative damage but not immunosuppression by organic tin compounds in 699 natural populations of Daubenton's bats (*Myotis daubentonii*). Comparative Biochemistry and 700 Physiology C-Toxicology & Pharmacology 157:298-305

701 Lilley TM, Meierjohann A, Ruokolainen L, Peltonen J, Vesterinen E, Kronberg L, Nikinmaa M (2012) 702 Reed beds may facilitate transfer of tributyltin from aquatic to terrestrial ecosystems through insect 703 vectors in the Archipelago Sea, SW Finland. Environmental Toxicology and Chemistry 31:1781-1787

704 Lucan RK, Hanak V (2011) Population structure of Daubenton's bats is responding to microclimate of 705 anthropogenic roosts. Biologia 66:690-695

706 Méndez-Rodríguez LC, Alvarez-Castañeda ST (2016) Assessment of Trace Metals in Soil, Vegetation 707 and Rodents in Relation to Metal Mining Activities in an Arid Environment. Bull Environ Contam Toxicol 708 97:44-49

709 Munshi-South J, Wilkinson GS (2010) Bats and birds: Exceptional longevity despite high metabolic 710 rates. Ageing Res Rev 9:12-19

711 Naidoo S, Vosloo D, Schoeman MC (2013) Foraging at wastewater treatment works increases the 712 potential for metal accumulation in an urban adapter, the banana bat (*Neoromicia nana*). Afr Zool 713 48:39-55

714 Naidoo S, Vosloo D, Schoeman MC (2016) Pollutant exposure at wastewater treatment works affects 715 the detoxification organs of an urban adapter, the Banana Bat. Environmental Pollution 208:830-839

716 Naidoo S, Vosloo D, Schoeman MC (2015) Haematological and genotoxic responses in an urban
717 adapter, the banana bat, foraging at wastewater treatment works. Ecotoxicol Environ Saf 114:304-311

718 Nam D, Yates D, Ardapple P, Evers DC, Schmerfeld J, Basu N (2012) Elevated mercury exposure and
719 neurochemical alterations in little brown bats (*Myotis lucifugus*) from a site with historical mercury
720 contamination. Ecotoxicology 21:1094-1101

721 Ngamprasertwong T, Piertney SB, Mackie I, Racey PA (2014) Roosting habits of Daubenton's bat
722 (*Myotis daubentonii*) during reproduction differs between adjacent river valleys. Acta
723 Chiropterologica 16:337-347

724 Nieminen A, Lemasters JJ (1996) Hepatic injury by metal accumulation. In: Chang LW, Magos L, Suzuki
725 T (eds) . Lewis Publishers, USA, pp 887-899

726 Ossola J, Groppa M, Tomaro M (1997) Relationship between oxidative stress and heme oxygenase
727 induction by copper sulfate. Arch Biochem Biophys 337:332-337

728 Park KJ, Müller CT, Markman S, Swinscow-Hall O, Pascoe D, Buchanan KL (2009) Detection of endocrine
729 disrupting chemicals in aerial invertebrates at sewage treatment works. Chemosphere 77:1459-1464

730 Peraza MA, Ayala-Fierro F, Barber DS, Casarez E, Rael LT (1998) Effects of micronutrients on metal
731 toxicity. Environ Health Perspect 106:203-216

732 Pilosof S, Korine C, Moore MS, Krasnov BR (2014) Effects of sewage-water contamination on the
733 immune response of a desert bat. Mamm Biol 79:183-188

734 Podlutsky A, Khritankov A, Ovodov N, Austad S (2005) A new field record for bat longevity. Journals of
735 Gerontology Series A-Biological Sciences and Medical Sciences 60:1366-1368

736 Principalle A, Iavicoli I, Cerpelloni M, Franceschi A, Manno M, Perbellini L (2017) Biological monitoring
737 of cobalt in hard metal factory workers. Int Arch Occup Environ Health 90:243-254

738 Propst T, Lochmiller R, Qualls C, McBee K (1999) In situ (mesocosm) assessment of immunotoxicity
739 risks to small mammals inhabiting petrochemical waste sites. Chemosphere 38:1049-1067

740 Rautio A, Kunnasranta M, Valtonen A, Ikonen M, Hyvärinen H, Holopainen IJ, Kukkonen JVK (2010)
741 Sex, Age, and Tissue Specific Accumulation of Eight Metals, Arsenic, and Selenium in the European
742 Hedgehog (*Erinaceus europaeus*). Arch Environ Contam Toxicol 59:642-651

743 Reeves P, Chaney R (2004) Marginal nutritional status of zinc, iron, and calcium increases cadmium
744 retention in the duodenum and other organs of rats fed rice-based diets. Environ Res 96:311-322

745 Regoli F, Giuliani ME, Benedetti M, Arukwe A (2011) Molecular and biochemical biomarkers in
746 environmental monitoring: A comparison of biotransformation and antioxidant defense systems in
747 multiple tissues. Aquatic Toxicology 105:56-66

748 Ruiz S, Espín S, Rainio M, Ruuskanen S, Salminen J, Lilley T, Eeva T (2016) Effects of dietary lead
749 exposure on vitamin levels in great tit nestlings - an experimental manipulation. Environ Pollut
750 213:688-697

751 Ryan JM, Stilwell DA, Morse NJ (1992) An evaluation of contaminant exposure of three endangered
752 bat species in Virginia. U S Fish and Wildlife Service, Environmental Contaminants Branch, Virginia
753 Field Office, White Marsh, Virginia

