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RESEARCH ARTICLE

# Large-scale geographical variation in eggshell metal and calcium content in a passerine bird (*Ficedula hypoleuca*)

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**Abstract** Birds have been used as bioindicators of pollution, such as toxic metals. Levels of pollutants in eggs are especially interesting, as developing birds are more sensitive to detrimental effects of pollutants than adults. Only very few studies have monitored intraspecific, large-scale variation in metal pollution across a species' breeding range. We studied large-scale geographic variation in metal levels in the eggs of a small passerine, the pied flycatcher (*Ficedula hypoleuca*), sampled from 15 populations across Europe. We measured 10 eggshell elements (As, Cd, Cr, Cu, Ni, Pb, Zn, Se, Sr, and Ca) and several shell characteristics (mass, thickness, porosity, and color). We found significant variation among populations

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in eggshell metal levels for all metals except copper. Eggshell lead, zinc, and chromium levels decreased from central Europe to the north, in line with the gradient in pollution levels over Europe, thus suggesting that eggshell can be used as an indicator of pollution levels. Eggshell lead levels were also correlated with soil lead levels and pH. Most of the metals were not correlated with eggshell characteristics, with the exception of shell mass, or with breeding success, which may suggest that birds can cope well with the current background exposure levels across Europe.

**Keywords** Heavy metals · Lead · Biomonitoring · Bird · Reproductive success · Ficedula · Flycatcher

## Introduction

The intensive use of metals in industrial and technological applications, and agriculture has resulted in a significant metal contamination globally. Accumulation of toxic metals influences the health and fitness of animals (Burger 1993). In birds particularly, exposure to toxic metals can affect reproduction by causing for example smaller clutch sizes, reduced fertility, and nestling mortality (Belskii et al. 2005; Eeva et al. 2009; Eeva and Lehikoinen 1995, 1996; Janssens et al. 2003).

Birds have been successfully used in biomonitoring of metals in the environment (e.g., Dauwe et al. 1999; Furness 1993; Gochfeld 1997; Swaileh and Sansur 2006). One widely used indicator is the egg and its shell (e.g., Dauwe et al. 1999; Furness 1993; Mora 2003; Swaileh and Sansur 2006), as eggs can be collected without need to catch the birds. Eggshell metal levels should reflect circulating levels in the blood of the female at the time of egg-laying (Burger 1993) and positive correlations between metal levels in blood and other tissues of parents and their eggs have been found (Burger and Gochfeld 1991, 1996; Dauwe et al. 2005; Franson et al. 2000). Importantly, it has been shown that in migratory species, levels (e.g., in liver) rise shortly after arriving to polluted sites (Berglund et al. 2011), strongly implying that egg metal levels reflect recent and local exposure (in the breeding grounds) to pollution of the adults that have laid them (Burger 1993). The eggshell should be an especially good indicator of lead pollution, as lead has similar properties to calcium, for example, competing for binding sites and being transported and stored similarly as calcium (Barton et al. 1978; Scheuhammer 1987). Furthermore, since bird embryos use the eggshell as a source of calcium for their own development (e.g., Blom and Lilja 2004), metals stored in the shell have a potential to negatively affect offspring development, especially as young animals are known to be more sensitive to pollutants than adults (e.g., Burger and Gochfeld 2000; DeSesso et al. 1998; Dietert et al. 2002).

Generally, studies using birds as bioindicators are rather biased towards wetland species and raptors (Furness 1993), while there is much less data on terrestrial passerines, except on heavily polluted sites and pollution gradients near active smelters (e.g., Belskii et al. 2005; Dauwe et al. 2004b; Eens et al. 1999; Nyholm 1998; Swiergosz et al. 1998), and large-scales studies are lacking (Eens et al. 2013; Mora et al. 2011). Furthermore, in addition to anthropogenic emissions, geographical variation in accumulation of heavy metals may also depend on the availability of other essential nutrients and minerals. For example, acidification and low calcium availability increases mobility and accumulation of several heavy metals (Dauwe et al. 2006; Eeva and Lehikoinen 2004; Scheuhammer 1996), increasing metal toxicity (Barton et al. 1978). Different habitats may differ in calcium availability (more acidic coniferous habitats potentially showing lower calcium availability than deciduous habitats, e.g., Wäreborn 1992). Thus, while monitoring heavy metals and their toxicity, their interactions with other elements should be investigated.

We studied large-scale geographic variation in eggshell metal levels in the pied flycatcher (*Ficedula hypoleuca*), by collecting egg samples from 15 populations across Europe and analyzing them for several elements (As, Cd, Cr, Cu, Ni, Pb, Zn, Se, Sr, Ca). Firstly, we investigated if metal levels in eggshell can be used as indicators of metal contamination in the environment at a large scale. Given that toxic metal emissions in densely populated Central Europe are generally higher than in sparsely populated areas of northern and southern Europe (e.g., Harmens et al. 2010), we predicted that metal levels, especially the levels of lead in eggshells, are higher in Central Europe and decrease towards north. Furthermore, we correlated actual soil metal levels with eggshell metal levels. Secondly, we studied variation in eggshell metal levels in different habitats and in relation to soil acidity, and predicted that purely coniferous habitats would show higher metal levels than mixed or deciduous forests. We also studied geographical and between-habitat variation in eggshell calcium levels. Thirdly, we studied the possible covariation between metal levels, eggshell quality, and breeding success. With the relatively large dataset and in-depth analysis of egg traits, we aim to study both the correlates and potential detrimental consequences of metal exposure.

## Methods

### Study species

The pied flycatcher is a migratory, insectivorous bird that breeds throughout a large range over Eurasia (Lundberg and

Alatalo 1992). Flycatchers are exposed to metals mainly via their insect food: metals from soils accumulate to plants and then to herbivorous insects (Dauwe et al. 2004a; Eeva et al. 2005). Toxic metals are known to affect the breeding success of the study species (e.g., Belskii et al. 2005; Eeva and Lehikoinen 1995, 1996). Furthermore, calcium deficiency is known to interact with metals in flycatchers (Eeva and Lehikoinen 2004).

