Experimental manipulation of dietary arsenic levels in great tit nestlings: accumulation pattern and effects on growth, survival and plasma biochemistry

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Abstract

Arsenic (As) is a ubiquitous metalloid classified as one of the most hazardous substances, but information about its exposure and effects in free-living passerines is lacking. The aim of this study is to elucidate the effect of an As manipulation experiment on survival, growth and physiology of great tits (*Parus major*). Wild *P. major* nestlings inhabiting an unpolluted area were dosed with water, 0.2 or 1 μg g^{-1} d^{-1} of sodium arsenite (Control, Low and High As groups), whereas those living in a metal-polluted area were dosed with water (Smelter group). Birds accumulated As in tissues (liver, bone and feathers) in a dose-dependent way. Nestlings exposed to 1 μg g^{-1} d^{-1} of sodium arsenite showed reduced number of fledglings per successful nest, and those exposed to 0.2 μg g^{-1} d^{-1} had reduced wing growth, which could have post-fledging consequences such as increased predation risk. These results suggest that the LOAEL for effects on nestling survival and development in great tits is likely equal to or below 1 μg g^{-1} d^{-1}. However, limited effects on the biochemical parameters evaluated were found. It has been shown that As may produce oxidative stress and tissue damage, so further research exploring this issue will be carried out in a future study.

Capsule: Biochemistry, growth and survival of wild *Parus major* nestlings dosed with arsenic

Key-words: breeding success; insectivorous passerines; *Parus major*; vitamins; pollution.

Introduction

Arsenic (As) is a common component of the soil and is present in different rock types. Some industrial activities such as metallurgical processes and combustion of coal are important anthropogenic sources of As and other metals into the environment (Pacyna and Pacyna, 2001). In addition, some arsenical pesticides such as sodium arsenite have been widely used, although they are now prohibited in most countries (WHO, 2000). The Agency for toxic substances and disease registry (ATSDR), has ranked arsenic as the first compound in the Substance priority list 2015 based on its frequency, toxicity, and potential for human exposure (ATSDR, 2015), which points out the fact that As is of great toxicological concern.

Birds have been successfully used as biomonitoring tools of environmental pollution all over the world (Furness et al., 1993). However, the scientific community has prioritized studies on other elements such as lead (Pb), mercury (Hg) and cadmium (Cd) (e.g. Burger and Gochfeld, 2000; Scheuhammer, 1987), whereas very few (and correlative) field studies have assessed the effects of As in birds so far (Sánchez-Virosta et al., 2015). Moreover, in the wild, birds are generally exposed to a mixture of metals and other stressors. Thus, proving a relationship between a specific contaminant and its associated health effects is very challenging. In addition, long-term pollution may disturb biological communities, which may end up causing secondary effects on bird species due to changes in food availability and quality (Eeva et al., 1997). Arsenic is of particular concern for mammalian exposure and toxicity, however, it is not clear whether the same applies for wild birds. Therefore, As manipulation experiments providing environmentally-relevant levels are needed to explore the specific effects of As on growth, survival and physiology in wild bird populations.

Experimental studies providing As compounds to bird species have mainly found developmental and reproductive effects (Sánchez-Virosta et al., 2015). Arsenic-treated mallard (*Anas platyrhynchos*) ducklings and zebra finch (*Taeniopygia guttata*) showed decreased weight gain and growth and reduced tarsus and wing length upon fledging (Albert et al., 2008a, 2008b; Camardese et al., 1990). Stanley et al. (1994) found that As altered mallard reproduction and ducklings' growth, decreased egg weight and produced eggshell thinning. Several physiological parameters, such as calcium (Ca), alkaline phosphatases (ALPs), vitamins D3 (cholecalciferol), E (tocopherol), K, A (retinol) and carotenoids are involved in different ways in nestling growth and development (Bügel, 2008; Chin and Ima-Nirwana, 2014; Cranenburg et al., 2007; Deeming and Pike, 2013; Espín et al., 2016a; Khazai et al., 2008; Zile, 2004). Regarding the effects of As in biochemistry, Albert et al. (2008a) suggested that its interaction with the mineral fraction of the bone may explain the effects on bone development, whereas Ortiz-Santaliestra et al. (2015) observed that As was associated with decreased retinol in plasma and increased creatine phosphokinase activity in Bonelli's eagle (*Aquila fasciata*) nestlings.

The main objective of this study is to explore if environmentally relevant As levels affect growth, survival and physiological biomarkers of great tits (*Parus major*). For this purpose, during the breeding season of 2015, nestlings were orally dosed with sodium arsenite daily (from day 3 to day 13 post-hatching) and were measured in terms of brood size, nestling survival, number of fledglings, body size and growth rate. Concentrations of As in feces of nestlings were analyzed to be used as indicators of As dietary intake, and a set of physiological biomarkers (hematocrit, vitamins, carotenoids and other biochemical parameters) that are expected to be potential indicators of health and/or As toxicity were measured in the blood. Dead nestlings were necropsied to

investigate As accumulation in liver, bone and feathers. The responses to three experimental manipulations (Control, Low and High groups) carried out in a great tit population with low metal exposure levels are compared with those in a population breeding in the vicinity of a copper-nickel (Cu-Ni) smelter, an anthropogenic As source (Smelter group). Thus, we will be able to compare the effects of dietary As levels to those caused by exposure to a mixture of As and metals, other pollutants and potential associated resource limitations. Based on the developmental and reproductive effects reported in As-manipulation experiments, we hypothesize that As will interfere with one or several physiological parameters and decrease growth and survival.

Material and methods

Experimental set-up

The As-manipulation experiment was performed during the breeding season 2015 in the proximities of a Cu-Ni smelter in Harjavalta (61°20′ N, 22°10′ E), SW Finland. There is an accumulation of heavy metals (mainly Cu, Ni, Pb, Cd, As, and zinc, Zn) in the area of the smelter (polluted zone) as a result of present and previous emissions. Metal concentrations decrease with distance to the smelter. The study area is described in detail by Eeva and Lehikoinen (1995). The As-manipulation was done on a great tit population using nest boxes placed in 11 different sites along the pollution gradient. The area was divided into the polluted and the unpolluted zone (0-2 km and 4.5-11 km, respectively, from the smelter). This study is part of a long-term (since 1991) follow-up of hole-breeding passerines in this area. The study sites have been selected to represent similar habitat, i.e. relatively barren pine dominated forests. Variation in tree species composition has been a very weak explanatory factor for clutch size or fledgling number, and no significant effects were found in our earlier studies (Eeva and

Lehikoinen 2000, 2013). On the other hand, long-term pollution has changed some habitat characteristics, like ground layer vegetation, which can be considered as one of the secondary effects of pollution. Tit population densities have been similar between study areas (Eeva and Lehikoinen 2013). Details of the study species are given in Supplementay Material (Document S1).

