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RESEARCH ARTICLE

Effects of calcium supplementation on growth and biochemistry in two passerine species breeding in a Ca-poor and metal-polluted area

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Abstract Several studies provide evidence of calcium (Ca)-limited reproduction in birds. A Ca-supplementation experiment was carried out in 2014 in a Ca-poor area associated with metal pollution in SW Finland. We aimed to evaluate the relationship between Ca availability and heavy metal exposure in free-living passerines, and to compare Ca levels in plasma and feces and the effects of Ca supplementation and metals on breeding, nestling growth, and plasma biochemistry in great tits and pied flycatchers. Although the Ca supplement was used by parents, in general the treatment had limited effects on growth and biochemistry, suggesting that parents are capable of finding sufficient Ca-rich foods to allow nestlings to grow properly. Snail shells were an abundant Ca source in the moderately polluted zone for pied flycatcher, and great tits likely combines the intake of snail shells and other anthropogenic Ca-rich items. Great tits had higher Ca concentrations in feces and plasma than pied flycatcher nestlings, suggesting that they need and sustain higher Ca levels and seem to be more opportunistic in search for Ca than pied flycatcher, since they consumed more of the supplemented Ca. Negative effects of pollution in nestling size and fledgling number were found in great tit. This species may suffer especially from the lower food quality and quantity in the polluted area. The pied flycatcher seems to be adapted to low Ca availability and they can successfully breed when metal concentrations are not too high. Our results show that great tits and pied flycatchers may employ different strategies in response to low Ca availability.

Keywords Calcium availability · Metal pollution · Breeding success · Food availability · Snail abundance · *Ficedula hypoleuca* · *Parus major*

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Introduction

Calcium (Ca) is an important macronutrient for birds, being essential, e.g., for successful breeding. It is required for shell formation during egg laying since up to 98 % of the dry mass of eggshell in birds is calcium carbonate (Reynolds et al. 2004). In addition, many bird species require Ca during post-natal development of nestlings, when skeletal growth continues (Starck 1998). Small passerine birds cannot store enough Ca in their body for successful reproduction, but they need to consume Ca-rich food in addition to their regular diet, since the latter contains insufficient Ca (Graveland and van Gijzen 1994). Snail shells are one of the main sources of dietary Ca for many passerine bird species (Graveland 1996; Graveland and Drent 1997; Tilgar et al. 1999; Mänd et al. 2000; Reynolds and Perrins 2010).

Considering the essential role of Ca in breeding and nestling development, birds may be susceptible to environmental changes that reduce the availability of Ca-rich material. Several studies have provided evidence of Ca-limited reproduction in birds, observing thin-shelled eggs, reduced clutch and egg size, and reduced number of fledglings (reviewed in Reynolds et al. 2004; Reynolds and Perrins 2010). This issue

is of special concern when birds inhabit acidified or polluted environments. Soil acidification may affect the amount of exchangeable Ca, and consequently the snail abundance (Graveland and van der Wal 1996). In addition, metal-polluted environments may affect diversity, abundance and quality of land snails, and birds may not have enough Ca-rich food items for successful breeding (Eeva et al. 2010). Furthermore, Ca deficiency in the diet is known to increase the absorption and accumulation of heavy metals, such as lead (Pb) and cadmium (Cd), in birds (Scheuhammer 1996; Dauwe et al. 2006b). The enhanced Pb absorption in low-dietary Ca species is related to the synthesis of intestinal Ca-binding proteins, when Ca is replaced by Pb (Fullmer et al. 1985). On the other hand, Pb and Cd may also alter the homeostasis and function of Ca (e.g., by interfering with Ca transport or storage processes, such as Ca^{2+} transport proteins; or by substitution of Ca^{2+} with Pb^{2+}/Cd^{2+} at functionally important Ca-binding sites, such as calmodulin; Pounds 1984; Suzuki et al. 1985).

Along with this, some species may be more effective in finding Ca-rich foods and/or in Ca absorption or may have different Ca requirement for successful breeding. Thus, in metal-polluted areas, species may differ in their susceptibility to accumulate and their ability to endure the adverse effects of metals. For example, studies of great tits (*Parus major*) and pied flycatchers (*Ficedula hypoleuca*) in the vicinity of a copper (Cu) smelter showed that great tit nestlings had four times higher Ca contents in their feces than pied flycatcher nestlings, suggesting differential need and/or ability to acquire Ca (Eeva and Lehikoinen 2004). It is likely that pied flycatchers need less Ca for successful breeding than great tits, since the latter have larger clutch-sizes, egg shell masses, and skeletal masses. However, species with naturally low dietary Ca content may also be more sensitive to adverse effects of metals (Eeva and Lehikoinen 2004). In that sense, some previous studies have found depressed eggshell thickness, egg size, clutch size and hatchability, and delayed ossification in pied flycatcher nestlings near the smelter site (Eeva and Lehikoinen 1995; Eeva and Lehikoinen 1996; Belskii and Grebennikov 2014). In contrast, the great tit laid relatively normal eggs and clutches even in the most polluted areas. These results show that heavy metals have detrimental effects on pied flycatcher nestlings especially when Ca availability is poor (Eeva and Lehikoinen 2004). Therefore, it is important to take account of the Ca availability and Ca requirement in different passerine species that may respond to Ca shortage in different ways, especially when breeding in metal-polluted areas, since these may affect the bioavailability of metals and alter the breeding success of natural bird populations.

The main objectives of the present study are the following: (i) to evaluate the relationship between Ca availability and heavy metal exposure by manipulating Ca availability in free-living passerines variably exposed to metals, and (ii) to compare Ca levels in plasma and feces and the effects of Ca supplementation and heavy metal exposure on breeding, nestling growth, and biochemistry in great tits and pied flycatchers. For this purpose, we performed a Ca-supplementation experiment in 2014 with great tits and pied flycatchers in Harjavalta, Finland, a Ca-poor area associated with metal pollution (Eeva et al. 2010). Although a number of Ca supplementation studies have been performed, equivocal results have been found (Reynolds et al. 2004). Some experiments show positive effects on clutch size, eggshell thickness, brood size, chick growth, and fledgling number in passerines, while other studies failed to find effects on these parameters (reviewed in Reynolds et al. 2004). In addition, some studies highlight the importance of evaluating Ca availability in conjunction with environmental pollution (Poulin and Brigham 2001; Reynolds 2001). However, to the best of our knowledge, only one experimental study has been carried out relating Ca deficiency and metal pollution in wild passerine populations (Eeva 1996). Therefore, the Ca supplementation of birds during the breeding season in the present study may help to detect Ca-limited reproduction in relation to metal exposure in two different passerine species. We hypothesize that pied flycatchers would have a good and better breeding success than great tits in a Ca-deficient area, since the former may have lower Ca requirement for successful breeding. However, great tits should deal better with a situation that combines Ca deficiency and heavy metal exposure, because of higher sensitivity of flycatchers to metal exposure. So far, this hypothesis has not been experimentally tested.