## Field protocol

The egg collection protocol is outlined in detail in Ruuskanen et al. (2011). Briefly, egg samples were collected from 15 different nest-box study populations across the breeding range of the pied flycatcher during the spring of 2007. Given that previous studies on our study species and other species indicate that internal metal levels rise shortly after arriving to the breeding grounds (Berglund et al. 2011), and because all resources for egg formation (including for example calcium and proteins) need to be gathered during laying, and not from storages of internal tissues (e.g., Pahl et al. 1997), we are confident that levels measured in eggs reflect local exposure in the environment birds are breeding. In each population, nest-boxes were checked at 3-day intervals to monitor the progress of nesting, as flycatchers lay one egg per day. When any eggs were found, we marked them with a nontoxic marker (one to three eggs) and came back the next days and marked the new eggs until we could collect the unmarked third or fourth egg of a clutch. The position of the egg in the laying sequence, laying date, and fresh mass (~0.01 g) were recorded. The eggs were thereafter stored in clean plastic containers at −20 °C. The nests were monitored throughout the breeding season to record final clutch size and number of hatchlings and fledglings. Egg collection was conducted under licenses from environmental authorities in each country. From each population, ca. 20 eggs were acquired, and approximately 10 eggs per population were used for metal analyses (see below and Table 3 of the Appendix). The sampling area covers large parts of the breeding range of pied flycatchers in Europe (see locations of the sampling populations in Fig. 1). From a population in Harjavalta (Finland), half of the eggs were collected near a copper smelter (e.g., Eeva and Lehikoinen 1995) and thus these are included as a separate population in the spatial analyses, as some of their metal levels most likely differ from the natural level in the area. In the laboratory, frozen eggs were thawed, and yolk, albumen, and shell separated. Shells were washed with distilled water, air-dried, and stored in darkness in clean plastic containers until analyses of shell traits and eggshell metals (see below).

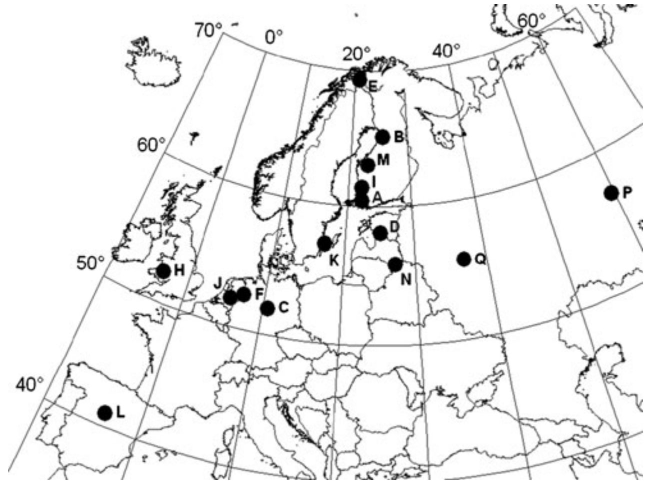


**Fig. 1** A map showing the 15 populations where *F. hypoleuca* eggs were collected for eggshell metal and eggshell quality analyses. *Letters* refer to populations listed in Appendix 1

## Metal and calcium analyses

The analyses were conducted at the Analytical Chemistry at Åbo Akademi, Turku, Finland. In total, 156 eggs were selected for analyses (see above), trying to maximize the number samples from which both metals and other eggshell traits were measured. We analyzed several metals and metalloids: arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), nickel (Ni), lead (Pb), selenium (Se), strontium (Sr), zinc (Zn), and also calcium (Ca) levels in the eggshells. Later, we use metals to refer to both metalloids and metals. Of these elements, As, Cd, Ni, and Pb are toxic whereas Cr, Cu, Zn, Se, and Sr are essential in small concentrations but toxic at higher levels. Eggshell samples were first weighed to 0.01 mg accuracy (shell mass 10–100 mg). We used acid and microwave digestion to process the samples: we added 3 or 5 ml (3 ml when sample mass was 10–30 mg, 5 ml when sample mass was 30–100 mg, respectively) of $HNO_3$ and 0.5 or 1 ml of $H_2O_2$ (Mercks Supra pure) and then used a microwave system (Anton Paar, Microwave Sample preparation System, Multiwave 3000). After digestion, the samples were diluted to 50 or 100 ml with deionized water. The determination of metal concentrations was done with ICP-MS (PerkinElmer-Sciex 6100 DRC Plus, quantitative determination). The calibration of the instrument was done with certified solution (Ultra scientific IMS -201, ICP-MS calibration standard 2). Ca was measured at 317.933 nm and Sr at 421.552 nm with inductively coupled plasma–optical emission spectrometry with PerkinElmer, Optima 5300 DV, by using standard plasma parameters. Certified reference material (DOLT-4 dogfish tissue; Sr and Ca as information values) was used for method validation. The mean recoveries (±SD) in four reference samples were as follows: As, 91.2±0.7 %; Cd, 99.0±0.4 %; Cr, 122.0±22.4 %; Cu, 103.8±0.6 %; Ni, 136.0±18.0 %; Pb, 145.9±34.8 %; Zn, 92.3±0.7 %; Se, 95.0±2.2 %. The mean recovery (±SD) of Ca for seven reference samples was 109.0±16.0 %. We are aware that recoveries for Pb and Ni are relatively high and variable, which is probably due to their low levels in the reference samples. However, this should not cause any systematic bias in analyzing spatial trends in metal levels. Detection limits varied between 5 and 160 ppt, depending on metal. Concentrations of Cd and Se were low and below the detection limit in most cases, and results are thus not further used in the statistical analyses. Mean (±SD) in milligram per kilogram for Cd was 0.005 (0.003) and for Se 0.340 (0.080). The levels of these metals are thus not further used in the statistical analyses. Additionally, two very extreme values (100-fold) of chromium (according to Cook's distance, following Fox 1997) from Estonian population were excluded (see also "Discussion," Table 3 of the Appendix).

