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# Experimental manipulation of dietary lead levels in great tit nestlings: limited effects on growth, physiology and survival

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**Abstract** We manipulated dietary lead (Pb) levels of nestlings in wild populations of the great tit (*Parus major* L) to find out if environmentally relevant Pb levels would affect some physiological biomarkers (haematocrit [HT], fecal corticosterone metabolites [CORT], heat shock proteins [HSPs], erythrocyte delta-aminolevulinic acid dehydratase activity [ALAd]), growth (body mass, wing length), phenotype (plumage coloration) or survival of nestlings. The responses to three experimental manipulation (control, low and high: 0, 1 and 4 μg/g body mass/day) are compared with those in a *P. major* population breeding in the vicinity of a heavy metal source, a copper smelter. Our Pb supplementation was successful in raising the fecal concentrations to the levels found in polluted environments (high: 8.0 μg/g d.w.). Despite relatively high range of exposure levels we found only few effects on growth rates or physiology. The lack of blood ALAd inhibition suggests that the circulating Pb levels were generally below the toxic level despite that marked accumulation of Pb in femur (high: 27.8 μg/g d.w.) was observed. Instead, birds in the metal polluted environment around the smelter showed decreased growth rates, lower HT, higher CORT, less colorful plumage and lower survival probabilities than any of the Pb treated groups. These effects are likely related to decreased food quality/quantity for these insectivorous birds at the smelter site. In general, the responses of nestlings to metal exposure and/or associated resource limitation were not gender specific. One of the stress proteins (HSP60), however, was more strongly induced in Pb exposed males and further studies are needed to explore if this was due to higher accumulation of Pb or higher sensitivity of males. In all, our results emphasize the importance of secondary pollution effects (e.g. via food chain disruption) on reproductive output of birds.

**Keywords** Biomarkers · Breeding success · Carotenoids · Heavy metals · Nestling growth · Stress hormones

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## Introduction

Lead (Pb) is a common environmental pollutant and a variety of detrimental effects on health and reproduction have been documented in Pb exposed animals in captivity and in the wild (Scheuhammer 1987c; Burger and Gochfeld 2000). Numerous cases of Pb poisoning and related health effects have been reported in waterfowl and birds of prey that have eaten Pb shot or sinkers (Kendall et al. 1996; Mateo et al. 1998; Fisher et al. 2006; Guitart et al. 2009). Some studies have indicated physiological or reproductive effects in urban environments or roadsides affected by traffic exhaust gases (Grue et al. 1984, 1986; Hutton 1980; Schilderman et al. 1997; Nam and Lee 2006). In general, however, reports on Pb toxicity in free-living terrestrial bird populations are relatively uncommon and the environmental levels may rarely be high enough to cause acute

toxic effects or impair reproduction (Scheuhammer 1991). Exceptions to this are metal mining and smelting sites where such effects have been documented (Nyholm et al. 1995; Janssens et al. 2003a; Belskii et al. 2005; Janssens et al. 2003b; Berglund et al. 2010). In the wild, however, it is not often straightforward to demonstrate a causal link between ambient Pb levels and impaired health or reproductive failure. Firstly, at point sources of pollutants, birds are often exposed to mixtures of heavy metals and other pollutants, making it difficult to link the symptoms to one specific pollutant. Furthermore, long-term pollution will change plant and animal communities, causing secondary effects on individual species via changed resources like food quantity and quality (Eeva et al. 2005b). In correlative studies, such secondary pollution impacts are difficult to separate from more direct effects of metal exposure. Therefore, experimental studies are necessary to explore the effects of Pb on physiology and reproduction in free-living populations at levels relevant to ones occurring in our environment.

We manipulated dietary Pb levels of nestlings in wild populations of the great tit (*Parus major* L) to find out if environmentally relevant Pb levels would affect growth, some physiological biomarkers, phenotype (plumage coloration) or survival of *P. major* nestlings. The experimental exposure levels were based on information found in the literature of dietary and fecal Pb levels reported for this species at some metal polluted sites. Our field experiment was performed in a *P. major* population with otherwise low metal exposure levels (Eeva et al. 2012). However, we wanted to include in the experimental setup also a *P. major* population breeding in the vicinity of a lead source, a non-ferrous smelter. This gives us a possibility to directly compare the effects of dietary Pb levels to those caused by exposure to mixtures of metals, other pollutants and associated resource limitation. Such information will help us to better separate between direct toxic and indirect secondary effects of pollution that is difficult with correlative data.

We selected a set of physiological biomarkers (hematocrit, delta-aminolevulinic acid dehydratase, corticosterone metabolites and heat shock proteins) that we considered as potential indicators of health and/or Pb toxicity. Hematocrit [HT] is a commonly used measure of condition in avian ecology (Fair et al. 2007) but it also has a special toxicological interest since Pb may cause anemia in birds by affecting haem synthesis, potentially decreasing haemoglobin and haematocrit levels (Hutton 1980; Grue et al. 1986; Scheuhammer 1991). Relating to this, delta-aminolevulinic acid dehydratase [ALAd] is an important enzyme in the haem biosynthetic pathway, and is sensitive to inhibition by lead (Scheuhammer 1987b, 1989a). ALAd is not readily inhibited by other heavy metals and can thus be used as a specific biomarker of lead toxicity (Scheuhammer 1987a; Peakall 1992; Gil and Pla 2001). Corticosterone [CORT] is the primary energy regulating hormone in birds but also an important stress hormone (Harvey et al. 1984), and the most common measure of individual stress response in bird studies (Stöwe et al. 2008 and references therein). Among many other stressors Pb exposure has been found to increase CORT levels in birds (Baos et al. 2006). Heat shock proteins (HSPs) are a family of proteins helping cells to recover from stress situations by correcting misconfigurations in protein structures (Morimoto et al. 1990). Many ecologically relevant factors may induce HSPs (e.g. temperature, parasites, diseases or physical activity), among them various toxicants and heavy metals (Bauman et al. 1993; Delmas et al. 1996; Sørensen et al. 2003; Tomás et al. 2004; Martinez-de la Puente et al. 2011). HSPs have therefore been also used as bioindicators of xenobiotic exposure, though seldom in bird studies (Eeva et al. 2000; Gil and Pla 2001). As compared to glucocorticoids, HSPs are considered more appropriate for detecting chronic or long-term exposure to stressors (Tomás et al. 2004).

We expect that supplemental lead will decrease ALAd activities and possibly also HT, and increase CORT metabolite and HSP levels. We also hypothesize that lead may retard nestling growth since young altricial birds are considered to be especially vulnerable to the effects of lead (Hoffman et al. 1985a; Hoffman et al. 1985b). On the basis of previous correlative studies at the smelter site we further expect that nestlings in this group would show inferior growth rates and less yellow plumage coloration than those in a less polluted environment (Eeva and Lehikoinen 1996; Eeva et al. 2009a). Slower growth and less intensive carotenoid colorations in this area has been associated to suboptimal food quality/food shortage, ambient pollution decreasing the numbers of lepidopteran larvae which are one of the main food items and an important source of carotenoid pigments for *P. major* nestlings (Eeva et al. 2009b). Experimental supplementing of food to nestlings near the smelter did not, however, raise the growth rates to the level of unpolluted environments (Eeva et al. 2003). We are therefore especially interested if lead as such, i.e. without an associated food chain disruption, would retard nestling growth in our experiment.