Material and methods

Experimental set-up

Our Ca-supplementation experiment was conducted during the breeding season 2014 in the surroundings of a Cu-Ni smelter in Harjavalta (61°20′ N, 22°10′ E), southwestern Finland. Heavy metals (mainly Cu, Ni, Pb, Zn, and As) are common contaminants in the polluted area due to current and long-term deposition, and metal levels decrease with increasing distance to the smelter, approaching background levels at sites farther than 5 km from the smelter (Eeva and Lehikoinen 1996; Koivula et al. 2011). In the vicinity of the smelter, the amount of exchangeable Ca in the soil has decreased due to leaching, but soil pH is similar and relatively low in the polluted and unpolluted areas (Jussila 1997; Derome and Nieminen 1998). The study was carried out on populations of great tit and pied flycatcher using nest boxes situated along the pollution gradient in the three main directions (SW, SE, and NW) away from the smelter complex up to the distance of 11 km. A more detailed description of the study area is given by Eeva and Lehikoinen (1995). Old nest material was removed before the start of nest building. Nest boxes were checked in mid-April, and then

frequently enough to monitor the progress in the nest building and to record the laying date, clutch size, hatching date, brood size, and number of fledglings. When new nests in an advanced building stage were found, it was first identified for its species and assigned by turns either to the Ca-supplemented group or to the control group. In that moment, feeders (small cylindrical plastic cups) with 5 g of crushed mussel shells (Versele Laga) were placed inside the experimental nest boxes. Previous studies confirmed that tits and pied flycatcher consume snail shell and egg shell fragments supplied in the feeders (Graveland et al. 1994; Graveland 1996; Graveland and Drent 1997; Tilgar et al. 1999; Tilgar et al. 2002; Mänd and Tilgar 2003). The feeders were checked during the visits to the nests and refilled when needed, so there was always a source of Ca material (*ad libitum* supplementation). Empty feeders were also placed in control nest boxes because of the possible influence of the feeder per se on breeding parameters. The 5 g of crushed shells were replaced and the mussel shells remains were weighed at the beginning of the incubation period and at day 0 during nestling period (just after hatching). At the age of 12 days in pied flycatchers (hereafter d12) and 14 days in great tits (d14) during nestling growth period, the feeder was removed and the leftover Ca material was weighed. Ca consumption during the laying, incubation, and chick-rearing periods was calculated with these measurements. In total, 10 different sites with nest boxes were used in this study (5 in the polluted and 5 in the unpolluted area). We set 29 Ca-supplemented nests (14 in polluted and 15 in unpolluted area) and 38 control nests (17 in polluted and 21 in unpolluted area) of great tit. Regarding pied flycatchers, we had 35 Ca-supplemented nests (17 in polluted and 18 in unpolluted area) and 30 control nests (15 in each area).

The experiment was conducted under licenses from the Animal Experiment Committee of the State Provincial Office of Southern Finland (license number ESAVI/1650/04.10.03/2012) and the Centre for Economic Development, Transport and the Environment, ELY Centre Southwest Finland (license number VARELY/319/07.01/2014).

Sampling and measurements

One egg from each clutch was collected (usually the third egg) the day that it was laid in order to evaluate the effect of the Ca supplementation on egg parameters and yolk vitamins that will be the scope of a different publication. On d7 after hatching, birds were ringed with individually numbered metal rings and combined feces of several nestlings from the same brood were collected in tubes and conserved at −20 °C for metal analyses. Feces were collected by inducing birds to defecate using a round-ended hairpin. Nestlings were weighed on d7 for both species and on d12 (pied flycatcher) or d14 (great tit) post-hatching with a precision of 0.1 g using a Pesola spring balance. Different dates for the second measurements were decided based upon pied flycatchers growing and fledging faster than great tits (own unpublished data on wing growth; Cramp and Perrins 1993). Wing length was measured to the nearest 0.5 mm using a ruler, and minimum tarsus length (according to the "alternative method" in Svensson, 1992) and total head (bill + head) length were measured with a digital caliper to the nearest 0.01 mm. Blood samples (approximately 75 μl) were collected on d7 (pied flycatcher) or d9 (great tit) and d12/14 by venipuncture of the brachial vein with a needle and using sodium-heparinized microhematocrit capillary tubes (80 iu/ml, Marienfeld). Blood samples from d7/9 were collected for vitamin analyses that will be the scope of another publication. Tubes were centrifuged in the field (4400 g, 5 min) and hematocrit was measured (% of red blood cells from total sample volume). Plasma and red cells were split in different tubes and kept in liquid nitrogen and then conserved at −80 °C in the laboratory. It was not always possible to collect blood from all the nestlings in a brood.

The mass of spilled natural snail shells found in the nest material of pied flycatcher after fledging ($n = 63$ nests; nest material was not collected in two nest boxes where nestlings died before fledging) was used as an index of natural Ca availability in a territory as described by Eeva et al. (2010). Since great tits clean dropped items from the nest, this index cannot be calculated for this species. Briefly, nests were collected after fledging and they were frozen, air-dried, and sieved to remove most of the nest material. Snail shells removed from the extracted nest substrate were weighed to provide the mass of shell material per nest.

At the end of the experiment (end of July), 40 soil samples were collected from the organic layer in the polluted (0 − 4 km from the smelter) and unpolluted areas (4.1 − 11 km from the smelter) for pH measurement (20 samples/area). Samples were collected from the 10 separate sites (4 samples were collected next to 4 nest boxes in each site) located at different distance zones around the smelter. Before sampling, the forest litter was removed and soil samples were collected from ground surface down to 5 cm. Samples were collected with a soil-sampling auger and conserved in plastic bags frozen at −20 °C until pH was measured in 1:5 (volume fraction) suspension of air-dried soil in water, following the ISO 10390:2005 standard.

Metal and biochemical analyses

Feces from the 7-day-old nestlings were dried over 72 h at 45 °C. Fecal samples from nestlings of the same brood were combined to assess metal exposure at brood level. Ca and metal concentrations (As, Pb, Cd, Cu, Ni, Zn, and selenium, Se) were determined with an inductively coupled plasma optical emission spectrometer (ICP-OES) in which the quantification limit is 1 ppm for Ca and 0.01 ppm for the other metals. Fecal samples (0.1 − 0.2 g, dry weight) were placed in

digestion tubes to which a mixture of 4 ml HNO_3 (70 %) and 1 ml H_2O_2 (33 %) was added. The sample was then submitted to a progressive thermal treatment and, after the microwave procedure, the sample was diluted in ultrapure water before analysis. Precision and accuracy of the method were tested using certified reference material (CRM) (TORT-2, lobster hepatopancreas reference material for trace metals, National Research Council Canada). Recovery of metals from three replicates of CRM ranged 77–90 %. The coefficient of variation (CV) for the repeatability was lower than 10 %.

Total mercury (Hg) was analyzed in a Milestone DMA-80 direct Hg analyzer by atomic absorption spectrophotometry with a detection limit of 0.005 ng. Fecal samples (0.01 − 0.1 g, dry weight) were loaded in a nickel boat and analyzed. Recovery of total Hg from three replicates of CRM (DORM-3, fish protein certified reference material for trace metals, National Research Council Canada) was 90 %, and the CV for the repeatability was 6 %. Metal concentrations were referred to dry weight. The mean (±SD) percentage for water content in feces was 75.7 ± 7.6 % in great tits and 79.5 ± 3.7 % in pied flycatchers.