## Eggshell trait analyses

A full description of the eggshell trait analyses from these eggs are detailed in Morales et al. (2012). Briefly, dry *shell mass* was measured in milligram. *Shell thickness* (~0.001 mm) was measured including shell membranes in three places of the eggshell with a Mitutoyo Digimatic Micrometer (Coolant Proof IP65) with a ball-point end. Shell thickness was repeatable within samples ($r=0.65$; $F_{357,\ 709}=6.56$, $P<0.001$, Lessells and Boag 1987). *Eggshell porosity* (no. of pores/square milliliter) was quantified by counting pores of three different shell pieces of the equatorial region of each eggshell under ×200 magnification with a Scanning Electron Microscope FEI INSPECT. Repeatability of pore density was low but significant ($r=0.19$; $F_{161,\ 324}=1.68$, $P<0.001$), showing that variation was higher among eggs than within eggs. *Biliverdin pigment* (nanomole per gram of dry weight of eggshells) was measured with high-performance liquid chromatography following the protocol described by Mateo et al. (2004), with few modifications (see also Moreno et al. 2006). Pore density and biliverdin concentration were collected from 10 eggshells from each population, but both pore density and metal data were available from 74 shells only and biliverdin concentration and metal data from 69 shells, respectively. *Eggshell color* intensity was measured in all collected eggs with a MINOLTA CM-2600d portable spectrophotometer (Minolta Co. Ltd., Osaka, Japan). From the reflectance spectra, we calculated blue-green chroma as the proportion of total reflectance that is in the blue-green region of the spectrum ($R_{400\text{-}570}/R_{360\text{-}700}$), following Siefferman et al. (2006). Blue-green chroma was highly repeatable within samples ($r=0.85$, $F_{351,\ 699}=17.28$, $P<0.001$). For eggshell thickness, porosity, and color, we used the average of the three measurements. Analyses of variation in eggshell quality (including geographical variation) have been reported in Morales et al. (2012). Thus here, we only report covariation among shell traits and metals.

## Population background data

In addition to data from the individual nests from which eggs were collected, background data from geographic coordinates of the populations and habitat type was collected (see Table 3 of the Appendix). For simplicity, habitats were classified as either purely coniferous forest ($N=7$ populations) or deciduous/mixed forest ($N=8$ populations), assuming that potentially more acidic pure coniferous habitats would show lower calcium availability than mixed or deciduous habitats. We also collected data on soil acidity (pH), as it may affect accumulation of metals and their toxicity (see Introduction). The data on soil acidity were acquired from maps at the European soil portal (http://eusoils.jrc.ec.europa.eu/library/data/ph/Resources/ph2.pdf) and Reuter et al. (2008). In these maps, soil pH data was classified in four classes (pH <4.5, 4.5–5.5, 5.5–6, 6–6.5) and we acquired a value for each population, which was further recoded as 1–4 for analyses. pH data for the Russian populations were acquired from Vorob'eva and Avdon'kin (2006) (Moscow region) and Vorobeichik and Pishchulin (2009) (Revda region, background area). Using pH either as a class or as a continuous variable produced qualitatively similar results, and for simplicity, we only present analyses where pH is included as a continuous variable. We also investigated if eggshells metal levels were correlated with soil metal levels. Soil metal data was acquired from the European Institute of Environment and Sustainability (Land Management and Natural Hazards Unit, FOREGS Geochemical database, http://eusoils.jrc.ec.europa.eu/foregshmc, on 20 January 2012). This dataset includes the laboratory measurements of extractable metal concentrations (HMC) in topsoil and floodplains determined for As, Cr, Cu, Ni, Pb, and Zn (in milligram per kilogram) by ICP-AES using the Aqua Regia method (see details in Lado et al. 2008). We used metal measurements from locations closest to the population (but if two measurements were available, the average was used). From the Moscow region, data using the same analysis methods were acquired from Koptsik et al. (2011), and from the Revda region, we used data from Vodyanitskii et al. (2011). The mean distance between nest-box populations and sampling sites of soil data was 36.8 km (SD 17.7 km, range 1.8–59.0 km).

### Statistical analyses

All statistical analyses were conducted with SAS 9.2. Pb, Cr, Cd, Zn, and Sr were log-transformed whereas Ca was squared for normality. First, we wanted to estimate among and within population variation in eggshell metal levels and to get simple statistics, with easily interpretable $R^2$ values. Thus, we used General Linear Models (proc GLM) in which the element concentration was the response variable and population the explanatory variable. As the Harjavalta population included eggs from both a polluted and a control area, these were analyzed as separate populations.

We then studied geographic variation in eggshell metal levels with linear mixed models (proc MIXED). The independent factors in the models were latitude, longitude, quadratic terms of latitude and longitude, and habitat (pure coniferous or mixed/deciduous). In a separate model, we tested the effect of soil pH and soil metal concentration (data available from As, Cu, Cr, Cd, Ni, Pb, and Zn) on eggshell metal levels. This was done because soil pH and latitude were negatively correlated. From soil metal measurements, only soil Pb was negatively correlated with latitude and longitude, and soil As negatively with longitude. The polluted area of the Harjavalta population was excluded from the spatial analyses, as it represents a known outlier, i.e., other areas were not selected close to any pollution source. Population was included as a random effect in all models. We also conducted a separate analysis to investigate if metal levels differ between the polluted and control area in the Harjavalta population ($N$=7 and 10, respectively). We further analyzed covariation among metals using mixed models with each metal as the response variable and another metal as the explanatory variable at a time, including population as a random factor.

In addition to models of individual metals, we also conducted a Principal Component Analysis as several metals were correlated, and their combined effects and interactions may be more important than individual loads. In the PCA, we included the toxic or potentially toxic metals that were strongly correlated (see Table 4 of the Appendix). This approach was used because including all elements, or separately, toxic vs. essential metals produced PC1s that explained only 20 % of the variation, which we consider too low. The selected metals were As, Cr, Ni, and Pb. The first PC explained 48 % (eigenvalue 1.9) and had positive loadings from all the selected metals. Later, we refer to “PC1 of contamination load.” We conducted the same analysis of geographical variation (see above) also using PC1.

Covariation among eggshell traits (shell mass, thickness, pore density, color, and biliverdin concentration) and eggshell metal levels were analyzed using mixed models with each shell trait as the response variable and each metal as the explanatory variable at a time. This method was selected as some metal levels were correlated with each other (see Table 4 of the Appendix). Associations between metal levels and either hatching and fledging success were analyzed using generalized linear mixed models with hatching or fledging success (hatched/eggs or fledged/hatched, when removed eggs were not taken into account, proc GLIMMIX, binomial distribution, and events/trials type syntax) as the response variable, and each model included one metal at the time as the explanatory factor. Population was included as a random effect, and laying date of the first egg (standardized within populations) as a covariate because timing of breeding may affect reproductive parameters. Data on hatching success was available from 129 nests and fledging success from 126 nests, respectively. In addition to models of individual metals, we also used the PC1 of contamination load to explore the combined effects of contamination load on shell traits and breeding success.

