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1 **Consequences of trace metal cocktail exposure in zebra finch (*Taeniopygia***  
2 ***guttata*) and effect of calcium supplementation**

3 Agnès Saulnier<sup>a,\*</sup>, Josefa Bleu<sup>a</sup>, Anne Boos<sup>a</sup>, Islah El Masoudi<sup>a</sup>, Pascale Ronot<sup>a</sup>, Sandrine Zahn<sup>a</sup>, Mireille Del  
4 Nero<sup>a</sup> & Sylvie Massemin<sup>a</sup>

5 <sup>a</sup> Université de Strasbourg, CNRS, IPHC UMR 7178, F-67000 Strasbourg, France

6 \*Corresponding author: Université de Strasbourg, CNRS, IPHC UMR 7178, 23 rue du Loess, F-67000 Strasbourg,  
7 France [agnes.saulnier@iphc.cnrs.fr](mailto:agnes.saulnier@iphc.cnrs.fr)

8  
9  
10 **Abstract:**

11 Trace metal elements are significant stressors in urban areas. Their harmful effects on physiological parameters  
12 are demonstrated, but current laboratory studies are not representative of wild chronic exposure to a trace metal  
13 cocktail. Calcium can reduce the accumulation and toxicity of several metals, but soil acidification in cities leads  
14 to a decrease in bioavailability of this mineral. The objective of this study was to investigate the accumulation and  
15 toxicity of a trace metal cocktail representative of urban exposure on passerine birds, and test the importance of  
16 calcium availability on these toxic effects. We exposed zebra finches (*Taeniopygia guttata*) to a cocktail of seven  
17 metals and one metalloid in drinking water, with or without calcium supplementation. We monitored the  
18 concentration of metals in the blood and feathers, and their effects on oxidative status and telomere length. The  
19 metal cocktail led to higher concentration of all elements in the feathers, and of arsenic and lead in the blood. Birds  
20 with a higher concentration of cadmium, arsenic and lead in the feathers had shorter telomeres, but no impact of  
21 the cocktail was detected on oxidative status. Birds of the ‘calcium’ group and the ‘calcium and metal’ group  
22 accumulated higher concentrations of zinc, chromium and nickel in feathers. The ‘calcium and metal’ group also  
23 accumulated lower concentrations of arsenic and lead in feathers compared to the ‘metal’ group. Our results  
24 suggest that chronic exposure to a cocktail of metals at low concentrations has deleterious effects on birds, which  
25 can be limited through calcium intake.

26  
27 **Keywords:** Urban pollution; oxidative stress; telomeres; metal toxicity; birds

## 33 Introduction

34 Trace metal elements (TME), including those commonly called ‘heavy metals’ by misuse of language (Duffus,  
35 2002) in reference to their potential toxicity (e.g. lead (Pb), cadmium (Cd)), are by definition naturally present in  
36 the environment in low concentration (under 1000 ppm in the earth’s crust). However, anthropogenic activities  
37 such as the combustion of fossil fuels, road traffic or industry, lead to massive releases of these metals into the  
38 environment (Järup, 2003). These activities are mainly concentrated in urban areas, leading to higher levels of  
39 TME in cities than in natural habitats. Multiple studies in polluted areas revealed that TME-pollution at very high  
40 concentrations could be related to several damages to birds physiology, primarily oxidative balance (Berglund et  
41 al., 2014; Espín et al., 2017; Sánchez-Virosta et al., 2019). Chronic exposure to TME leads to their accumulation  
42 in different tissues such as the kidneys, liver, muscles, or for birds, in the feathers (Dauwe et al., 2005; Espín et  
43 al., 2016a). Accumulation can lead to the production of reactive oxygen species (ROS) via direct (Fenton) or  
44 indirect (mitochondria dysfunction) reactions (Koivula and Eeva, 2010; Leonard et al., 2004). In response to the  
45 production of ROS, the organism could increase the production of antioxidants such as enzymes and radical  
46 scavengers (Berglund et al., 2007; Rainio et al., 2015). Glutathione (GSH) is a major antioxidant in metal-related  
47 oxidative stress. GSH can reduce ROS, but can also chelate pro-oxidant metal ions through a reaction catalysed  
48 by glutathione peroxidase (GPx) to make them biologically inert and facilitate their elimination (Koivula and Eeva,  
49 2010). Inversely, some metals, notably Pb and cadmium Cd, can also deplete this antioxidant (Espín et al., 2014b;  
50 Fraga, 2005). Glutathione metabolism is a reliable marker of pollutant exposure in urban areas (Isaksson, 2010)  
51 and is frequently used as a metal-stress biomarker (Berglund et al., 2014; De la Casa-Resino et al., 2015; Espín et  
52 al., 2014a; Rainio et al., 2013). Non-neutralised ROS leads to oxidative stress and can cause macromolecular  
53 damage on proteins and lipids leading to the apparition of reactive oxygen metabolite (ROM), but also on DNA.  
54 In particular, it can lead to accelerated erosion of telomeres, which results in early ageing of organisms  
55 (Boonekamp et al., 2017; Epel et al., 2004). Stauffer *et al.* (2017) showed that great tits’ nestlings (*Parus major*)  
56 living in a polluted environment had shorter telomeres than chicks living in an unpolluted environment. However,  
57 in urban environments, a previous study on the same species showed that telomere length is a predictor of birds’  
58 survival, with a selective disappearance of individuals who had shorter telomeres (Salmón et al., 2017). Yet, the  
59 correlation with metal exposure was not demonstrated.

60 Laboratory studies, in a controlled environment, are necessary to tease apart the effects of TME pollution on  
61 oxidative balance and telomere length. Laboratory studies have shown that the toxicity of metals are strongly  
62 regulated by competition processes between metals (Goyer, 1997; Levander, 1978; Wah Chu and Chow, 2002;  
63 Yenice et al., 2015). Some TME, such as zinc (Zn) and copper (Cu), are crucial for biological processes and are  
64 therefore called ‘essential’. Others, such as lead (Pb) and mercury (Hg), are non-essential to biological processes  
65 and are directly toxic to organisms. Antagonistic relationships between these two types of metals have been  
66 demonstrated. Essential TME can limit the accumulation and thereby, the deleterious effects of non-essential  
67 metals (Goyer, 1997; Levander, 1978). For example, Zn has an antagonistic impact on tissue accumulation and  
68 toxicity of Pb (Prasanthi et al., 2010). There may also be synergistic effects between metals, with increased toxicity  
69 of cocktail metals compared to the sum of the toxicities of each metal separately (Wah Chu and Chow, 2002). But  
70 only a few laboratory studies have been conducted on cocktails containing more than two metals (Stankevičiūtė et  
71 al., 2018; Wah Chu and Chow, 2002; Zhu et al., 2004) especially on a bird model (Scheuhammer, 1996; Yenice  
72 et al., 2015). However, in cities, animals are exposed to diffuse pollution, i.e. a cocktail of numerous TME at low

73 concentration, containing essential and non-essential metals and metalloid. Also, exposure to TME is often  
74 associated with emissions of Sulphur dioxide (SO<sub>2</sub>) and Nitrogen oxides (NO<sub>x</sub>) in urban areas, which leads to soil  
75 acidification and limits the bioavailability of essential elements such as calcium (Ca) in the environment  
76 (Scheuhammer, 1991). Ca is crucial for reproduction in birds but is also known to limit the absorption and toxicity  
77 of some metals such as Pb or Cd (Eeva and Lehtikoinen, 2004; Goyer, 1997). For example, Snoeijs *et al.* (2005)  
78 have shown that Ca supplementation in zebra finch resulted in lower Pb concentrations in the kidney and bones  
79 and prevented lead inhibition of second humoral responses. Several studies have also recently shown that Ca  
80 supplementation could increase the antioxidant capacity to cope with metal-related oxidative stress (Espín *et al.*,  
81 2016c; Sánchez-Virosta *et al.*, 2019).

82 In this study, we propose to test the effects of a TME cocktail on the physiology of a bird species, the zebra finch  
83 (*Taeniopygia guttata*), and in addition, the impact of Ca supplementation on the harmful effects of TME. For this  
84 purpose, we exposed birds to a cocktail of 7 trace metals (Cu, Zn, Pb, Cd, chromium (Cr), nickel (Ni) and cobalt  
85 (Co)) and 1 metalloid (arsenic (As)), whose concentrations are representative of exposure in a medium-sized city  
86 (337.61km<sup>2</sup>, 491 000 inhabitants). In the text below, we use TME to refer to both metalloids and metals. First, we  
87 measured the changes in blood and feather TME concentrations, molecular damages linked to ROS, the production  
88 of antioxidants such as GSH, and telomere length. Second, we evaluated the effect of Ca on these measures (pattern  
89 of accumulation, harmful effects on physiology) to evaluate the importance of its availability. Hence, half of the  
90 birds were supplemented with Ca. An increase of TME concentrations in the blood and feathers of the TME-  
91 exposed group was expected, in association with an increase of ROM, which are representative of oxidative  
92 damages on lipids and proteins, telomere shortening and a modification in antioxidants levels (increase or  
93 decrease). An antagonistic effect of Ca on TME concentration and less harmful impacts in Ca-supplemented birds  
94 were also predicted.

## 95 **Material and methods**

### 96 2.1 *Animals and experimental design*

97 Experimentation was performed from February to April during the non-breeding period. Forty adult male zebra  
98 finches in their first year were randomly distributed in four groups of 10 individuals in cages of 150 × 70 × 100  
99 cm<sup>3</sup> in a room with artificial lighting on a 14 h light – 10 h night cycle. To ensure homogeneity between cages,  
100 absence of significant differences in body mass and individual size (represented by tarsus size) between groups  
101 was verified at the beginning of the experiment (ANOVA test, p>0.05). Each group had *ad libitum* access to seed  
102 mix (tropical finches Prestige Versele laga©) which contains low levels of Ca (1 mg/g). After a two weeks  
103 habituation period, each group received a specific treatment provided in bottles of weakly mineralised drinking  
104 water (Montcalm©). Administration of the treatment via drinking water permits to control birds' consumption and  
105 to know with accuracy the amount of TME consumed. Indeed, zebra finch is granivorous. Administration of the  
106 treatment by diet borne would have required to spike the seeds with a solution of the TME cocktail. The TME  
107 would have been located on the surface of seeds. However, birds usually remove the outer layer of the seed before  
108 eating them. In these conditions, control of the exposure to a precise quantity of TME would not have been  
109 possible.

