No delayed behavioral and phenotypic responses to experimental early-life lead exposure in great tits (Parus major)

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Received: 30 March 2014 / Accepted: 20 August 2014 / Published online: 7 September 2014
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Abstract Early-life exposure to pollutants, such as lead, may have long-lasting consequences on health, behavior, and cognition. However, experiments on delayed effects of specific pollutants are very rare in wild animals. We experimentally exposed wild nestling great tits (Parus major) to dietary lead (high, low, or control group) in levels relevant to exposure levels of wild populations in Europe and studied delayed effects on phenotypic and behavioral traits in captivity. We also included a group of birds from a vicinity of a copper smelter, exposed to a mixture of toxic metals and altered food supply during development. This experimental setup allowed us to compare the strength of direct (exposure to lead per se) and indirect (pollution-related changes in diet) effects of pollutants. Our experimental lead treatment significantly increased lead levels in bone and feces compared with controls. However, we found no carry-over effect of early-life dietary lead on morphology, plumage coloration, or heat shock proteins. Treatment did not affect activity, exploration, neophobia, or success in learning and spatial memory task. We conclude that with the exposure levels and relatively short exposure period used, delayed effects on the measured traits were not found. However, it is important to further study other types of behavioral traits and ultimately fitness effects.

Keywords Heavy metal · Carry-over effect · Developmental stress · Behavioral changes · Bird ecotoxicology

Introduction

Intensive anthropogenic use of metals has resulted in a significant metal contamination globally. One major pollutant is lead (Pb), which has been found to cause both acute toxicity and delayed detrimental effects on health, especially on the nervous system, even at very low levels both in human and animal models (Goyer 1997; White et al. 2007). Pb toxicity is caused by Pb having similar properties to calcium, competing for binding sites, and being transported and stored similarly (Barton et al. 1978; Garza et al. 2006; Goyer 1997; Scheuhammer 1987). Pb can be stored in bone (a half life of decades, Needlemann 2009) and released over time, thus early-life exposure can create a body burden that can have long-lasting carry-over effects. Young animals are known to be more sensitive to detrimental effects of Pb and other pollutants than adults for example due to their underdeveloped digestion, and especially underdeveloped blood–brain barrier (Burger and Gochfeld 2000; Dietert and Piepenbrink 2006; Domingo 1994; Scheuhammer 1987). In the central nervous system, Pb disturbs synaptic and receptor functions and impairs synaptic growth (Bressler and Goldstein 1991; Gilbert et al. 2005; for birds, see: Muller et al. 2008).

In humans and captive animal models, low Pb exposure levels during early life have well-documented detrimental effects on cognitive abilities and altered behavior that persist into adulthood (e.g., impaired learning, memory, increased aggression, hyperactivity, and, Brown et al. 1971; Chen et al. 2012; Chiodo et al. 2007; Morgan et al. 2000; Needlemann 2009). However birds, especially in the wild, are little studied. The few existing studies in birds document multiple negative effects of early-life Pb exposure on behavior.
Eens 2008) and mercury pollution increased brightness in found larger in polluted sites than in control sites (Dauwe and 2014). Indeed, the black breast stripe of great tits has been high doses): (1) "high" group corresponds to levels measured close to active pollutant sources in Europe, (2) "low" group to moderate levels, and (3) control group, which was not exposed to Pb. The experiment was performed in a great tit population with "naturally" low exposure levels (Eeva et al. 2012). We also included a fourth group: a great tit population breeding in the vicinity of a nonferrous smelter in Harjavalta, where birds are not only exposed to moderate levels of Pb and other metals but also pollution-related decrease in food quality and quantity, i.e., indirect, secondary effects of pollution (Eeva et al. 2005). We can then compare how strong are the direct effects of Pb (experimental Pb dosing) in relation to a situation where both direct and indirect effects (metal exposure/resource limitation) occur—this comparison is not possible with correlative data. Both females and males were included in this study in order to explore the potential sex-specific responses to Pb exposure: gender-specific differences in metal accumulation have been acknowledged, but effects have rarely studied in ecological context (Burger 2007).

We predicted that birds from high Pb treatment and Harjavalta smelter would grow smaller and have higher heat shock protein levels (indicators of long-term stress) than controls. We further predicted potentially larger melanin ornaments in high Pb treatment and Harjavalta compared with controls, either due to the effect of early-life nutritional stress or Pb directly. For behavioral traits, based on data from previous studies (see above), we predicted that birds exposed to high levels of Pb during development and from Harjavalta would show lower activity and exploration behavior and lower performance in learning and memory test as juveniles, due to detrimental effects of metals on brain development.