It is well acknowledged that gender differences exist in metal accumulation and/or their physiological consequences and that gender should be better taken into account in ecotoxicological studies (Burger 2007). However, quite often it is not possible to sex bird nestlings by morphological characters and we are not aware of any study that had explored sex-related effects of heavy metals at nestling phase. This is why we determined the sex of nestlings with molecular methods to explore if metal accumulation, physiological responses, growth or survival would show

sex-specific treatment effects in the nestling phase. We have no a priori hypothesis on the differences in sensitivity of nestlings but in *P. major* males are slightly larger and grow faster than females and might therefore be expected to accumulate more metals.

## Materials and methods

### Experimental setup

The experiment was conducted in Turku (60°26′N, 22°10′E) and Harjavalta (61°20′N, 22°10′E), southwestern Finland. In March 2011 we settled ca. 200 nest boxes in the study area in Turku; in Harjavalta 260 nest boxes were already present (see Lambrechts et al. 2010 for description of nest boxes). We regularly monitored *P. major* nests to obtain basic breeding parameters: laying date of the first egg, clutch size and hatching date. At the age of 3 days (hereafter d3 etc.; d0 = hatching day), we randomly assigned each nest in Turku study area to either high lead (n = 15), low lead (n = 16) or control treatment (n = 15). Earlier measurements on blue tit (*Cyanistes caeruleus*) feces indicated low metal levels in this area (Eeva et al. 2012). All chicks in one nest received the same treatment. Lead treatment was executed by dosing distilled water with lead acetate (treatment) or distilled water (control) orally every day for twelve days (from d3 to d14). See details on dosages and dosing below. The fourth treatment group consisted of nests (n = 19) in the vicinity (<2 km) of Harjavalta copper smelter, where there is a long-term exposure of several heavy metals (e.g. As, Cd, Cu, Ni, Pb) (Kiikkilä 2003). In those nests chicks were also dosed with distilled water from d3 to d14. The breeding habitat was similar in both areas, representing pine dominated forests with mixed spruce and birch. However, in Turku some oaks were scattered in the forest and we presume that food availability (e.g. caterpillar numbers) for birds was better in Turku, though this was not measured in our study.

On d3 we weighed the chicks and coded them individually by nail cutting. On d7 chicks were measured (wing length and body mass), ringed and a small blood sample (max 70 µl) from brachial vein was collected with a capillary tube. We centrifuged the blood sample in the field to measure HT (% height of packed red blood cells from total sample in a capillary tube). Red blood cell fraction was used for analyses of oxidative stress markers and plasma was used for vitamin analyses (reported elsewhere). We also collected a small drop of blood on absorbent paper and used this for sexing the nestlings with molecular methods (see details below). On d7 also individual fecal samples were collected for non-invasive analyses of metal levels (As, Cd, Cu, Ni and Pb) and CORT metabolites. On d14 we measured the nestlings again (wing and body mass) and collected another blood sample (70–140 µl) for analyses of ALAd activity (a biomarker for Pb exposure) and HSPs. Samples were stored immediately (within few minutes) in liquid nitrogen. At d14 we also collected ca. ten yellow breast feathers for carotenoid color analysis.

At d15 some nestlings were moved to aviary for behavioral experiments (reported elsewhere) and this is why we do not analyze fledging success here. Unfortunately, some of the captive birds died later (from d33 onward) due to various reasons (hitting the wall, pecked by conspecifics, infections etc.). Since cases of death took place in all of the treatment groups (High 6, Low 8, Control 8, Harjavalta 9) we took an advantage of the carcasses to measure their bone Pb concentrations and compared the Pb accumulation among groups. Since absorbed Pb is known to concentrate in bone tissue where it remains relatively stable (half-life being several years) the bone levels can be used as a measure of cumulative exposure (Scheuhammer 1991; Dauwe et al. 2005). Therefore the femurs of dead birds (n = 31) were dissected and dried in laboratory for Pb analysis. Note that birds were not given lead after d14.

### Lead exposure

We estimated the levels of Pb exposure in our experiment by concentrations in nestling feces and food items in passerine populations (for *P. major* and *Ficedula hypoleuca*) near pollution sources and at control areas. Information on fecal concentrations was available for four smelter sites: Harjavalta, Finland (Eeva and Lehikoinen 1996; Eeva et al. 2005b; Eeva et al. 2009a), Antwerp, Belgium (Dauwe et al. 2000; Dauwe et al. 2004), Rönnskär, Sweden (Nyholm 1994; Berglund et al. 2010) and Revda, Russia (Belskii et al. 1995b). Some data on invertebrate concentrations was also available for the first three sites. Concentrations in fecal matter were expressed as µg/g Pb wet or dry mass and we converted dry to wet mass by a conversion factor 3.53 (Vanparys et al. 2008). Concentrations of lead in feces in polluted sites ranged from 4 to 43 µg/g (depending on site and species) and 0.5–1.3 µg/g in control areas. We estimated the daily lead dose from concentrations in fecal matter assuming that 10 % of lead in food is absorbed and the rest is excreted in feces (Scheuhammer 1991), and that 70 % of ingested food is digested, and 30 % excreted (Graveland and van Gijzen 1994). On the basis of data from van Balen (1973) we estimated that 8 days old nestlings (c.a. 13 g) would ingest 6.3 g of food and receive Pb on average 2.2–8.5 µg/g body mass daily in polluted and 0.2–0.5 µg/g in control areas. We also calculated total lead ingestion by using data from caterpillars (constitute around 60 % of diet, van Balen 1973). Those were reported to contain 4 µg/g d.w. (Eeva et al. 2005b) and 29 µg/g d.w.

(Dauwe et al. 2004) lead in polluted sites yielding a daily uptake of 0.4–2.8 μg/g body mass in polluted areas and 0.1–0.2 μg/g in control areas. We also searched the avian literature for appropriate and relevant experimental concentrations, but there was huge variation among experiments (ranging from 0.82 to 130 μg/g body mass) and developmental stages, and only few studies clearly reported dose per individual and per body mass unit (Lawler et al. 1991; Youssef et al. 1996; Lurie et al. 2006; Zhong et al. 2010). Based on the available data and our calculations, we decided to give a dose of 4 μg/g body mass as the high treatment as this corresponds to lead exposure in relatively highly polluted sites, but is still what birds are exposed to in the wild. We also wanted to avoid using unrealistically high doses for ethical reasons. We chose to give 1 μg/g as the low level of lead treatment to investigate effects at levels corresponding to those at less polluted sites. The long-term data on daily nestling body mass from Harjavalta was used to calculate a daily ascending dose of lead per nestling. This was given orally as diluted lead acetate solutions (High: 280 μg lead/ml; Low: 67 μg lead/ml; lead II acetate trihydrate, code 316512, Sigma), the daily dose gradually increasing from 60 to 240 μl from d3 to d14.

The experiment was conducted under licenses from the Animal Experiment Committee of the State Provincial Office of Southern Finland (license number ESAVI/846/04.10.03/2011) and the Centre for Economic Development, Transport and the Environment, ELY Centre Southwest Finland (license number VARELY/149/07.01/2011).

## Molecular sexing

Molecular sexing of nestlings was performed by the Center of Evolutionary Applications (University of Turku, Finland). DNA was extracted from dried blood spots on paper cards by salt extraction method modified from (Aljanabi and Martinez 1997). The sex identification was done by amplifying the sex specific CHD-W and CHD-Z genes using primers P2/P8 that produce one fragment in males and two different sized fragments in females (Griffiths et al. 1998). For each individual a single PCR was performed using QIAGEN Multiplex PCR Kit (Qiagen Inc. Valencia, CA, USA) following the manufacturer's instructions with an annealing temperature of 50 °C. Approximately 100 ng of genomic DNA was used as template in the reactions. After the PCR, in order to minimize the number of fragment analyses needed, four samples labeled with different fluorescent dyes (FAM, PET, VIC, NED) were pooled and analyzed together. The fragments were then visualized by capillary electrophoresis in Abi 3130xl Genetic Analyzer (Applied Biosystems). Samples were genotyped with GeneMapper 4.0 (Applied Biosystems). Peak intensity thresholds were set at 2,000 units for homozygotes and 200 units for heterozygotes. Some adults of known sex (n = 6) were used as positive controls for the molecular sexing and these samples were always correctly sexed. In addition, 17 replicate analyses from PCR step onwards were conducted, and these replicates resulted in the same sexes as in the first analyses.