110

111 Treatment lasted for 57 days, with fresh preparations supplied every two days. We measured the quantity of water  
112 drunk for each cage after each replacement of cocktail bottles (no difference between groups,  $p > 0.05$ ). The duration  
113 of treatment was based on previous laboratory studies, which show significant physiological effects for similar  
114 exposure (Chatelain et al., 2016a; Dauwe et al., 2006; Scheuhammer, 1991). Two groups received a TME cocktail  
115 associated with either low or high Ca diet. The two other groups received only low or high Ca diet, without the  
116 TME cocktail. There is a lack of data on the effects of urban TME cocktail in birds in a controlled laboratory  
117 situation. Thus, to prepare a cocktail representative of urban exposure, the composition and concentrations of the  
118 TME cocktail were determined on the basis of fecal TME concentrations measurement of great tits captured in  
119 the Eurometropolis of Strasbourg, France (see Table A.1. of Supplementary materials for more details). Among  
120 the detected elements in great tits' feces, TME chosen for the cocktail were the TME whose concentrations are  
121 regulated by European legislation and/or which are known to have deleterious effects on birds. Fecal TME  
122 concentrations are correlated with food TME concentrations (Bel'skii et al., 1995; Dauwe et al., 2004) and  
123 represent both the non-assimilated portion of TME in food and the portion that has been assimilated and excreted.  
124 Thus, it represents the minimal exposure of birds. Moreover, these measures of fecal TME concentrations allow  
125 to assess the proportion of each TME in a cocktail. These concentrations are representative of the minimal chronic  
126 exposure in a medium-size city. Only Pb concentration was increased in the cocktail (1ppm measured but 10ppm  
127 in the cocktail) to work on values closer than those used in previously published experiments (Chatelain et al.,  
128 2016b; Dauwe et al., 2006; Snoeijs et al., 2005). Finally, the TME cocktail contained 10 ppm (48.3  $\mu\text{M}$ ) of Pb  
129 (pure metal concentration prepared from  $\text{PbCl}_2$ ), 0.7 ppm (13.5  $\mu\text{M}$ ) of Cr (as  $\text{Cr}(\text{NO}_3)_3$ ), 1 ppm (13.3  $\mu\text{M}$ ) of As  
130 (as  $\text{NaAsO}_2$ ), 100 ppm (1.5 mM) of Zn (as  $\text{ZnCl}_2$ ), 24 ppm (0.4 mM) of Cu (as  $\text{CuCl}_2$ ), 0.5 ppm (8.5  $\mu\text{M}$ ) of Ni  
131 ( $\text{NiCl}_2$ ), 0.2 ppm (3.4  $\mu\text{M}$ ) of Co (as  $\text{CoCl}_2$ ) and 0.1 ppm (0.9  $\mu\text{M}$ ) of Cd ( $\text{CdCl}_2$ ). Groups with high Ca diet  
132 received 1 % Ca in food (seed + water as  $\text{CaCl}_2$ ) associated with *ad libitum* cuttlefish bones. Low Ca diet groups  
133 received 0.3 % Ca in food, in accordance with the minimum recommended intake for this species and to avoid  
134 deprivation effects (Tully et al., 2000). Ca supplementation on water is regularly used in both wild (Poulin and  
135 Brigham, 2001) and laboratory (Damron and Flunker, 1995) studies. In brief, we had four groups: (1) 'TME'  
136 group: TME cocktail + Ca 0.3 %, (2) 'TME + Ca' group: TME cocktail + Ca 1%, (3) 'Ca' group: Ca 1%, (4) 'Ctrl'  
137 group: Ca 0.3 %. Three birds died on the first 30 days of treatment (1 individual in 'TME+Ca' group, 1 in 'Ca'  
138 group and 1 in 'Ctrl' group) and were not included in analyses. To avoid an effect of cage position, we switched  
139 top cages with bottom cages after 29 days of experimentation. This experiment has been approved by the French  
140 national ethical committee n°35 (project APAFIS#12020-2017101116394831 v5).

141

## 142 2.2 Sampling

143 Before the treatment, tarsus length was measured with a calliper ( $\pm 0.1$  mm) and body mass ( $\pm 0.1$  g) with a  
144 precision scale. Blood and feathers were collected to measure physiological parameters and concentrations of TME  
145 in different tissues. During the experiment, birds and collected blood samples were weighed every two weeks. For  
146 blood samples, 100  $\mu\text{L}$  was taken from the brachial vein using 100  $\mu\text{L}$  heparinised capillaries and separated into  
147 two tubes of 50  $\mu\text{L}$ . One tube was immediately frozen at  $-20$  °C for TME concentration analyses. The other one  
148 was centrifuged at 1700 RCF for 10 min to separate plasma and erythrocytes before being conserved at  $-20$  °C for  
149 two months. Laboratory analyses were performed immediately after the end of the experimentation. On each bird,

150 the right fourth primary feather was collected at 0 and 29 days of treatment. In this species, feathers are typically  
151 fully regrown in 26 days (Zann, 1996). Removed feathers were immediately frozen at -20 °C for further TME  
152 concentration analyses.

### 153 2.3 TME analyses in feather and blood

154 TME concentrations were monitored in feathers and blood of birds. These two compartments were selected to  
155 realise non-invasive and longitudinal sampling. Moreover, the concentrations of TME in feather and blood are  
156 good indicators of metal exposure (Lodenius and Solonen, 2013).

157 Blood samples were weighted for digestion to assure a lower uncertainty on the TME concentration measurements  
158 than with volume sampling. Nitric acid solution (HNO<sub>3</sub> 68%, trace quality) was added and the mixture was heated  
159 to 60°C for two hours. Then, digested samples were diluted with deionized water before analyses. The added  
160 quantity of nitric acid and deionized water was calculated for each sample following this proportion: 0.025 g of  
161 blood + 0.25 mL of HNO<sub>3</sub> + deionized water QSP 2.5 g. Using the same proportions of acid and water for all  
162 samples whatever the sample weight leads to better control of matrix effects during the measurements.

163 Total feathers (3 to 6 mg depending on samples) were dehydrated overnight at 80 °C. Dried feathers were weighted  
164 and washed in 0.25 M NaOH and three ultrapure water baths according to the protocol described in Frantz *et al.*  
165 (2012). Then, feathers were fully covered with 1 mL of pure HNO<sub>3</sub> solution (68%, TraceMetal grade, Fischer  
166 Chemicals) and digested overnight at 40 °C before dilution with ultrapure water to a final volume of 5 mL. The  
167 concentration of each TME of the cocktail was determined by inductively coupled plasma mass spectrometry (ICP-  
168 MS Agilent 7700) and was expressed in µg/kg of blood samples or in µg/kg dry mass for feather samples. TME  
169 concentration was determined using a calibration curve prepared by dilutions of a multi-element certified standard  
170 (10 µg/mL, CPI International). Indium 10 ppb (1000 µg/mL, CPI International) was used as an internal standard  
171 (See Table A.2 for the operational conditions of ICP-MS analysis). Analyses were performed on day 0, day 14,  
172 day 29, day 43 and day 57 for blood samples and on day 0 and day 29 for feather samples.

173 For both method, blanks were prepared and measured in the same way as blood and feather samples. Numerous  
174 values were under the limit of detection (LOD) of the instrument for blood analyses (See Table A.2 of  
175 Supplementary Material for the LOD of the ICP-MS analysis). For each TME, the instrumental LOD was  
176 calculated as the mean + 3 standard deviation of 9 blank sample measurements. The method LOD reported in  
177 Tables A.2.1 and A.2.1 could change between the samples as the method LOD is impacted by the dilution factor,  
178 which depends on the sample mass available for analysis. Indeed, blood mass depends on the blood collected and  
179 is different according to individuals. Specificity of the analytical methods was checked by recovery measurements  
180 of spiked samples. Recovery values were 90-110% for all elements.

181

### 182 2.4 Physiological analyses in erythrocytes and plasma

183 Relative telomere length (RTL) was measured on erythrocytes (day 0, day 29 and day 57), by quantitative real-  
184 time amplification method (qPCR) adapted for birds, following the method of Criscuolo *et al.* (2009). DNA was

185 extracted and purified with Nucleospin® Blood QuickPure kits (from Macherey-Nagel©). Then, the quantity and  
186 purity of the extracted DNA were measured using a microvolume spectrometer, the Nanodrop 1000 (Thermo  
187 scientific©). The control used was glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, specific of the  
188 zebra finch. Primers used for this control was GAPDH-F: 5'-AACCAGCCAAGTACGATGACAT-3' and  
189 GAPDH-R: 5'-CCATCAGCAGCAGCCTTCA-3' diluted to 5 nM. Primers used for telomere were Tel1b: 5'-  
190 CGGTTTGTGGGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3' and Tel2b 5'-  
191 GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3' diluted to 5 nM. Amplification cycle was  
192 performed with 5 ng of DNA per well, and we used the GoTaq® qPCR mix (Promega, Madison, USA). Conditions  
193 for telomere amplification started with 2 min at 95°C followed by 30 cycles of 30 sec at 56°C, 30 sec at 72°C and  
194 1 min 15 sec at 95°C. For GAPDH, amplification started with 2 min at 95°C followed by 40 cycles of 1 min at 60  
195 °C and 1 min at 95°C. Each plate includes a serial dilution of referent individual's DNA (20 ng, 10 ng, 5 ng, 2.5  
196 ng and 1.25 ng). These are used as a standard curve to control the amplification efficiency, which was 102.6 % for  
197 GAPDH and 98.0 % for telomere. Both negative control (water) and melting curves were run for each plate to  
198 check for non-specific amplification and primer-dimer artefacts. Each sample was run in duplicate. The RTL  
199 corresponds to the ratio (T/S) of telomere repeat copy number (T) to control gene copy number (S). The sample  
200 corresponding to the referent individual was designated 'golden sample' with a ratio (T/S) arbitrarily set to 1.  
201 Mean intra-individual Cq (number of cycles necessary to detect amplified DNA above basal signal) variation was  
202  $0.57 \pm 0.04$  % for GAPDH assay and  $1.62 \pm 0.12$ % for telomere assay. Mean inter-run Cq variation was  $0.48 \pm$   
203  $0.05$ % and  $1.95 \pm 0.27$ % for GAPDH and telomere respectively.

#### 204 2.5 Oxidative stress measurement

205 To evaluate oxidative damage, we measured ROM such as hydroperoxides concentration in bird's plasma (day 0,  
206 day 29, day 57). Hydroperoxides are a result of oxidation of several molecules like lipid or glucoside by ROS.  
207 Measures were performed using the d-ROM test (diacron- reactive oxygen metabolite test, Diacron, Italy), adapted  
208 in 96 wells plate. Briefly, 200 µL of the provided acid buffer combined to a chromogenic substrate was added to  
209 4 µL of plasma to cause iron salting-out and incubated at 37°C during 75 min. With iron, hydroperoxide was  
210 converted in alkoxy and peroxy radicals by Fenton reaction. Free radicals will then react with the chromogenic  
211 substrate that turns pink when oxidised. The absorbance of the samples is then measured at 510 nm. The value  
212 obtained is directly proportional to the ROM in the sample. Concentration was determined with a standard sample  
213 with known absorbance and converted to mg of H<sub>2</sub>O<sub>2</sub>/dL (based on Carrateli Units) following the kit instructions.  
214 Samples were run in duplicates. Mean coefficient of intra-individual and inter-plate variation was  $4.99 \pm 1.96$   
215 and  $6.25$  % respectively.