Finally, we used the Variogram procedure to check whether there was spatial autocorrelation in the residuals of the final models (i.e., those that retained at least one significant term). Moran's $I$ coefficients ranged from 0.01 to 0.16, indicating weak positive autocorrelation in the data. Thus, we conducted again the final mixed models with a geospatial analysis that allows controlling for geographic coordinates and testing whether spatial covariance structure gives a better model fit than the default structure (variance components). This analysis was implemented in the final mixed models by specifying in the random statement the geographic coordinates of data and an exponential covariance structure (Littell et al. 2006). However, using geospatial analysis did not significantly increase the model fit (ΔAICC <4, and fit was often worse), and results were qualitatively unchanged. Thus, we report statistics from models with the default covariance structure.

## Results

### Among population variation in eggshell metal levels

The averages and standard deviations of eggshell metals and calcium for each population are presented in Table 3 of the Appendix. Concentrations of several metals in the eggshells were inter-correlated (see Table 4 of the Appendix) and most correlations were positive. There was high and statistically significant among-population variation in all eggshell metal concentrations except for copper (Table 1, Fig. 2a–I; Table 3 of the Appendix). Population explained around 25–50 % of the variation in most measured metal

**Table 1** Among-population variation in eggshell elements (in milligram per kilogram, Ca as milligram per gram). Results are from GLMs with population as the explanatory variable. Arsenic (As), chromium (Cr), copper (Cu), nickel (Ni), lead (Pb), strontium (Sr), zinc (Zn), and calcium (Ca)

| Metal | $R^2$ | $F$ | $P$ value |
|---|---|---|---|
| As | 0.50 | 9.36 | <0.0001 |
| Cr (log) | 0.26 | 3.19 | 0.002 |
| Cu | 0.13 | 1.36 | 0.1771 |
| Ni | 0.71 | 22.89 | <0.0001 |
| Pb (log) | 0.42 | 6.89 | <0.0001 |
| Sr (log) | 0.81 | 37.70 | <0.0001 |
| Zn (log) | 0.44 | 7.46 | <0.0001 |
| Ca (squared) | 0.27 | 3.5 | <0.0001 |

concentrations in the eggshells. For nickel and strontium, population explained around 70–80 % of the variation. In particular, two Finnish populations (Ruissalo and Oulu) showed lower nickel levels in the eggshells than all the other populations (Fig. 2d; Tukey post hoc tests Ruissalo vs others: $P<0.001$, $t<-4.5$; Oulu vs others, $P<0.01$, $t<-4.63$, except Germany $P>0.05$). Also, arsenic levels were lower in Ruissalo and Oulu than most other populations (Fig. 2a; Ruissalo vs others $P<0.01$, $t<-4.2$; Oulu vs others, $P<0.02$, $t<-3.77$, except Germany and Estonia $P>0.05$). In strontium, about half of the populations had much lower values than the other half, but for unknown reason (Fig. 2f).

In a Finnish population (Harjavalta), we found that copper and zinc levels in eggshells were higher in the polluted area than in the control area, whereas eggshell strontium levels were lower (Cu: $F_{1,\ 15}=9.44$, $P=0.0077$; Zn: $F_{1,\ 15}=5.68$, $P=0.03$; Sr: $F_{1,\ 15}=5.69$, $P=0.03$; Fig. 2 and Table 3 of the Appendix). There were no differences in other metal levels in eggshells between the control and polluted areas ($P$ values >0.12).

### Geographical variation in eggshell metal levels

We found several geographical patterns in eggshell metal concentrations: eggshell lead and zinc level decreased towards the north (Fig. 2f, g; Table 2). There was a quadratic effect of latitude on chromium concentration (Fig. 2b, Table 2): chromium levels in the eggshell decreased from Central Europe to north and were also low in the most southern population (Spain). The PC1 of contamination load was also negatively correlated with latitude ($F_{1,\ 12}=7.22$, $P=0.02$, Fig. 2i). There was also a quadratic effect of longitude on nickel concentration: levels were higher in furthest western and eastern populations (Fig. 2d, Table 2).

Habitat affected eggshell copper and nickel concentrations: copper levels were ca 7 % lower in pure coniferous forests than in deciduous/mixed forests (marginal means mg/kg, dw± SE: coniferous, 2.36±0.06; mixed/deciduous forest, 2.54± 0.05; Table 2, Fig. 2c). Eggshells collected from pure coniferous forests had 29 % higher levels of nickel than eggshells of birds breeding in mixed/deciduous (marginal means mg/kg, dw±SE: coniferous, 42.80±1.12; mixed/deciduous, 33.30± 1.10, Table 2, Fig. 2d). Also, the PC1 of contamination load tended to be higher in coniferous forests than in deciduous forests ($F_{1,\ 12.1}=4.24$, $P=0.06$).

Eggshell lead concentration was positively correlated with soil lead levels and soil pH (Fig. 3a, b; Table 2). No other significant correlations between eggshell metal levels and soil metal levels or pH were found (Table 2). Arsenic and strontium concentration in eggshells were not associated with any of the explanatory variables (Fig. 2a, f, Table 2).

### Covariation between toxic metals and eggshell traits

Eggshell nickel concentration was negatively correlated with shell mass ($F_{1,\ 34.7}=9.83$, $P=0.0035$, Fig. 4a). Eggshell arsenic concentration was positively correlated with pore density ($F_{1,\ 59.2}=7.25$, $P=0.0092$, Fig. 4b). None of the metals were associated with eggshell color, thickness, or biliverdin concentration ($P$ values >0.05). PC1 of contamination load was negatively correlated with eggshell mass (Fig. 4d, $F_{1,\ 52.3}=6.2$, $P=0.016$), but not with other egg shell traits. The correlations between Ni and PC1 and shell mass remained significant when corrected for egg mass.

### Covariation between toxic metals and breeding success

Hatching and fledging success were negatively correlated with shell zinc concentration (hatching success: $\beta$±SE, −1.42±0.67; $F_{1,\ 127}=4.23$, $P=0.041$; fledging success: $\beta$± SE, −2.26±0.82; $F_{1,\ 114}=7.54$, $P=0.007$). There were no other significant correlations among eggshell metal levels, PC1 of contamination load, and breeding success (all $P>0.15$).