## Metal analyses

Fresh feces (including urine) were collected individually from defecating nestlings on d7 directly to plastic Eppendorf tube, stored in liquid nitrogen and later preserved at −80 °C in laboratory. Fecal and bone samples for metal analyses were later dried at 50 °C for 72 h. Due to relatively costly analyses we did not measure the fecal samples individually but two samples (one male and one female) from the same brood were always combined to assess brood level metal exposure (n = 94 samples from 47 broods). We considered this reasonable since all the nestlings in a brood were treated in similar way. Bones were, however, measured individually. Samples (n = 47 for feces; n = 31 for bones) were digested with a microwave digestion system (Anton Paar Microwave Sample preparation System, Multiwave 3000) in a mixture of Suprapure acids, 5 ml $HNO_3$, 0.5 ml HCl and 3 ml $H_2O_2$. Afterwards the samples were diluted to 50 ml (feces) or 100 ml (bones) with de-ionized water. The determination of metal concentrations (feces: As, Cd, Cu, Ni, Pb; bones: Pb) was done with ICP-MS (Elan 6100 DRC+ from PerkinElmer-Sciex), by using a quantitative standard mode. The detection limit for most of the elements was around 1 ppt (ng/l) and below. The calibration of the instrument was done with a commercial multi-standard from Ultra Scientific, IMS–102, ICP-MS calibration standard 2. Certified reference materials (mussel tissue ERM-CE278 for As, Cd, Cu and Pb in feces; dogfish liver DOLT-4 for Ni in feces; mussel tissue SRM-2976 for Pb in bones) were used for method validation. For feces, the mean recoveries (± SE) in seven reference samples were as follows: As 95 ± 0.84 %, Cd 86 ± 0.53 %, Cu 96 ± 1.62 %, Ni 104 ± 5.47% and Pb 94 ± 4.16 %. The recovery of Pb in bone analysis was 104 ± 0.97 %. The results are expressed as μg/g dry weight.

## Corticosterone metabolites

A subset of fresh fecal samples were determined for their CORT metabolite concentrations (n = 145 samples from 59 broods, 1–5 samples/brood trying to include samples from male and female nestlings when possible). For the steroid analysis we followed the protocol for enzyme immunoassay described in Stöwe et al. (2008). In short, 0.05 g of wet droppings was shaken in a mixture of

methanol (0.3 ml) and distilled water (0.2 ml) for 15 min (Palme et al. 2013). Next, 0.1 ml of this extract were evaporated, sediments dissolved in 0.1 ml Na-acetate buffer with 0.2 µl of β-glucuonidase-arylsulfatase (Merck 1.04114.0002) and hydrolyzed at 38 °C for 18 h. The enzyme immunoassay assay used had been validated for *P. major* adults previously (Carere et al. 2003) and proved to be appropriate also for nestling samples (Stöwe et al. 2010). It shows cross-reactions not only with $C_{19}O_3$ steroids but also with $C_{21}O_4$ metabolites that have a 3α-ol,11-oxo structure, therefore measuring 3α,11oxo-CM (detailed description in Möstl et al. 2002). Samples were assayed in duplicate. Intra-assay variation was 8.1 % and inter-assay 15.4 % for low level pool and 7.8 % for high level pool. The results are given as ng/g w.w. One exceptionally low value (0.55 ng/g) was omitted from the analyses as an outlier.

## Heat shock proteins

Western blot analysis was used to determine the relative amounts of HSP60- and HSP70-proteins in d14 whole blood samples following modified protocol from Tomás et al. (2004). Blood was diluted in 0.9 % NaCl and 6× SDS-sample buffer. Samples were heated 5 min at 95 °C. Protein concentration was determined spectrophotometrically using the Bio-Rad Protein Assay (Bio-Rad Laboratories). Proteins (40 µg/well) were separated on 10 % SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Whatman Protran Nitrocellulose Transfer Membrane). Membranes were blocked for 1 h in 4 % nonfat powdered milk in PBS with 0.3 % Tween 20 at room temperature and incubated with primary antibodies overnight at 4 °C. The primary monoclonal antibodies were anti-HSP60 (clone LK2, Sigma) and anti-HSP70 (clone BRM22, Sigma) diluted 1/1,000 and 1/5,000 in buffer with 1 % bovine serum albumin (BSA) and 0.02 % $NaN_3$ in phosphate buffered saline (PBS). Anti-mouse (W4021, Promega) diluted 1:20,000 in 4 % nonfat powdered milk in PBS with 0.3 % Tween 20 was used as secondary antibody. Membranes were incubated with secondary antibody 1.5 h at room temperature. Three washes with PBS with 0.3 % Tween 20 were performed after each step. The proteins were detected using enhanced chemiluminescence (Pierce ECL Western Blotting Substrate, Thermo Scientific) according to the manufacturer's instructions. The signals were captured on X-ray film, and the relative optical density of protein bands was quantified with ChemiImagerTMReady software (Alpha Innotech Corporation). HSP levels are expressed in arbitrary units indicating the density of the bands. Different blots may show variation so every effort was made to avoid gel-to-gel variation in the present experiments. First, samples from different treatment groups were evenly distributed among several gels, every gel containing samples from most groups. Equal protein loading and transfer was confirmed by staining membranes with Ponceau S. Results were adjusted to detected amount of α-actin protein (clone AC-40, Sigma, dilution 1:5,000), a highly conserved protein which was used for the quantification of the protein amount. The HSP60 and HSP70 levels were calculated dividing the protein band intensity (0 = white, 255 = black) with the α-actin protein band intensity of the same sample. Moreover, every gel contained a control sample, and variation among gels was further controlled by adjusting the results to variation in control sample.

## ALAd

We measured ALAd activities in whole blood (at d14) according to a modified version of the method of Pain (1989). Blood samples (n = 347 samples from 57 broods) were diluted 1:6 with water and kept at 38 °C for 10 min. Diluted blood samples containing 5-aminoleuvulinic acid hydrochlorine (ALA) were incubated for 1 h in darkness at 38 °C. After incubation, trichloroacetic acid (TCA) was added to stop the reaction, and samples were centrifuged at 10,000×g for 10 min. Next, 100 ml Ehrlich's reagent (0.3 g dimethylaminobenzaldehyde, 2.95 ml 70 % perchloric acid made up to 12 ml with glacial acetic acid) was added to wells containing 25 µl samples or reference blanks in triplicate in 384 well plates. The absorbance at 555 nm was determined on a microplate reader after 5 min. Intra-assay variation was <10 %. The protein content was measured with the Bradford method (Bradford 1976). ALAd activities were calculated using $6.2 \times 10^4$/mM/cm as the molar extinction coefficient for porphobilinogen (PBG) and expressed as nmol PBG/h/mg.