216 Associated with oxidative damage, we performed some antioxidant measures. We measured total (GSH<sub>tot</sub>), free  
217 (GSH) and oxidised glutathione (GSSG) concentration and glutathione peroxidase activity (GPx) on erythrocytes.  
218 Glutathione concentration (GSH<sub>tot</sub>, GSH and GSSG), in µmol/L, was determined using a 5 × 96 well Glutathione  
219 Fluorescence kit (Arbor Assay, USA) on 1:100 diluted erythrocytes in 5 % of 5-sulfosalicylic acids (SSA) ( as  
220 recommended by the fabricant instructions. GPx, in U/mg erythrocytes, was measured with a Randox kit (Ransel,  
221 UK) adapted in 96 wells plate, in 5 µL of 1:40 diluted erythrocytes using the diluting buffer provided with the kit.  
222 Samples were run in duplicates. of intra-individual variations were of  $1.75 \pm 0.75$ %,  $3.78 \pm 2.33$ %,  $2.25 \pm$

223 1.67% for GSHtot , GSH and GPx respectively. Mean coefficients of inter-plate variation was of 2.70 % and 4.30%  
224 for GSHtot and GPx respectively.

## 225 2.6 Statistical analyses

226 Statistical analyses were conducted with R software (3.4.3 version) with ‘nlme’, ‘car’, ‘lme4’ and ‘multicomp’  
227 packages (R Development Core Team, 2016).

### 228 2.6.1 Effect of TME and Ca supplementation on TME concentrations in blood and feathers

229 Statistical approaches were different between blood and feather concentrations. For feathers, all TME  
230 concentrations were above the LOD except Co concentrations. We performed statistical tests taking into account  
231 the “cocktail” effect of metals on physiology and allowing the visualisation of accumulation patterns. On the  
232 contrary, in blood, there were many metal concentrations under the LOD, except for Cu and Zn. Thus, calculations  
233 have been done for each TME considered individually. Data under the LOD indicate that these concentrations are  
234 very low and cannot be determined. But this data is different from a missing value. This kind of dataset, with both  
235 exact values (above the LOD) and non-exact values (below the LOD), is a left-censored dataset and can be analysed  
236 with specific statistical tools such as those developed in the NADA package (Helsel, 2011). More precisely, for  
237 blood, we used a non-parametric score test that determines whether distribution function differs among groups of  
238 censored data. This is called a generalized Wilcoxon test or a Peto-Prentice test (cendiff function in the NADA  
239 package). The advantage of score tests is that they can handle data censored at multiple reporting limits (Helsel,  
240 2011).

241 Differences between treatments were tested at each exposure time. When Wilcoxon scores were significant,  
242 multiple comparison tests were performed between each pair of groups with a Bonferroni correction of the p-value:  
243  $p\text{-value}_{\text{pairwise}} = 0.05/\text{number of comparison}$ . Corrected p-value was equal to 0.0083 (6 pairs of comparisons) for  
244 multiple treatment comparisons. We considered as significant only the tests with a p-value less than or equal to  
245 this value.

246 For TME concentrations in feathers, principal component analyses (PCA) were performed to observe the  
247 relationships between metals and differences in accumulation patterns between groups. Co was excluded from the  
248 PCA because numerous values were under the LOD. Before analyses, feather TME concentrations were scaled  
249 and centred to normalize the distribution. The Bartlett’s test for sphericity ( $\chi^2_{\text{ddl}(28)} = 190.5$ ,  $p\text{-value} < 0.01$ ) and  
250 correlations were ran to ensure data appropriateness for PCA ( $p < 0.05$ ). For each individual, the composite  
251 variables PC1Feather and PC2Feather corresponding to the first and second principal components of the PCA,  
252 respectively, were extracted. To test the differences of PCA components (PC1Feather and PC2Feather)  
253 between treatments, mixed effect linear models were performed followed by ANOVA of type III for each  
254 component. Models included treatment, exposure time and their interaction as explanatory variables and individual  
255 as a random effect (Bird ID) to take into account the non-independence of repeated measures on the same  
256 individual. When ANOVA were significant, Tukey multiple comparison tests (‘multicomp’ package) were used  
257 to perform post-hoc tests. Normality and homoscedasticity of residuals were verified with Kolmogorov-Smirnov  
258 and Bartlett test, respectively. These variables (PC1Feather and PC2Feather) were also used as explanatory  
259 variables in models for physiological analyses.



## 260 2.6.2 Effect of TME and Ca supplementation on body mass and physiological parameters

261 Before treatment, the homogeneity in body mass and tarsus length (representative of body size) between groups  
262 were checked with ANOVA type III test. There were no differences between groups (body mass:  $F_{3,34}=1.12$ , p-  
263 value=0.36, log(tarsus):  $F_{3,33}=0.87$ , p-value=0.47).

264 Influence of the treatment on physiological parameters and body mass were analysed using mixed effects linear  
265 models followed by type III ANOVA. We tested seven models: body mass, telomere length, GSH, GSSG, GShtot  
266 concentration, GPx activity and ROM concentration. The best model for each parameter was selected using a  
267 stepwise method based on the p-value. For each model tested, the variable or interaction with higher p-value was  
268 deleted until only significant variables were left ( $p < 0.05$ ). When none of the variables was significant, the last  
269 non-significant variable was kept. In all models, the random effect was the individual (bird ID) to take into account  
270 the non-independence of repeated measures on the same individual. Concerning fixed effects, full models included  
271 treatment, time of treatment and its interaction as explanatory variables with body mass and tarsus length as  
272 covariates. For body mass model, only tarsus length was a covariate. When variables were significant, Tukey  
273 multiple comparison tests ('multicomp' package) were used to perform post-hoc tests. Direct effect of TME  
274 exposure on physiological parameters was also tested. For each physiological parameter, linear mixed models were  
275 run with variables PC1Feather and PC2Feather as an explanatory variable (with bird ID as a random effect). For  
276 blood, we performed linear mixed models between each physiological parameter and single TME concentration  
277 above the LOD (with bird ID as random effect). For all linear models, normality and homoscedasticity of residuals  
278 were checked with Kolmogorov-Smirnov and Bartlett test respectively. To validate these conditions, telomere  
279 length, GSH (free, total and GSSG), ROM and GPx were log-transformed. For free GSH and body mass, we  
280 applied treatment-dependent variance (option varIdent) in our models (Zuur et al., 2009) because homoscedasticity  
281 was not respected.

## 282 **Results and discussion**

283 In blood, statistical analyses on Co could not be performed because Co concentrations above LOD represent only  
284 13.9% of all values (Table 1). When values above LOD represent less than 20% of dataset, statistical tests are  
285 unreliable (Helsel, 2011). In blood, less than 50% of values were above the detection limits for Cr, Cd, Ni and As  
286 (22.8% for Cr, 26.1% for Cd, 35.3% for Ni and 37.7% for As). Pb concentrations above LOD represented 66.1%  
287 of total values, and Zn and Cu concentrations were all above the detection limits. In feathers, all values were above  
288 the LOD except for Co (75%). Nevertheless, we did not include the Co in the statistical analysis because we did a  
289 PCA analysis that cannot handle censored values (see material and methods).

### 290 3.1 *Effect of the exposition to TME cocktail and Ca supplementation on blood and feather TME* 291 *concentrations*

292 Concerning the concentration of TME in blood, generalised Wilcoxon tests show no significant difference between  
293 treatments at day 0, except for As (Table 2). Nevertheless, pairwise comparisons revealed no significant  
294 differences for this TME (Table 1, p-values  $>0.0083$ , See Table A.3 of Supplementary material for values of  
295 pairwise tests and p-values). Several significant effects of groups (for example at day 14 for Cr or Cd) were not  
296 supported by significant differences in the pairwise comparisons. As and Pb concentrations in 'TME' group were

297 higher for the period from day 14 to day 57 compared to day 0. The 'TME' group presented higher concentrations  
298 than the three other groups at day 14, 29, 43 and 57 (Table 2, p-values >0.0083) for Pb (except for day 14) and As.  
299 Rich-calcium diet seemed to limit the increase of these two TME concentration in the blood of 'TME+Ca' birds.  
300 At the end of the experiment (day 57), As concentration was higher in the 'TME' group compared to the other  
301 groups and the Pb concentration was higher in the 'TME' group and 'Ca' group (only one value) than in other  
302 groups. Otherwise, Zn concentration was higher in both TME-supplemented groups ('TME' and 'TME+Ca') at  
303 day 29 and only in 'Ca' group compared to "ctrl" group at day 43.

304 The PCA for TME in feathers explained 54.81% of the total variation of TME concentration in feathers (Fig. 1).  
305 The first principal component PC1Feather (36.94% of the variation) was mostly explained and positively  
306 correlated with Cu and also with Zn, Cr and Pb concentrations, but all TME were significantly associated with this  
307 component ( $p < 0.05$ ) (see Table A.4 of supplementary material for contributions and p-values). The second  
308 principal component PC2Feather (18.17% of the variation) was positively correlated with Cd, Pb and As and  
309 negatively with Ni, Zn and Cr. At day 0, the four groups were superposed and presented low concentrations of  
310 TME along both axes. PC1Feather separates feather TME concentrations of 'TME' and 'TME+Ca' groups at day  
311 29 from the initial time and the other groups (see Table A.5 of Supplementary material for TME concentrations in  
312 feathers). This pattern indicates that birds in TME-exposed groups ('TME' and 'TME+Ca') presented higher  
313 concentrations of TME in their regrown feathers (day 29) than in the initial feather and in all feathers of non-TME  
314 exposed birds, especially for Cu, Zn, Cr and Pb. These differences are significant (Table 3; Tukey test, p-  
315 values < 0.05, see Table A.4 of supplementary material for all Tukey p-values), except between groups 'TME' at  
316 day 29 and 'Ctrl' at day 0 (Tukey,  $p\text{-value}_{\text{CtrlT0-TMET29}} = 0.07$ ) and between groups 'TME' at day 29 and 'Ca' at day  
317 29 (Tukey test,  $p\text{-value}_{\text{CaT0-TMET29}} = 0.37$ ). Interestingly, PC2Feathers separates Ca supplemented groups ('Ca'  
318 and 'TME+Ca') and 'TME' group in regrown feathers (day 29), and their TME concentrations were significantly  
319 different (Table 3, Tukey test,  $p\text{-value}_{\text{TMET29-CaT29}}$  and  $p\text{-value}_{\text{TMET29-TME+CaT29}} < 0.001$ ,  $p\text{-value}_{\text{CaT29-TME+CaT29}} = 0.95$ ).  
320 Ca supplemented groups ('Ca' and 'TME+Ca') accumulated a higher concentration of Zn, Ni and Cr than 'TME'  
321 group, which accumulated a higher concentration of Cd, Pb and As.