Methods

General experimental setup

The experiment was conducted in Ruissalo, Turku (60° 26′ N, 22° 10′ E) and Harjavalta (61° 20′ N, 22° 10′ E), southwestern Finland. 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. In March 2011, we settled ca. 200 nest boxes in the study area in Turku; in Harjavalta, 260 nest boxes were already present. We regularly monitored P. major nests to obtain some basic breeding parameters, including 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 Pb (n=15), low Pb (n=16), or control treatment (n=15). Thus, all chicks in one nest received the same treatment. Pb treatment was executed by dosing distilled water with Pb acetate (treatment) or distilled water only (control) orally every day for 12 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 metals (e.g., arsenic, cadmium, copper, nickel, and Pb) (Kiikkilä 2003). In those nests, chicks were also dosed with distilled water from d3 to d14. As we specifically wanted to study the delayed effects of Pb exposure during important early-life developmental period, we decided to expose birds during posthatching development, which is a critical developmental period in altricial birds. On d3, we weighed the chicks and collected a blood sample for molecular sexing (see details in Eeva et al. 2014) and a fecal sample for noninvasive analyses of Pb levels on d7.

At the age of 15 days (1 day after cessation of Pb treatment), i.e., shortly before fledging (16–20 days), two chicks, a female and a male (using molecular sexing from 7-day samples) from approximately eight nests per treatment were brought to captivity to aviaries located in Ruissalo. These were arranged into eight foster nests of four to ten nestlings, each foster nest containing nestlings of same (±1 day) age and multiple treatments. Brood size of the foster nest in captivity was originally planned to be balanced, i.e., eight nestlings per nest, but it was logistically not possible as sometimes there were not enough nests with nestlings of similar age. We caught foster parents for these captive broods from randomly chosen treatment nests in Ruissalo. Other nestlings from foster parents’ original nests were distributed to other nests with similar stage around the study site. The foster parents then fed the foster nestlings in captivity and were released after the young could forage independently (at the age of ca. 40 days). Each foster nest was kept in an aviary of size 2 m × 2 m × 2.5 m. Temperature was +15 °C during the day and +10 °C during the night. Light was provided from two full-spectrum fluorescent tubes per aviary with natural photoperiod. Aviaries contained perches to sit, some fresh branches for enrichment and fresh water ad libitum. Birds were fed daily (ad libitum) with live food (mealworms and fly larvae) and a mixture of egg yolk, peanuts, breadcrumbs, oat, fresh apple, calcium, and vitamins. Unfortunately, some of the captive birds died (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; and 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; the bone levels can be used as a measure of cumulative exposure (Dauwe et al. 2005; Scheuhammer 1991). Therefore, the femurs of dead birds (n=31) were dissected and dried in laboratory for Pb analysis. Note that birds were not given Pb after d14. Sick birds were held in quarantine and were not used in behavioral tests. Birds in good condition were released to the wild in September. The experiments were 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 Environmental Center of Southwestern Finland (license number VARELY/149/07.01/2011).

Lead exposure

Detailed description on the choice of Pb exposure dose is presented in Eeva et al. (2014). Briefly, 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, including Harjavalta, Finland (Eeva and Lehikoinen 1996; Eeva et al. 2005, 2009), Antwerp, Belgium (Dauwe et al. 2000, 2004), Rönnskär, Sweden (Berglund et al. 2010; Nyholm 1994), and Revda, Russia (Belskii et al. 1995). Concentrations of Pb 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. This corresponds to a Pb dose of 2.2–8.5 μg/g body mass daily in polluted and 0.2–0.5 μg/g in control areas. Calculating daily Pb dose from food Pb content, we estimated 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 large variation among experiments (ranging from 0.8 to 130 μg/g body mass) and developmental stages (Lawler et al. 1991; Lurie et al. 2006; Youssef et al. 1996; 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 Pb 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 Pb treatment to investigate effects at levels corresponding to those in less-polluted sites. The long-term data on daily nestling body mass from Harjavalta was used to calculate a daily ascending dose of Pb per nestling. Pb dose was given orally as diluted Pb acetate solutions (high, 280 μg Pb/ml; low, 67 μg Pb/ml; Pb II acetate trihydrate, code 316512, Sigma), the dose gradually increases from 60 to 240 μl from d3 to d14.