## Plumage color

Plumage color was measured spectrophotometrically, following Eeva et al. (2009b). Ten yellow breast feathers (n = 439 individuals from 58 broods) were placed on top of each other and reflectance was measured close to the feather tips in a range 400–700 nm with AvaSpec-2048 spectrometer, AvaLight-DHS halogen light source and FCR-7UV400-2-ME reflection probe. The probe was placed perpendicularly to feather surface by using a black PVC sleeve to keep the distance between probe and feather surface constant (5 mm). Five repeated measurements were taken from all samples by lifting the probe between measurements. As a measure of yellowness we use the value of carotenoid chroma:$(R_{700} - R_{450})/R_{700}$,where R is percentual reflectance (Andersson and Prager 2006). The repeatability of chroma values was calculated according to the

recommendations of Lessells and Boag (1987) by using the repeated chroma measurements in each sample as replicates. The repeatability of chroma values was good, 0.82 (ANOVA: $F_{441,1769} = 10.1$, $p < 0.0001$).

### Statistics

All analyses were performed with SAS statistical software (SAS 2008). We first analysed the differences in Pb levels (feces at d7 and bones at ≥d33) and response variables between treatment groups with general or generalized linear mixed models (GLM or GLMM; Glimmix procedure in SAS). The response variables were: growth rates (change in body mass and wing length between d3 and d14), HT (d7), CORT metabolite level in feces (d7), ALAd activity (d14), HSP60 and HSP70 (d14), carotenoid chroma of plumage (d14) and nestling survival (d3 to d14). Growth rates were calculated for each individual nestling per time interval from the hour of the first measurement (on d3) to the hour of the last measurement (on d14), and they are expressed as g/day (body mass) and mm/day (wing length) in the results. Treatment and sex were used as independent factors and their interaction was also tested, except for brood level variables (fecal Pb and nestling survival) and for bone Pb level due to low sample numbers (3 to 5 per group). Brood was used as a random factor when necessary (to account for non-independency of measurements within broods). Normality of variables was checked from the model residuals and some of the variables were log-transformed to normalize them (i.e. fecal Pb, bone Pb, CORT metabolites, HSPs and ALAd). For haematocrit we used Poisson error distribution. Survival was modelled by using binomial error distribution and events/trial type syntax in the Glimmix procedure of SAS (trials = nestling number at d3, events = nestling number at d14). Pairwise comparisons among treatment groups were made with Tukey's test.

As a second step, we ran linear models by using *brood level* data to explore the effects of measured levels of Pb and other metals on growth and physiological markers. Using brood level data (brood means calculated separately for males and females) was reasonable because, as with faecal Pb levels, measuring multiple physiological parameters was relatively expensive and it was often not possible to measure all nestlings. Furthermore, due to relatively small sample volumes different markers could not always be measured for the same nestlings. In these models we used sex and metal levels as explanatory factors together with some possibly confounding variables, such as brood size (at d3) and hatching date. Log-transformed faecal Pb level was included in the model as such. Since the rest of the metals (As, Cd, Cu, Ni) were strongly positively correlated we calculated the principal components on them. The first principal component (PC1, eigenvalue 3.2) explained 80 % of the variation in data with positive loadings from all metals, describing well the general level of non-Pb metal exposure. Non-significant terms were dropped from all the models one-by-one. They were added again in the final model one-by-one and kept if significant. For all models containing random factors the degrees of freedom were calculated with Kenward-Rogers method. We were also interested in relationships among our response variables (especially between growth parameters and physiological markers), but because in many cases the causality between them is not clear, these associations were explored with Pearson correlations. The significance level was set at $p < 0.05$ in all analyses.

## Results

### Lead in feces and bones

Fecal Pb level at d7 was highest in the High treatment group and intermediate in Harjavalta group as compared to relatively low levels in Low and Control groups (Table 1; Fig. 1). Bone Pb level after d33 showed still more prominent differences among treatments ($F_{3,19.9} = 26.1$, $p < 0.0001$; Fig. 1), but did not differ between males and females ($F_{1,6.50} = 0.01$, $p = 0.94$). While fecal Pb level in High treatment was 3.7 times higher as compared to control, in bones it was 26 times higher, indicating that Pb accumulated in bone tissue over time (Fig. 1). Furthermore, in bones, the Low treatment group showed 5.4 times higher values than the Control group, despite that relatively small and statistically non-significant difference was observed in feces (Fig. 1). Brood mean Pb levels in feces and bones correlated positively ($r_P = 0.47$, $p = 0.032$, $n = 21$). Since Pb levels in Harjavalta and Low groups showed opposite patterns for feces and bones we ran one more model to test a posteriori if there is an interaction between treatment group (Harjavalta vs. Low) and sample type (feces vs. bone). This interaction was significant ($F_{1,16.4} = 13.8$, $p = 0.0018$), suggesting opposite patterns in two sample types.

### Growth and haematocrit

Nestlings in Harjavalta group grew more slowly by body mass (18 %) and wing length (12 %) than in the other treatments (Table 2; Fig 2). Interestingly, wing growth rate was also 7.9 % smaller in High treatment than in Low treatment, the Control group showing intermediate rates (Fig. 2). Females were further gaining body mass and wing length more slowly than males, as expected on the basis of somewhat smaller final size of females in this species

**Table 1** Metal concentration (μg/g, dry weight) in feces of 7 day old *Parus major* nestlings in the four treatment groups

| Metal | High n = 12 | Harjavalta n = 11 | Low n = 12 | Control n = 12 | $F_{ndf,\ ddf}$ | p |
|---|---|---|---|---|---|---|
| Pb | 8.0 (4.8–13.3)a | 4.4 (2.6–7.6)ab | 3.0 (1.8–5.0)b | 2.2 (1.3–3.6)b | $4.97_{3,43}$ | 0.0047 |
| As | 0.40 (0.25–0.62)a | 4.3 (2.7–6.9)b | 0.29 (0.19–0.46)a | 0.60 (0.38–0.95)a | $27.0_{3,43}$ | <0.0001 |
| Cd | 0.73 (0.51–1.04)a | 1.93 (1.34–2.78)b | 0.56 (0.39–0.80)a | 0.54 (0.38–0.76)a | $11.0_{3,43}$ | <0.0001 |
| Cu | 34.6 (27.1–44.1)a | 111 (86–144)b | 25.2 (19.8–32.2)a | 29.6 (23.2–29.6)a | $29.2_{3,43}$ | <0.0001 |
| Ni | 2.24 (1.6–3.1)a | 20.6 (14.5–29.2)b | 1.74 (1.2–2.4)a | 1.99 (1.4–2.8)a | $46.9_{3,43}$ | <0.0001 |

The values are geometric means with 95 % CIs. GLM and Tukey's test: means with the same letter are not significantly different

*N* indicates number of broods

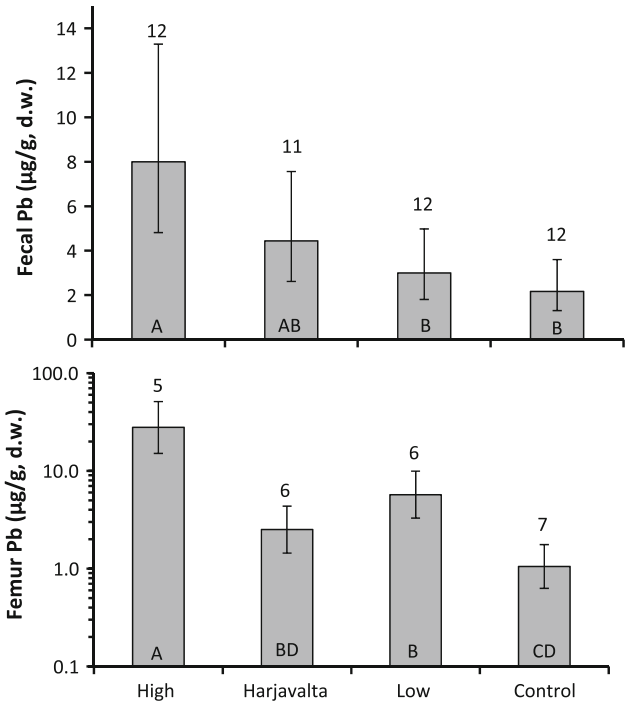



**Fig. 1** The least squares means (±95 % CL) of lead levels in feces (age 7 days) and bones (femurs; age ≥33 days) of *Parus major* nestlings in four treatment groups. *Letters* inside the *bars* denote statistical differences: means with the *same letter* are not statistically different (Tukey's test). The numbers above the *error bars* indicate the numbers of broods