322 These results confirm that chronic exposure to a low concentration TME cocktail leads to the increase of TME in  
323 blood and feathers notably in the non-essential TME Cd (feathers), Pb (blood and feathers) and As (blood and  
324 feathers). Interestingly, these results also suggest that Ca supplementation leads to an increase of Zn, Cr, and Ni  
325 concentrations in the feathers of TME exposed birds but also for Zn in TME-unexposed birds. On the contrary,  
326 the fact that we found less As and Pb in blood and less As, Pb and Cd in the feathers of Ca-rich diet birds confirmed  
327 our initial hypothesis about the antagonistic effects of Ca supplementation on non-essentials TME. Indeed, the  
328 interaction between Pb and Ca is already well documented. For example in mammals, Six and Goyer (1970) found  
329 similar results in Pb-exposed rat: blood levels of Pb is lower for animals with normal Ca diet (0.7%) compared to  
330 those with low-Ca diet (0.1%). The antagonistic effect was demonstrated in organs like the kidney, bones and liver  
331 (Dauwe et al., 2006; Snoeijs et al., 2005). In zebra finches exposed to 20 ppm of Pb, Pb accumulation in organs  
332 (bones, livers, kidney, muscle and brain) is 2.2 to 3.1 times lower in oyster shell grit supplemented birds (Dauwe  
333 et al. 2006). The same mechanism was observed for Cd, in which low Ca availability leads to higher accumulation  
334 of this metal in tissues, especially in bones (Jaishankar et al., 2014; Scheuhammer, 1996; Stohs and Bagchi, 1995).  
335 However, the relation between a calcium-rich diet and As accumulation is less documented and seems to be

336 dependent on the tissue. For example, in pollution-exposed birds, As was found in eggshell, mainly composed of  
337 Ca, but not in the content of the eggs (Dauwe et al., 2005, 1999; Mora, 2003). Similarly, As supplementation leads  
338 to an accumulation of this element in the bones (composed largely of Ca) of great tits (Sánchez-Virosta et al.,  
339 2018), but supplementation of Ca did not reduce the concentration of As in the blood and kidneys of As-exposed  
340 rats (Srivastava et al., 2010). The mechanism of interaction between As and Ca could be potentially explained by  
341 complexation mechanisms of Ca on As (Arsenic V form) leading to neutral and non-bioavailable species  $\text{CaHAsO}_4$   
342 and  $\text{Ca}_3(\text{AsO}_4)_2$  (Wang et al., 2008). However, the increase of essential-TME in the feathers of Ca supplemented  
343 groups was unexpected but several hypotheses can be suggested. The accumulation of Zn, Ni and Cr is favoured  
344 by Ca pathway. Some studies have shown that the divalent cation form of Ni and Zn metals can compete with Ca  
345 for binding sites on Ca channels or calcium-binding proteins, for example (Komjarova and Blust, 2009). Zn also  
346 contributes to the keratinization process explaining its high concentrations in feathers (Ek et al., 2004). In the same  
347 way, the accumulation of Cr in Ca-exposed bird is potentially linked to Zn homeostasis as revealed in zinc-  
348 deficient rats supplemented with Cr (Hahn and Evans, 2017).

### 349 3.2 Effect of the exposition to TME cocktail and Ca supplementation on body mass and physiological 350 parameters

351 There was no significant effect of TME exposure or Ca supplementation on the body mass or telomere length of  
352 birds (p-value > 0.05, statistics in Table 4, see Table A.6 of supplementary material for estimates values).  
353 Concerning the oxidative status, exposure time had a significant effect on ROM, GSSG and GShtot concentrations  
354 (Tables 4 and 5). ROM concentration of birds was lower at 57 days of treatment compared to the two previous  
355 periods T0 and T29 (Tukey:  $p_{T57-T0}=0.002$ ,  $p_{T57-T29}=0.0003$ , and  $p_{T0-T29}=0.84$ ). On the contrary, GSSG and GShtot  
356 values were higher after 57 days of treatment compared to values measured after 0 and 29 days of treatment (Tukey  
357 test, GSSG:  $p_{T57-T0} = 0.001$ ,  $p_{T57-T29} < 0.001$  et  $p_{T0-T29}=0.92$  and GShtot:  $p_{T57-T0} < 0.001$ ,  $p_{T57-T29}=0.01$  and  $p_{T0-}$   
358  $T29}=0.58$ ). For GSH and GPx activity, the interaction between treatment and exposure time was significant.  
359 However, pairwise comparison only revealed that GSH concentration of 'TME' group at 57 days was higher than  
360 the concentration in the 'Ca' group at day 0 (Tukey test,  $p_{\text{ETM57-CaT0}} = 0.04$ ). Similarly, GPx activity in 'Ca' group  
361 at 29 days of treatment was higher than day 0 (Tukey test,  $p_{\text{CaT29-CaT0}} = 0.015$ ).

362 There was also no significant correlation between physiological parameters and concentration of single TME in  
363 blood (Table 6). However, linear models with composite variables PC1Feather and PC2Feathers revealed a  
364 significant negative effect of PC2Feather on telomere length ( $F_{1,35}=4.23$ , p-value=0.047) but no significant effects  
365 of PC1Feathers ( $F_{1,35}=1.08$ , p-value=0.31). Telomere length was shorter in individuals with high concentrations  
366 of Pb, Cd and As and lower concentrations of Zn, Cr and Ni in feathers (Fig. A.1 of supplementary material).  
367 There was no significant effect of composite variables of PCA on the other physiological parameters (Table 4).

368 The fact that birds with higher accumulated concentrations of Pb, As and Cd in feather had shorter telomeres, is  
369 concordant with the work of Stauffer *et al.* (2017). They found shorter telomeres in great tits nestling living in a  
370 metal-polluted area compared to a non-polluted area. Nevertheless, contrary to their study, our results suggest a  
371 direct correlation between TME concentration in tissue and telomere length. Several studies have shown the  
372 genotoxicity of Pb, As and Cd exposure (Koivula and Eeva, 2010). Genotoxicity of these TME is for a major part  
373 linked to an increase of ROS production, which can interact with oxidised DNA bases and leads to strand breaks,

374 but, it is also linked to the inhibition of DNA repair mechanisms (Stojs and Bagchi, 1995). For example,  
375 Japanese quails (*Coturnix japonica*) exposed to 100 ppm of Pb exhibit higher plasmatic concentrations of 8-  
376 hydroxy-2-deoxyguanosine (oxidised by-product of guanine) indicating more DNA strand breaks compared to  
377 unexposed birds (Farag et al., 2018). In the same way, after 40 days of exposure to 1 and 10 ppm Cd, Lucia *et al.*  
378 (2009) observed expression of the DNA damage repairing gene, *ggsh*, of 6.5 and 19.5 times greater respectively  
379 in Muscovy duck (*Cairina moschata*). In Spain, the blood of white stork (*Ciconia ciconia*) nestlings present a  
380 similar concentration of As (i.e. 0.02 ppm) and lower concentrations of Cd (i.e. 0.00031) than our birds and they  
381 find a significant correlation between DNA damages and As and Cd concentration (Baos et al., 2006). Our results  
382 suggest that even low concentration exposure could lead to accelerated telomere attrition on birds living in metal-  
383 polluted areas, which could affect their longevity (Epel et al., 2004). However, telomere length of birds in 'TME'  
384 group was not different from length for birds exposed to other treatments or at the initial time. This absence of  
385 significant effect can be explained by the fact that birds which exhibited high concentrations of As, Pb, or Cd were  
386 not necessarily in the 'TME' group. For example, there was no significant difference between 'TME' at day 29  
387 and 'Ctrl' at day 0 and 'TME' at day 29 and 'Ca' at day 29 along the axis PC1Feather. In the first case, it is  
388 probably because birds of 'Ctrl' group at day 0 exhibited similar levels of Pb and Cd in feathers as the 'TME'  
389 group at day 29. The concentrations of these TME in 'Ctrl' group decreased during the experiment and thus  
390 concentrations at day 29 in 'Ctrl' group were lower than concentration in initial feathers. In the second case, it  
391 could be linked to the fact that 'Ca' group exhibited higher Zn concentration at day 0 compared to day 29.

392 There was no significant relationship between feather and blood TME concentration and antioxidants measured  
393 (GSH, GSSG and GShtot and GPx) nor between treatment and measured antioxidants. Results only showed a  
394 time-dependent increase of GShtot and GSSG whatever the treatments. Several field-studies exhibit a significant  
395 correlation between TME concentration in tissues and antioxidant production (De la Casa-Resino et al., 2015;  
396 Espín et al., 2014a; Stauffer et al., 2017). For example, in wild griffon vulture (*Gyps fulvus*), a blood concentration  
397 higher than 0.15 ppm of Pb leads to an inhibition of GPx activity (Espín et al., 2014a). In the same way, the blood  
398 GSH concentration of white stork nestlings living in a TME-polluted area (in blood, 21 µg/L of As and 0.103µ/L  
399 of Pb respectively) is 3.9 times higher than birds living in non-polluted areas (De la Casa-Resino et al. 2015). In  
400 great tits females, high concentration of As and Pb in feces is correlated with GPx inhibition, but not with GSH  
401 and GShtot (Stauffer et al. 2017) whereas in pied flycatcher females (*Ficedula hypoleuca*) As and Pb in feces is  
402 positively correlated with GSH and GShtot concentration. (Berglund et al., 2007). However, other studies show  
403 no significant effect of TME-exposition on GPx or GShtot (Berglund et al., 2007, 2014; Koivula et al., 2011). For  
404 example, pied flycatcher nestlings living in TME-polluted areas present higher catalase, glutathione reductase and  
405 glutathione-S-transferase activity than birds in non-polluted areas but no significant differences in GPx and GShtot  
406 was observed between the two areas (Berglund et al., 2007). GSH and GPx activity are a strong marker of oxidative  
407 stress linked to TME exposure (Koivula and Eeva, 2010), but the antioxidant mechanism against TME exposure  
408 seems to be different between species (Berglund et al., 2011; Espín et al., 2016b; Rainio et al., 2013; Stauffer et  
409 al., 2017). It is possible that other biomarkers, such as superoxide dismutase activity or catalase activity, would  
410 have been more impacted by exposure to TME. The time-dependent increase of GShtot and GPx in our study was  
411 associated with a time-dependent decrease of ROM. This result could be an answer to physiological stress due to  
412 frequent manipulation of birds during the experiment. The stress linked to the manipulation could lead to the  
413 increase of ROM, which in turn, have led to the production of GShtot to neutralize them.

414 There was no significant correlation between ROM and TME concentration in tissue. If telomere length attrition  
415 is linked to ROS production, ROS has no visible impacts on lipid and protein damages nor glutathione metabolism.  
416 Several studies have shown that TME exposure leads to an increase in oxidative damage including lipid  
417 peroxidation (Espín et al., 2014a; Kurhalyuk et al., 2009; Prasanthi et al., 2010; Stauffer et al., 2017) but it also  
418 seems to depend on the species. For example, lipid peroxidation is measured in blood (at concentrations higher  
419 than 0.15 ppm of Pb) in the griffon vulture (Espín et al., 2014a) but not in white storks (De la Casa-Resino et al.,  
420 2015). Moreover, some studies show that the correlative negative effects of TME-polluted environments on wild  
421 birds could be linked to other factors such as food availability or food quality (Eeva et al., 2005, 2003; Koivula et  
422 al., 2011). A decrease of carotenoid content in prey is detected in polluted sites (Koivula et al., 2011). Oxidative  
423 stress in great tit chicks is related to the prey quality but not to TME-concentration exposure. In the present study,  
424 the absence of a significant effect on glutathione metabolism may be the result of the use of dietary antioxidant  
425 such as carotenoids by birds. Animals were in a protected laboratory environment with ad libitum food of high  
426 quality (except for Ca in two groups of birds). They have no physiological and ecological constraint and can  
427 allocate more resources to overcome TME and their consequences.