### Variation in eggshell calcium levels

There was significant variation in eggshell calcium levels among populations (Table 1, Table 3 of the Appendix). Calcium tended to increase towards east but was not affected by habitat type or by soil pH (Fig. 2h, Table 2). In a Finnish population (Harjavalta) where we had egg samples from both a polluted and a control area, we found no differences in calcium levels from eggshells collected from the two areas ($P$ values >0.12). Calcium decreased with increasing shell mass ($F_{1,\ 126}=42.1$, $P<0.001$, Fig. 4c), but was not associated with other eggshell traits ($P$ values >0.4). Shell

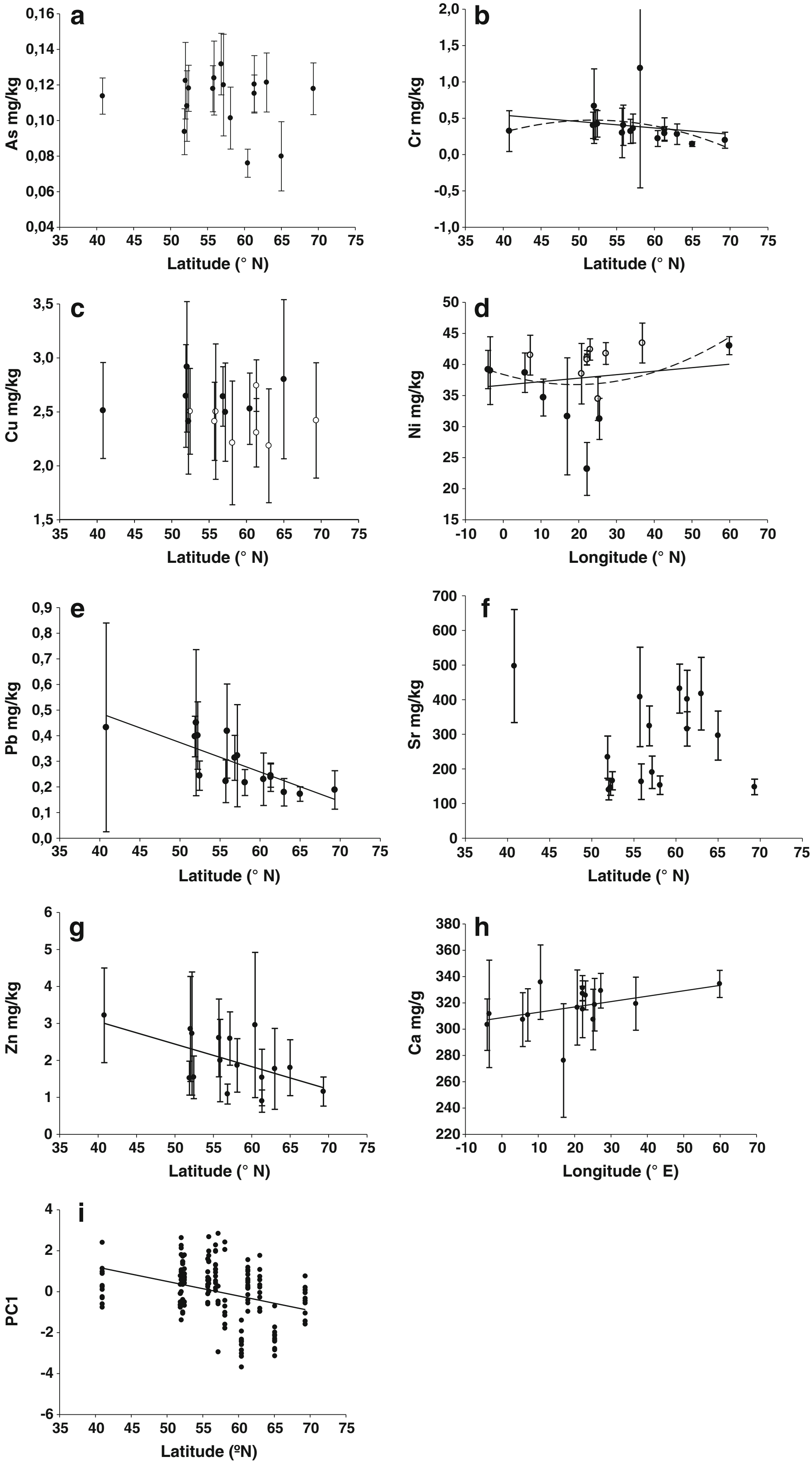


**Fig. 2** Among population variation and associations between eggshell metals and latitude and longitude. **a** Arsenic (As); **b** chromium (Cr); **c** copper (Cu); **d**; nickel (Ni); **e** lead (Pb); **f** strontium (Sr); **g** zinc (Zn); **h** calcium (Ca); and **i** PC1 of metals (As, Cr, Ni, Pb). If no significant longitudinal patterns were found, we plotted values in relation to latitude. Regression lines are shown for metals with statistically significant associations with latitude or longitude. Mean±SD from untransformed data is shown for each population. In **c** and **d**, *black symbols* represent mixed/deciduous and *white symbols* pure coniferous habitats (in other figures, habitats are not specified). From Harjavalta (Finland), both the control and the polluted population are shown for illustrative purposes, but only the control site was included in the statistical analyses

**Table 2** Geographical variation in eggshell metal and calcium levels. Results are from two linear mixed models with population as a random effect. Model 1: latitude, longitude, quadratic terms of latitude and longitude, habitat (coniferous = CON or mixed/deciduous). Model 2: pH and metal concentration in soil (when available). Non-significant terms were dropped from the model. Model estimates (SE) are shown

| | Estimate (SE) | Den DF | *F* | *P* value |
|---|---|---|---|---|
| As | | – | – | – |
| Cr (log) | | | | |
| Latitude | 0.115 (0.052) | 11.0 | 4.89 | 0.049 |
| Latitude$^2$ | −0.001(0.0005) | 11.1 | 6.04 | 0.032 |
| Cu | | | | |
| Habitat | CON: −0.182 (0.079) | 12.5 | 5.27 | 0.039 |
| Ni | | | | |
| Longitude | −0.603 (0.157) | 11.1 | 14.7 | 0.003 |
| Longitude$^2$ | 0.012 (0.003) | 10.9 | 18.17 | 0.0014 |
| Habitat | CON: 9.79 (2.08) | 11.2 | 22.02 | 0.0006 |
| Pb (log) | | | | |
| Latitude | −0.015 (0.004) | 12.7 | 16.87 | 0.0013 |
| Soil Pb | 0.005 (0.002) | 12.5 | 9.06 | 0.01 |
| pH | 0.061 (0.027) | 11.8 | 5.23 | 0.04 |
| Sr (log) | | – | – | – |
| Zn (log) | | | | |
| Latitude | −0.013 (0.006) | 12.7 | 5.61 | 0.034 |
| Ca (squared) | | | | |
| Longitude | 248.18 (117.92) | 8.91 | 4.18 | 0.072 |