Plasma from 213 individuals (121 great tits and 92 pied flycatchers) collected on d12/14 was used to measure the enzyme activities of alkaline phosphatase (ALP; Enzyme Commission (EC) number 3.1.3.1) and creatine kinase (CK; EC 2.7.3.2), and the plasma constituents uric acid and Ca. The evaluation of biochemical parameters is considered a useful tool for diagnosis of health status and cellular changes (Hochleithner 1994). Increased activity of bone-ALP in plasma is a marker for skeletal calcification and growth in great tit (Tilgar et al. 2004a; Tilgar et al. 2004b). CK is central to the maintenance of intracellular energy supplies and is released into circulation mostly in response to muscle cell damage due to environmental stressors or inflammation (Hochleithner 1994; Mitchell and Sandercock 1995). Uric acid is the most abundant circulating antioxidant, and it is also the main form of nitrogen excretion in birds (Koivula and Eeva 2010). Uric acid levels in plasma may increase because of renal damage (Hochleithner 1994) and protein catabolism associated with starvation, so it is considered a good indicator of nutritional status (Ferrer 1994; Alonso-Alvarez and Ferrer 2001). These analyses were performed in plasma of d12 (pied flycatchers) or d14 (great tits) from two nestlings randomly selected per brood. Samples were defrosted and analyzed using a microplate reader (EnSpire, Perkin-Elmer). All measurements were performed in triplicate using 384-well microplates to minimize the sample volume, and an average was produced from the triplicated values. A reduction of reagent volumes was required as compared to the method instructions of the commercial kits from BioSystems S.A.

Statistical analyses

Statistical analyses were performed with SAS 9.4 and SPSS 22.0 statistical packages. Generalized linear models (GLMs; Glimmix procedure in SAS) were performed to study the following: (i) the differences in Ca and metal concentrations in feces among areas (polluted zone = 0 − 4 km from the smelter and unpolluted zone = 4.1 − 11 km from the smelter); (ii) the effects of the treatment (Ca-supplement/control) and the zone (polluted/unpolluted) on breeding, growth, and biochemical parameters (fledgling number, size, growth rate, hematocrit, and Ca, uric acid, ALP, and CK in plasma); and (iii) the effects of fecal metal concentrations and plasmatic Ca levels on breeding, growth, and biochemical parameters (laying date and brood size were included as possible confounding variables). Wing, tarsus, head, and body mass growth rates were calculated for each individual from the hour of the first measurement on d7 to the hour of the last measurement on d12/14, and they are expressed as millimeters per day (wing, tarsus, and head growth) or grams per day (body mass growth). For all the parameters individually measured (biometric and biochemical parameters), the mean value per brood was considered in the models because of the non-independency of measurements from nestlings of the same brood. Since body size parameters (wing, tarsus, head length, and body mass on d7), growth parameters (wing, tarsus, head, and body mass growth), and metal concentrations (As, Cd, Ni, Pb, Cu, and Hg) in feces were positively correlated, we carried out Principal Component Analysis (PCA; Princomp procedure in SAS), using the first principal component (PC1) in the models. The PC1 for the size parameters (eigenvalue 3.38 and 3.64 in great tit and pied flycatcher, respectively) explained 85 and 91 % of the variation in our data in great tit and pied flycatcher; the PC1 for the growth parameters (eigenvalue 2.61 and 2.68 in great tit and pied flycatcher) explained 65 and 67 % of the variation; and the PC1 for metal concentrations (eigenvalue 3.80 and 4.30 in great tit and pied flycatcher) explained 63 and 72 % of the variation in our data in great tit and pied flycatcher, respectively; so they were used in the models as response variables to describe the size on d7, the growth rate, and the general level of metal exposure. Normality of data was checked from the model residuals and some of the variables were normalized using log-transformation (i.e., fecal As, Pb, Cd, Cu, Ni, Ca, Se, and Hg concentrations in great tit and/or pied flycatcher; and uric acid, ALP and CK in both species). The Poisson error distribution was used for fledgling number. Terms in the model were retained if they were significant ($p < 0.05$). Non-significant variables were dropped one by one from the model starting from interactions, and they were added in the final model one by one and kept if significant.

For the evaluation of the potential effect of metal pollution in the use of Ca supplements and Ca availability, we decided to consider four different categories according to the distance to the smelter (0–1, 1.01–4, 4.01–8, and >8 km) instead of polluted/unpolluted area based on previous findings (see Eeva and Lehikoinen 2004). Pairwise comparisons among distance categories were made with Tukey's test.

Pearson (r_p) or Spearman's (r_s) correlation coefficient was used to analyze correlations among response variables depending on the normality of data (checked using the Kolmogorov-Smirnov test). For correlations between pH in soil samples and metal concentrations (PC1) or mass of snail shells in the nest after fledging, a mean pH value for each site was assigned to each nest. The significance level was set at $p < 0.05$ in all analyses.

Results

Concentrations of metals and use of Ca supplements and local Ca sources (snail shells): effect of metal pollution and differences between species

Both bird species showed higher fecal concentrations in the polluted zone for almost all metals (Table 1). The distance to the smelter (as calculated for each nest) was strongly and negatively correlated with PC1 from metal concentrations in both great tit ($r_s = -0.78$, $p < 0.001$, $n = 65$) and pied flycatcher ($r_s = -0.79$, $p < 0.001$, $n = 60$).

No significant differences were found in consumption of Ca-supplemented material along the pollution gradient in either species (Fig. 1a). However, pied flycatchers breeding in the unpolluted area (4.1 − 11 km) had lower amount of snail shells in their nests ($p < 0.05$, Fig. 1b) and, respectively, consumed higher amount of Ca supplements (Fig. 1a). On the contrary, pied flycathers breeding in nests situated in the moderately polluted area (1–4 km) had the highest mass of snail shells in their nest ($p < 0.05$; Fig. 1b) and the lower Ca consumption (Fig. 1a). The mass of snail shells in the nests and the consumption of Ca supplements (both total Ca consumption and the consumption during the nestling period) were not significantly correlated ($r_s = -0.15$, $p = 0.45$ and $r_s = -0.15$, $p = 0.44$, for total Ca consumption and the consumption during the nestling period, respectively). Interestingly, Ca levels in feces of pied flycatcher were relatively low in the unpolluted areas ($p < 0.05$; Table 1, Fig. 1c), but Ca concentrations in their plasma were similar along the pollution gradient (Fig. 1d). In the most heavily polluted sites (0–1 km), both Ca consumption and mass of snail shells in nests were low (Fig. 1a, b). However, the Ca levels in feces were the highest among distance categories (Fig. 1c). Positive correlations were found between Ca concentrations in feces of pied flycatcher and PC1 from metals in feces ($r_s = 0.46$, $p < 0.001$, $n = 60$) and mass of snail shells in nest ($r_s = 0.39$, $p = 0.002$, $n = 61$).

Regarding great tits, we found a tendency of lower (75 % lower) consumption of Ca supplements in the moderately polluted area (1–4 km) as compared to the most polluted and unpolluted sites, although no significant differences were found (Fig. 1a). Ca concentrations in excrement and plasma were similar in the four distance zones from the pollution source (Fig. 1c, d).

In general, great tits consumed 12 times more Ca supplements from the feeders than pied flycatchers (0.88 ± 1.04 and 0.07 ± 0.10 g/nestling considering the brood size at d7 in great tit and pied flycatcher, respectively; $F = 37.58$, $p < 0.001$, $n = 52$ across all the periods). The total amount of Ca supplements consumed in each period was (mean ± SD): 1.83 ± 2.04 and 0.05 ± 0.10 g during laying period; 0.68 ± 0.82 and 0.06 ± 0.15 g during incubation period; 1.69 ± 2.10 and 0.19 ± 0.32 g during nestling period in great tit and pied flycatcher, respectively. Besides, we found that 68 % of pied flycatcher nests checked after fledging had snail shells collected by the parents (2.54 ± 3.30 snail shells per nest, 25.8 ± 40.7 mg of shells per nest, $n = 63$).