428 Limited As and Pb concentrations in blood in Ca-supplemented birds could also explain the absence of effects  
429 caused by the TME cocktail on physiological parameters, especially on telomere length. Ca bioavailability  
430 indirectly protects against the toxicity of TME by preventing their accumulation. Moreover, a Ca supplementation  
431 can enhance the antioxidant capacity of birds, as has been shown experimentally in wild great tits (Sánchez-Virosta  
432 et al. 2019). The increase of these essential TME in feathers in the case of Ca supplementation could also explain  
433 the higher telomere length. The essentials TME could thus protect the organism against the harmful effects of non-  
434 essential TME. Previous studies have shown the beneficial effects of Zn supplementation on oxidative stress and  
435 accumulation of non-essential TME in the brain (Goyer, 1997; Kucuk, 2008; Prasanthi et al., 2010). In addition,  
436 mice exposed to 2000 ppm of Pb showed less lipid peroxidation and radical formation with supplementation of  
437 200 ppm of Zn (Prasanthi et al., 2010).

## 438 **Conclusion**

439 The aim of this study was to experimentally evaluate the effects of a TME cocktail, representative of urban  
440 exposure, on the concentration of these TME in the blood and feathers, and on the physiology of a passerine bird.  
441 The second aim was to test the role of the availability of Ca on the effects of this cocktail. There are numerous  
442 factors in urban areas, which can influence the physiology of birds. In the wild, it is difficult to tease apart the  
443 effect of each pollutant. Laboratory experiments are crucial to understand the impact of each category of pollutant,  
444 but it should be representative of natural phenomenon. Our results suggest that chronic exposure to a TME cocktail  
445 at low concentrations, representative of minimal exposure in a medium-size city, could have a deleterious effect  
446 on physiological parameters of birds. Exposure to TME seems to lead to an increase of telomere attrition and  
447 therefore could reduce the longevity of birds. Low effect of TME cocktail on the measured oxidative status  
448 biomarker suggests that TME concentrations are potentially too low to activate the glutathione metabolism  
449 pathway, or that birds can compensate with other antioxidants, such as dietary antioxidants. Ca bioavailability  
450 seems to be important to fight against the harmful effects of TME exposure, in limiting the accumulation of non-  
451 essential metals and metalloids promoting the accumulation of essential metals. Future studies should be conducted  
452 to decrypt the effects of TME on telomere length and longevity in urban birds, notably the effect of early-life

453 exposure. In laboratory, TME cocktail exposure during reproduction or combined with food limitation could  
454 provide a more realistic simulation of natural conditions at certain moments in the life cycle. In addition, it seems  
455 to be essential to take into account Ca bioavailability when studying the consequences of exposure to TME.

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#### 466 **REFERENCES**

467 Baos, R., Jovani, R., Pastor, N., Tella, J.L., Jiménez, B., Gómez, G., González, M.J., Hiraldo, F., 2006. Evaluation  
468 of genotoxic effects of heavy metals and arsenic in wild nestling white storks (*Ciconia ciconia*) and black  
469 kites (*Milvus migrans*) from Southwestern Spain after a mining accident. *Environ. Toxicol. Chem.* 25, 2794–  
470 2803. <https://doi.org/10.1897/05-570R.1>

471 Bel'skii, E.A., Bezel, V.S., Lyakhov, A.G., 1995. Characteristics of the reproductive indices of birds nesting in  
472 tree hollows under conditions of technogenic pollution. *Russ. J. Ecol.* 26, 126–131.

473 Berglund, Å.M.M., Koivula, M.J., Eeva, T., 2011. Species- and age-related variation in metal exposure and  
474 accumulation of two passerine bird species. *Environ. Pollut.* 159, 2368–2374.  
475 <https://doi.org/10.1016/j.envpol.2011.07.001>

476 Berglund, Å.M.M., Sturve, J., Förlin, L., Nyholm, N.E.I., 2007. Oxidative stress in pied flycatcher (*Ficedula*  
477 *hypoleuca*) nestlings from metal contaminated environments in northern Sweden. *Environ. Res.* 105, 330–  
478 339. <https://doi.org/10.1016/j.envres.2007.06.002>

479 Berglund, Å.M.M.M., Rainio, M.J., Kanerva, M., Nikinmaa, M., Eeva, T., 2014. Antioxidant status in relation to  
480 age, condition, reproductive performance and pollution in three passerine species. *J. Avian Biol.* 45, 235–  
481 246. <https://doi.org/10.1111/j.1600-048X.2013.00126.x>

482 Boonekamp, J.J., Bauch, C., Mulder, E., Verhulst, S., 2017. Does oxidative stress shorten telomeres? *Biol. Lett.*  
483 13, 20170164. <https://doi.org/10.1098/rsbl.2017.0164>

484 Chatelain, M., Gasparini, J., Frantz, A., 2016a. Do trace metals select for darker birds in urban areas? An  
485 experimental exposure to lead and zinc. *Glob. Chang. Biol.* 22, 2380–2391.  
486 <https://doi.org/10.1111/gcb.13170>

487 Chatelain, M., Gasparini, J., Haussy, C., Frantz, A., 2016b. Trace metals affect early maternal transfer of immune  
488 components in the feral pigeon. *Physiol. Biochem. Zool.* 89, 206–212. <https://doi.org/10.1086/685511>

489 Criscuolo, F., Bize, P., Nasir, L., Metcalfe, N.B., Foote, C.G., Griffiths, K., Gault, E.A., Monaghan, P., 2009. Real-  
490 time quantitative PCR assay for measurement of avian telomeres. *J. Avian Biol.* 40, 342–347.  
491 <https://doi.org/10.1111/j.1600-048X.2008.04623.x>

492 Damron, B.L., Flunker, L.K., 1995. Calcium supplementation of hen drinking water. *Poult. Sci.* 74, 784–787.  
493 <https://doi.org/10.3382/ps.0740784>

494 Dauwe, T., Bervoets, L., Blust, R., Pinxten, R., Eens, M., 1999. Are eggshells and egg contents of great and blue  
495 tits suitable as indicators of heavy metal pollution? *Belgian J. Zool.* 129, 439–447.

496 Dauwe, T., Janssens, E., Bervoets, L., Blust, R., Eens, M., 2005. Heavy-metal concentrations in female laying  
497 great tits (*Parus major*) and their clutches. *Arch. Environ. Contam. Toxicol.* 49, 249–256.  
498 <https://doi.org/10.1007/s00244-003-0209-z>

499 Dauwe, T., Janssens, E., Bervoets, L., Blust, R., Eens, M., 2004. Relationships between metal concentrations in  
500 great tit nestlings and their environment and food. *Environ. Pollut.* 131, 373–380.  
501 <https://doi.org/10.1016/j.envpol.2004.03.009>

502 Dauwe, T., Snoeijs, T., Bervoets, L., Blust, R., Eens, M., 2006. Calcium availability influences lead accumulation  
503 in a passerine bird. *Anim. Biol.* 56, 289–298. <https://doi.org/10.1163/157075606778441822>

504 De la Casa-Resino, I., Hernández-Moreno, D., Castellano, A., Soler Rodríguez, F., Pérez-López, M., 2015.  
505 Biomarkers of oxidative status associated with metal pollution in the blood of the white stork (*Ciconia*  
506 *ciconia*) in Spain. *Toxicol. Environ. Chem.* 97, 588–598. <https://doi.org/10.1080/02772248.2015.1051484>

507 Duffus, J.H., 2002. “Heavy metals” - A meaningless term? (IUPAC technical report). *Pure Appl. Chem.* 74, 793–  
508 807. <https://doi.org/10.1351/pac200274050793>

509 Eeva, T., Hasselquist, D., Langefors, Å., Tummeleht, L., Nikinmaa, M., Ilmonen, P., 2005. Pollution related effects  
510 on immune function and stress in a free-living population of pied flycatcher *Ficedula hypoleuca*. *J. Avian*  
511 *Biol.* 36, 405–412. <https://doi.org/10.1111/j.0908-8857.2005.03449.x>

512 Eeva, T., Lehikoinen, E., 2004. Rich calcium availability diminishes heavy metal toxicity in Pied Flycatcher.  
513 *Funct. Ecol.* 18, 548–553. <https://doi.org/10.1111/j.0269-8463.2004.00875.x>

514 Eeva, T., Lehikoinen, E., Nikinmaa, M., 2003. Pollution-induced nutritional stress in birds: An experimental study  
515 of direct and indirect effects. *Ecol. Appl.* 13, 1242–1249. <https://doi.org/10.1890/01-5375>

516 Ek, K.H., Morrison, G.M., Lindberg, P., Rauch, S., 2004. Comparative tissue distribution of metals in birds in  
517 Sweden using ICP-MS and laser ablation ICP-MS. *Arch. Environ. Contam. Toxicol.* 47, 259–269.  
518 <https://doi.org/10.1007/s00244-004-3138-6>

519 Epel, E.S., Blackburn, E.H., Lin, J., Dhabhar, F.S., Adler, N.E., Morrow, J.D., Cawthon, R.M., 2004. Accelerated  
520 telomere shortening in response to life stress. *Proc. Natl. Acad. Sci.* 101, 17312–17315.

521 <https://doi.org/10.1073/pnas.0407162101>

522 Espín, S., García-Fernández, A.J., Herzke, D., Shore, R.F., van Hattum, B., Martínez-López, E., Coeurdassier, M.,  
523 Eulaers, I., Fritsch, C., Gómez-Ramírez, P., Jaspers, V.L.B., Krone, O., Duke, G., Helander, B., Mateo, R.,  
524 Movalli, P., Sonne, C., van den Brink, N.W., 2016a. Tracking pan-continental trends in environmental  
525 contamination using sentinel raptors—what types of samples should we use? *Ecotoxicology* 25, 777–801.  
526 <https://doi.org/10.1007/s10646-016-1636-8>

527 Espín, S., Martínez-López, E., Jiménez, P., María-Mojica, P., García-Fernández, A.J., 2016b. Interspecific  
528 differences in the antioxidant capacity of two Laridae species exposed to metals. *Environ. Res.* 147, 115–  
529 124. <https://doi.org/10.1016/j.envres.2016.01.029>

530 Espín, S., Martínez-López, E., Jiménez, P., María-Mojica, P., García-Fernández, A.J., 2014a. Effects of heavy  
531 metals on biomarkers for oxidative stress in Griffon vulture (*Gyps fulvus*). *Environ. Res.* 129, 59–68.  
532 <https://doi.org/10.1016/j.envres.2013.11.008>

533 Espín, S., Martínez-López, E., León-Ortega, M., Martínez, J.E., García-Fernández, A.J., 2014b. Oxidative stress  
534 biomarkers in Eurasian eagle owls (*Bubo bubo*) in three different scenarios of heavy metal exposure.  
535 *Environ. Res.* 131, 134–144. <https://doi.org/10.1016/j.envres.2014.03.015>

536 Espín, S., Ruiz, S., Sánchez-Virosta, P., Eeva, T., 2016c. Effects of calcium supplementation on growth and  
537 biochemistry in two passerine species breeding in a Ca-poor and metal-polluted area. *Environ. Sci. Pollut.*  
538 *Res.* 23, 9809–9821. <https://doi.org/10.1007/s11356-016-6219-y>