754 Salmon AB, Leonard S, Masamsetti V, Pierce A, Podlutsky AJ, Podlutskaya N, Richardson A, Austad SN,
755 Chaudhuri AR (2009) The long lifespan of two bat species is correlated with resistance to protein
756 oxidation and enhanced protein homeostasis. Faseb Journal 23:2317-2326

757 Scheuhammer AM (1987) The Chronic Toxicity of Aluminum, Cadmium, Mercury, and Lead in Birds - a
758 Review. Environmental Pollution 46:263-295

759 Schneeberger K, Czirják GA, Voigt CC (2013) Inflammatory challenge increases measures of oxidative
760 stress in a free-ranging, long-lived mammal. J Exp Biol 216:4514-4519

761 Senthilkumar K, Kannan K, Subramanian A, Tanabe S (2001) Accumulation of organochlorine pesticides
762 and polychlorinated biphenyls in sediments, aquatic organisms, birds, bird eggs and bat collected from
763 South India. Environmental Science and Pollution Research 8:35-47

764 Sies H (1999) Glutathione and its role in cellular functions. Free Radic Biol Med 27:916-921

765 Skerratt L, Speare R, Berger L, Winsor H (1998) Lyssaviral infection and lead poisoning in black flying
766 foxes from Queensland. J Wildl Dis 34:355-361

767 Stauffer J, Panda B, Eeva T, Rainio M, Ilmonen P (2017) Telomere damage and redox status alterations
768 in free-living passerines exposed to metals. Sci Total Environ 575:841-848

769 Studier EH, Sevick SH, Ridley DM, Wilson DE (1994) Mineral and Nitrogen Concentrations in Feces of
770 some Neotropical Bats. J Mammal 75:674-680

771 Studier EH, Viele DP, Sevick SH (1991) Nutritional Implications for Nitrogen and Mineral Budgets from
772 Analysis of Guano of the Big Brown Bat *Eptesicus fuscus* (Chiroptera: Vespertilionidae). Comparative
773 Biochemistry and Physiology A-Physiology 100:1035-1039

774 Sutton RH, Wilson PD (1983) Lead Poisoning in Grey-headed Fruit Bats (*Pteropus poliocephalus*). J
775 Wildl Dis 19:294-296

776 Tchounwou PB, Yedjou CG, Patlolla AK, Sutton DJ (2012) Heavy metal toxicity and the environment.
777 Molecular, Clinical and Environmental Toxicology 101:133-164

778 US-EPA (1998) Sources of lead in soil: a literature review 747-R-98-001a

779 Valko M, Morris H, Cronin M (2005) Metals, toxicity and oxidative stress. Curr Med Chem 12:1161-
780 1208

781 Vesterinen EJ, Ruokolainen L, Wahlberg N, Peña C, Roslin T, Laine VN, Vasko V, Sääksjärvi IE, Norrdahl
782 K, Lilley TM (2016) What you need is what you eat? Prey selection by the bat *Myotis daubentonii*. Mol
783 Ecol 25:1581-1594

784 Viegas-Crespo AM, Lopes PA, Pinheiro MT, Santos MC, Rodrigues PD, Nunes AC, Marques C, Mathias
785 ML (2003) Hepatic elemental contents and antioxidant enzyme activities in Algerian mice (*Mus
786 spretus*) inhabiting a mine area in central Portugal. Sci Total Environ 311:101-109

787 Wada H, Yates DE, Evers DC, Taylor RJ, Hopkins WA (2010) Tissue mercury concentrations and
788 adrenocortical responses of female big brown bats (*Eptesicus fuscus*) near a contaminated river.
789 Ecotoxicology 19:1277-1284

790 Walker LA, Simpson VR, Rockett L, Wienburg CL, Shore RF (2007) Heavy metal contamination in bats
791 in Britain. Environmental Pollution 148:483-490

792 Webb P, Speakman J, Racey P (1993) Defecation, apparent absorption efficiency, and the importance
793 of water obtained in the food for water balance in captive brown long-eared (*Plecotus auritus*) and
794 Daubenton's (*Myotis daubentonii*) bats. J Zool 230:619-628

795 Wilhelm Filho D, Althoff SL, Dafré AL, Boveris A (2007) Antioxidant defenses, longevity and
796 ecophysiology of South American bats. Comparative Biochemistry and Physiology C-Toxicology &
797 Pharmacology 146:214-220

798 Yates DE, Adams EM, Angelo SE, Evers DC, Schmerfeld J, Moore MS, Kunz TH, Divoll T, Edmonds ST,
799 Perkins C, Taylor R, O'Driscoll NJ (2014) Mercury in bats from the northeastern United States.
800 Ecotoxicology 23:45-55

801 Zocche JJ, Leffa DD, Damiani AP, Carvalho F, Mendonça RÁ, Iochims dos Santos CE, Boufleur LA, Dias
802 JF, Moraes de Andrade V (2010) Heavy metals and DNA damage in blood cells of insectivore bats in
803 coal mining areas of Catarinense coal basin, Brazil. Environ Res 110:684-691

804 Zook BC, Sauer RM, Garner FM (1970) Lead Poisoning in Australian Fruit Bats (*Pteropus poliocephalus*).
805 J Am Vet Med Assoc 157:691-&

806 Zukal J, Pikula J, Bandouchova H (2015) Bats as bioindicators of heavy metal pollution: history and
807 prospect. Mammalian Biology 80:220-227

808