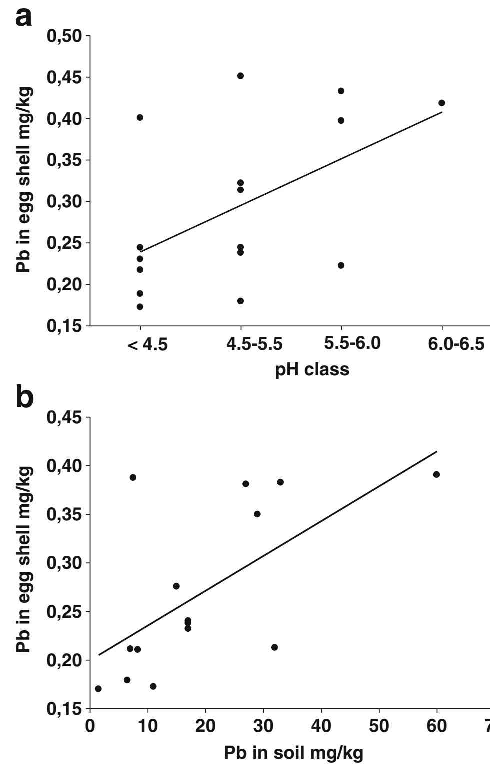


**Fig. 3** Association between eggshell lead (Pb) concentration (untransformed data) and **a** soil pH (class); **b** soil lead. Mean concentration for each population is shown (as there is only one value for pH or soil lead per population)

calcium concentration did not affect hatching and fledging success ($P>0.65$). Calcium was positively correlated with arsenic, nickel, and strontium levels, and negatively with copper levels (Table 4 of the Appendix).

## Discussion

### Geographical variation in eggshell metal levels

Metal levels in our flycatcher eggs were similar to those found in great tits (*Parus major*) in western Europe (Dauwe et al. 1999, 2005), except for nickel which were five times higher in our data (40 vs 9 ppm) and lead which was 80 % lower in our data (0.4 vs 2 ppm). However, other studies on passerines (Mora 2003; Swaileh and Sansur 2006) recorded somewhat different concentrations (lead concentration: in our data 0.4 vs 3.3 ppm in *Passer domesticus*, and zinc concentration: 2 vs 20 ppm, respectively), and generally, comparable data is scarce.

As predicted, lead concentration in the eggshell decreased from densely human populated Central Europe northwards. This suggests that lead levels in eggshells indeed indicate local lead exposure, and thus eggshells may be considered as an indicator for lead pollution, both in small and large scales. Interestingly, also zinc and to a lesser extent chromium as well as the PC1 of contamination load showed similar latitudinal trends. Indeed, atmospheric and soil levels of zinc and chromium in Central and Western Europe are clearly higher than in Northern Europe and in the Baltic region (Harmens et al. 2010; Lado et al. 2008). Thus, eggshell metal levels can also be used as an indicator of excess exposure to essential metals. Furthermore, the findings of significant differences in eggshell metal levels (especially copper and zinc) between a polluted and a control area in Harjavalta (similarly as in other reference and polluted sites, Dauwe et al. 1999) support the usefulness of eggshell metals in biomonitoring. An interesting detail in our data was that few samples from Estonia from adjacent nests showed up to 100-fold chromium levels compared to others in the same population. We do not know the origin of this pollution source, but one

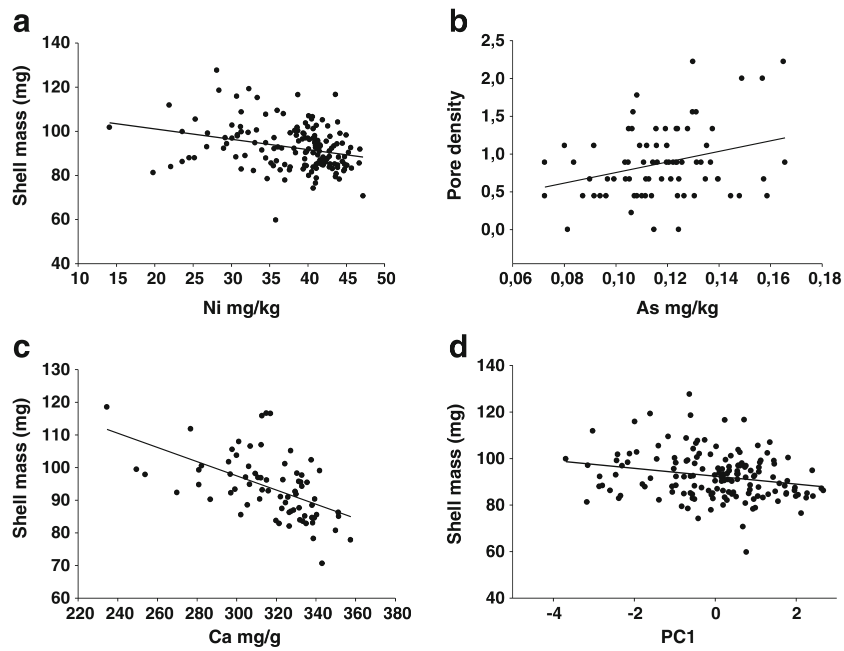


**Fig. 4** Correlations between **a** eggshell mass and nickel (Ni); **b** eggshell pore density and arsenic (As); **c** eggshell mass and the PC1 of metals; and **d** eggshell mass and calcium (Ca). Untransformed data is presented

possibility is intensive tree logging potentially using some chromium-containing timber protecting agent.

We found some habitat differences associated with eggshell metal levels: The higher levels of nickel and the PC1 of contamination load in eggshells collected from coniferous forests is supportive of our hypothesis of higher accumulation and mobility of metals in more acidic and calcium-poor habitat. However, for nickel, the result must be interpreted with caution, as it may be driven by low levels in two deciduous habitats (see Fig. 2d). As similarly classified habitats may differ at different latitudes (see e.g., Mägi et al. 2009), for example, due to soil quality and tree species composition, more detailed analyses of habitats within a region are needed to confirm habitat differences in accumulation of metals in eggshells.