539 Espín, S., Ruiz, S., Sánchez-Virosta, P., Lilley, T., Eeva, T., 2017. Oxidative status in relation to metal pollution  
540 and calcium availability in pied flycatcher nestlings – A calcium manipulation experiment. *Environ. Pollut.*  
541 229, 448–458. <https://doi.org/10.1016/j.envpol.2017.05.094>

542 Farag, M.R., Alagawany, M., Abd El-Hack, M.E., El-Sayed, S.A.A., Ahmed, S.Y.A., Samak, D.H., 2018. *Yucca*  
543 *schidigera* extract modulates the lead-induced oxidative damage, nephropathy and altered inflammatory  
544 response and glucose homeostasis in Japanese quails. *Ecotoxicol. Environ. Saf.* 156, 311–321.  
545 <https://doi.org/10.1016/j.ecoenv.2018.03.010>

546 Fraga, C.G., 2005. Relevance, essentiality and toxicity of trace elements in human health. *Mol. Aspects Med.*  
547 <https://doi.org/10.1016/j.mam.2005.07.013>

548 Frantz, A., Pottier, M.A., Karimi, B., Corbel, H., Aubry, E., Haussy, C., Gasparini, J., Castrec-Rouelle, M., 2012.  
549 Contrasting levels of heavy metals in the feathers of urban pigeons from close habitats suggest limited  
550 movements at a restricted scale. *Environ. Pollut.* 168, 23–28. <https://doi.org/10.1016/j.envpol.2012.04.003>

551 Goyer, R.A., 1997. Toxic and essential metal interactions. *Annu. Rev. Nutr.* 17, 37–50.  
552 <https://doi.org/10.1146/annurev.nutr.17.1.37>

553 Hahn, C., Evans, G., 2017. Absorption of trace metals in the zinc-deficient rat. *Am. J. Physiol. Content* 228, 1020–  
554 1023. <https://doi.org/10.1152/ajplegacy.1975.228.4.1020>

555 Helsel, D.R., 2011. *Statistics for censored environmental data using Minitab® and R*. John Wiley & Sons, Inc.,



556 Hoboken, NJ, USA. <https://doi.org/10.1002/9781118162729>

557 Isaksson, C., 2010. Pollution and its impact on wild animals: A meta-analysis on oxidative stress. *Ecohealth* 7,  
558 342–350. <https://doi.org/10.1007/s10393-010-0345-7>

559 Jaishankar, M., Tseten, T., Anbalagan, N., Mathew, B.B., Beeregowda, K.N., 2014. Toxicity, mechanism and  
560 health effects of some heavy metals. *Interdiscip. Toxicol.* <https://doi.org/10.2478/intox-2014-0009>

561 Järup, L., 2003. Hazards of heavy metal contamination. *Br. Med. Bull.* 68, 167–82.  
562 <https://doi.org/10.1093/bmb/ldg032>

563 Koivula, M.J., Eeva, T., 2010. Metal-related oxidative stress in birds. *Environ. Pollut.*  
564 <https://doi.org/10.1016/j.envpol.2010.03.013>

565 Koivula, M.J., Kanerva, M., Salminen, J.-P., Nikinmaa, M., Eeva, T., 2011. Metal pollution indirectly increases  
566 oxidative stress in great tit (*Parus major*) nestlings. *Environ. Res.* 111, 362–370.  
567 <https://doi.org/10.1016/j.envres.2011.01.005>

568 Komjarova, I., Blust, R., 2009. Effect of Na, Ca and pH on simultaneous uptake of Cd, Cu, Ni, Pb, and Zn in the  
569 water flea *Daphnia magna* measured using stable isotopes. *Aquat. Toxicol.* 94, 81–86.  
570 <https://doi.org/10.1016/j.aquatox.2009.05.018>

571 Kucuk, O., 2008. Zinc in a combination with magnesium helps reducing negative effects of heat stress in quails.  
572 *Biol. Trace Elem. Res.* 123, 144–153. <https://doi.org/10.1007/s12011-007-8083-6>

573 Kurhalyuk, N., Hetmański, T., Antonowicz, J., Tkachenko, H., 2009. Oxidative stress and protein oxidation  
574 affected by toxic metals in feral pigeons (*Columba livia*) from northern Poland. *Balt. Coast. Zo.* 187–197.  
575 [https://doi.org/DOI 10.1002/ana.410360714](https://doi.org/DOI%2010.1002/ana.410360714)

576 Leonard, S.S., Harris, G.K., Shi, X., 2004. Metal-induced oxidative stress and signal transduction. *Free Radic.*  
577 *Biol. Med.* <https://doi.org/10.1016/j.freeradbiomed.2004.09.010>

578 Levander, O.A., 1978. Metabolic interactions between metals and metalloids. *Environ. Health Perspect.* VOL. 25,  
579 77–80.

580 Lodenius, M., Solonen, T., 2013. The use of feathers of birds of prey as indicators of metal pollution.  
581 *Ecotoxicology* 22, 1319–1334. <https://doi.org/10.1007/s10646-013-1128-z>

582 Lucia, M., Andre, J.M., Gonzalez, P., Baudrimont, M., Gontier, K., Maury-Brachet, R., Davail, S., 2009. Impact  
583 of cadmium on aquatic bird *Cairina moschata*. *BioMetals* 22, 843–853. <https://doi.org/10.1007/s10534-009-9232-5>  
584

585 Mora, M.A., 2003. Heavy metals and metalloids in egg contents and eggshells of passerine birds from Arizona.  
586 *Environ. Pollut.* 125, 393–400. [https://doi.org/10.1016/S0269-7491\(03\)00108-8](https://doi.org/10.1016/S0269-7491(03)00108-8)

587 Poulin, R.G., Brigham, R.M., 2001. Effects of supplemental calcium on the growth rate of an insectivorous bird,  
588 the purple martin (*Progne subis*). *Ecoscience* 8, 151–156. <https://doi.org/10.1080/11956860.2001.11682640>

589 Prasanthi, R.P.J., Devi, C.B., Basha, D.C., Reddy, N.S., Reddy, G.R., 2010. Calcium and zinc supplementation  
590 protects lead (Pb)-induced perturbations in antioxidant enzymes and lipid peroxidation in developing mouse  
591 brain. *Int. J. Dev. Neurosci.* 28, 161–167. <https://doi.org/10.1016/j.ijdevneu.2009.12.002>

592 R Development Core Team, 2016. R: A language and environment for statistical computing. Vienna, Austria.

593 Rainio, M.J., Eeva, T., Lilley, T., Stauffer, J., Ruuskanen, S., 2015. Effects of early-life lead exposure on oxidative  
594 status and phagocytosis activity in great tits (*Parus major*). *Comp. Biochem. Physiol. Part - C Toxicol.*  
595 *Pharmacol.* 167, 24–34. <https://doi.org/10.1016/j.cbpc.2014.08.004>

596 Rainio, M.J., Kanerva, M., Salminen, J.P., Nikinmaa, M., Eeva, T., 2013. Oxidative status in nestlings of three  
597 small passerine species exposed to metal pollution. *Sci. Total Environ.* 454–455, 466–473.  
598 <https://doi.org/10.1016/j.scitotenv.2013.03.033>

599 Salmón, P., Nilsson, J.F., Watson, H., Bensch, S., Isaksson, C., 2017. Selective disappearance of great tits with  
600 short telomeres in urban areas. *Proc. R. Soc. B Biol. Sci.* 284. <https://doi.org/10.1098/rspb.2017.1349>

601 Sánchez-Virosta, P., Espín, S., Ruiz, S., Salminen, J.P., García-Fernández, A.J., Eeva, T., 2018. Experimental  
602 manipulation of dietary arsenic levels in great tit nestlings: Accumulation pattern and effects on growth,  
603 survival and plasma biochemistry. *Environ. Pollut.* 233, 764–773.  
604 <https://doi.org/10.1016/j.envpol.2017.10.113>

605 Sánchez-Virosta, P., Espín, S., Ruiz, S., Stauffer, J., Kanerva, M., García-Fernández, A.J., Eeva, T., 2019. Effects  
606 of calcium supplementation on oxidative status and oxidative damage in great tit nestlings inhabiting a metal-  
607 polluted area. *Environ. Res.* 484–492. <https://doi.org/10.1016/j.envres.2019.01.047>

608 Scheuhammer, A.M., 1996. Influence of reduced dietary calcium on the accumulation and effects of lead,  
609 cadmium, and aluminum in birds. *Environ. Pollut.* 94, 337–343. [https://doi.org/10.1016/S0269-](https://doi.org/10.1016/S0269-7491(96)00084-X)  
610 [7491\(96\)00084-X](https://doi.org/10.1016/S0269-7491(96)00084-X)

611 Scheuhammer, A.M., 1991. Effects of acidification on the availability of toxic metals and calcium to wild birds  
612 and mammals. *Environ. Pollut.* 71, 329–375. [https://doi.org/10.1016/0269-7491\(91\)90036-V](https://doi.org/10.1016/0269-7491(91)90036-V)

613 Six, K.M., Goyer, R.A., 1970. Experimental enhancement of lead toxicity by low dietary calcium. *J. Lab. Clin.*  
614 *Med.* 76, 933–942. <https://doi.org/10.5555/URI:PII:0022214370902301>

615 Snoeijs, T., Dauwe, T., Pinxten, R., Darras, V.M., Arckens, L., Eens, M., 2005. The combined effect of lead  
616 exposure and high or low dietary calcium on health and immunocompetence in the zebra finch (*Taeniopygia*  
617 *guttata*). *Environ. Pollut.* 134, 123–132. <https://doi.org/10.1016/j.envpol.2004.07.009>

618 Srivastava, D., Subramanian, R.B., Madamwar, D., Flora, S.J.S., 2010. Protective effects of selenium, calcium,  
619 and magnesium against arsenic-induced oxidative stress in male rats. *Arh. Hig. Rada Toksikol.* 61, 153–159.  
620 <https://doi.org/10.2478/10004-1254-61-2010-1993>

621 Stankevičiūtė, M., Sauliūtė, G., Makaras, T., Markuckas, A., Virbickas, T., Baršienė, J., 2018. Responses of  
622 biomarkers in Atlantic salmon (*Salmo salar*) following exposure to environmentally relevant concentrations  
623 of complex metal mixture (Zn, Cu, Ni, Cr, Pb, Cd). Part II. *Ecotoxicology* 27, 1069–1086.