Measurements

Metal measurements in fecal matter and bone using ICP-MS

Fecal samples were collected from 7-day-old nestlings (two nestlings per brood if possible; one male and one female, n=65 samples, including only nestlings reared in captivity). Bone tissue samples (n=31) were collected from the birds that died (see above) in captivity. After drying (at 50 °C for 72 h),
samples were digested with a microwave digestion system (Anton Paar Microwave Sample preparation System, Multiwave 3000) and diluted to 50 ml (feces) or 100 ml (bones) with de-ionized water. Instrument calibration was performed using a commercial multistandard from Ultra Scientific, IMS-102, ICP-MS calibration standard 2, and the samples were prepared according to the method instructions. The concentrations of Pb were determined with ICP-MS (Elan 6100 DRC, PerkinElmer-Sciex, USA) with a detection limit of 1 ng/l. Certified mussel tissues (ERM-CE278 for Pb in feces and SRM-2976 for Pb in bones) were used for method validation. The mean recovery (±SE) in five reference samples for Pb in feces was 94.0±4.16 %, and the recovery of Pb in bones was 104±0.97 %. The results are expressed as micrograms per gram of dry weight.

**Morphology and plumage measurements**

At the age of ca. 40 days (mean, 39.3 days; SD, 4.0), all birds were measured for their body mass (~0.1 g), tarsus length (~0.1 mm), and wing length (~1 mm). A blood sample was taken (~0.1 ml) from the brachial vein for HSP measurements (see below). From each bird, we took a digital photo of the ventral side for plumage trait measurements, following Figuerola and Senar (2000): the bird was placed on its back against grey background, beak stretched with millimeter scale at the background. The size of their black bib was measured against grey background, beak stretched with millimeter scale at the background. The size of their black bib was measured by manual selection of the edges (program Image J), always by one person. Postjuvenile molt starts at the age of ca. 50 days in great tits: we did not find intensively molting individuals, and thus our plumage measurements represent juvenile plumage.

**Heat shock proteins**

Western blot analysis was used to determine the relative amounts of HSP60 and HSP70 proteins in 40-day whole blood samples (see above) following modified protocol from Tomás et al. (2004). The analysis is detailed in Eeva et al. (2014). Briefly, blood was diluted in 0.9 % NaCl and six times SDS sample buffer and heated at 95 °C. Protein concentration was determined spectrophotometrically using the Bio-Rad Protein Assay (Bio-Rad Laboratories). Proteins were separated on 10 % SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were blocked in powdered milk in PBS and incubated with primary antibodies (diluted 1/1,000 and 1/5,000, anti-HSP60: clone 6H4, Sigma and anti-HSP70: clone BRM22, Sigma). Secondary antibody was anti-mouse (W4021, Promega) diluted 1:20,000. The proteins were detected using enhanced chemiluminescence, and signals were captured on X-ray film. The relative optical density of protein bands was quantified with ChemiImager™ Ready software. 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 (dilution 1:5,000, clone AC-40, Sigma), 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 and 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.

**Behavioral measurements**

**Novel environment test**

The novel environment test was conducted to measure exploration and activity by individual birds in a novel environment. The test was conducted when birds were ca. 1.5 months old. We followed a slightly modified protocol from Verbeek (1994). The experiment was conducted in an observation room 4 m×2 m×2.5 m in size with five artificial wooden trees (1.5 m height, with four 20-cm-long cylindrical branches placed on opposite sides of the trunk). One bird at a time was captured from the aviary or cage and weighed. It was allowed to calm down from the stress of capture in a cloth bag for ca. 30 min, and after it was released to the novel room the test started immediately. The birds' behavior was recorded with video camera and observed for 10 min. We recorded the time and duration of every flight or hop the bird made. In contrast to the previous results in great tits (see, e.g., Verbeek et al. 1994), only few birds visited all five trees; many explored the room very little during the 10-min period. Thus, we used a categorical variable, (1) exploring/not exploring the room and (2) the sum of all hops and flights during the 10-min period in the subsequent analyses (hereafter “activity in the novel environment”). Experiments were conducted between 8 a.m. and 6 p.m., and time of the day was controlled in the analyses.