Eggshell lead level was positively correlated with soil lead level, which indicates that although our soil data were not collected at the exact locations of the egg collection, eggshell data can reveal large-scale patterns in lead pollution to which the birds are exposed during egg-laying. However, for other metals, the lack of correlation may be due to small-scale variation in soil metal levels (originating from point-like sources), and such variation cannot be captured with our sampling procedure. Comparable data on soil metal and egg or shell metal concentrations is rare. However, for example, Hui (2002) did not find correlations among air pollution levels and egg metal levels in rock doves (*Columba livia*) living in urban areas, which suggests that food is a more important exposure route for metals than inhalation.

The positive correlation between lead levels in eggshells and soil pH was contrary to expectations, as in previous studies acidity and low calcium availability have rather increased metal accumulation (Dauwe et al. 2006). However, this association may just reflect the fact that forest soils in the north are relatively acidic and less polluted by lead, hence both variables being correlated with latitude. Still, eggshell lead levels and soil pH were positively correlated in models including or excluding latitude and thus this result therefore needs further investigation. One explanation may be that in calcareous environments birds need to use more grit as it is more rapidly dissolved in the acidic medium of gizzard (Mateo and Guitart 2000), and larger amount of ingested grit may lead to higher metal exposure (Bendell 2011).

## Toxic metals, eggshell quality, and breeding success

We found that eggshell metal levels measured at our scale were not strongly correlated with eggshell traits, with the exception of shell mass. Shell mass decreased with increasing nickel concentration and general contamination load (PC1), suggesting that females may have reduced shell material when exposed to higher contamination load at laying. Similar results have been previously found around Harjavalta smelter in Finland, where the amount of shell material decreased with decreasing distance to pollution source: Thinner/smaller shells in polluted sites may be due to lower calcium availability or metals interfering with calcium metabolism (Eeva and Lehikoinen 1995). Egg porosity increased with increasing arsenic levels, although levels were generally very low and a causal relationship cannot be confirmed here. For comparison, rook (*Corvus frugilegus*) eggshells in Poland were reported to have almost 300 times the levels of arsenic than found in our study (Orlowski et al. 2010). However, it is difficult to compare absolute levels among species as ecology (e.g., diet, breeding habitat type, foraging range) most likely determines much of the pollution levels species are exposed to. Also, egg coloration may be affected by pollution, potentially via altered calcium uptake (Jagannath et al. 2008), or metals altering heme synthesis (porphyrins), and therefore biliverdin production (e.g., Hanley and Doucet 2012; Mateo et al. 2006). We did not find any associations between shell metal levels and color. In a recent study in herring gulls *Larus argentatus*, eggshell UV chroma was found to be more strongly associated with egg contamination load in egg contents than blue-green chroma (Hanley and Doucet 2012). Thus, we cannot discard that shell metal levels correlate with UV chroma in our data. On the other hand, it is possible that shell color better reflects contamination load in egg contents (as previously found by Hanley and Doucet 2012; Jagannath et al. 2008) rather than metal loads in the eggshell.

We found that hatching and fledging success decreased with increasing zinc levels in the eggshell, supporting previous results in red-winged blackbirds exposed to much higher pollution load (Sparling et al. 2004). The fact that pollution levels in our study were relatively low (see e.g., Dauwe et al. 1999) may indicate that embryos are particularly susceptible to small amounts of zinc in the environment. In general, there are few estimates of which levels for each metal in eggshell or egg content could affect embryo development. Furthermore, as there might be differences in accumulation of a metal in the yolk and the shell, it is difficult to directly extrapolate our eggshell metal levels to comparable levels in the yolk.

### Variation in eggshell calcium level

Eggshell calcium levels found in our study are similar (ca. 300 mg/g) to those reported in Mora et al. (2011) for several bird species. Calcium levels somewhat increased from west to east. The fact that shell calcium concentration was not affected by habitat or soil pH (as expected) may be due to calcium deficiency leading to thinner/smaller shells rather than lower calcium concentration. But we also note that our rough estimation method (soil data not collected in the exact location of the study population) may interfere with in the interpretation. The negative correlation between calcium concentration and shell mass was also unexpected. Given that calcium is limited during laying, it may be that females laying larger eggs (i.e., large shell mass) may have still the same amount of calcium to distribute over the shell as birds laying small eggs, leading to lower calcium *concentration* in large eggs (see also Tilgar et al. 1999). If toxic metals are interfering with calcium metabolism, we would have further expected that low calcium levels would be associated with high metal concentrations. However, contrary to our expectations, levels of calcium were positively correlated with several elements (As, Ni, and Sr) and one of the potent calcium disruptors, lead, showed no association with shell calcium levels.

## Conclusions

Our data shows that for several toxic metals, levels decreased from Central Europe to the north, in line with the gradient of pollution levels over Europe, thus suggesting that eggshell can be used as an indicator of local exposure to both non-essential (especially lead) and essential metals. Also, contamination load tended to be higher in coniferous forests, which supports that there is a higher accumulation and mobility of metals in more acidic habitats. Nickel and contamination load were negatively related to egg shell mass, but other metals were not strongly correlated with other egg shell traits. With the exception of zinc, eggshell metal levels measured at our scale were not strongly correlated with breeding success, which may suggest that birds can cope well with the current background exposure levels across Europe.