(Table 2). HT at d7 correlates positively with growth rates of mass ($r_P = 0.36$, $p = 0.0061$, $n = 56$) and wing length ($r_P = 0.49$, $p = 0.0001$, $n = 56$) and in general shows similar variation among treatment groups than growth parameters (Fig. 2). There was further a nearly significant interaction between treatment and sex so that the HT of males was especially high in the Low treatment group, corresponding similar but likewise non-significant trend in wing growth rate (Table 2). Fecal Pb levels did not explain growth rates or HT but both growth parameters were negatively associated with the level of the other metals (Table 3). These associations, however, were driven by distinctly higher metal load and slower growth rates in Harjavalta group, and metal level and growth were not significantly correlated within areas (body mass: Ruissalo $r_P = 0.011$, $p = 0.92$, $n = 68$, Harjavalta $r_P = 0.34$, $p = 0.16$, $n = 18$; wing length: Ruissalo $r_P = -0.21$, $p = 0.082$, $n = 68$, Harjavalta $r_P = 0.21$, $p = 0.40$, $n = 18$). Wing growth rate and HT further depended on hatching date, wings growing 20 % faster and HTs being 19 % higher in latest as compared to earliest broods (range 18 days).

## Corticosterone metabolites

The concentrations of CORT metabolites in droppings at d7 showed an interaction between sex and treatment, females showing two times higher levels in the Control group than males while there were no significant differences in the other groups (Table 2; Fig. 2). For both sexes the levels were highest in the Harjavalta group (Table 2; Fig. 2). High stress hormone levels in the polluted area seems to reflect the poor growth of nestlings, since CORT metabolite levels correlate negatively with growth rates of mass ($r_P = -0.32$, $p = 0.015$, $n = 57$) and wing length ($r_P = -0.38$, $p = 0.0041$, $n = 55$) between d3 and d7, though not significantly with HT ($r_P = -0.15$, $p = 0.27$, $n = 58$). CORT level was not associated with fecal Pb levels, but showed a positive association to the other metals (Table 3). Again, however, there was no such association within areas (Ruissalo $r_P = 0.14$, $p = 0.27$, $n = 62$, Harjavalta $r_P = -0.067$, $p = 0.80$, $n = 17$). Amounts of excreted CORT metabolites did not depend on hatching date or brood size (Table 3).

## Heat shock proteins

At d14, HSP60 showed higher values in High treatment as compared to Low and Control treatments, but only for males (Table 2; Fig. 2). Instead, no significant treatment or sex effect was found for HSP70 (Table 2; Fig. 2). HSP70 levels decreased in the course of summer, being 49 %

**Table 2** Test statistics (general or generalized linear models) for variation in lead levels, growth, physiological measures, plumage color and survival of *Parus major* nestlings in the four treatment groups

| Response variable | Treatment | | Sex | | Treatment × sex | |
|---|---|---|---|---|---|---|
| | $F_{df}$ | *p* | $F_{df}$ | *p* | $F_{df}$ | *p* |
| Body mass growth d3–d14 (g/day)[a] | **$13.5_{3,61.6}$** | **<0.0001** | **$47.6_{1,380.8}$** | **<0.0001** | $0.62_{3,381.0}$ | 0.60 |
| Wing length growth d3–d14 (mm/day)[a] | **$18.9_{3,64.4}$** | **<0.0001** | **$12.6_{1,377.2}$** | **0.0004** | $2.19_{3,377.2}$ | 0.089 |
| Hematocrit d7 (%)[b] | **$3.26_{3,59.7}$** | **0.027** | $0.14_{1,377.6}$ | 0.71 | $2.60_{3,379.4}$ | 0.052 |
| CORT metabolites d7 (ng/g, w.w.)[a] | **$4.11_{3,57.3}$** | **0.010** | **$1.23_{1,101.5}$** | **0.27** | **$2.89_{3,103.0}$** | **0.039** |
| HSP60 d14 (arbitrary unit)[a] | **$0.73_{3,37.5}$** | **0.54** | **$0.12_{1,36.8}$** | **0.73** | **$4.35_{3,34.8}$** | **0.0011** |
| HSP70 d14 (arbitrary unit)[a] | **$0.53_{3,36.0}$** | **0.67** | $0.09_{1,42.4}$ | 0.76 | $1.72_{3,36.2}$ | 0.18 |
| ALAd (nmol PBG/h/mg)[a] | $1.28_{3,50.5}$ | 0.29 | **$1.55_{1,348.7}$** | **0.21** | $0.90_{3,343.1}$ | 0.44 |
| Carotenoid chroma d14[a] | **$12.4_{3,56.6}$** | **<0.0001** | $0.00_{1,397.1}$ | 0.98 | $0.71_{3,398.3}$ | 0.54 |
| Survival probability d3–d14[c] | **$6.49_{3,57}$** | **0.0007** | – | – | – | – |

The age of nestlings is indicated as d3 (=3 days), d7 and d14

Terms left in the final model are shown bold

[a] GLMM with normal error distribution and brood as a random factor

[b] GLMM with Poisson error distribution and brood as a random factor

[c] GLM with binomial error distribution

smaller in latest broods than in the earliest ones (range 17 days). Such change was not observed for HSP60. No associations were found between HSPs and metal PC1 or brood size (Table 3). Nor did the HSPs correlate with growth rates between d3 and d14, HT or CORT metabolite levels at d7 (all $p > 0.05$).

### ALAd

No significant differences were found among the treatment groups or sexes in blood ALAd activity at d14 (Table 2; Fig. 2). Nor did we find any association between ALAd level and fecal Pb, PC1 of the other metals, hatching date or brood size (Table 3). ALAd did not either show any correlation with growth rates between d3 and d14 (mass: $r_P = 0.0023$, $p = 0.99$, $n = 56$; wing: $r_P = 0.011$, $p = 0.93$, $n = 56$).