**Novel object test**

The novel object test was conducted to measure the boldness of individuals with regard to novel objects, i.e., neophobia and neophilia, and the activity in the presence of a novel object. The test was conducted at the age of ca. 2 months. The test took place in individual cages (size, 35×70×60 cm) where each bird was transferred at least 1 day before testing. The cages were covered on two sides as well as on the top and bottom, so that the birds had no visual contact with each other.
One bird was tested at a time. The novel object was a wooden tray (ca. 9×20 cm; see Fig. 1), placed on the bottom of the cage in the middle part, standing on 10-cm poles. It contained in total 28 holes (10 mm deep, 15 mm in diameter) in four rows, and in addition to each row, there was a string of green Velcro (glued to the tray). Attached to the Velcro, there were 14 small red felt flaps, which covered half of the holes in the tray. Under each flap, there was a mealworm and additional seven mealworms were placed in uncovered holes. The behavior of the bird was recorded with a video camera for 30 min after the object was introduced. To quantify the bird’s reaction, we calculated: (1) latency of the bird to land on the tray and (2) total number of hops and flights during the test period (converted to hops and flights/minute, hereafter “activity in the novel object test”). Experiments were conducted between 12 and 4 p.m.

**Learning and spatial memory test**

The effect of early-life exposure to Pb on learning and spatial memory was tested following slightly modified protocols from Hodgson and Healy (2005) and Arnold et al. (2007) where a food tray was used. Only spatial cues (location of the reward) were tested. The training and learning tasks were performed at the age of ca. 2–3 months. First, birds were trained to find food (dead mealworms) hidden within an array of 28 wells in a wooden tray (see description above; Fig. 1). The bird first foraged for mealworms in the tray and then learnt to pull back red flaps of felt (all flaps were of similar color and form) covering the wells in order to access mealworms hidden underneath in the following protocol (see timeline and contents of the tray in Fig. 1): first, half of the holes in a tray were covered with flaps and a hidden mealworm and seven of the uncovered holes also contained a mealworm. After eating all visible mealworms, the tray was replaced, and only two worms were placed visible and half of the covered holes were filled. After eating all hidden mealworms in this trial, food was only inserted under the flaps (i.e., hidden), and we gradually reduced the number of flaps from 14 flaps and 7 hidden mealworms to 7 flaps and 1 mealworm (three setups, each presented twice). The training took ca. 3 h every day (in the afternoon), and success was checked every 30 min. If bird had completed the task (i.e., eaten all mealworms under the flaps) in 30 min, the next task was given. If the task was not completed, birds were checked in 30-min intervals until the task was completed. When the birds had successfully found the one worm under the seven flaps in the last training trial, it was considered successfully trained. Training was generally stopped after eighteen 30-min periods if birds was not interested in the task at all, and thus then it was considered not learning the task (n=5). An exception was three birds, which were interested but took more than 20 training periods to complete training.

On the next day after successfully completing training, memory test was conducted as follows: after 60 min of food deprivation, the tray (see above) was placed in the cage (trial 1). Of the 28 wells, seven were covered with red felt flaps and mealworm was hidden under one flap. The bird was allowed to investigate for up to 15 min or until it removed the flap covering the food. After 5 min, during which the flaps and food were replaced in their original positions, the tray was returned to the cage (trial 2). A record was made of all incorrect flaps lifted, and the trial lasted 15 min or until the bird found the worm. After trial 2, the same setup of flaps and worms was presented once more (trial 3). On the next day, the same three trials were conducted with a different spatial arrangement of flaps and food. We defined three measures of learning: (1) general learning ability: whether or not the bird learnt to remove flaps to find food and was successfully trained (passed the last training session); (2) number of 30-min periods it took to complete the training (birds that did not learn were excluded); (3) success in the spatial memory test as follows: the bird was defined as having succeeded in the spatial memory test if it learnt the location of the food, i.e., made three or fewer mistakes both in trials 2 and 3 on either
test day (as in Hodgson and Healy 2005). These measures of learning were used because there was a high degree of variability in learning performance, since some birds never learnt to use the tray. All trials were recorded with video cameras, and videos from all behavioral experiments were analyzed blindly of the treatment and by one person (PK).