There are ca. 500 nest boxes in the study area that were checked in April and then periodically to track the progress in the nest building. When newly hatched nestlings were found in a nest in the unpolluted area, the nest was assigned randomly either to the Control (distilled water), Low (0.2 µg g^{-1} d^{-1} of liquid sodium arsenite), or High (1 µg g^{-1} d^{-1} of liquid sodium arsenite) As-supplemented groups. In the polluted area, all the nests received distilled water (hereafter called Smelter group).

We aimed to provide environmentally relevant As doses in order to achieve As concentrations at which wild passerines are currently exposed at polluted sites. After preliminary trials of 8 µg g^{-1} d^{-1} and 2 µg g^{-1} d^{-1}, we set 1 µg As g^{-1} d^{-1} as the high treatment (hereafter called High As) corresponding to As exposure in relatively highly polluted areas, and another dose at 0.2 µg As g^{-1} d^{-1} as the low treatment (hereafter called Low As). A more detailed explanation on the selection of these dosing levels is provided in Document S2. Sodium arsenite (Sigma S7400, Batch SLBH5736V, 98% pure) was used to prepare dilutions to dose the nestlings. Sodium arsenite was used because it is one of the most common trivalent inorganic As compounds (WHO, 2000) and because of its well-known toxic effects. Moreover, Moriarty et al. (2009) found that most of the As in terrestrial invertebrates is inorganic, with the proportion as arsenite versus arsenate varying by invertebrate type. Lepidoptera, particularly in larval form, is the main invertebrate group in the diet of great tits in our study area (Eeva et al., 2010). In this sense, Moriarty et al. (2009) showed that the As speciation in their study was

60% arsenite versus 34% arsenate in mature Lepidoptera and 29% arsenite versus 64% arsenate in larval Lepidoptera in contaminated zones, while larval Lepidoptera in the background zone had 55% arsenite and 45% arsenate. Two different dilutions of 100 µg As mL^{-1} and 20 µg As mL^{-1} in distilled water were used.

At 3 days of age (d3), we started providing As or distilled water daily for eleven days (until d13). We established d14 as the end of the experiment (last sampling and measurements) to avoid handling and dosing birds too close to the fledging date. Nestlings were dosed with increasing volumes of the corresponding treatment (Control, Low or High As) in order to receive the appropriate dose (0, 0.2 or 1 µg g^{-1} d^{-1}) according to their body mass. The volumes were provided orally with pipettes (from 50 to 170 µL from d3 to d13). For volume calculations, we used the long-term data on daily nestling body mass from nestlings of the same area. All nestlings from the same brood received the same treatment. In exceptional cases, if one sibling was clearly smaller than the other nestlings in the brood (around half weight), we provided half of the volume.

The ideal number of nests in the experiment was set at 60 (15 nests per treatment group: Control, Low As, High As and Smelter). Since some nests could fail later, we took a higher number of nests at the beginning of the experiment. In total, the experiment was carried out on 70 nests (16 Control nests, 17 Low As nests, 16 High As nests, and 21 Smelter nests) with a total of 400 nestlings dosed.

The study was approved by the Centre for Economic Development, Transport and the Environment, ELY Centre Southwest Finland (VARELY/593/2015) and the Animal Experiment Committee of the State Provincial Office of Southern Finland (ESAVI/11579/04.10.07/2014).

Parents were not captured in this experiment because this species is relatively sensitive for capturing and handling, and there is a small risk for nest desertion even during the late nestling period. Moreover, data (n = 508 nests) from previous years showed that even though old females (age > 2 calendar years) lay c.a. 0.3 eggs (mean = 9.23 eggs) more than young ones (mean = 8.93), the age effects on fledgling numbers (old, mean = 5.45; young, mean = 5.20) or nestling survival (fledglings/clutch size; old, mean = 47%; young, mean = 0.43%) are statistically non-significant, indicating that female age is a weak explanatory factor for fledgling number or nestling survival (unpublished data).

Sampling, measurements and metal analysis

Details on sampling and measurements are provided in Document S3. Briefly, on d7 birds were ringed, and on d8 feces were collected for metals analysis. On d8 and d14 post-hatching, nestlings were weighed, wing, tarsus and total head length were measured and blood samples were collected. Some birds in all the treatment groups died during the experiment, especially in June, probably due to a combination of relatively low food availability, low temperatures and high rainfall during that period (Figure 1). All the dead nestlings found in the nests were collected and frozen at −20 °C until necropsies could be performed in July 2015. We used the carcasses to measure As and metal concentrations in liver, bone and feathers in order to compare As accumulation among groups and its distribution among tissues. Prior to dissection, the carcasses were completely thawed and morphometric measurements taken (wing length and body mass). During necropsy, the liver and the two femurs were weighed and collected in different tubes. Wing feathers were removed and collected. The lengths of the 4th primary feathers from both wings were determined. The necropsies were performed on 80 nestlings (16 from Control nests, 14 from Low As nests, 21 from High As nests, 18 from Smelter nests, 9 from the trial of 2 μg g^{-1} d^{-1}, and 2 from the trial of 8 μg g^{-1} d^{-1}).

Carcasses of nestlings from different ages (3-15 days old) evenly distributed among the treatment groups were selected to evaluate As accumulation in liver and bone along time. Note that birds did not receive As after d13. Since younger nestlings have not developed the wing feathers yet, feathers were collected from 13-19 day-old nestlings (n = 42: 10 from Control nests, 10 from Low As nests, 11 from High As nests, and 11 from Smelter nests).

Feces collected on d8, and liver, bone and feathers from dead nestlings were dried for As and other elements (Ca, Cd, Cu, Ni, Pb, selenium, Se and Zn) determination by inductively coupled plasma optical emission spectrometry (ICP-OES). Further information on metal analysis is provided in Document S4. Arsenic concentrations were also analyzed in 9 samples of moth larvae, spiders and beetles collected directly from parent great tits feeding their nestlings in the polluted area in 2000 and 2002 (sampling is described in Eeva et al., 2005).

Caterpillar index, rainfall and temperature

The frass-fall method (Southwood, 1978) was used to measure the abundance of caterpillars and sawfly larvae using round plastic funnels (diameter 34 cm, 4 collectors per site, 10 sites). Temperature also affects the falling frass, since caterpillars develop faster in warmer weather, producing more frass (Eeva et al., 1997). Daily mean temperature data was downloaded from the database provided by the Finnish Meteorological Institute (Kokemäki Tulkkila, 61°15’ N, 22.21’ E). We also measured rainfall at each of the 10 sites using rain gauges placed close to one of the funnels. More details are provided in Document S5.

206 **Vitamins and biochemistry analyses**

207 Plasma collected at d8 was pooled by brood (n = 69) to obtain a volume of 90 µL for
208 vitamin and carotenoid analysis. Vitamins and carotenoids were analyzed with an
209 Acquity ultra-performance liquid chromatography system (UPLC; Waters Corp.,
210 Milford, MA, USA) coupled to a Xevo TQ triple-quadrupole mass spectrometer with
211 electrospray ionization (ESI). Details of the technique are given in Document S6.