### Plumage color

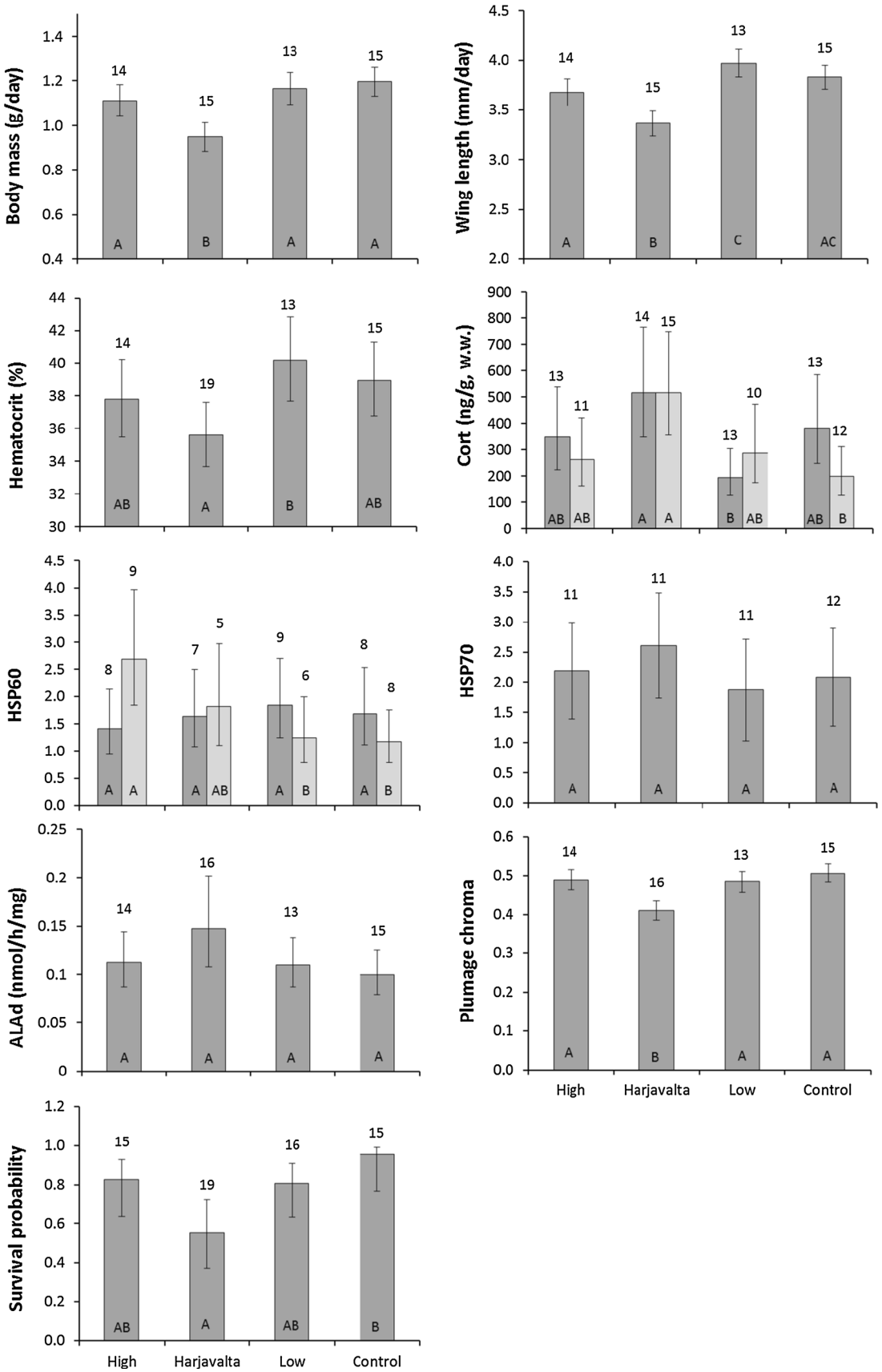
Carotenoid chroma of breast feathers at d14 was 17 % lower in Harjavalta group than in the other groups, nestlings being less yellow in Harjavalta (Table 2; Fig. 2). Chroma showed no association with sex, fecal Pb level, hatching date or brood size (Table 3). It was negatively associated with PC1 of metals, though again this association was mainly driven by Harjavalta study area, no association being found within birds in Ruissalo ($r_P = 0.065$, $p = 0.60$, $n = 68$) and negative but non-significant association taking place in Harjavalta ($r_P = -0.41$, $p = 0.074$, $n = 20$). Plumage chroma correlated positively with growth rates of mass ($r_P = 0.47$, $p = 0.0002$, $n = 56$) and wing length ($r_P = 0.28$, $p = 0.034$, $n = 56$) between d3 and d14.

### Nestling survival

Nestling survival between d3 and d14 was 42 % lower in the Harjavalta group than in the Control group, the two lead treated groups showing intermediate values, though not significantly different from the Control (Table 2; Fig. 2). Survival probability was not dependent on sex, hatching date or fecal Pb level at d7, but it was generally higher in initially large broods (Table 3). However, the latter was not true within Harjavalta group where initially large broods suffered higher mortality (survival vs. brood size at d3: $r_s = -0.63$, $p = 0.012$, $n = 15$). Nestling survival decreased with increasing PC1 of metals (Table 3; Fig. 3). There was, however, no significant correlations between survival and PC1 of metals within sites (Ruissalo: $r_P = -0.26$, $p = 0.12$, $n = 36$; Harjavalta: $r_P = -0.13$, $p = 0.70$, $n = 11$).

## Discussion

Our Pb supplementation was successful in raising the fecal concentrations to the levels that have been measured in relatively heavily polluted environments. To facilitate comparison of our experimental levels to values published elsewhere we present here arithmetic means (c.f. Table 1). The mean Pb level in the fecal samples of High treatment


Body mass (g/day)
Wing length (mm/day)
Hematocrit (%)
Cort (ng/g, w.w.)
HSP60
HSP70
ALAd (nmol/h/mg)
Plumage chroma
Survival probability
High
Harjavalta
Low
Control

◀**Fig. 2** The least squares means (±95 % CL) of growth rates (body mass, wing length), physiological measures (hematocrit, fecal corticosterone metabolites, heat shock proteins 60 and 70 and delta-aminolevulinic acid dehydratase), plumage color and survival (from d3 to d14) of *Parus major* nestlings in four treatment groups. Estimates are based on the final models presented in Table 1. *Letters* inside the *bars* denote statistical differences: means with the *same letter* are not statistically different (Tukey's test). The numbers above the *error bars* indicate the numbers of broods

group (10 µg/g, d.w.) was higher than in the polluted area of Harjavalta (7.0 µg/g, d.w.), but still below what has been measured for *P. major* nestlings in Harjavalta in early 1990s (28 µg/g, d.w.; Eeva and Lehikoinen 1996), at a copper smelter in Russia (26 µg/g, d.w.; Belskii et al. 1995b), and at a lead smelter in Belgium (61 µg/g, d.w.; Dauwe et al. 2004). Unlike the levels in feces at d7, the Pb concentration in femurs is indicative of accumulation of lead over the whole 12 day exposure period and showed relatively high values in the High treatment group (31 µg/g, d.w.), exceeding the levels in *P. major* nestlings at Harjavalta smelter in early 1990s (femur: 9.5 µg/g, d.w.; Eeva and Lehikoinen 2000) and levels at a copper smelter in Russia (skeleton: 21 µg/g, d.w.; Belskii et al. 1995a), but being lower than those measured for *F. hypoleuca* nestlings at Rönnskär copper smelter (sternum: 85 µg/g, d.w.; Berglund and Nyholm 2011). Our experimental exposure is therefore relevant as regards to the levels found at Pb polluted sites.