When comparing Ca concentrations among species, it was found that the mean Ca levels in feces and plasma were 61 and 29 % significantly higher in great tit than in pied flycatcher (10.67 ± 7.32 mg/g and 4.13 ± 4.87 mg/g in feces of great tits and pied flycatchers, $F = 51.84$, $p < 0.001$, $n = 130$; 13.49 ± 2.04 mg/dl and 9.59 ± 0.81 mg/dl in plasma of great tit and pied flycatcher, $F = 183.38$, $p < 0.001$, $n = 109$). Metal concentrations in feces of pied flycatchers were significantly higher than those found in great tits ($p < 0.05$ for all metals, Table 1).

Soil pH was generally low and higher in the polluted area (4.36 ± 0.35 and 3.99 ± 0.38 in polluted and unpolluted area, respectively; $F = 10.1$, $p = 0.003$, $n = 40$). The pH in soil samples was positively correlated with PC1 from metals ($r_s = 0.52$, $p < 0.001$, $n = 65$ and $r_s = 0.58$, $p < 0.001$, $n = 60$ in great tits and pied flycatchers, respectively). In addition, pH in soil was positively correlated with the mass of snail shells in the flycatcher nests after fledging ($r_s = 0.37$, $p = 0.003$, $n = 63$).

Effects of calcium and heavy metals on breeding, nestling growth, and biochemistry of great tits and pied flycatchers

The breeding, growth, and biochemical parameters in great tits and pied flycatchers in the polluted and unpolluted areas are presented in Supplementary Material (Table S1). Correlations among variables (growth and biochemical parameters) are shown in Supplementary Material (Table S2).

Ca treatment had no effect on the majority of the measured parameters (Table 2). Ca-supplemented great tit nestlings had slightly higher hematocrit (42.03 ± 5.13 %) than control nestlings (40.22 ± 3.34 %) ($p = 0.055$; Table 2, Fig. 2). Ca concentrations in feces (Table 1) and plasma (Table S1) and the mass of snail shells collected by parents (only in pied flycatcher; Fig. 1b) did not differ among treatments in either species (all $p > 0.50$; Table 2).

Great tits had more fledglings in the unpolluted (4.97 ± 2.32) than in the polluted (3.58 ± 1.57) area ($p < 0.01$; Table 2 and Table S1, Fig. 2; note that one egg was removed

Table 1 Metal concentrations (dry weight, dw) in feces of 7-day-old great tits and pied flycatchers according to zone (polluted and unpolluted) in Harjavalta, Finland. *N* = number of broods

Element[a]	Great tit					Pied flycatcher				
	Polluted area		Unpolluted area			Polluted area		Unpolluted area		
	N	Mean ± SD Median (min-max)	*N*	Mean ± SD Median (min-max)	$F_{ndf,ddf}$[b] *p*	*N*	Mean ± SD Median (min-max)	*N*	Mean ± SD Median (min-max)	$F_{ndf,ddf}$[b] *p*
As[d]	31	7.08 ± 4.99 5.53 (1.27–20.93)	36	0.54 ± 0.46 0.41 (0.07–1.99)	$170.14_{1.65}$ <0.0001	31	6.54 ± 3.25 6.69 (2.59–16.38)	32	0.52 ± 0.34 0.48 (0.07–1.31)	$213.07_{1.61}$ <0.0001
Pb[d]	31	6.21 ± 4.49 5.16 (1.93–24.80)	36	2.20 ± 1.47 1.69 (0.44–6.85)	$50.40_{1.65}$ <0.0001	31	9.92 ± 7.17 7.63 (1.34–35.17)	32	2.18 ± 1.55 1.77 (0.91–9.58)	$107.43_{1.61}$ <0.0001
Cd[d]	31	4.01 ± 1.90 3.65 (1.03–7.46)	36	1.68 ± 1.11 1.34 (0.52–5.18)	$46.02_{1.65}$ <0.0001	31	6.40 ± 3.58 5.59 (0.54–19.68)	32	3.17 ± 1.47 2.81 (0.99–6.13)	$22.15_{1.61}$ <0.0001
Cu[d]	31	149.88 ± 56.41 147.55 (78.14–264.69)	36	72.30 ± 39.50 62.33 (27.85–227.85)	$57.63_{1.65}$ <0.0001	31	221.85 ± 95.92 202.04 (27.19–435.90)	32	79.80 ± 31.31 73.07 (47.52–169.12)	$63.26_{1.61}$ <0.0001
Ni[d]	31	16.89 ± 8.10 14.68 (8.03–48.26)	36	3.77 ± 1.84 3.45 (1.13–8.45)	$185.37_{1.65}$ <0.0001	31	33.61 ± 29.22 23.63 (2.53–132.97)	32	3.32 ± 1.88 2.82 (1.52–10.16)	$185.46_{1.61}$ <0.0001
Zn	31	284.40 ± 82.50 271.91 (135.80–520.21)	36	272.89 ± 83.32 248.55 (110.74–499.20)	$0.32_{1.65}$ 0.5732	31	361.08 ± 116.54 350.46 (88.08–606.23)	32	314.37 ± 73.87 312.69 (139.62–469.44)	$3.64_{1.61}$ 0.061
Ca[c]	31	10.46 ± 6.61 8.46 (1.59–23.59)	36	10.86 ± 7.98 9.43 (1.25–32.73)	$0.05_{1.65}$ 0.8240	31	5.96 ± 5.07 4.54 (0.36–21.82)	32	2.35 ± 3.99 0.92 (0.15–21.12)	$18.02_{1.61*}$ <0.0001
Se[d]	31	0.82 ± 1.01 0.52 (0.07–4.96)	36	0.19 ± 0.20 0.07 (0.07–0.80)	$16.85_{1.65}$ <0.0001	31	0.59 ± 0.78 0.34 (0.07–3.50)	32	0.14 ± 0.16 0.07 (0.07–0.77)	$22.21_{1.61}$ <0.0001
Hg[d]	30	0.12 ± 0.05 0.11 (0.05–0.25)	35	0.08 ± 0.04 0.07 (0.02–0.18)	$21.37_{1.63}$ <0.0001	29	0.15 ± 0.05 0.15 (0.08–0.24)	31	0.10 ± 0.04 0.11 (0.05–0.19)	$21.45_{1.58}$ <0.0001

[a] Element concentrations in micrograms per gram, dw except for Ca (mg/g, dw)

[b] GLM with normal error distribution

[c] Since Ca concentrations in feces did not differ among treatments in either species, all nests (Ca-supplemented and control) were used for the analysis

[d] Concentration was log-transformed before analysis. All metal concentrations were determined with an inductively coupled plasma optical emission spectrometer (ICP-OES) except for total mercury that was analyzed in a Milestone DMA-80 direct Hg analyzer by atomic absorption spectrophotometry