212 Creatine kinase (CK) and ALP activities, and the plasma components uric acid and Ca
213 were measured from plasma from 120 individuals (2 nestlings randomly selected per
214 brood) collected on d14. A microplate reader (EnSpire, Perkin-Elmer) was used to
215 analyze the samples. All measurements were done in triplicate using 384-well
216 microplates to minimize the sample volume. Reagent volumes were adjusted according
217 to this miniaturization.

218 **Statistical analysis**

219 The statistical packages SAS 9.4 and SPSS 22.0 were used to perform the statistical
220 analyses. A detailed explanation on the statistics is provided in Document S7. Briefly,
221 generalized linear mixed models (GLMMs) were run to study the differences in As and
222 metal concentrations between treatment groups and to evaluate the effect of the
223 experiment on different response variables: (i) survival and growth parameters, (ii)
224 biochemical parameters from d8 and d14. Tukey's test was used to make pairwise
225 comparisons between treatment groups. The site was included as random factor in the
226 models. GLMMs were also used to evaluate the effect of the zone (polluted vs.
227 unpolluted) and period on the caterpillar index. During the experiment, we lost 14 nests
228 after day 8 (ca. June 5-June 17), which was likely related to a combination of relatively
229 low food availability, low temperatures and high rainfall during that period (Figure 1).

Therefore, we also ran the models removing those 14 nests with no fledglings since they could have an important effect on the survival and growth parameters. When working with all the broods, the treatment had no effect on those variables (fledging success, nestling survival, brood size, number of fledglings, body size and growth). However, when excluding the 14 failed nests, few but some significant effects were found, suggesting that these nests may mask the effect of the treatment. Therefore, we provide results from the models excluding these possibly confounding nests for the survival and growth parameters.

GLMMs were also used to analyze the effects of As and metals on survival and growth parameters, biochemistry and vitamin/carotenoid concentrations. Since metal concentrations (Cd, Cu, Ni, Pb) in feces were positively correlated to each other, we performed principal component analysis. The first principal component ($PC1_{met}$) from metals and log-transformed fecal As levels were included as explanatory variables in the model, as well as hatching date and brood size at d3, since they could be confounding variables. Explanatory variables were retained when significant. For all the parameters individually measured (biometric and biochemical parameters on d14), the mean value per brood was considered in the models due to the non-independence of measurements. For statistical analyses, metal concentrations below the detection limit were substituted with a value equal to limit of quantitation (LOQ)/$\sqrt{2}$.

The correlations among response variables were tested with the Pearson (r_p) or Spearman's (r_s) correlation coefficients. Normality of data was checked with the Kolmogorov-Smirnov test. The significance level was set at $p \leq 0.05$ in all analyses.

Results

Arsenic and metals in feces, liver, bone and feathers

Arsenic concentrations in feces at d8 varied significantly among the four treatment groups (Table 1, Figure 2), and were highest in the Smelter group, followed by the High As group, and then the Low group, with levels respectively 16.6, 9.6 and 3.7 times higher than the Control group. Fecal Cd, Cu, Ni and Pb concentrations tend to be higher in the Smelter group (significantly higher for Ni) as compared to the groups from the unpolluted area (Control, Low As and High As; Table 1).

Arsenic levels in liver, bone and feathers also showed significant differences among treatment groups (Table 2, Figure 2), showing the same trend in all three matrices. The highest As concentrations were found in the High As group, followed by the Low As and the Smelter group with similar levels among them, and then the Control group with the lowest As concentrations (Table 2, Figure 3). The mean ratio of As concentrations for Control, Low, High and Smelter groups was 1:13:72:16 in liver and 1:9:46:10 in bone. Arsenic concentrations in liver and bone from the trial nestlings are also reported (Table 2), and concentrations in liver and bone of nestlings receiving 8 μg g^{-1} d^{-1} and 2 μg g^{-1} d^{-1} were significantly higher than those found in the High As group (Table 2).

Arsenic concentrations were positively correlated between liver and bone ($r_s = 0.87$, $p < 0.001$, $n = 80$), liver and feathers ($r_s = 0.50$, $p = 0.014$, $n = 23$) and bone and feathers ($r_s = 0.75$, $p < 0.001$, $n = 23$; Table S1). The prediction equations of hepatic and bone As concentrations (d.w.) to use feathers as non-destructive samples, obtained via GLMs, are described below [Eqs. (1) and (2)].

$$\text{Log}_e \text{ As Liver } (\mu g\ g^{-1}) = -1.8015 + 0.5899 * \text{Log}_e \text{ As Feathers } (\mu g\ g^{-1}) \quad (1)$$

$$\text{Log}_e \text{ As Bone } (\mu g\ g^{-1}) = -1.040 + 0.4038 * \text{Log}_e \text{ As Feathers } (\mu g\ g^{-1}) \quad (2)$$

Arsenic concentrations in liver and bone were correlated with different element concentrations such as Cd ($r_s = 0.26$, $p = 0.021$ and $r_s = 0.43$, $p < 0.001$, $n = 80$), Ni ($r_s = 0.39$, $p < 0.001$ and $r_s = 0.45$, $p < 0.001$, $n = 80$), Pb ($r_s = 0.24$, $p = 0.034$ and $r_s = 0.24$, $p = 0.031$, $n = 80$), and Se ($r_s = -0.23$, $p = 0.037$ and $r_s = -0.39$, $p < 0.001$, $n = 80$) in liver and bone, respectively (Table S1). In feathers, As concentrations were correlated with levels of Cd ($r_s = 0.35$, $p = 0.023$, $n = 42$). The other elements were also correlated between them in the different tissue types (see Table S1).

Arsenic concentrations in samples of moth larvae, spiders and beetles collected in the polluted area in 2000 and 2002 were 5.22 ± 7.89 (n = 5), 0.50 ± 0.63 (n = 2) and 6.49 ± 4.72 (n = 2) $\mu g\ g^{-1}$, respectively.

Effects of arsenic on growth, survival and plasma biochemistry

Nestlings in the Low As group showed a slower wing growth rate than nestlings from the other treatment groups (12% slower than the Control group). A similar tendency was found in the High As group compared to the Control, but the effect was not significant (Figure 3, Table S2). The number of fledglings per successful nest was smaller in the High As group compared to the Low As group (2.91 vs. 4.49 fledglings) but it did not significantly differ compared to the Control group (2.91 vs. 4.19 fledglings; Figure 3, Table S2).

Vitamin K1 concentrations in plasma at d8 were slightly lower in nestlings from the Low and High As groups compared to the Control group (13 and 15% lower, respectively), although not significantly, but nestlings from the Smelter group showed significantly higher vitamin K1 levels compared to the Low and High As groups (90 and 95% higher, respectively; Figure 3, Table S2). Nestlings from the Smelter group

also showed slightly higher vitamin A levels in plasma compared to the other treatment groups, although not significantly (Figure 3, Table S2). The $PC1_{met}$ was negatively associated with vitamin K1 and vitamin D3, while fecal As concentrations were positively related to hematocrit (Table S3). The correlations for growth, survival and biochemical parameters, and single metals in feces are shown in Table S4.