Interestingly, nestlings in the Low treatment group tended to show lower fecal concentrations than those at Harjavalta smelter, but bone concentrations were still higher in the Low group (a significant interaction between sample type and treatment). At first this was surprising considering that nestlings at the smelter received polluted food continuously while the supplemental Pb was dosed just once a day. A likely explanation for the opposite patterns between sample types is that Pb is more efficiently absorbed from Pb acetate than from invertebrates containing biologically incorporated lead (see Custer et al. 1984). Food items (e.g. caterpillars, spiders and beetles) contain chitinous parts where Pb is incorporated (Hare 1992; Borowska et al. 2004) but which are not fully digested by *P. major* nestlings, though they are dissolved when Pb is measured in the laboratory. Since fecal samples were taken one day after the previous oral lead exposure, and food retention times are relatively short (few hours) in

**Table 3** Test statistics (general or generalized linear mixed models) for variation in growth, physiological measures, plumage color and survival of *Parus major* nestlings

| Response variable | Sex | | Fecal Pb | | Sex × Pb | | Metals (PC1) | | Hatching date | | Brood size | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $F_{df}$ | p | $F_{df}$ | *p* | $F_{df}$ | p | $F_{df}$ | p | $F_{df}$ | *p* | $F_{df}$ | *p* |
| Body mass growth d3–d14 (g/day)[a] | **$19.2_{1,39.0}$** | **<0.0001** | $1.03_{1,40.3}$ | 0.32 | $2.68_{1,37.6}$ | 0.11 | **$10.6_{1,43.2}$** | **0.0022** | $1.00_{1,41.7}$ | 0.32 | $1.71_{1,41.8}$ | 0.20 |
| Wing length growth d3–d14 (mm/day)[a] | **$12.5_{1,39.0}$** | **0.0011** | $0.00_{1,40.4}$ | 0.96 | $2.96_{1,37.7}$ | 0.094 | **$10.0_{1,42.2}$** | **0.0028** | **$17.0_{1,42.0}$** | **0.0002** | $0.85_{1,41.9}$ | 0.36 |
| Hematocrit d7 (%)[b] | $0.14_{1,82.0}$ | 0.71 | $0.46_{1,41.0}$ | 0.50 | $0.00_{1,81.0}$ | 0.95 | $3.84_{1,45.2}$ | 0.056 | **$18.4_{1,61.5}$** | **<0.0001** | $1.92_{1,43.6}$ | 0.17 |
| CORT metabolites d7 (ng/g, w.w.)[a] | $1.38_{1,38.1}$ | 0.25 | $0.47_{1,42.8}$ | 0.50 | $0.77_{1,40.3}$ | 0.38 | **$5.89_{1,44.8}$** | **0.019** | $0.39_{1,38.9}$ | 0.54 | $0.55_{1,43.4}$ | 0.46 |
| HSP60 d14 (arbitrary unit)[a] | **$2.97_{1,28.6}$** | **0.096** | **$0.04_{1,29.0}$** | **0.85** | **$5.53_{1,28.1}$** | **0.026** | $0.20_{1,32.4}$ | 0.66 | $0.84_{1,32.0}$ | 0.37 | $0.80_{1,22.7}$ | 0.38 |
| HSP70 d14 (arbitrary unit)[a] | $0.33_{1,34.4}$ | 0.57 | $0.09_{1,28.6}$ | 0.77 | $2.88_{1,29.1}$ | 0.10 | $2.00_{1,32.6}$ | 0.17 | **$4.75_{1,34.5}$** | **0.036** | $0.11_{1,23.9}$ | 0.74 |
| ALAd (nmol PBG/h/mg)[a] | $1.48_{1,45.2}$ | 0.23 | $1.42_{1,43.0}$ | 0.24 | $2.04_{1,41.4}$ | 0.16 | $1.17_{1,47.4}$ | 0.28 | $0.06_{1,41.7}$ | 0.81 | $0.02_{1,45.5}$ | 0.88 |
| Carotenoid chroma d14[a] | $0.57_{1,40.8}$ | 0.46 | $0.48_{1,43.0}$ | 0.49 | $0.03_{1,39.0}$ | 0.87 | **$18.2_{1,44.4}$** | **0.0001** | $0.00_{1,41.4}$ | 0.99 | $0.02_{1,43.0}$ | 0.89 |
| Survival probability d3–d14[c] | $0.57_{1,84.0}$ | 0.45 | $0.00_{1,51.5}$ | 0.97 | $0.00_{1,81.0}$ | 0.97 | **$4.32_{1,45.4}$** | **0.043** | $0.05_{1,37.8}$ | 0.83 | **$5.34_{1,59.8}$** | **0.024** |

Brood means (calculated separately for males and females) are explained by fecal Pb concentration, 1st principal component of the other metals (As, Cd, Cu, Ni) in feces, hatching date and brood size in the beginning of the experiment

The age of nestlings is indicated as d3 (=3 days), d7 and d14. Terms left in the final model are shown bold

Sample sizes vary according to the model

[a] GLMM with normal error distribution and brood as a random factor

[b] GLMM with Poisson error distribution and brood as a random factor

[c] GLMM with binomial error distribution and brood as a random factor

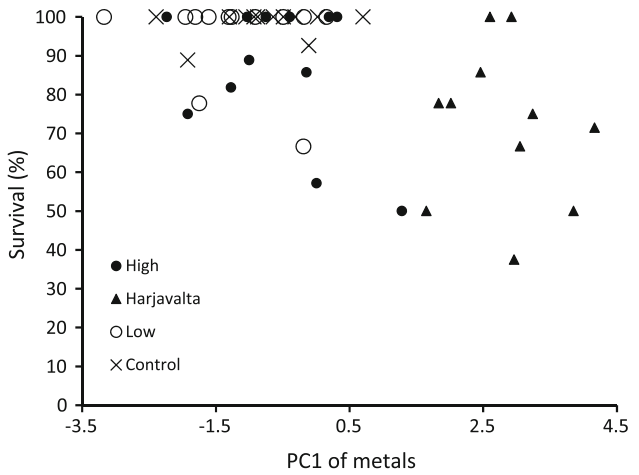



**Fig. 3** Proportion (%) of nestling surviving from day 3 to 14 as a function of metal levels (1st principal component of fecal As, Cd, Cu and Ni) in their feces at day 7. The four Pb treatment groups shown in different symbols. N = 47 broods

nestlings of passerine birds (Brzek et al. 2009), it is also likely that a great deal of Pb measured in feces comes from the body by active excretion to feces/urine (note that bird droppings include urine).

Lead treatment had no effect on the growth rate of body mass, nestlings growing relatively well in all three experimental groups in Ruissalo. Wing growth rate, however, was higher in the Low Pb group as compared to the High Pb group. Since the control group showed intermediate growth rate the result could mean that small amount of lead would stimulate growth while higher exposure would retard it, though these effects were relatively small in our experiment. Growth inhibition has been considered as one of the primary signals of lead toxicity in mammals (Edens et al. 1976). High experimental lead doses have been found to retard growth rates also in birds (Edens et al. 1976; Hoffman et al. 1985a), but studies in wild birds exposed to environmental Pb levels generally have shown no or only weak effects (Grue et al. 1984; Grue et al. 1986; Janssens et al. 2003a). Lead exposure retarded growth of bones in kestrels (Hoffman et al. 1985a), which is a well conceivable effect since Pb is known to accumulate especially in bone tissue (Scheuhammer 1991; Dauwe et al. 2005). Furthermore, epidemiological studies in humans have shown an inverse relationship between Pb level and bone growth, though the exact mechanism is not well understood (Berglund et al. 2000). The length of bird wing is a combination of growing limb (bones) and feathers, the development of both being dependent on minerals such as Ca (Tilgar et al. 2004; Dawson and Bidwell 2005). Lead is known to interfere with Ca metabolism by competing with calcium binding sites in body (Goyer 1997). On the other hand, studies in humans and rats suggest that Pb may stimulate DNA synthesis by mimicking the action of Ca in activating protein kinases (Goyer 1997; Lu et al. 2001). The Pb levels in our experiment were obviously too low to have major negative effects on growth but the results suggests that low levels may stimulate growth. However, since the differences were relatively small, and we could not demonstrate a direct association between fecal Pb levels and wing growth, the latter possibility should be confirmed with further studies.