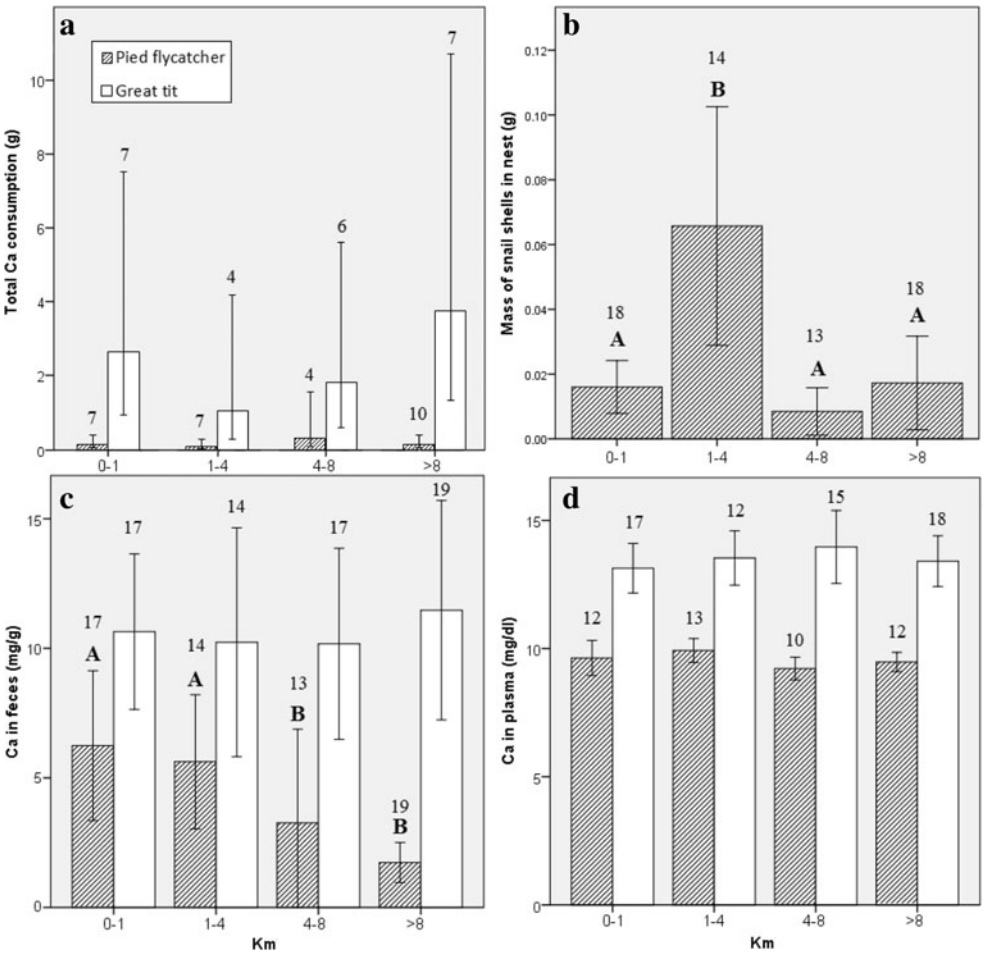

Fig. 1 Mean (±95 % CI) consumption of supplemented Ca (mussel shell) during breeding (**a**), mass of natural snail shells in pied flycatcher nest after fledging (not applicable to great tits) (**b**), and Ca concentrations (dry weight) in feces (**c**) and plasma (**d**) of great tit and pied flycatcher nestlings at the age of 7 and 14/12 days, respectively, along a pollution gradient in Harjavalta, Finland. X-axis represents the distance to the smelter in Km. *Letters* above the bars denote significant differences between distance categories (means with *different letter* are statistically different). *Numbers* above the bars indicate the number of broods

Table 2 Generalized linear models of the effects of zone (polluted vs. unpolluted) and Ca treatment on breeding, growth and biochemical parameters of great tits and pied flycatchers in Harjavalta, Finland. Means and sample sizes are given in the supplementary material Table S1

	Great tit						Pied flycatcher					
	Zone		Treatment		Zone x Treatment		Zone		Treatment		Zone x Treatment	
Dependent variable	$F_{ndf,ddf}$	*p*	$F_{ndf,ddf}$	*p*	$F_{ndf,ddf}$	*p*	$F_{ndf,ddf}$	*p*	$F_{ndf,ddf}$	*p*	$F_{ndf,ddf}$	*p*
Fledgling number[a]	*$7.39_{1.65}$*	*0.0084*	$1.07_{1.64}$	0.31	$0.85_{1.63}$	0.36	$0.00_{1.62}$	0.87	$0.03_{1.63}$	0.87	$0.79_{1.61}$	0.38
Size (d7) (PC1)[b]	*$17.76_{1.65}$*	*0.0001*	$2.10_{1.64}$	0.15	$0.06_{1.63}$	0.81	$1.50_{1.63}$	0.23	$0.01_{1.62}$	0.94	$0.14_{1.61}$	0.71
Growth rate (PC1)[b]	$0.17_{1.60}$	0.69	$0.25_{1.61}$	0.62	$0.03_{1.59}$	0.86	$1.73_{1.50}$	0.19	$0.00_{1.49}$	0.97	$3.86_{1.48}$	0.055
Hematocrit (d12/14) (%)[b]	*$9.13_{1.59}$*	*0.0037*	*$3.83_{1.59}$*	*0.055*	$0.91_{1.58}$	0.35	$1.22_{1.45}$	0.27	$0.42_{1.44}$	0.52	$0.20_{1.43}$	0.66
Ca in plasma (d12/14) (mg/dl)[b]	$0.48_{1.60}$	0.49	$0.31_{1.59}$	0.58	$1.48_{1.58}$	0.23	$3.33_{1.45}$	0.075	$0.14_{1.44}$	0.71	$0.22_{1.43}$	0.64
Uric acid in plasma (d12/14) (mg/dl)[b, c]	$0.91_{1.60}$	0.35	$0.62_{1.59}$	0.43	$1.19_{1.58}$	0.28	$0.02_{1.45}$	0.90	$0.01_{1.44}$	0.93	$1.97_{1.43}$	0.17
ALP in plasma (d12/14) (U/l)[b, c]	$0.99_{1.60}$	0.32	$0.00_{1.59}$	0.97	$0.08_{1.58}$	0.77	$1.07_{1.44}$	0.31	$2.16_{1.45}$	0.15	$0.10_{1.43}$	0.76
CK in plasma (d12/14) (U/l)[b, c]	$0.39_{1.60}$	0.53	$0.02_{1.59}$	0.90	$0.14_{1.58}$	0.71	$0.56_{1.44}$	0.46	$0.24_{1.43}$	0.63	$1.48_{1.42}$	0.23

The age of nestlings is indicated as d7 and d12/14. PC1 for size is based on wing, tarsus, head length, and body mass on d7. PC1 for growth rate is based on wing, tarsus, head, and body mass growth. Terms left in the final model are shown in italics

ALP alkaline phosphatase, *CK* creatine kinase

[a] GLM with Poisson error distribution

[b] GLM with normal error distribution (brood mean was used)