624 <https://doi.org/10.1007/s10646-018-1960-2>

625 Stauffer, J., Panda, B., Eeva, T., Rainio, M., Ilmonen, P., 2017. Telomere damage and redox status alterations in  
626 free-living passerines exposed to metals. *Sci. Total Environ.* 575, 841–848.  
627 <https://doi.org/10.1016/j.scitotenv.2016.09.131>

628 Stohs, S.J., Bagchi, D., 1995. Oxidative mechanisms in the toxicity of metal ions. *Free Radic. Biol. Med.*  
629 [https://doi.org/10.1016/0891-5849\(94\)00159-H](https://doi.org/10.1016/0891-5849(94)00159-H)

630 Tully, T.N., Lawton, J.M., Dorrestein, G.M., 2000. Handbook of avian medicine Second Edition, Handbook of  
631 avian medicine. Elsevier/Saunders. <https://doi.org/10.1016/B978-0-7506-3598-1.50012-6>

632 Wah Chu, K., Chow, K.L., 2002. Synergistic toxicity of multiple heavy metals is revealed by a biological assay  
633 using a nematode and its transgenic derivative. *Aquat. Toxicol.* 61, 53–64. [https://doi.org/10.1016/S0166-](https://doi.org/10.1016/S0166-445X(02)00017-6)  
634 [445X\(02\)00017-6](https://doi.org/10.1016/S0166-445X(02)00017-6)

635 Wang, J., Wang, T., Burken, J.G., Chusuei, C.C., Ban, H., Ladwig, K., Huang, C.P., 2008. Adsorption of  
636 arsenic(V) onto fly ash: A speciation-based approach. *Chemosphere* 72, 381–388.  
637 <https://doi.org/10.1016/j.chemosphere.2008.02.045>

638 Yenice, E., Mizrak, C., Gültekin, M., Atik, Z., Tunca, M., 2015. Effects of dietary organic or inorganic manganese,  
639 zinc, copper and chrome supplementation on the performance, egg quality and hatching characteristics of  
640 laying breeder hens. *Ankara Üniv Vet Fak Derg* 62, 63–68.

641 Zann, R., 1996. Zebra finch: a synthesis of field and laboratory studies, *Zebra Finch: A Synthesis of Field and*  
642 *Laboratory Studies*. Oxford University Press.

643 Zhu, Y., Wang, J., Bai, Y., Zhang, R., 2004. Cadmium, Chromium, and Copper induce polychromatocyte  
644 micronuclei in carp (*Cyprinus carpio L.*). *Bull. Environ. Contam. Toxicol.* 72, 78–86.  
645 <https://doi.org/10.1007/s00128-003-0243-6>

646 Zuur, A.F., Ieno, E.N., Walker, N.J., Saveliev, A.A., Smith, G.M., 2009. Mixed effects models and extension in  
647 ecology with R, First Edit. ed. Springer. <https://doi.org/10.1017/CBO9781107415324.004>

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**Table 1.** Concentration of metal in blood (in ppm) at each exposure time

Metal	Treatment	T0		T14		T29		T43		T57					
		>LOD/n	mean ± SD	>LOD/n	mean ± SD	>LOD/n	mean ± SD	>LOD/n	mean ± SD	>LOD/n	mean ± SD				
<b>Arsenic</b>	Ctrl	0/9	<i>a</i>	1/9	0.007	<i>a</i>	1/9	0.003	<i>a</i>	1/9	0.002	<i>a</i>	0/9	<i>a</i>	
	TME	0/8	<i>a</i>	10/10	0.016±0.009	<i>b</i>	9/9	0.014±0.008	<i>b</i>	10/10	0.021±0.011	<i>b</i>	10/10	0.018±0.011	<i>b</i>
	Ca	0/9	<i>a</i>	0/9		<i>a</i>	3/9	0.004±0.003	<i>a</i>	1/9	0.005	<i>a</i>	0/9	<i>a</i>	
	TME+Ca	2/7	0.012±0.002	<i>a</i>	7/9	0.004±0.002	<i>a</i>	0/9	<i>a</i>	4/9	0.008±0.002	<i>a</i>	9/9	0.004±0.003	<i>a</i>
<b>Cadmium</b>	Ctrl	0/9		0/9			0/9		<i>a</i>	2/9	0.01±0.002	<i>ab</i>	0/9		
	TME	0/8		0/10			4/9	0.005±0.0006	<i>ab</i>	2/10	±0.00005	<i>a</i>	2/10	0.005±0.0003	
	Ca	1/9	0.007	0/9			0/9		<i>a</i>	1/9	0.009	<i>b</i>	1/9	0.068	
	TME+Ca	1/7	0.23	3/9	0.005±0.003		9/9	0.007±0.001	<i>b</i>	1/9	0.004	<i>a</i>	0/9		
<b>Chromium</b>	Ctrl	5/9	0.006±0.007	6/9	0.011±0.006		4/9	0.106±0.331		2/9	0.014±0.012		2/9	0.014	
	TME	1/8	0.002	1/10	0.029		1/9	0.156		2/10	0.02±0.012		2/10	0.020	
	Ca	4/9	0.013±0.032	6/9	0.013±0.022		4/9	0.006±0.003		2/9	0.032±0.019		2/9	0.032	
	TME+Ca	1/7	0.011	1/9	0.012		0/9			1/9	0.027		1/9	0.027	
<b>Cobalt</b>	Ctrl	0/9		0/9			3/9	0.003±0.004		1/9	0.002		0/9		
	TME	0/8		0/10			0/9			5/10	0.003±0.003		6/10	0.003±0.001	
	Ca	0/9		0/9			2/9	0.003±0.003		1/9	0.002		0/9		
	TME+Ca	0/7		0/9			0/9			4/9	0.034±0.105		3/9	0.004±0.002	
<b>Copper</b>	Ctrl	9/9	0.52±0.441	9/9	0.398±0.167		9/9	0.292±0.337	<i>a</i>	9/9	0.245±0.037	<i>a</i>	9/9	0.32±0.061	<i>a</i>
	TME	8/8	0.296±0.048	10/10	0.283±0.088		9/9	0.34±0.121	<i>a</i>	10/10	0.326±0.091	<i>ab</i>	10/10	0.239±0.062	<i>a</i>
	Ca	9/9	0.364±0.149	9/9	0.355±0.074		9/9	0.552±1.206	<i>a</i>	9/9	0.274±0.077	<i>ab</i>	9/9	0.326±0.053	<i>a</i>
	TME+Ca	7/7	0.325±0.147	9/9	0.403±0.153		9/9	0.358±0.146	<i>a</i>	9/9	0.326±0.052	<i>b</i>	9/9	0.286±0.03	<i>a</i>
<b>Lead</b>	Ctrl	4/9	0.031±0.043	5/9	0.034±0.043	<i>a</i>	5/9	0.016±0.007	<i>a</i>	<b>9/9</b>	0.076±0.131	<i>a</i>	2/9	0.04±0.032	<i>ac</i>
	TME	0/8		10/10	0.2±0.063	<i>b</i>	9/9	0.218±0.084	<i>b</i>	<b>10/10</b>	0.276±0.105	<i>b</i>	10/10	0.226±0.089	<i>b</i>
	Ca	3/9	0.055±0.17	8/9	0.024±0.01	<i>a</i>	2/9	0.006	<i>a</i>	<b>9/9</b>	0.032±0.054	<i>a</i>	1/9	0.240	<i>c</i>
	TME+Ca	2/7	0.186±0.453	6/9	0.093±0.043	<i>ab</i>	6/9	0.055±0.026	<i>a</i>	<b>9/9</b>	0.057±0.024	<i>a</i>	9/9	0.064±0.036	<i>a</i>
<b>Nickel</b>	Ctrl	2/9	0.024±0.013	9/9	0.01±0.005		6/9	0.028±0.03	<i>a</i>	3/9	0.004		0/9		
	TME	2/8	0.001±0.005	1/10	0.184		1/9	0.013	<i>a</i>	0/10			0/10		
	Ca	3/9	0.007±0.002	8/9	0.009±0.003		6/9	0.156±0.419	<i>a</i>	1/9	0.012		0/9		
	TME+Ca	1/7	0.015	2/9	0.059±0.133		2/9	0.01±0.003	<i>a</i>	0/9			0/9		
<b>Zinc</b>	Ctrl	9/9	4.448±1.338	9/9	5.7±2.909		9/9	2.401±0.54	<i>a</i>	9/9	3.789±0.45	<i>a</i>	9/9	3.649±1.036	

TME	8/8	4.415±1.135	10/10	4.136±0.916	9/9	3.41±0.519	<b>ab</b>	10/10	4.508±0.428	<b>ab</b>	10/10	3.722±0.695
Ca	9/9	4.89±2.018	9/9	5.124±1.04	9/9	2.683±0.525	<b>c</b>	9/9	4.38±0.495	<b>b</b>	9/9	4.164±1.164
TME+Ca	7/7	4.645±1.413	9/9	4.25±1.049	9/9	3.42±0.617	<b>bc</b>	9/9	4.491±0.8	<b>ab</b>	9/9	5.184±2.868

Notes : 'n' =number of individuals for the treatment at each exposure time (days), '>LOD/n' = number of values above the limit of detection/number of values in the group, 'Ctrl'= control group with low-Ca diet and no TME, 'TME'= birds with low-Ca diet exposed to a TME cocktail, 'Ca'= rich-Ca diet and no TME and 'TME+Ca'= rich-Ca diet and TME cocktail. Post-hoc tests were performed when differences between treatments were significant (Table 2). Different letters represent significant differences using the Bonferroni correction (p<0.0083) and are represented in bold. The absence of letter indicates that differences between treatments were not significant.

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652 **Table 2.** Results of statistical models comparing metal concentration in blood between treatments at different  
 653 times (from 0 to 57 days) (Generalized Wilcoxon tests)

	T0		T14		T29		T43		T57	
	X <sup>2</sup>	p	X <sup>2</sup>	p	X <sup>2</sup>	p	X <sup>2</sup>	p	X <sup>2</sup>	p
<b>Arsenic</b>	8	<b>0.05</b>	43.8	<b>&lt;0.001</b>	44.1	<b>&lt;0.001</b>	47.9	<b>&lt;0.001</b>	40.6	<b>&lt;0.001</b>
<b>Cadmium</b>	2.3	0.5	2.4	0.1	27.8	<b>&lt;0.001</b>	8.9	<b>0.03</b>	4.6	0.2
<b>Chromium</b>	4.8	0.2	1.1	0.8	4.2	0.2	0.6	0.9	not detected	
<b>Copper</b>	2.2	0.5	4.7	0.2	18	<b>&lt;0.001</b>	9.5	<b>0.02</b>	9.8	<b>&lt;0.001</b>
<b>Lead</b>	3.8	0.3	39.7	<b>&lt;0.001</b>	50.3	<b>&lt;0.001</b>	39.2	<b>&lt;0.001</b>	40.3	<b>&lt;0.001</b>
<b>Nickel</b>	1.1	0.8	1.5	0.7	12	<b>0.007</b>	2.2	0.5	not detected	
<b>Zinc</b>	0.4	0.9	4.5	0.2	16.9	<b>&lt;0.001</b>	8.1	<b>0.04</b>	5.1	0.2

Notes : Significant differences between treatments are represented in bold.

654

655 **Table 3.** Effects of treatment and exposure time on PCA component (mixed linear models and type III ANOVAs).

	Variable	F-value	DF	p-value	
657	PC1Feather	<b>Treatment*Exposure time</b>	27.53	3,33	<b>&lt;0.001</b>
		<b>Treatment</b>	9.85	3,33	<b>&lt;0.001</b>
		<b>Exposure time</b>	66.07	1,33	<b>&lt;0.001</b>
658	PC2Feather	<b>Treatment*Exposure time</b>	17.06	3,33	<b>&lt;0.001</b>
		<b>Treatment</b>	4.95	3,33	<b>0.0060</b>
		<b>Exposure time</b>	12.40	1,33	<b>0.0013</b>

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Full models included treatment ('Ctrl': control group with low-Ca diet and no TME, 'TME': birds with low-Ca diet exposed to a TME cocktail, 'Ca':rich-Ca diet and no TME and 'TME+Ca': rich-Ca diet and TME cocktail), exposure time (T0 and T29) and the interaction term. In each model, bird ID was the random factor. Significant variables are represented in bold. Post-hoc tests are in Table A.4.