Finally, the zone (polluted vs. unpolluted) had no effect on the caterpillar index ($F_{ndf, ddf} = 2.27_{1,38.7}$, $p = 0.14$), while it varied significantly among periods ($F_{ndf, ddf} = 30.67_{10,318.4}$, $p < 0.001$; Figure 1).

Discussion

Arsenic in feces, liver, bone and feathers

Our experiment aimed to provide environmentally relevant As doses in order to achieve As concentrations at which wild passerines are actually exposed at polluted sites. Since some nestlings died during the experiment, they were necropsied to investigate internal accumulation and distribution of As among tissues. The liver is clearly the most commonly used internal tissue to analyze As in dead passerines (Sánchez-Virosta et al., 2015), and it was selected as an appropriate organ to determine As accumulation. Since data on As concentrations in bone tissue is scarce, bone As concentrations were also measured. Moreover, feathers have been shown to be suitable matrices to evaluate As exposure in passerines, but studies evaluating the relationship between As levels in internal tissues and feathers in wild passerines are scarce, so As levels in wing feathers were also analyzed. Our results are indicative of an As accumulation in liver, bone and feathers over time. The Low As treatment resulted in significantly higher As concentrations in liver, bone and feathers than the Control group and similar As concentrations than those found in the Smelter group, while the High As treatment

showed significantly higher As concentrations than the Control and Low As groups. In addition, As concentrations were positively correlated between liver, bone and feathers. The accumulation pattern in liver, bone and feathers shows that we were successful in achieving concentrations that have been measured in polluted environments. In this sense, Berglund et al. (2012) found that emission reductions in the Cu/Ni smelter in Harjavalta resulted in decreased hepatic As concentrations in great tit nestlings, from 3.2 μg g^{-1} d.w. in 1991 in nestlings within 2 km from the smelter, being slightly lower than those found in the High As group in our experiment, to 0.24 μg g^{-1} d.w. in 2009. On the other hand, As concentrations in liver, bone and feathers in the Low As group are similar to those currently found in the smelter zone. However, nestlings receiving the trial doses (2 and 8 μg g^{-1} d^{-1}) reached hepatic As levels far above those reported in polluted environments in previous studies (Sánchez-Virosta et al., 2015), suggesting that those trial doses were too high taking into account our aim of providing environmentally relevant As doses at which wild passerines are currently exposed at polluted sites.

Although internal tissues have been traditionally used as indicators of contaminant exposure, non-destructive sampling is becoming the trend in recent years (Espín et al., 2016c; García-Fernández et al., 2013). In this sense, As is integrated in feathers during their growth (Janssens et al., 2001). In unpolluted sites, As concentrations in feathers of great tit nestlings are generally lower than 0.3 μg g^{-1}, as found in our Control group, whereas in polluted sites, concentrations are within 0.6-1.1 μg g^{-1} (Dauwe et al., 2004; Eeva et al., 2006; Janssens et al., 2002), as observed in our Low As and Smelter groups. Our experiment backs feathers of nestling passerines as a good type of sample for As monitoring. Thus, we provide prediction equations for estimating hepatic and bone As concentrations in great tit nestlings using the As concentrations in wing feathers.

Newly-grown feathers from nestlings should well represent the load of As in the organism, as pollutant concentrations in the first plumage are unaffected by migration or molting, and the external contamination should be negligible (Sánchez-Virosta et al., 2015).

Feces are a good alternative to blood samples in small animals where a limited amount of blood is available for sampling (Sánchez-Virosta et al., 2015). Fecal As concentrations in passerines from different areas range from 0.1 to 1.4 μg g^{-1} d.w. in unpolluted sites and from 5 to 16 μg g^{-1} d.w. in polluted areas (see articles reviewed by Sánchez-Virosta et al., 2015). In the present experimental study, nestlings from the High As group reached fecal As concentrations ranging 0.7-18.8 μg g^{-1} d.w., so this group could represent levels found in polluted sites, while our Control group (0.01-1.8 μg g^{-1} d.w.) has similar levels to those found in unpolluted sites in the literature. Interestingly, concentrations found in the Smelter group (ranging 0.1-48.7 μg g^{-1} d.w.) were higher than those observed in nestlings in the other experimental groups, although significantly higher only compared to the Control and Low As groups. Feces do not reflect the As accumulated in the organism, but As that was not absorbed and As that was excreted after absorption (Sánchez-Virosta et al., 2015). It is likely that As is better absorbed from sodium arsenite dissolved in water than from prey (i.e. invertebrates such as caterpillars, spiders and beetles) that have accumulated As, thus resulting in higher fecal As levels from excretion in the Smelter group. In this sense, Moriarty et al. (2012) used a physiologically based extraction test and estimated that 47% of invertebrate As was bioaccessible in the shrew (*Sorex cinereus*) gastrointestinal tract. In addition, while sodium arsenite was dosed once a day in As-treated birds, nestlings in the Smelter group are continuously exposed to As and metal-polluted food. Feces were collected ca. 24 h after the previous As dosing, so it is likely that a higher proportion of sodium arsenite is

excreted in the first droppings after exposure and feces collected 24 h after dosing are mostly reflecting the body active excretion to feces of absorbed As. It is notable that As concentrations in feathers relative to those in liver and bone were higher in the As-dosed nestlings than in either the Control or the Smelter group. This is consistent with greater As absorption in the As-dosing treatments, as depuration into feathers is an elimination mechanism for absorbed As only, whereas fecal elimination applies to both non-absorbed and absorbed As. Regarding the As concentrations in food items, this study provides some results in moth larvae, spiders and beetles collected in the polluted area in previous years. Since those samples were collected directly from parent great tits feeding their nestlings (Eeva et al., 2005), they should be very relevant indicators of As exposure for great tit nestlings. Another study in the same area showed that moth (*Epirrita autumnata*) larvae collected in 2014 in the polluted area had 12 times higher As concentrations than larvae from the unpolluted area (geometric means and 95% confidence limit): 0.48 (0.36-0.64) (n = 12) and 0.039 (0.029-0.052) (n = 12) μg g^{-1} d.w., respectively ($F_{df} = 322.2_{1,20}$, $p < 0.0001$; unpublished data).

Correlation tests revealed the presence of significant relationships between different element concentrations in all three tissues and between tissues. The positive correlations described between As, Cd, Ni and Pb, and the negative correlation found between all those elements and Se in both liver and bone tissue, may be indicative of shared uptake and accumulation pathways and similar regulation and detoxification mechanisms, as suggested before by different researchers (e.g. Ribeiro et al., 2009). In this sense, the negative relationship between the different elements and Se indicates that, in addition to metalothioneins, Se is important in the storage and detoxification of those elements, since it protects against Cd, Pb, As and Hg toxicity (Schwalfenberg et al., 2015).