Nestlings grew markedly slower near the Harjavalta copper smelter than in Ruissalo. This difference could be because of higher total metal load in the polluted area of Harjavalta (Table 1), because of food limitation for birds in Harjavalta (Eeva et al. 2005b), or for a combined effect. Though metal levels in Harjavalta are markedly higher than in Ruissalo, single-element concentrations in Harjavalta are currently below critical levels associated with subclinical effects (Berglund et al. 2012), and various physiological markers have shown poor correlations with individual metal levels, suggesting that in general toxic levels do not exceed (Eeva et al. 2000, 2003, 2005a; Koivula et al. 2011; Eeva et al. 2009b). Therefore we consider direct toxic effects unlikely though element-specific threshold levels, and their possible interactions, should be experimentally studied.

At Harjavalta smelter, reduced growth rates and higher nestling mortality has been reported regularly for *P. major* during the past two decades, together with associated decrease in abundance of some important food items like caterpillar larvae (Eeva and Lehikoinen 1996; Eeva et al. 1998, 2009b). Though food availability was not assessed in our Pb manipulation experiment we presume that it explains most of the differences in growth, condition and fitness variables between the two sites. Reduced food quantity or quality in Harjavalta would explain slower growth rates, lower HT (see also Laaksonen et al. 2004), higher CORT metabolite levels (see also Loiseau et al. 2008) and lower survival probabilities of nestlings. Lower availability of carotenoid-rich caterpillars would further explain less yellow plumage color of nestlings, as found in the Harjavalta population in previous studies (e.g. Eeva et al. 1998). The primary yellow carotenoid pigment in great tit feathers is lutein, which moves unmodified in the food chain from tree leaves to herbivorous caterpillars and to bird feathers (Partali et al. 1985; Sillanpää et al. 2008). Experimental studies on carotenoids have shown that primary reason for pollution-related variation in plumage color is carotenoid deficiency in diet, not metal-related disturbance of carotenoid metabolism (Eeva et al. 2008). Furthermore, despite relatively high variation in fecal metal levels (PC1) among broods we found no direct associations with metal level and growth, HT, plumage color or survival within sites. Harjavalta group shows

further one basic difference from the others: in Harjavalta not just nestlings but also their parents are exposed. In theory, this could produce some behavioral differences e.g. affecting nestling feeding rates and having negative consequences for the offspring. Feeding rates, however, were not found to be reduced in the polluted area and we consider inferior food quality as more likely explanation (Eeva et al. 2005b).

Of the used physiological markers only HSP60 responded to Pb exposure, showing increased values for heavily Pb exposed male nestlings. In males of High Pb group, HSP60 levels were more than double to those in the control group and almost double to the level of females in the High Pb group. HSPs are considered as good biomarkers for detecting early exposure to metals (Bauman et al. 1993) and induction of both HSP60 and HSP70 by metal have been found in laboratory and field e.g. in earthworms (Mariño et al. 1999). However, they have rarely been used in studies of avian ecotoxicology and we are aware of only one study using HSPs as biomarkers of metal pollution, showing no HSP70 induction in nestlings of *F. hypoleuca* and *P. major* near the copper smelter in Harjavalta (Eeva et al. 2000). We did not either find associations between growth and levels of HSPs, though poor growth have been associated with increased HSP60 levels in *F. hypoleuca* nestlings (Moreno et al. 2002). Higher HSP60 levels in male nestlings of High Pb treatment could be a response to higher Pb accumulation, e.g. due to their slightly higher growth rates. Bone Pb levels, however, did not differ between sexes and are therefore unlikely to explain the gender difference in HSP60 response though it should be recalled that our sample size for bone concentration was too small to detect small differences in Pb levels. Higher internal metal concentrations have been found in adult males of Parids in winter (Hogstad 2001; Deng et al. 2007) but we are not aware of any comparative studies in nestlings. In our study, the reason for gender specific response of HSP60 to Pb exposure remains therefore open. In general, this and many other aspects between avian HSP response and metal exposure are poorly known and call for further studies, for example to evaluate threshold metal levels for stress protein induction in birds. On the basis of our results HSP70 is not a good biomarker for Pb exposure at the levels in our experiment, but being associated with hatching date it likely responds to some temporal changes in growing conditions, such as ambient food availability or temperature. We did not, however, find direct correlations between growth rates and HSP levels. The HSP levels of *P. major* nestlings did not correlate with fecal CORT metabolite levels though such correlation has been found in adult *F. hypoleuca* males (Lobato et al. 2010). In our study, however, CORT metabolites were measured at d7 and HSPs at d14, and they were analyzed at brood level, both factors likely diminishing the association between the two stress parameters, although stress responses of nestlings likely differ from adults as well.

Against our expectation ALAd levels of *P. major* nestlings were not suppressed by Pb exposure, although this enzyme is considered a sensitive and specific biomarker for Pb. A marked suppression of ALAd activity (<20 % of control) was found in *P. major* adults near a smelter in Belgium where Pb levels in *P. major* livers were ten times higher (11 μg/g d.w.; Vanparys et al. 2008) than at Harjavalta smelter (1.1 μg/g d.w.; Berglund et al. 2011). Similarly, decreased ALAd activities (c.a. 60 % from control) were observed in *F. hypoleuca* nestlings at a lead mine in Sweden, again with much higher liver Pb concentrations (15 μg/g d.w., using w.w.–d.w. conversion factor of 3.53; Berglund et al. 2010) than in Harjavalta (0.9 μg/g d.w. for *F. hypoleuca* nestlings; Berglund et al. 2011). Vanparys et al. (2008) further reported blood Pb concentrations of 2.3 μg/g d.w. for *P. major* adults in a metal polluted environment and found that lowest Pb concentration in blood corresponding with obvious ALAd inhibition was 0.13 μg/g d.w. Henny et al. (1994) reported 55 % ALAd depression in nestling American kestrels (*Falco sparverius*) accompanied by lower hematocrits at a mining site where blood lead was 1.6 μg/g d.w. (using w.w. to d.w. conversion factor of 6.66). Scheuhammer (1989) concluded that blood Pb concentrations below 0.66 μg/g d.w. (using w.w.–d.w. conversion factor of 6.66) do not typically cause ALAd inhibition in wild birds. Because of many physiological measures and limited sample volumes we could not include blood metal analyses in our study protocol but four blood samples (whole blood) from the High Pb group at d14 were still measured for their Pb levels, giving an average (±SD) of 0.42 ± 0.12 μg/g d.w. Therefore it seems likely that the highest Pb levels in our manipulation study were at a boundary of negative effects while the majority of levels were below that. A failure of relationship between ALAd activity and Pb exposure in our study is therefore likely due to relatively low exposure levels as compared to some other studies showing negative relationships. In accordance with this and the responses of Pb exposed passerine nestlings in some other studies (Grue et al. 1984; Fair and Myers 2002) we found no effect of Pb on haematocrit levels, though reduced HTs have been reported in Pb exposed birds (Hoffman et al. 1985b; Grue et al. 1986).

## Conclusions

Taken together, despite relatively high range of exposure levels in our Pb supplementation experiment we found only few effects on growth rates or physiology of *P. major* nestlings. The lack of ALAd inhibition in erythrocytes suggests that the circulating Pb levels were generally below

the toxic level despite that marked accumulation of Pb in bone tissue was observed. Birds in the metal polluted environment around the copper smelter, instead, showed decreased growth rates, lower HT levels, higher CORT metabolite levels, less colorful plumage and lower survival probabilities. These effects are most likely related to decreased food quality/quantity for these insectivorous birds at the smelter site. In general, the responses of nestlings to metal exposure and/or associated resource limitation were not gender specific. One of the heat shock proteins (HSP60), however, was more strongly induced in Pb exposed males and further studies are needed to explore if this was due to higher accumulation of Pb or higher sensitivity of males. In all, our results emphasize the importance of secondary pollution effects (e.g. via food chain disruption) on reproductive output of birds.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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