[c] The variables were log-transformed before analysis

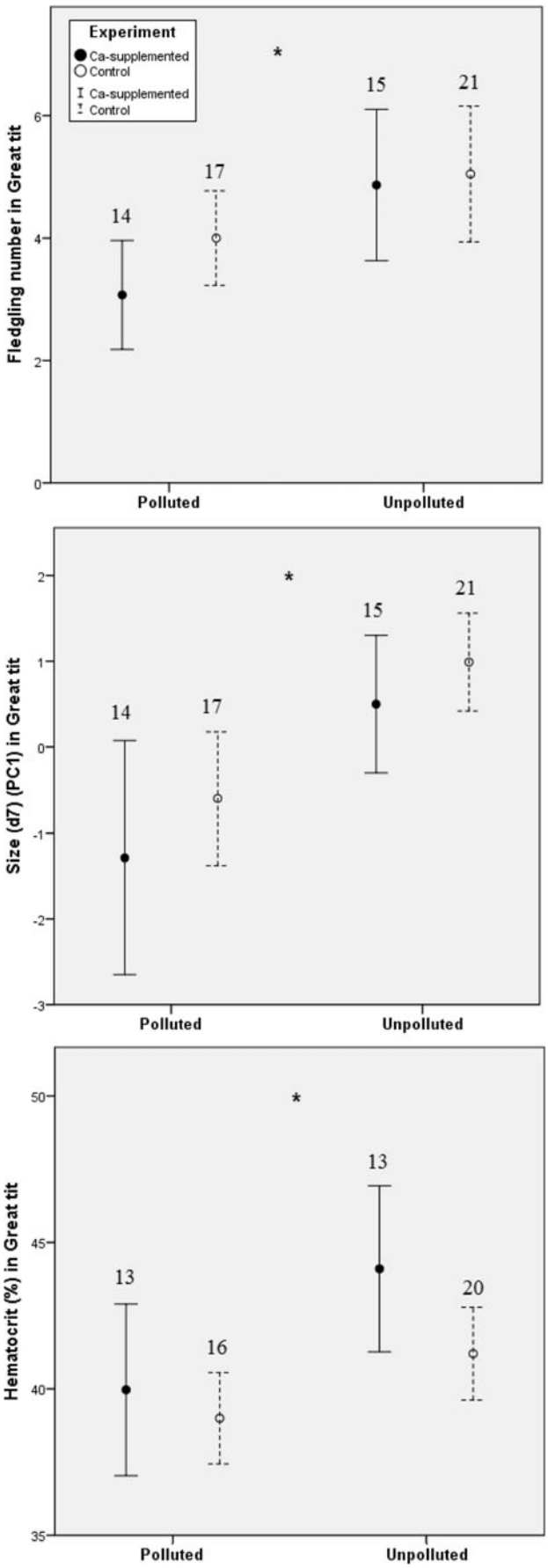

Fig. 2 Mean (±95 % CIs) fledgling number, size (d7) (PC1) and hematocrit (d14) in great tit according to zone (polluted and unpolluted) and experiment (Ca-supplemented and control) in Harjavalta, Finland. (*Asterisk*) significant differences among zones. *Numbers* above the bars indicate the number of broods

from each clutch). Regarding the biometric measurements, great tits from the polluted area were smaller (13, 9, 4, and 3 % lower wing, tarsus, head length, and body mass at d7, respectively) than birds from the unpolluted sites (Table 2 and Table S1, Fig. 2). Accordingly, fecal metal concentrations (PC1) had a negative association to fledgling number and size on d7 (PC1), together with laying date (Table 3). Great tits breeding in the polluted area showed 7 % lower hematocrit at d14 than in the unpolluted area (Table 2 and Table S1, Fig. 2). In pied flycatchers, the zone and treatment had no effect on the parameters under study (Table 2).

Ca concentration in plasma was positively associated with fledgling number, growth rate (PC1), and ALP activity in great tit (Table 3). In pied flycatchers, Ca in plasma had a positive association with the growth rate (PC1) and a negative association with CK activity (Table 3). Brood size and/or laying date explained some variation in the growth and biochemical parameters in both species (Table 3). Ca concentrations in feces of pied flycatcher was positively correlated with growth rate ($r_p = 0.33$, $p = 0.020$, $n = 50$), and the mass of snail shells in the nest was positively correlated with fledgling number ($r_s = 0.28$, $p = 0.03$, $n = 63$) in this species.

Discussion

Concentrations of metals and use of Ca supplements and local Ca sources (snail shells): effect of metal pollution and differences between species

Feces provide information on the metal levels in the diet or metal exposure (Berglund et al. 2015; Sánchez-Virosta et al. 2015). As expected, for both species, concentrations of most elements were higher in the polluted area (Table 1). This pollution gradient has been clearly observed in previous studies in the same subject area (Eeva and Lehikoinen 1996; Koivula et al. 2011; Berglund et al. 2015). Higher fecal concentrations of Pb, Ni, Cd, Cu, Zn, and Hg in nestling pied flycatchers as compared to great tits were found (Table 1), which has been suggested before as indicative that food items of pied flycatcher may contain higher metal concentrations (Berglund et al. 2011).

Regarding fecal Ca concentrations, the similar Ca levels in the feces of great tits along the pollution gradient (Fig. 1c) suggest that the Ca intake in the different sites may be similar, although the sources are not necessarily the same. However, pied flycatchers showed higher Ca levels in feces in polluted

Table 3 Generalized linear models for variation in fledgling number, growth, and biochemical parameters of great tit and pied flycatcher nestlings in Harjavalta, Finland

Response variable	Metals (PC1)			Ca in plasma			Metals (PC1) x Ca in plasma			Laying date			Brood size (d7)		
	$F_{ndf,ddf}$	*p*	*E*	$F_{ndf,ddf}$	*p*	*E*	$F_{ndf,ddf}$	*p*	*E*	$F_{ndf,ddf}$	*p*	*E*	$F_{ndf,ddf}$	*p*	*E*
Great tit															
Fledgling number[a]	*$4.96_{1.59}$*	*0.030*	*−0.072*	*$4.43_{1.59}$*	*0.040*	*0.059*	$0.02_{1.57}$	0.89	0.002	$0.10_{1.58}$	0.75	−0.003	Ni	Ni	Ni
Size (d7) (PC1)[b]	*$9.47_{1.62}$*	*0.0031*	*−0.307*	$0.75_{1.57}$	0.39	0.095	$0.05_{1.56}$	0.83	−0.012	*$4.47_{1.62}$*	*0.039*	*0.066*	$3.28_{1.61}$	0.075	0.210
Growth rate (PC1)[b]	$1.06_{1.57}$	0.31	−0.101	*$12.34_{1.60}$*	*0.0009*	*0.330*	$0.22_{1.56}$	0.64	−0.025	$1.90_{1.58}$	0.17	−0.047	$2.33_{1.59}$	0.13	−0.181
Hematocrit (d14) (%)[b]	$0.67_{1.58}$	0.42	−0.221	$0.10_{1.57}$	0.75	0.094	$0.04_{1.56}$	0.85	0.030	*$4.52_{1.59}$*	*0.038*	*0.176*	*$4.52_{1.59}$*	*0.038*	*0.681*
Uric acid in plasma (d14) (mg/dl)[b, c]	$0.34_{1.59}$	0.56	−0.015	$3.41_{1.60}$	0.070	0.046	$2.65_{1.58}$	0.11	−0.023	$0.02_{1.56}$	0.89	0.001	$0.02_{1.57}$	0.89	0.005
ALP in plasma (d14) (U/l)[b,c]	$0.34_{1.58}$	0.56	−0.007	*$9.99_{1.59}$*	*0.0025*	*0.038*	$0.00_{1.56}$	0.95	−0.0004	$0.07_{1.57}$	0.79	−0.001	*$5.54_{1.59}$*	*0.022*	*0.035*
CK in plasma (d14) (U/l)[b, c]	$1.73_{1.59}$	0.19	0.020	$0.29_{1.57}$	0.59	−0.009	$0.14_{1.56}$	0.71	−0.003	*$6.58_{1.60}$*	*0.013*	*0.012*	$1.09_{1.58}$	0.30	0.020
Pied flycatcher															
Fledgling number[a]	$0.00_{1.58}$	0.95	−0.003	$0.05_{1.42}$	0.83	0.022	$0.06_{1.41}$	0.81	−0.017	$0.01_{1.40}$	0.91	0.002	Ni	Ni	Ni
Size (d7) (PC1)[b]	$2.16_{1.57}$	0.15	−0.154	$2.07_{1.40}$	0.16	−0.412	$0.67_{1.39}$	0.42	0.159	$2.75_{1.63}$	0.10	−0.114	$3.16_{1.56}$	0.081	−0.384
Growth rate (PC1)[b]	$3.21_{1.41}$	0.081	0.166	*$10.60_{1.44}$*	*0.0022*	*0.774*	$2.85_{1.40}$	0.099	−0.273	*$7.42_{1.44}$*	*0.0092*	*0.139*	$0.30_{1.39}$	0.59	0.112
Hematocrit (d12) (%)[b]	$1.19_{1.42}$	0.28	0.366	$0.90_{1.40}$	0.35	0.789	$0.37_{1.39}$	0.55	0.344	$0.01_{1.38}$	0.92	−0.019	$2.24_{1.45}$	0.14	−1.021
Uric acid in plasma (d12) (mg/dl)[b, c]	$0.09_{1.40}$	0.77	0.009	$0.30_{1.43}$	0.59	−0.045	$0.00_{1.39}$	0.98	0.001	$2.04_{1.45}$	0.16	−0.024	$1.28_{1.44}$	0.26	−0.075
ALP in plasma (d12) (U/l)[b,c]	$0.16_{1.42}$	0.69	0.005	$2.44_{1.45}$	0.13	−0.047	$2.24_{1.41}$	0.14	−0.031	$0.00_{1.39}$	0.99	−0.00006	$0.40_{1.40}$	0.53	−0.017
CK in plasma (d12) (U/l)[b,c]	$0.03_{1.39}$	0.85	0.005	*$6.52_{1.44}$*	*0.014*	*−0.142*	$0.01_{1.38}$	0.91	−0.004	$2.40_{1.43}$	0.13	−0.018	$0.39_{1.42}$	0.54	−0.030