Effects of arsenic on growth, survival and plasma biochemistry

Our As experiment aimed to explore if growth, survival and physiology are affected by environmentally relevant As levels in great tits. Experimental studies on birds dosed with different As forms have reported decreased body weight, tarsi and wing length, and rate of growth (see articles reviewed by Sánchez-Virosta et al., 2015). Our As treatment had no effect on body mass, tarsus or head growth. However, As-dosed nestlings had depressed wing growth rate compared to Control nestlings, only significant for the Low As group. As suggested by other researchers (Albert et al., 2008a), this could be related to an interaction between the mineral fraction of the bone and As, most likely with As substituting phosphate in the hydroxyapatite (Kretshmer et al., 2002). Thus, wild nestlings exposed to similar doses to those provided in this experiment could suffer skeletal growth problems which could lead in longer term effects such as increased predation risk after fledging. In this respect, skeletal growth problems were observed in *F. hypoleuca* nestlings in the vicinity of the smelter in the beginning of the 1990's, before metal emissions decreased (Eeva and Lehikoinen, 1996). However, although a tendency of slower wing growth rate in the High As group compared to the Control is observed, this result was not significant. The lack of a dose-response relationship for this endpoint suggests that further research would be needed to support this result.

In addition, nestlings in the High As group showed lower fledging success, nestling survival and number of fledglings per successful nest compared to the other experimental groups, although significant differences were only found between the Low and High As group for the number of fledglings per successful nest. According to the results found in our experiment, we consider that the lowest-adverse-effect level (LOAEL) for effects on size and growth in great tit nestlings is likely equal to or below our higher dose of 1 μg g^{-1} d^{-1}. Since the trial dose of 8 μg g^{-1} d^{-1} resulted in the death

of 4 nestlings after the first dose and the death of the other 3 nestlings after the second dose, this was clearly a high lethal dose for great tit nestlings. The trial dose of 2 μg g^{-1} d^{-1} also resulted in the death of 1 nestling per day after the first 2 or 3 doses depending on the brood, and the rest of the nestlings also died in one of the broods at different ages. Thus, the oral LD50 of sodium arsenite for nestling of this species could fall between 2 and 8 μg g^{-1}.

The As treatment showed limited effects on the biochemical parameters evaluated. Fecal As concentrations were positively related only to hematocrit (the percentage of red blood cells in a blood sample). Previous studies have found that As exposure may result in hemolysis and reduced hematocrit in experimental animals (e.g. Antonio Garcia et al., 2013; Hong et al., 1989). However, in accordance with our results, blood hematocrit was increased in rats after sodium arsenite administration (single dose of 0.1 or 1 μg g^{-1}; Mitchell et al., 2000). This result could suggest a hormetic effect of As, showing that exposure to relatively low doses of As could stimulate erythropoiesis as a protective effect, increasing hematocrit levels, but at certain As exposure, this metalloid would produce its hemolytic effect and consequent anemia. In this sense, sodium arsenite has been found to induce ABCB6 expression, a mitochondrial porphyrin transporter essential for heme biosynthesis (Krishnamurthy et al., 2006), in mice and cells *in vitro*, which might be indicative of a hormetic mechanism triggered to protect cells against the oxidative stress induced by As (Chavan et al., 2011). In addition, Kajiguchi et al. (2005) observed that the expression of erythropoietin, a glycoprotein that promotes the proliferation and differentiation of erythrocyte precursors (Lacombe et al., 1991), markedly increased in cells exposed to a therapeutic concentration (0.5 μM) of arsenic trioxide, while a bigger concentration (2.5 μM), was found to inhibit cell growth and decrease the erythropoietin expression to the baseline.

Regarding the bigger levels of vitamin K1 or phylloquinone in the polluted environment, we hypothesize that, since it is synthesized by green leafy plants (Basset et al., 2016), a putative source of this difference could be the diet, with birds in the polluted area consuming more vitamin K1-rich food items. Dietary differences have been documented in great tit nestlings between the polluted and reference areas (Eeva et al., 2005) but we cannot evaluate this hypothesis due to the lack of knowledge on the vitamin K1 content in their food items.

Vitamin K1 is primarily involved in coagulation and vascular and skeletal metabolism (Basset et al., 2016). Different studies have described that metals decrease whole-blood coagulation time (Lim et al., 2010; Sangani et al., 2010). This metal-related anticoagulant activity supports our higher vitamin K1 levels in metal-exposed nestlings from the Smelter group. Metal-induced oxidative stress is one of the defining means of metal toxicity (Ercal et al., 2001). Thus, a possible mechanism underlying the procoagulative changes following metal exposure in the Smelter group is an association between oxidative stress and coagulation responses (Bind et al., 2014). In addition, evidence suggests that hepatic stores of phylloquinone are very mobile and, under dietary scarcity conditions, these stores are diminished (Usui et al., 1990). Thus, birds facing poor food quality and/or quantity could activate a mechanism to stimulate vitamin K1 absorption. On the other hand, our results showed that $PC1_{met}$ was negatively associated with vitamin K1. This negative association between vitamin K1 and metals seems to contradict our finding of higher vitamin K1 levels in the Smelter group. However, it should be noted that the negative association depends quite much on two broods with high metal concentrations. The generally higher vitamin K1 values in the Smelter group cannot be explained by metals, or at least not solely by them, but maybe by a different diet composition or an indirect effect of metals on diet quality and

quantity resulting in a mechanism to enhance vitamin K1 absorption. We encourage further studies on vitamin K1 levels in birds to better understand the metal-related effects on its metabolism.

Additionally, fecal concentrations of Cu and Pb were negatively correlated with vitamin K2. This vitamin is important for regulating the Ca deposition and proper Ca use in the organism (Maresz, 2015). However, metal effects on vitamin K2 have been poorly evaluated in free-living birds. A previous study by our research group showed that great tit nestlings exposed to low doses of Pb showed the lowest vitamin K concentrations among treatment groups (Ruiz et al., 2016). Although further studies are needed to understand the effects of metals on vitamin K2 homeostasis, a possible mechanism explaining its relationship with Pb could be related to the negative effects of this metal on Ca metabolism (Pounds, 1984).

In addition, $PC1_{met}$ negatively affected vitamin D3 levels, which is consistent with previous studies on the same area where we reported a negative association between metals and yolk vitamin D3 in great tit (Espín et al., 2016b). It is known that metals (particularly Cd and Pb) disrupt vitamin D3 metabolism by interfering with renal 1,25-dihydroxyvitamin D synthesis (Moon, 1994; Smith et al., 1981). However, in a previous study in the same area, additional Pb increased vitamin D3 levels (Ruiz et al., 2016). Thus, future studies should assess in detail the effect of metals on vitamin D3.