660 **Table 4.** Physiological parameters and body mass depending on A) treatment, exposure time and interaction term  
 661 and B) composite variables of metal concentration from the PCA: PCAfeather1 and PCAfeather2.

	<b>Variable</b>	<b>F-value</b>	<b>DF</b>	<b>p-value</b>
<b>A) Models with treatment groups and exposure time</b>				
RTL (log)	Exposure time	3.07	2,72	0.052
Body mass	Treatment	1.38	3,32	0.26
ROMs	<b>Exposure time</b>	8.83	2,69	<b>&lt;0.001</b>
GSHtot (log)	<b>Exposure time</b>	7.81	2,72	<b>0.001</b>
GSH (log)	<b>Treatment*Exposure time</b>	2.27	6,66	<b>0.047</b>
	Treatment	2.21	3,33	0.106
	Exposure time	1.37	2,66	0.262
GSSG (log)	<b>Exposure time</b>	9.43	2,72	<b>&lt;0.001</b>
GPx (log)	<b>Treatment*Exposure time</b>	3.71	6,66	<b>0.003</b>
	Treatment	1.75	3,33	0.175
	Exposure time	2.17	2,66	0.122
<b>B) Models with PCA composite variables of metal concentration</b>				
RTL (log)	PC1Feather	1.082	1,35	0.305
	<b>PC2Feather</b>	4.226	1,35	<b>0.047</b>
Body mass	PC1Feather	0.34	1,35	0.561
	PC2Feather	0.002	1,35	0.961
ROMs (log)	PC1Feather	0.0002	1,33	0.99
	PC2Feather	0.933	1,33	0.34
GSH (log)	PC1Feather	0.298	1,35	0.588
	PC2Feather	0.370	1,35	0.547
GSSG (log)	PC1Feather	0.077	1,35	0.783
	PC2Feather	0.463	1,35	0.500
GSHtot (log)	PC1Feather	0.393	1,35	0.534
	PC2Feather	0.774	1,35	0.384
GPx (log)	PC1Feather	0.01	1,35	0.929
	PC2Feather	1.19	1,35	0.284

Note : RTL= Relative telomere length (ratio copy telomere/ copy control gene); ROMs= Reactive oxygene metabolites ; GSSG= oxidised glutathione; GSH= free glutathione GSHtot = total glutathione; GPx= glutathione peroxidase. Best models were selected with a stepwise procedure from the full model that included treatment. In each model (A and B), bird ID was the random factor. The significant results are in bold.

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668 **Table 5.** Means and standard deviation of body mass and physiological parameters in the different treatments at  
 669 different exposure times (day 0, day 29, and day 57).

		<b>RTL</b>	<b>Body mass</b>	<b>ROM</b>	<b>GSH</b>	<b>GSSG</b>	<b>GShtot</b>	<b>GPx</b>
Treatment		<i>Mean ± sd</i>	<i>Mean ± sd</i>	<i>Mean ± sd</i>	<i>Mean ± sd</i>	<i>Mean ± sd</i>	<i>Mean ± sd</i>	<i>Mean ± sd</i>
<i>T0</i>	<b>Ctrl</b>	0.65 ± 0.24	17.77 ± 2.25	4.18 ± 2.14	674.97 ± 954.56	543.14 ± 846.82	1761.26 ± 2323.36	792.22 ± 160.96
	<b>TME</b>	0.75 ± 0.26	16.38 ± 1.84	3.96 ± 1.62	188.24 ± 164.33	143.77 ± 135.06	475.8 ± 400.32	723.96 ± 162.07
	<b>Ca</b>	0.7 ± 0.17	16.18 ± 2.39	4.86 ± 2.77	271.48 ± 446.11	254.34 ± 332.75	780.19 ± 1095.95	839.71 ± 211.61
	<b>TME +Ca</b>	0.76 ± 0.18	17.23 ± 2	4.54 ± 3.7	401.01 ± 426.13	96.72 ± 111.61	594.46 ± 573.27	683.71 ± 134.05
<i>T29</i>	<b>Ctrl</b>	0.85 ± 0.38	18.91 ± 2.41	3.74 ± 2.17	377.18 ± 260.39	226.44 ± 203.9	830.05 ± 591.91	741.76 ± 90.32
	<b>TME</b>	0.75 ± 0.22	16.85 ± 1.74	5.74 ± 4.61	349.96 ± 276.77	218.16 ± 303.68	786.27 ± 828.51	656.53 ± 82.16
	<b>Ca</b>	0.9 ± 0.31	16.56 ± 2.35	5.31 ± 1.85	647.6 ± 765.06	296.06 ± 469.21	1239.73 ± 1623.15	636.84 ± 122.88
	<b>TME +Ca</b>	1 ± 0.47	17.09 ± 1.93	3.82 ± 1.05	197.44 ± 163.56	98.16 ± 82.93	393.73 ± 237.16	782.08 ± 133.01
<i>T57</i>	<b>Ctrl</b>	0.63 ± 0.24	17.96 ± 2.01	2.18 ± 1.41	372.11 ± 665.34	572.6 ± 584.78	1517.33 ± 1679.75	684.44 ± 149.96
	<b>TME</b>	0.7 ± 0.19	17.01 ± 1.73	3.86 ± 2.38	569.06 ± 691.44	383.31 ± 299.51	1335.67 ± 663.81	759.95 ± 192.36
	<b>Ca</b>	0.85 ± 0.2	16.09 ± 1.88	3.1 ± 1.67	729.12 ± 897.1	883.29 ± 893.29	2495.69 ± 2507.2	775.7 ± 237.53
	<b>TME +Ca</b>	0.71 ± 0.26	17.14 ± 2.23	3.68 ± 2.25	369.31 ± 427.55	436.67 ± 473.45	1242.63 ± 1284.69	691.85 ± 102.07

Notes : 'Ctrl'= control group with low-Ca diet and no TME; 'TME'= birds with low-Ca diet exposed to a TME cocktail; 'Ca'=rich-Ca diet and no TME; 'TME+Ca'= rich-Ca diet and TME cocktail; RTL= Relative telomere length (copy telomere /copy control gene); body mass (g); ROM= Reactive oxygene metabolites (mg H2O2/dl) ;GSH = free glutathione (µM) ; GSSG= oxidised glutathione (µM); GShtot= total glutathione (µM);GPx= glutathione peroxidase (U/L hemolysat)

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672 **Table 6.** Physiological parameters and body mass depending on each single metal concentration in blood (mixed  
 673 linear models, type III ANOVAs).

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		RTL (log)	Body mass	ROM (log)	GSH (log)	GSSG (log)	GSHtot (log)	GPx(log)
<b>Arsenic</b>	Fvalue(df)	1.53(1, 10)	0.83(1, 10)	1.35(1, 9)	1.67(1, 10)	3.95(1, 10)	0.13(1, 22)	0.22(1, 10)
	n= 34 p-value	0.24	0.38	0.27	0.23	0.08	0.73	0.65
<b>Chromium</b>	Fvalue(df)	0.36(1, 5)	0.93(1, 5)	3.49(1, 5)	0.01(1, 5)	3.32(1, 5)	0.43(1, 5)	0.02(1, 5)
	n=20 p-value	0.58	0.38	0.12	0.94	0.13	0.54	0.88
<b>Copper</b>	Fvalue(df)	2.92(1, 68)	0.2(1, 68)	3.66(1, 65)	1.67(1, 68)	0.21(1, 68)	1.12(1, 68)	0.6(1, 68)
	n=106 p-value	0.09	0.65	0.06	0.20	0.65	0.29	0.44
<b>Nickel</b>	Fvalue(df)	4.37(1, 5)	0.05(1, 5)	0.32(1, 5)	0.01(1, 5)	1.71(1, 5)	1(1, 5)	0.01(1, 5)
	n=23 p-value	0.09	0.84	0.60	0.91	0.25	0.36	0.91
<b>Lead</b>	Fvalue(df)	1.68(1, 22)	0.21(1, 22)	0.2(1, 21)	1.77(1, 22)	0.38(1, 22)	1.27(1, 22)	0.16(1, 22)
	n=53 p-value	0.21	0.65	0.66	0.20	0.54	0.27	0.69
<b>Zinc</b>	Fvalue(df)	0.31(1, 68)	2.3(1, 68)	0.19(1, 65)	3.93(1, 68)	0.53(1, 68)	0.56(1, 68)	1.59(1, 68)
	n=106 p-value	0.58	0.13	0.67	0.05	0.47	0.46	0.21
<b>Cadmium</b>	Fvalue(df)	0.05(1, 1)	0.93(1, 1)	0.02(1, 1)	No normal	34.73(1, 1)	97.33(1, 1)	0.23(1, 1)
	n=18 p-value	0.86	0.51	0.91		0.11	0.06	0.72

Note : ROMs= Reactive oxygene metabolites ; GSSG= oxidised glutathione; GSH= free glutathione GSHtot =glutathione total; GPx= glutathione peroxidase; RTL= Relative telomere length (copy telomere/ copy control gene ratio)

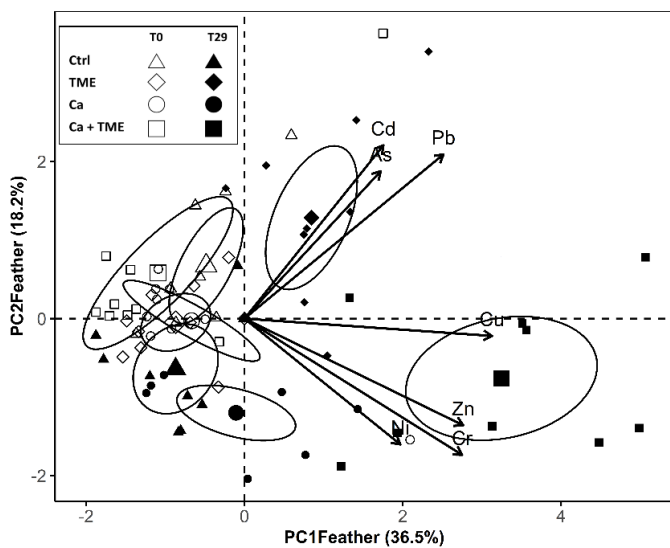
In each model, bird ID was the random factor. 'n' corresponds to the number of observations for each metal. The exposure time was T0, 29 and 57 for physiological parameters and T0, 29, 14, 43 and 57 for body mass

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677 **Fig. 1. Representation of the PCA analysis of metal concentrations in feathers.** Empty shapes represent mean  
678 coordinates of metal concentration in initial feather (day 0) for each treatment ('Ctrl': control group with low-Ca  
679 diet and no TME, 'TME': birds with low-Ca diet exposed to a TME cocktail, 'Ca': rich-Ca diet and no TME and  
680 'TME+Ca': rich-Ca diet and TME cocktail) and the full shapes represent mean coordinates of metal concentration  
681 in regrown feathers (day 29). Ellipses represent confidence intervals

682 Fig.1. (single column fitting)



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