The age of nestlings is indicated as day 7 and day 12/14. PC1 for size is based on wing, tarsus, head length, and body mass on d7. PC1 for growth rate is based on wing, tarsus, head, and body mass growth. Terms left in the final model are shown in italics. Ni = variables not included in the initial model

E estimate, *ALP* alkaline phosphatase, *CK* creatine kinase

[a] GLM with Poisson error distribution

[b] GLM with normal error distribution (brood mean was used)

[c] The variables were log-transformed before analysis

(and more anthropogenic habitat, 0–4 km) than unpolluted areas (Fig. 1c). A lower Ca excretion in unpolluted sites may be indicative of lower Ca availability or dietary Ca intake. This result is reinforced by the lower mass of snail shells in nests in the unpolluted sites (4.1–11 km; Fig. 1b) when comparing with the moderately polluted area that showed the highest mass of snail shells in nests from this species (1.01–4 km; Fig. 1b). Our results agree with previous studies carried out in the same subject area (Eeva and Lehikoinen 2004; Eeva et al. 2010), observing the most diverse and abundant snail shells in nests from the moderately polluted areas (1.01–4 km), while they were scarce in remote unpolluted areas and in the most heavily polluted sites. The general acidity of the forest soils in our study (Derome and Lindroos 1998) may explain the relatively low snail shell populations even in the unpolluted areas (Eeva et al. 2010). Accordingly, we found significant differences in soil pH among areas, the soil in the unpolluted area being even more acidic than in the polluted sites. In addition, pH in soil was positively correlated with the mass of snail shells in the nests of pied flycatchers after fledging, indicating the importance of soil acidity to the snail abundance (Graveland and van der Wal 1996). Moreover, the most polluted sites in our study area have lower exchangeable Ca in soil due to leaching (Derome and Nieminen 1998), which further explains that this environment does not support abundant land snail populations (Eeva et al. 2010). However, human habitation may produce habitats suitable for snails and their number may be higher in such anthropogenic environments, while Ca sources may be scarce in the less human-populated background sites with more uniform forests (Eeva et al. 2010). Furthermore, the positive correlations found between Ca levels in feces and mass of snail shells or PC1 from metals are indicative of a higher Ca excretion rate in polluted areas with higher land snail availability. The highest Ca levels in feces in the most heavily polluted sites could also be related to an adverse effect of metals on birds. Hypercalciuria or excessive urinary Ca excretion has been related to metal (especially Cd) exposure (Wu et al. 2001). Cd induces renal tubular damage and decreases the Ca reabsorption, thus resulting in hypercalciuria (Staessen et al. 1994) and low bone mineral density (Staessen et al. 1999; Järup and Alfvén 2004). In spite of relatively large variation in natural Ca availability (Fig. 1b), the pied flycatchers are able to sustain similar plasma Ca concentrations in all locations (Fig. 1d), and no differences among treatments were found in breeding, growth, and biochemical parameters evaluated (Table 2). Therefore, this species seems to be adapted to naturally low dietary Ca availability.

No significant differences were found in the consumption of supplemental Ca along the pollution gradient for either species. As suggested by Reynolds et al. (2004), Ca supplementation will normally be ineffective if natural food provides sufficient dietary Ca for the birds' breeding requirements. This could be the case with the low consumption of supplemented Ca in pied flycatchers in the present study, especially in the polluted area (0–4 km from the smelter) (Fig. 1a). Thus, snail numbers and some other Ca-rich food as woodlice (Graveland 1995; Eeva and Lehikoinen 2004) near the smelter seem to be high enough for pied flycatchers. Pied flycatchers breeding in nests from the unpolluted areas showed a tendency of consuming slightly, though non-significantly, higher amount of Ca supplements, probably related to the low natural snail availability (Fig. 1a, b). As above, supplemented Ca consumption of great tits tended to be lower in the moderately polluted sites (1.01–4 km) (Fig. 1a). Thus, in the polluted and more human-populated areas, other Ca-rich items are possibly available. Some birds may acquire snails from more moist and grassy habitats near their territories, or may consume anthropogenic Ca sources in areas where snails are scarce (Graveland 1996; Reynolds et al. 2004; Eeva et al. 2010).

Previous studies have found that great tits and pied flycatchers respond to Ca supplementation in a different way, since Ca supplements are less consumed by pied flycatcher than by great tit (Graveland 1995). In accordance with this, although both species seem to show a similar trend of higher consumption of supplemented Ca in unpolluted sites, in our experiment great tits consumed much more Ca-rich material than pied flycatchers (Fig. 1a). Different species may differ in their strategy or effectiveness in finding Ca-rich material and/or in Ca absorption or may have different Ca requirement for successful breeding. Great tits could be a more opportunistic species in habitats with Ca limitation, thus they might prefer saving energy and time by consuming the readily available Ca supplements. In this sense, Graveland (1995) found that great tits facing low snail shell availability responded by collecting anthropogenic materials (domestic chicken eggshell and grit). In contrast, pied flycatchers consumed more millipedes and woodlice, richer in Ca compared with other forest arthropods, and fewer snail shell fragments (Graveland 1995). Eeva and Lehikoinen (2004) also hypothesized that female great tits may use anthropogenic sources of Ca (e.g., plaster, ash, and Ca leaching from concrete) in the polluted area that may be not used by pied flycatchers. In addition, as it has been suggested before, it is likely that pied flycatchers need less Ca for successful breeding than great tits, since the latter has larger clutch-sizes, egg shell masses, and skeletal masses (Eeva and Lehikoinen 2004). In this sense, great tit nestlings showed 61 and 29 % higher Ca contents in their feces and plasma, respectively, than pied flycatcher nestlings. Similar results in feces have been reported before, suggesting differential need and/or ability to acquire Ca between species (Eeva and Lehikoinen 2004).