**Statistical analyses**

All analyses were conducted with SAS version 9.3. Fecal and bone Pb concentration (log-transformed, fecal data using captive individuals only) were analyzed with linear mixed models (normal error distribution) with treatment and sex (if applicable) as factors. Wing and tarsus length, area of the black breast stripe, and HSPs were analyzed with linear mixed models (normal error distribution) with treatment, sex, and their interaction as explanatory factors and hatching date (range, 52–66 April days) as a covariate. In all models, nest of origin was included as a random factor (ddfm Kenward-Roger) to control for the nonindependence of siblings. To analyze body condition, we included wing length as a covariate in the analysis of body mass (including tarsus as a covariate yielded similar results). In the model for black stripe, we also included wing length to control for possible size differences. We removed nonsignificant interactions, covariates, and main effects, starting from the least significant, but always included the main effect of treatment, as this was our main study question. Each variable was further added separately to the reduced model (statistics shown in Tables 1 and 2).

The probability to explore the novel environment (explored/did not explore, \(n=54\)), the probability to approach a novel object (approached/did not approach, \(n=34\)), probability to succeed training (1/0, \(n=30\)), and success in solving the memory task (1/0 for individuals that succeeded training, \(n=24\)) were analyzed with generalized linear mixed models (binomial error distribution and logit link function) with treatment, sex and their interaction, time of day, and body mass (if applicable) as fixed factors. Activity in the novel environment (no. of hops and flights, log-transformed, including birds that explored the room \(n=32\)) and activity in novel object experiment (no. of hops and flights/min) were analyzed with a similar model except using normal error distribution. Results on activity in the novel environment were similar if also nonmoving individuals were included (by adding one hop to all scores to enable log-transformation; results not shown). Training duration, i.e., the number of 30 min training periods that it took for each bird to complete training was analyzed with similar models (Poisson distribution and log link). In models of learning and memory, origin of birds (nest identity) was not included as a random factor due to small sample size.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pb in feces (7 days)</th>
<th>Pb in bones (&gt;33 days)</th>
<th>Wing length (mm)</th>
<th>Tarsus length (mm)</th>
<th>Body mass (g)</th>
<th>Breast stripe (cm²)</th>
<th>HSP60</th>
<th>HSP70</th>
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<tbody>
<tr>
<td>High</td>
<td>13.2 (9.9)a</td>
<td>30.7 (10.6)a</td>
<td>75.7 (2.9)</td>
<td>22.7 (0.8)</td>
<td>17.0 (1.2)</td>
<td>2.75 (0.60)</td>
<td>2.11</td>
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<td>Low</td>
<td>2.9 (2.8)b</td>
<td>8.9 (3.2)b</td>
<td>76.7 (2.5)</td>
<td>23.0 (0.7)</td>
<td>17.5 (1.3)</td>
<td>1.82 (0.54)</td>
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<td>Control</td>
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<td>1.1 (0.8)</td>
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<td>23.2 (0.5)</td>
<td>17.5 (1.2)</td>
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<td>Harjavalta</td>
<td>5.6 (4.0)ab</td>
<td>4.4 (6.5)bd</td>
<td>75.2 (2.6)</td>
<td>22.7 (0.8)</td>
<td>16.7 (0.9)</td>
<td>1.93 (0.71)</td>
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<th>Factors</th>
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<th>Treatment × Hatch date</th>
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<th>Hatch date</th>
<th>Wing length (mm)</th>
<th>Body mass (g)</th>
<th>Pb in bones (μg/g)</th>
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<td>F 3, 18.9=22.01***</td>
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<td>F 3, 24.6=1.90</td>
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<td>F 3, 20=0.36</td>
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<td>F 3, 25=2.27</td>
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Table 1: Means (±SD) and associated statistics from linear mixed models of juvenile great tit phenotypic traits at the age of ca. 40 days in relation to experimental lead exposure during the nestling period.
to the low sample size (only six sibling pairs in the data) and convergence problems. Furthermore, few birds started to show potential signs of illness during the behavioral experiments. All models were run with and without these individuals to ascertain that potentially different behavior did not affect the outcome. However, as results from models excluding these individuals were the same, data on all individuals is presented.

To understand the effect of early growth conditions in more detail, in a second set of models, we analyzed the effect of realized Pb exposure using brood average (separately for males and females) of fecal Pb levels measured at 7 days, sex, and their interaction on the behavioral measurements. To investigate if nestling size, indicating nutritional conditions, or nestling dominance would affect behavior later in life we also included the following covariates: body size (nestling wing length at d14), relative body size (absolute deviation from brood mean wing length at d14), brood size at d3, and hatching date.