Furthermore, the single metals Cd and Cu were negatively correlated with vitamin E. These metals induce oxidative stress and damage by increasing ROS generation (Koivula and Eeva, 2010), which can deplete the levels of antioxidants such as vitamin E, suggesting that the defense mechanism is removing reactive species in the organism (Halliwell and Gutteridge, 2007).

As reported in previous studies in the same study area (Espín et al., 2016a), since the bone isoform of ALP is related to active skeletal growth, the positive relationship between ALP and nestling size, growth and number of fledglings in great tit nestlings seems logical (Viñuela and Ferrer, 1997). The number of fledglings was positively correlated with the nestling size, wing and head growth, hematocrit, lutein + zeaxanthin and vitamin K1, while nestling size and growth were positively correlated with hematocrit. These results show that hematocrit and carotenoid levels, which are closely related to food quality and quantity (Eeva et al., 2009), play a major role in the nestling growth and survival. The positive correlation observed between Ca and vitamin K2 or menaquinone in plasma is due to vitamin K2 being important for regulating the Ca deposition and proper Ca use (Maresz, 2015). Moreover, some vitamins were positively intercorrelated, probably due to their role in essential physiological mechanisms in growing birds. In this sense, vitamin E has different functions: it is a potent antioxidant that inhibits the production of ROS, it is an important anti-inflammatory agent, it can be beneficial to bone health, and it stimulates humoral and cell immune responses and phagocytic functions (Chin and Ima-Nirwana, 2014; Rizvi et al., 2014). Vitamin A is involved in cell differentiation, growth and immune function (Tanumihardjo, 2011; Zile, 2004), and vitamins K1 and K2 are essential in blood coagulation and in bone and vascular metabolism (Bügel, 2008; Cranenburg et al., 2007; Maresz, 2015). Finally, vitamin D3 (cholecalciferol) is well known for its role in Ca homeostais and proper skeletal growth (Khazai et al., 2008). Therefore, it is not surprising that these vitamins are positively correlated with each other during nestling development.

Conclusions

Great tit nestlings receiving sodium arsenite through the diet accumulate As in liver, bone and feathers in a dose-dependent manner. Broods exposed to 1 μg g^{-1} d^{-1} of

sodium arsenite showed reduced number of fledglings per successful nest compared to broods exposed to 0.2 µg g^{-1} d^{-1} but not to the Control group. Lower concentrations (0.2 µg g^{-1} d^{-1}) resulted in sublethal effects as reduced wing growth, which could have post-fledging consequences such as increased predation risk. Due to the lack of a dose-response relationship for this endpoint, further research would be needed to support an effect on the wing growth rate. These results suggest that the LOAEL for effects on nestling survival and development in great tits is likely below 1 µg g^{-1} d^{-1}.

In spite of the clear As accumulation, limited effects on the biochemical parameters evaluated were found. This could suggest that nestlings exposed to similar doses in the wild will not suffer effects on Ca, uric acid, ALP, CK, carotenoids and vitamin levels in plasma. However, this should be interpreted prudently, especially when As exposure is associated with exposure to other contaminants or resource limitations (e.g. low calcium availability or restrictions in food intake).

It is known that As may produce oxidative stress, and other physiological effects with potential long-term consequences cannot be discarded. Further research on this issue will be carried out in our future studies.

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References

Albert, C., Williams, T.D., Morrissey, C.A., Lai, V.W.-M., Cullen, W.R., and Elliott, J.E. (2008a). Tissue uptake, mortality, and sublethal effects of monomethylarsonic acid (MMA(V)) in nestling zebra finches (*Taeniopygia guttata*). J. Toxicol. Environ. Health A *71*, 353–360.

Albert, C.A., Williams, T.D., Morrissey, C.A., Lai, V.W.M., Cullen, W.R., and Elliott, J.E. (2008b). Dose-dependent uptake, elimination, and toxicity of monosodium methanearsonate in adult zebra finches (*Taeniopygia guttata*). Environ. Toxicol. Chem. SETAC *27*, 605–611.

Antonio Garcia, M.T., Herrera Dueñas, A., and Pineda Pampliega, J. (2013). Hematological effects of arsenic in rats after subchronical exposure during pregnancy and lactation: the protective role of antioxidants. Exp. Toxicol. Pathol. Off. J. Ges. Toxikol. Pathol. *65*, 609–614.

ATSDR (2015). The ATSDR 2015 Substance Priority List (U.S.A.: Agency for Toxic Substances and Disease Registry, U.S. Public Health Service).

Berglund, Å.M.M., Rainio, M.J., and Eeva, T. (2012). Decreased metal accumulation in passerines as a result of reduced emissions. Environ. Toxicol. Chem. SETAC *31*, 1317–1323.

Bind, M.-A., Coull, B., Suh, H., Wright, R., Baccarelli, A., Vokonas, P., and Schwartz, J. (2014). A Novel Genetic Score Approach Using Instruments to Investigate Interactions between Pathways and Environment: Application to Air Pollution. PLoS ONE *9*.

Bügel, S. (2008). Vitamin K and bone health in adult humans. Vitam. Horm. *78*, 393–416.

Burger, J., and Gochfeld, M. (2000). Effects of lead on birds (Laridae): a review of laboratory and field studies. J. Toxicol. Environ. Health B Crit. Rev. *3*, 59–78.

Camardese, M.B., Hoffman, D.J., LeCaptain, L.J., and Pendleton, G.W. (1990). Effects of arsenate on growth and physiology in mallard ducklings. Environ. Toxicol. Chem. *9*, 785–795.

Chavan, H., Oruganti, M., and Krishnamurthy, P. (2011). The ATP-Binding Cassette Transporter ABCB6 Is Induced by Arsenic and Protects against Arsenic Cytotoxicity. Toxicol. Sci. *120*, 519–528.

Chin, K.-Y., and Ima-Nirwana, S. (2014). The Effects of α-Tocopherol on Bone: A Double-Edged Sword? Nutrients *6*, 1424–1441.

575 Cranenburg, E.C.M., Schurgers, L.J., and Vermeer, C. (2007). Vitamin K: the coagulation vitamin that
576 became omnipotent. Thromb. Haemost. *98*, 120–125.

577 Dauwe, T., Janssens, E., Bervoets, L., Blust, R., and Eens, M. (2004). Relationships between metal
578 concentrations in great tit nestlings and their environment and food. Environ. Pollut. Barking Essex 1987
579 *131*, 373–380.

580 Deeming, D.C., and Pike, T.W. (2013). Embryonic growth and antioxidant provision in avian eggs. Biol.
581 Lett. *9*, 20130757.

582 Eeva, T., and Lehikoinen, E. (1995). Egg shell quality, clutch size and hatching success of the great tit
583 (Parus major) and the pied flycatcher (*Ficedula hypoleuca*) in an air pollution gradient. Oecologia *102*,
584 312–323.

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

587 Eeva, T., and Lehikoinen, E. (2000). Recovery of breeding success in wild birds. Nature *403*, 851–852.

588 Eeva, T., and Lehikoinen, E. (2013). Density effect on great tit (*Parus major*) clutch size intensifies in a
589 polluted environment. Oecologia *173*, 1661–1668.