Effects of calcium and heavy metals on breeding, nestling growth, and biochemistry of great tits and pied flycatchers

Ca supplementation of birds during the breeding season may help to detect if breeding is constrained by insufficient Ca

availability. In general, providing nestlings with supplemental Ca had no significant effect on the parameters examined, suggesting that the great tits and pied flycatchers were not Ca-limited at the nestling stage (Table 2). In addition, Ca concentrations in feces and plasma did not differ in Ca-supplemented and control nestlings. The most likely explanation for the lack of treatment effects is that control parents were able to obtain sufficient natural or anthropogenic sources of Ca to allow chicks to grow properly (see previous section). Supplemental Ca could allow the parents to increase the time for searching for macronutrient-rich food, which might result in a higher intake of protein and other nutrients because egg laying great tit females on a Ca-deficient diet spent 43 % of the daylight hours searching for Ca-rich material, almost twice as much as females with sufficient Ca (Graveland and Berends 1997). This could explain how supplemental Ca indirectly resulted in an increase of hematocrit in great tit nestlings in both the polluted and unpolluted areas, since females could provide more macronutrient-rich food for nestlings (Table 2, Fig. 2). Anemia is a feature of protein-energy deficiency, and hematocrit values may increase with increases in the level of dietary protein (Anthony and Edozien 1975; Edozien and Switzer 1977). Ca limitation may be hard to detect, since birds may modify their behavior in Ca-poor areas by increasing their search effort for Ca, which may carry a lower intake of protein and other nutrients or a higher energy expenditure (Graveland and Drent 1997). As a result, birds might produce smaller or fewer eggs, with good shells, and Ca limitation is not shown so clearly. Another thing to consider is that the removal of one egg per clutch could reduce the parental effort and parents could compensate for the local Ca deficiency by increasing the care of the young (Tilgar et al. 1999). Although Ca availability did not constrain the growth of nestlings, it may constrain other parameters during early stage reproduction such as egg volume or shell thickness (Tilgar et al. 1999), so further research is needed in this regard.

It is well known that heavy metals may produce direct and indirect effects in health and growth in passerines (Eeva and Lehikoinen 1996; Dauwe et al. 2006a; Eeva et al. 2009; Sánchez-Virosta et al. 2015), and species with naturally low dietary Ca content may be more sensitive to adverse effects of metals (Eeva and Lehikoinen 2004). Although we found that pied flycatchers had lower Ca concentrations in feces and plasma and lower consumption of Ca supplement than great tits, showing lower Ca intake, in general we did not find signs of higher sensitivity to metals in the parameters evaluated. A likely reason for this is that metal exposure is currently below a toxic level. Metal emissions and the subsequent metal exposure in passerines have been reduced in the last 25 years (Berglund et al. 2012; Berglund et al. 2015), improving breeding success in the vicinity of the smelter (Eeva and Lehikoinen 2000). Thus, it seems that pied flycatchers can successfully breed at low Ca availability when metal concentrations are not too high. It is possible that 25 years ago, birds also had enough Ca available for successful breeding, but at that time metal concentrations exceeded the tolerance of pied flycatchers leading to poor breeding success (Eeva and Lehikoinen 1995; Eeva and Lehikoinen 1996). In this study, great tits had lower fledgling numbers and hematocrit values, and nestlings were smaller in the polluted than in the unpolluted area (Table 2 and Table S1, Fig. 2). In addition, significant negative effects were found on fledgling number and nestling size on d7 (Table 3). Close to the smelter, reduced growth rates, higher nestling mortality and lower hematocrit values have been extensively reported in this species, primarily associated with a decrease in abundance of some important foods (Eeva and Lehikoinen 1996; Eeva et al. 2005; Eeva et al. 2014). As suggested before in the same study area, reduced growth is most probably related to a combination of higher metal concentrations (Table 1) and reduced food quantity or quality in the polluted area (Eeva et al. 2005; Eeva et al. 2009). In connection with this, Eeva et al. (2005) suggested that a more opportunistic forager (pied flycatcher) is less vulnerable to a changing invertebrate prey base caused by metal pollution than a caterpillar specialist (great tit).

According to the results in the present study, it is clear that Ca has an essential role in breeding, growth, and biochemistry in both species. Tits consumed more Ca during the egg-laying period, less during incubation and raised consumption again during the nestling period. The same pattern has been found in a Ca provisioning experiment in the same species in the Netherlands (Graveland and Drent 1997), showing the special Ca requirement during these periods for eggshell formation and skeletal growth (Starck 1998; Reynolds et al. 2004). Ca concentration in plasma had positive association to growth in both species and on fledgling number in great tit (Table 3). These results seem logical since Ca is essential for skeletal development (Mänd and Tilgar 2003). Similarly, Ca concentrations in feces of pied flycatcher, which may be indicative of Ca availability, were positively correlated with growth rate too. The mass of snail shells in the nest, an important Ca source for pied flycatcher, was positively correlated with fledgling number in this species (see also Eeva and Lehikoinen 2004). ALPs are a family of glycoproteins that are present in plasma in different isoforms, liver-ALP and bone-ALP being the two major and diagnostically most relevant isoforms of this enzyme (Romagnoli et al. 1998; Tilgar et al. 2004a). The positive relationship between total ALP and plasmatic Ca levels (Table 3), growth rate (PC1), or fledgling number (Table S2) in great tit nestlings seems logical because the bone isoform is directly related to active bone growth which is dependent upon Ca (Viñuela et al. 1991; Viñuela and Ferrer 1997). However, these results were not observed in pied flycatchers (Table 3), which could be due to the fact that we analyzed total ALP instead of the more specific bone-ALP isoform (Tilgar et al. 2004b). It is remarkable that pied

flycatchers showed two times higher ALP activity than great tits (Table S1). The higher ALP activity may indicate more intensive Ca metabolism in pied flycatchers allowing them more effective use of limited Ca resources. In this sense, different authors have found that Ca-deficient diets lead to elevated serum ALP in chicks (see references in McComb et al. 1979). On the other hand, Ca levels in plasma had a negative effect on CK in pied flycatchers (Table 3). Jasmin et al. (1975) found that serum CK was higher in Ca-deficient hamsters. Similarly, Weishaar and Simpson (1987) found a significant increase in serum CK in vitamin D_3-deficient rats, which is indicative of muscle damage. Vitamin D_3 is essential for maintaining plasma levels of Ca, and the increase in CK appeared to correlate with the onset of hypocalcemia (Weishaar and Simpson 1987). This result suggests that circulating Ca levels may have metabolic consequences in CK activity.

In the light of the results of this study, it seems clear that bird species respond differently to low Ca availability. Great tit had markedly higher Ca concentrations in feces and plasma than pied flycatcher nestlings. Great tits may need higher Ca levels and seem to be more opportunistic in search for Ca than pied flycatchers, since they consumed more of the supplemented Ca, probably saving energy and time. In spite of this, we did not find signs of higher sensitivity to adverse effects of metals in pied flycatcher, but some negative effects of pollution were found in great tit nestlings. The most likely explanation for this is that we did not find high metal concentrations, and pied flycatchers seem to be adapted to low Ca availability and can successfully breed when metal concentrations are not too high. However, there may be a balance between Ca levels in the organism and the tolerance to metal-related effects. Therefore, it is important to consider the Ca concentrations in the organism in different bird species and its potential consequences in metal sensitivity, especially when birds inhabit areas with a higher metal exposure than that found in this study.

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Compliance with ethical standards The experiment was conducted under licenses from the Animal Experiment Committee of the State Provincial Office of Southern Finland (license number ESAVI/1650/04.10.03/2012) and the Centre for Economic Development, Transport and the Environment, ELY Centre Southwest Finland (license number VARELY/319/07.01/2014).

Conflict of interest The authors declare that they have no competing interests.

Ethical approval "All applicable international, national, and/or institutional guidelines for the care and use of animals were followed."

Informed consent "Informed consent was obtained from all individual participants included in the study."

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