### Results

#### Lead exposure

Experimental Pb treatment increased Pb levels in feces and especially in bone tissue (Table 1). Individuals from high Pb treatment had significantly higher Pb concentration in feces and bone than in the other groups (Table 1).

### Table 2 Means (±SD) of great tit behavioral measures in relation to lead exposure

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Novel environment test</th>
<th>Novel object test</th>
<th>Learning and memory test</th>
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<tbody>
<tr>
<td></td>
<td>Explored/nota</td>
<td>Activity=sum of hopsb</td>
<td>Approach/nota</td>
</tr>
<tr>
<td>High</td>
<td>11/3</td>
<td>14.6 (16.1)</td>
<td>3/7</td>
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<tr>
<td>Low</td>
<td>4/8</td>
<td>13.5 (11.1)</td>
<td>1/7</td>
</tr>
<tr>
<td>Control</td>
<td>10/5</td>
<td>8.20 (4.70)</td>
<td>5/6</td>
</tr>
<tr>
<td>Harjavalta</td>
<td>9/4</td>
<td>20.8 (22.1)</td>
<td>2/3</td>
</tr>
</tbody>
</table>

For the binomial variables, the first value is the number of birds (per group) exploring novel environment, approaching novel object, successful in training or memory test. The second value is the number of birds not succeeding these tasks. Activity in novel environment only includes birds that explored the room. Training duration = number of 30-min training periods to successfully complete training. Factors included in the final model are set in italics

*p<0.05; **p<0.01

- GLMM with binomial distribution, logit link
- GLMM with normal error distribution (log-transformed values)
- GLMM with Poisson distribution

#### Morphology, plumage, and physiology

Exposure to Pb at nestling stage did not affect wing length, tarsus length, or body condition of the juveniles. Males and females did not respond to the treatment differently (Table 1). At the age of ca. 40 days, males had longer wing and tarsus length than females (wing: marginal means±SE—females, 74.5±0.54; males, 77.6±0.54; tarsus: females, 22.7±0.1, males, 23.3±0.1; Table 1). Body mass increased with hatching date (β±SE, 0.092±0.034; Table 1), but hatching date did not affect the other measurements (p>0.05). Pb treatment also did not affect plumage melanin coloration (Table 1), but males had larger ornaments than females (marginal means±SE (cm²), males, 2.14±0.10; females, 1.91±0.10). Pb treatment did not affect HSP60 or HSP70 levels, and there was no interaction with treatment and sex (Table 1, Fig. 2).

#### Behavioral measurements

Probability of exploring the novel environment or activity (sum of hops and flights) was not affected by Pb exposure (Table 2, Fig. 3a). Also, probability to approach the novel object (neophilia/neophobia) or activity in the novel object test (no. of hops and flights/min) was not affected by treatment (Table 2, Fig. 3b). Females were more likely to approach the object than males (Table 2). Activity decreased across the day (β±SE, −0.13±0.035; Table 2). The probability of successfully completed training, duration of the training...
period, or probability to solve the spatial memory task were not significantly affected by Pb exposure (Table 2, Fig. 3c,d), although none of the birds from Harjavalta (n=4) succeeded in the memory task. Females and males did not respond differently to the Pb treatment (Table 2). Any of the behavioral measurements were not associated with fecal Pb concentration at 7 days of age, absolute or relative body size, brood size, or hatching date (GLMMs, p values >0.08)

Discussion

Using environmentally relevant Pb exposure levels in wild passerines, we found no long-lasting/delayed effects of nestling stage dietary exposure to Pb on phenotype or the measured behavioral parameters. Females and males did not respond differently to the Pb treatment either. Furthermore, exposure to moderate levels of several metals and lowered food quality/quantity (Harjavalta group) did not affect any of the measured parameters.