590 Eeva, T., Helle, S., Salminen, J-P., Hakkarainen, H. (2010). Carotenoid composition of invertebrates
591 consumed by two insectivorous bird species. J. Chem. Ecol. *36*, 608–613.

592 Eeva, T., Lehikoinen, E., and Pohjalainen, T. (1997). Pollution-related variation in food supply and
593 breeding success in two hole-nesting passerines. Ecology *78*, 1120–1131.

594 Eeva, T., Ryömä, M., and Riihimäki, J. (2005). Pollution-related changes in diets of two insectivorous
595 passerines. Oecologia *145*, 629–639.

596 Eeva, T., Belskii, E., and Kuranov, B. (2006). Environmental pollution affects genetic diversity in wild
597 bird populations. Mutat. Res. *608*, 8–15.

598 Eeva, T., Sillanpää, S., and Salminen, J.-P. (2009). The effects of diet quality and quantity on plumage
599 colour and growth of great tit *Parus major* nestlings: a food manipulation experiment along a pollution
600 gradient. J. Avian Biol. *40*, 491–499.

Ercal, N., Gurer-Orhan, H., and Aykin-Burns, N. (2001). Toxic metals and oxidative stress part I: mechanisms involved in metal-induced oxidative damage. Curr. Top. Med. Chem. *1*, 529–539.

Espín, S., Ruiz, S., Sánchez-Virosta, P., and Eeva, T. (2016a). Effects of calcium supplementation on growth and biochemistry in two passerine species breeding in a Ca-poor and metal-polluted area. Environ. Sci. Pollut. Res. *Online*, doi 10.1007/s11356-016-6219-y.

Espín, S., Ruiz, S., Sánchez-Virosta, P., Salminen, J.-P., and Eeva, T. (2016b). Effects of experimental calcium availability and anthropogenic metal pollution on eggshell characteristics and yolk carotenoid and vitamin levels in two passerine birds. Chemosphere *151*, 189–201.

Espín, S., García-Fernández, A.J., Herzke, D., Shore, R.F., van Hattum, B., Martínez López, E., Coeurdassier, M., Eulaers, I., Fritsch, C., Gómez-Ramírez, P., et al. (2016c). Tracking pan-continental trends in environmental contamination using sentinel raptors — what types of samples should we use? Ecotoxicology *25*, 777–801.

Furness, R.W., Greenwood, J.J.D., and Jarvis, P.J. (1993). Can birds be used to monitor the environment? In Birds as Monitors of Environmental Change, (London, UK: Furness R.W., Greenwood J.J.D. Eds.; Chapman and Hall), pp. 86–143.

García-Fernández, A.J., Espín, S., and Martínez-López, E. (2013). Feathers as a biomonitoring tool of polyhalogenated compounds: a review. Environ. Sci. Technol. *47 (7)*, 3028–3043.

Halliwell, B., and Gutteridge, J. (2007). Free Radicals in Biology and Medicine (Oxford University Press, USA).

Hong, H.L., Fowler, B.A., and Boorman, G.A. (1989). Hematopoietic effects in mice exposed to arsine gas. Toxicol. Appl. Pharmacol. *97*, 173–182.

Janssens, E., Dauwe, T., Bervoets, L., and Eens, M. (2001). Heavy metals and selenium in feathers of great tits (*Parus major*) along a pollution gradient. Environ. Toxicol. Chem. SETAC *20*, 2815–2820.

Janssens, E., Dauwe, T., Bervoets, L., and Eens, M. (2002). Inter- and Intraclutch Variability in Heavy Metals in Feathers of Great Tit Nestlings (*Parus major*) Along a Pollution Gradient. Arch. Environ. Contam. Toxicol. *43*, 323–329.

627 Jussila, I., and Jormalainen, V. (1991). Spreading of heavy metals and some other air pollutants at Pori-
628 Harjavalta district in SW-Finland. SYKEsarja B *4*, 1–58.

629 Kajiguchi, T., Yamamoto, K., Sawa, M., Emi, N., and Naoe, T. (2005). Increased erythropoietin level and
630 reticulocyte count during arsenic trioxide therapy. Leukemia *19*.

631 Khazai, N., Judd, S.E., and Tangpricha, V. (2008). Calcium and Vitamin D: Skeletal and Extraskeletal
632 Health. Curr. Rheumatol. Rep. *10*, 110–117.

633 Koivula, M.J., and Eeva, T. (2010). Metal-related oxidative stress in birds. Environ. Pollut. *158*, 2359–
634 2370.

635 Koivula, M.J., Kanerva, M., Salminen, J.-P., Nikinmaa, M., and Eeva, T. (2011). Metal pollution
636 indirectly increases oxidative stress in great tit (*Parus major*) nestlings. Environ. Res. *111*, 362–370.

637 Kretshmer, X., Pingitore, N.E., and Cruz-Jiménez, G. (2002). Incorporation of arsenic in mammal bone:
638 X-ray absorption spectroscopy. Am. Geophys. Union Fall Meet. 2002 Abstr. B51B-0728.

639 Krishnamurthy, P.C., Du, G., Fukuda, Y., Sun, D., Sampath, J., Mercer, K.E., Wang, J., Sosa-Pineda, B.,
640 Murti, K.G., and Schuetz, J.D. (2006). Identification of a mammalian mitochondrial porphyrin
641 transporter. Nature *443*, 586–589.

642 Lacombe, C., Da Silva, J.L., Bruneval, P., Casadevall, N., Camilleri, J.P., Bariety, J., Tambourin, P., and
643 Varet, B. (1991). Erythropoietin: sites of synthesis and regulation of secretion. Am. J. Kidney Dis. Off. J.
644 Natl. Kidney Found. *18*, 14–19.

645 Lim, K.-M., Kim, S., Noh, J.-Y., Kim, K., Jang, W.-H., Bae, O.-N., Chung, S.-M., and Chung, J.-H.
646 (2010). Low-level mercury can enhance procoagulant activity of erythrocytes: a new contributing factor
647 for mercury-related thrombotic disease. Environ. Health Perspect. *118*, 928–935.

648 Maresz, K. (2015). Proper Calcium Use: Vitamin K2 as a Promoter of Bone and Cardiovascular Health.
649 Integr. Med. Clin. J. *14*, 34–39.

650 Mitchell, R.D., Ayala-Fierro, F., and Carter, D.E. (2000). Systemic indicators of inorganic arsenic
651 toxicity in four animal species. J. Toxicol. Environ. Health A *59*, 119–134.

652 Moon, J. (1994). The role of vitamin D in toxic metal absorption: a review. J. Am. Coll. Nutr. *13*, 559–
653 564.

654 Moriarty, M.M., Koch, I., Gordon, R.A., and Reimer, K.J. (2009). Arsenic speciation of terrestrial
655 invertebrates. Environ. Sci. Technol. *43 (13)*, 4818–4823.