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## Appendix

**Table 3** Among population variation in *F. hypoleuca* eggshell metal and calcium levels. Metals are expressed as milligram per kilogram (dw) and Ca as milligram per gram

| Country | Area | Lat | Long | Habitat | No. | As | | Cr | | Cu | | Ni | | Pb | | Sr | | Zn | | Ca | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| E. Norway | Skibotn | 69 | 21 | Coniferous | 10 | 0.118 | 0.015 | 0.20 | 0.11 | 2.42 | 0.53 | 38.5 | 4.87 | 0.19 | 0.08 | 148.0 | 22.3 | 1.16 | 0.39 | 316.5 | 28.6 |
| B. Finland | Oulu | 65 | 25 | Mixed/deciduous | 11 | 0.008 | 0.019 | 0.14 | 0.03 | 2.72 | 0.74 | 31.3 | 3.31 | 0.17 | 0.03 | 296.3 | 70.9 | 1.80 | 0.76 | 318.6 | 20.0 |
| M. Finland | Kauhava | 63 | 23 | Coniferous | 11 | 0.121 | 0.017 | 0.28 | 0.14 | 2.19 | 0.53 | 42.4 | 1.72 | 0.18 | 0.05 | 417.2 | 105.0 | 1.77 | 1.09 | 325.7 | 11.0 |
| I. Finland | Harjavalta CO | 61 | 22 | Coniferous | 10 | 0.120 | 0.016 | 0.35 | 0.16 | 2.31 | 0.32 | 41.1 | 1.15 | 0.24 | 0.06 | 401.7 | 83.3 | 0.90 | 0.30 | 331.3 | 9.5 |
| I. Finland | Harjavalta PO | 61 | 22 | Coniferous | 7 | 0.115 | 0.010 | 0.29 | 0.09 | 2.74 | 0.24 | 40.8 | 0.90 | 0.24 | 0.05 | 315.6 | 49.6 | 1.54 | 0.76 | 327.1 | 5.6 |
| A. Finland | Turku | 60 | 22 | Mixed/deciduous | 10 | 0.076 | 0.008 | 0.22 | 0.11 | 2.44 | 0.33 | 23.2 | 4.26 | 0.23 | 0.10 | 432.0 | 70.7 | 2.96 | 1.96 | 315.2 | 21.6 |
| D. Estonia | Pärnu | 58 | 25 | Coniferous | 10 | 0.101 | 0.017 | 1.19[a] | 1.65 | 2.21 | 0.57 | 34.5 | 3.48 | 0.22 | 0.05 | 152.9 | 26.8 | 1.86 | 0.72 | 307.3 | 23.0 |
| K. Sweden | Öland | 57 | 17 | Mixed/deciduous | 6 | 0.121 | 0.029 | 0.36 | 0.20 | 2.41 | 0.46 | 31.6 | 9.42 | 0.32 | 0.20 | 190.1 | 46.9 | 2.59 | 0.72 | 276.2 | 43.2 |
| P. Russia | Revda | 57 | 60 | Mixed/deciduous | 13 | 0.132 | 0.017 | 0.32 | 0.17 | 2.56 | 0.28 | 43.0 | 1.45 | 0.31 | 0.09 | 324.2 | 57.6 | 1.09 | 0.27 | 334.4 | 10.3 |
| N. Latvia | Kraslava | 56 | 27 | Coniferous | 10 | 0.124 | 0.021 | 0.40 | 0.28 | 2.50 | 0.63 | 41.8 | 1.76 | 0.42 | 0.18 | 163.1 | 51.4 | 2.00 | 1.11 | 329.3 | 13.2 |
| Q. Russia | Moscow | 56 | 37 | Coniferous | 10 | 0.118 | 0.013 | 0.30 | 0.34 | 2.41 | 0.36 | 43.5 | 3.21 | 0.22 | 0.08 | 407.9 | 143.7 | 2.61 | 1.05 | 319.4 | 20.2 |
| F. Germany | Lingen | 52 | 7 | Coniferous | 10 | 0.118 | 0.013 | 0.42 | 0.18 | 2.51 | 0.40 | 41.5 | 3.21 | 0.24 | 0.06 | 165.7 | 26.2 | 1.54 | 0.58 | 310.8 | 19.9 |
| H. UK | Powys | 52 | −3 | Mixed/deciduous | 9 | 0.108 | 0.020 | 0.41 | 0.21 | 2.33 | 0.49 | 39.0 | 5.45 | 0.40 | 0.13 | 145.9 | 22.2 | 2.73 | 1.67 | 311.7 | 40.9 |
| J.Netherlands | Buunderkamp | 52 | 6 | Mixed/deciduous | 10 | 0.122 | 0.022 | 0.67 | 0.51 | 2.83 | 0.60 | 38.7 | 3.18 | 0.45 | 0.29 | 139.5 | 28.9 | 2.85 | 1.42 | 307.3 | 20.5 |
| C. Germany | Harz | 52 | 11 | Mixed/deciduous | 8 | 0.094 | 0.013 | 0.40 | 0.18 | 2.56 | 0.48 | 34.7 | 2.97 | 0.40 | 0.08 | 233.9 | 61.0 | 1.52 | 0.46 | 335.8 | 28.3 |
| L. Spain | Lozoya | 41 | −4 | Mixed/deciduous | 11 | 0.114 | 0.010 | 0.32 | 0.28 | 2.43 | 0.44 | 39.2 | 3.08 | 0.43 | 0.41 | 497.2 | 163.3 | 3.22 | 1.28 | 303.4 | 19.6 |

From Harjavalta population, eggs were collected from near smelter (PO = polluted) and control area (CO), which are analyzed separately

*Lat* latitude (°N), *Long* longitude (°E), *No*. sample size per population, *As* arsenic, *Cd* cadmium, *Cr* chromium, *Cu* copper, *Ni* nickel, *Pb* lead, *Se* selenium, *Sr* strontium, *Zn* zinc, *Ca* calcium

[a] If two extreme values are included in Estonian population, Cr (mean ±SD): 4.4±6.8

**Table 4** Correlations among eggshell metals. Results are from mixed models where each metal at the time was the response and explanatory factor and population as a random factor. $\beta$(± SE) and associated $F$ values are shown

| | Cr (log) | Cu | Ni | Pb (log) | Sr (log) | Zn (log) | Ca (squared) |
|---|---|---|---|---|---|---|---|
| As | ns | Ns | 0.002±0.0002 $F$=50.35*** | ns | ns | ns | 3.74E−07 $F$=14.05** |
| Cr (log) | | Ns | ns | 0.72±0.12 $F$=36.14*** | ns | 0.27±0.11 $F$=5.67* | ns |
| Cu | | | ns | ns | ns | ns | −0.00000693±0.0000003 $F$=6.18* |
| Ni | | | | | 6.73±2.52 $F$=7.14* | ns | 0.000197±0.00001 $F$=224.02*** |
| Pb (log) | | | | | Ns | 0.19±0.08 $F$=7.85* | ns |
| Sr (log) | | | | | | ns | 2.02E−06 $F$=9.36* |
| Zn (log) | | | | | | | ns |

*ns* nonsignificant

***$P$<0.0001; **$P$<0.001; *$P$<0.01

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