Lead exposure

Our Pb supplementation was successful in raising the fecal and bone concentrations higher than the control group and to levels that have been measured in relatively heavily polluted environments. However, the realized levels of Pb exposure measured from fecal and bone samples were still sometimes lower than those measured in very polluted environments close to active pollution sources: the mean Pb level in the fecal samples of the high-treatment group (13 μg/g, d.w.) was higher than in the polluted area of Harjavalta (5.6 μg/g, d.w.), but still below what has been measured for great tit 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. 1995), and at a Pb 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 Pb 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 great tit nestlings at Harjavalta smelter in

Fig. 2 Mean ±SD of heat shock proteins (HSP60 and HSP70) in relation to four experimental lead treatments in great tits (Parus major). Smelter= birds from nests close to Harjavalta smelter

Fig. 3 Results of behavioral tests in relation to experimental lead manipulation. Percentage of birds exploring novel environment (a), approaching the novel object (b), successful in learning the task and completing training (c), and successful in memory test (d) are shown. See Table 1 for details and statistics
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. 1995) but being lower than those measured for *F. hypoleuca* nestlings at Rönnskär copper smelter (sternum, 85 μg/g, d.w.; Berglund et al. 2011). Our experimental exposure was therefore relevant with regard to the levels found at Pb-polluted sites.

Lack of delayed effects of lead on behavior and learning

In contrast to previous studies in humans, rodents, and also some bird species, we did not find delayed/carry-over effects of developmental dietary Pb exposure on exploration, neophilia/neophobia, activity, learning, or spatial memory. For a general understanding why our Pb exposure did not show carry-over effects on behavior as found in previous studies, we need to consider the effects of (1) different exposure dosages, (2) continuous vs. single exposure and duration of dosing, (3) timing of exposure during development, and (4) route of exposure.

First, in laboratory experiments in various rodents, the exposure levels during development that are found to Pb to carry-over effects are relatively high, higher than concurrent circulating levels leading to behavioral changes (e.g., Garavan et al. 2000). Also in previous Pb exposure experiments in birds where behavioral or morphological changes were detected, higher levels than ours were often used (e.g., 100 μg, Burger and Gochfeld 2005; 130 mg/kg, Lurie et al. 2006; 28 g, Muller et al. 2008; and 40 mg/kg, Youssef et al. 1996; but for 5.5 mg/kg, see Zhong et al. 2010). Our experimental exposure is relevant with regard to the levels found at Pb-polluted sites but much lower than in many laboratory studies. However, to cause severe long-lasting effects on cognition, developmental levels of Pb may have to be relatively high and acutely toxic. In our case, the nestlings in the Pb treatment did not show symptoms of acute toxicity: in a companion paper from the same study setup (Eeva et al. 2014), we report that growth (body mass) was not impaired compared with the controls in our study (wing growth tended to even increase with low Pb exposure). Also, physiological biomarkers (hematocrite, fecal corticosterone, with the exception of HSP60) and especially a Pb-specific marker, Ala-D enzyme activity were not affected by Pb treatment in our study (results reported in Eeva et al. 2014), suggesting that exposure levels were too low to cause acute toxicity. On the other hand, Eeva et al. 2014 reported that nestlings grew worse in Harjavalta than in Ruissalo, and many of the biomarkers suggested developmental stress. However, in humans, it has been showed that that even very low (not acutely toxic) exposure levels during development can lead to detrimental effects for example on cognitive skills (Needleman 2009). Thus, it is still important to assess the behavioral components and delayed effects even with low, nonacutely toxic exposure levels.

Second, an associated question is the method/means of exposure: in several studies, one or two single large injections/loads were given, whereas our experiment tried to replicate natural situation where Pb is acquired via food items with a continuous/chronic low exposure level. These approaches most likely have different consequences due to the potential of acute toxic effects or rate or route of excretion and detoxification. Assumably, a single large Pb dose is more likely to cause acute toxicity as internal detoxification system is not able to detoxify large concentrations. Furthermore, the duration of the dosing was only 12 days, and even if it includes the most important period of posthatching development, we cannot rule out that a longer exposure period in early development (including exposure prehatching) may have stronger carry-over effects. Third, the developmental stage when embryos or young animals are exposed to Pb or other pollutants is most likely critical (see, e.g., Dietert and Piepenbrink 2006 for effects on immune function) as detoxification system is developing in young animals and due to lack of control in blood–brain barrier. We can only speculate that perhaps similar level of exposure at earlier stage of development, for example, during egg stage, could lead to long-lasting effects or permanent changes, as found in chickens (Zhong et al. 2010). Finally, also the route (ingested vs injected) of exposure potentially affects the rate of excretion vs accumulation in the body, and thus the potential of detrimental effects. Importantly, our experiment was explicitly designed to replicate and test natural situation where Pb is acquired via food (the most important route during nestling stage), and thus cannot be extrapolated or directly compared to injection studies.