656 Moriarty, M.M., Koch, I., and Reimer, K.J. (2012). Arsenic speciation, distribution, and bioaccessibility
657 in shrews and their food. Arch. Environ. Contam. Toxicol. *62*, 529–538.

658 Ortiz-Santaliestra, M.E., Resano-Mayor, J., Hernández-Matías, A., Rodríguez-Estival, J., Camarero, P.R.,
659 Moleón, M., Real, J., and Mateo, R. (2015). Pollutant accumulation patterns in nestlings of an avian top
660 predator: biochemical and metabolic effects. Sci. Total Environ. *538*, 692–702.

661 Pacyna, J.M., and Pacyna, E.G. (2001). An assessment of global and regional emissions of trace metals to
662 the atmosphere from anthropogenic sources worldwide. Environ. Rev. *9*, 269–298.

663 Pounds, J.G. (1984). Effect of lead intoxication on calcium homeostasis and calcium-mediated cell
664 function: a review. Neurotoxicology *5*, 295–331.

665 Ribeiro, A.R., Eira, C., Torres, J., Mendes, P., Miquel, J., Soares, A.M.V.M., and Vingada, J. (2009).
666 Toxic element concentrations in the Razorbill Alca torda (Charadriiformes, Alcidae) in Portugal. Arch.
667 Environ. Contam. Toxicol. *56*, 588–595.

668 Rizvi, S., Raza, S.T., Ahmed, F., Ahmad, A., Abbas, S., and Mahdi, F. (2014). The Role of Vitamin E in
669 Human Health and Some Diseases. Sultan Qaboos Univ. Med. J. *14*, e157–e165.

670 Ruiz, S., Espín, S., Rainio, M., Ruuskanen, S., Salminen, J.-P., Lilley, T.M., and Eeva, T. (2016). Effects
671 of dietary lead exposure on vitamin levels in great tit nestlings - An experimental manipulation. Environ.
672 Pollut. *213*, 688–697.

673 Sánchez-Virosta, P., Espín, S., García-Fernández, A.J., and Eeva, T. (2015). A review on exposure and
674 effects of arsenic in passerine birds. Sci. Total Environ. *512–513*, 506–525.

675 Sangani, R.G., Soukup, J.M., and Ghio, A.J. (2010). Metals in air pollution particles decrease whole-
676 blood coagulation time. Inhal. Toxicol. *22*, 621–626.

677 Scheuhammer, A.M. (1987). The chronic toxicity of aluminium, cadmium, mercury, and lead in birds: a
678 review. Environ. Pollut. 1987 *46*, 263–295.

679 Schwalfenberg, G.K., Genuis, S.J., Schwalfenberg, G.K., and Genuis, S.J. (2015). Vitamin D, Essential
680 Minerals, and Toxic Elements: Exploring Interactions between Nutrients and Toxicants in Clinical
681 Medicine. Sci. World J. Sci. World J. *2015*, *2015*, e318595.

682 Smith, C.M., DeLuca, H.F., Tanaka, Y., and Mahaffey, K.R. (1981). Effect of lead ingestion on functions
683 of vitamin D and its metabolites. J. Nutr. *111*, 1321–1329.

684 Southwood, T.R.E. (1978). Ecological methods. With particular reference to the study of insect
685 populations. (Chapman and Hall, London, UK.).

686 Stanley, T.R.J., Spann, J.W., Smith, G.J., and Rosscoe, R. (1994). Main and interactive effects of arsenic
687 and selenium on mallard reproduction and duckling growth and survival. Arch. Environ. Contam.
688 Toxicol. *26*, 444–451.

689 Tanumihardjo, S.A. (2011). Vitamin A: biomarkers of nutrition for development1234. Am. J. Clin. Nutr.
690 *94*, 658S–665S.

691 Usui, Y., Tanimura, H., Nishimura, N., Kobayashi, N., Okanoue, T., and Ozawa, K. (1990). Vitamin K
692 concentrations in the plasma and liver of surgical patients. Am. J. Clin. Nutr. *51*, 846–852.

693 Viñuela, J., and Ferrer, M. (1997). Regulation of growth in Red Kites and imperial eagles. Wilson Bull.
694 *109*, 92–101.

695 WHO (2000). Arsenic. In Air Quality Guidelines for Europe, (WHO Regional Publications, European
696 Series), p.

697 Zile, M.H. (2004). Vitamin A requirement for early cardiovascular morphogenesis specification in the
698 vertebrate embryo: insights from the avian embryo. Exp. Biol. Med. Maywood NJ *229*, 598–606.

699

700 **Table captions**

701 Table 1. Arsenic and metal concentrations (μg g^{-1}, d.w.) in feces of great tit nestlings
702 (age 8 days) in the four treatment groups. N = number of broods

703 Table 2. Arsenic concentrations (μg g^{-1}, d.w.) in liver, bone and feathers of dead great
704 tit nestlings (age 3-15 days). N = number of individuals

705 Table S1. Spearman correlation coefficients and their p-values for metal concentrations
706 in liver, bone and feathers of great tit nestlings. N = Number of samples

707 Table S2. Generalized linear mixed models for variation in growth, survival and
708 biochemistry of great tit nestlings in the four treatment groups

709 Table S3. Generalized linear models for variation in growth, survival and biochemistry
710 of great tit nestlings. PC1met includes fecal concentrations of Cd, Ni, Pb and Cu

711 Table S4. Correlation coefficients (number of broods) and their p-values for survival
712 and growth parameters and biochemistry in great tit nestlings

713 **Figure captions**

714 Figure 1. Mean (± 95% CI) caterpillar index or frass fall (bars, mg d^{-1}) in the polluted
715 and unpolluted zone, rain (top graph, bars, mm d^{-1}) and mean temperature (top graph,
716 black dots, °C) measured during the experiment (May 4-July 23). Each bar represents
717 the mean frass fall from 20 collectors. Dashed line denotes the whole nestling period of
718 great tits from this study from hatching (mean = June 5, min-max = May 21-June 29) to
719 the age of 14 days (mean = June 19, min-max = June 4-July 13)

720 Figure 2. Least square means (± 95% CI) of arsenic levels in feces (age 8 days), liver,
721 bone (age 3-15 days) and feathers of great tit nestlings in the four treatment groups.

722 Statistical differences within each tissue type are shown in Tables 1 and 2. The numbers
723 above the error bars indicate the number of broods (feces) or individuals (liver, bone,
724 feathers)

725 Figure 3. Least square means (± 95% CI) of number of fledglings per successful nest,
726 wing growth (mm d^{-1}), plasma vitamin A and plasma ∑Vitamin K1 (µg mL^{-1}) of great
727 tit nestlings in the four treatment groups. The numbers above the error bars indicate the
728 number of broods. Vitamins were log-transformed for GLMMs shown in Table S2 and
729 then back-transformed for representation. Letters in bars denote significant differences
730 among treatments (means with different letter are statistically different)