Furthermore, different pathways/stages of neuronal development, aspects of cognition, and behavior may not be equally sensitive to exposure to Pb and other toxins. Thus, the type of behavioral test selected may influence the outcome: for example, it has been found that low Pb levels during embryonic development in chickens had detrimental effects on long-term but not short-term memory retrieval (Zhong et al. 2010). In our experiment, only short-term spatial memory was tested, and as our protocol is also prone to the effects of motivation, it would be interesting to study different learning tasks to gain more comprehensive understanding on the effects of low level of Pb. We tried to test behaviors with ecological relevance, but it would also be important to further study potential delayed effects of Pb on, e.g., territorial aggression, singing activity, and song quality, as there is some evidence of altered behavior in metal-polluted sites (Gorissen et al. 2005; Hallinger et al. 2010; Janssens et al. 2003).

Importantly, we also did not find any differences in the behavior and learning between Pb treatment groups and birds near the smelter in Harjavalta, exposed to higher total load of metals and lower food quality and quantity. We could not measure food quality and quantity in the two areas (Ruissalo
vs. Harjavalta), but based on previous studies, both are known to be suppressed in the polluted areas in Harjavalta (Eeva et al. 2005). This indirect effect of metal pollution is known to be important for offspring growth and survival (e.g., Eeva et al. 2003). In our experiment, nestlings from Harjavalta were smaller and had lower hematocrit and higher stress hormone levels (as reported in a companion paper from the same study setup, see Eeva et al. 2014), suggesting such nutritional stress. Nutritional stress during development (often associated with high corticosterone levels) can have long-lasting effects on offspring performance, behavior (e.g., development of personality types), and fitness (e.g., Carere et al. 2005; Metcalfe and Monaghan 2001). For example, exploratory behavior and learning have been found to be affected by quantity (also via altered sibling competition and social environment) or quality of food during development in parids (Arnold et al. 2007; Carere et al. 2005; Naguib et al. 2011). In order to explore this hypothesis, we also analyzed the association between early growth conditions (absolute and relative body size at 14 days and brood size effect) and behavioral traits but did not find effects on behavior.

Effects of lead on morphology and physiology

We did not find effects of developmental Pb exposure on morphology (tarsus, wing, and body mass) at juvenile stage. We also found that stress protein levels (HSP60 and HSP70), often a sensitive indicator of sustained stress levels, including metals (Bauman et al. 1993), were not elevated, suggesting that birds were not suffering from long-lasting stress due to early-life exposure and/or potential body burden (i.e., release of accumulated Pb from bones). Similarly, no effect of metal pollution on HSP70 in adult birds was found in a previous study (Eeva et al. 2000). Furthermore, we did not find statistically significant differences in morphology between Harjavalta and Ruiissalo treatment groups, although growth rates were slower at nestling phase in Harjavalta (as reported in Eeva et al. 2014). However, similarly to nestlings, wing length as adults was slightly larger in low and control treatment compared to Harjavalta and high treatment (Eeva et al. 2014). The melanin ornament, black breast stripe, was also not affected by early life Pb exposure. Whether Pb or other metals can influence plumage ornaments, via metals disrupting melanin synthesis pathways or via general endocrine disruption (e.g., McGraw 2003; White and Cristol 2014) should be more studied.

Conclusions

Our experimental Pb treatment significantly increased Pb levels in feces and bone compared with controls. However, we found no carry-over effect of early-life dietary Pb on morphology, physiology, or melanin-based plumage coloration. Treatment did not affect activity, exploration of novel environment, neophobia to novel objects, or success in learning and spatial memory task. Birds from near smelter did not differ from controls in any of the measured traits. We conclude that with the exposure levels and relatively short exposure period used, we could not find delayed effects on the measured traits. However, it is important to further study potential carry-over effects of other toxic substances, other types of behavioral traits and ultimately their fitness effects.

Acknowledgments

We thank Salla Koskinen, Tarja Pajari, Marjo Aikkö, Orsolya Palfi, Åsa Berglund, and Jorma Nummi for their efforts in helping us with field work. Tuja Koivisto made the color measurements. Meri Lindquist is acknowledged for molecular sexing of birds. Paul Ek and Sten Lindholm (Åbo Akademi) are acknowledged for the metal analyzes. Our study was financed by KONE foundation (SR) and Academy of Finland (TE, project 265859).

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