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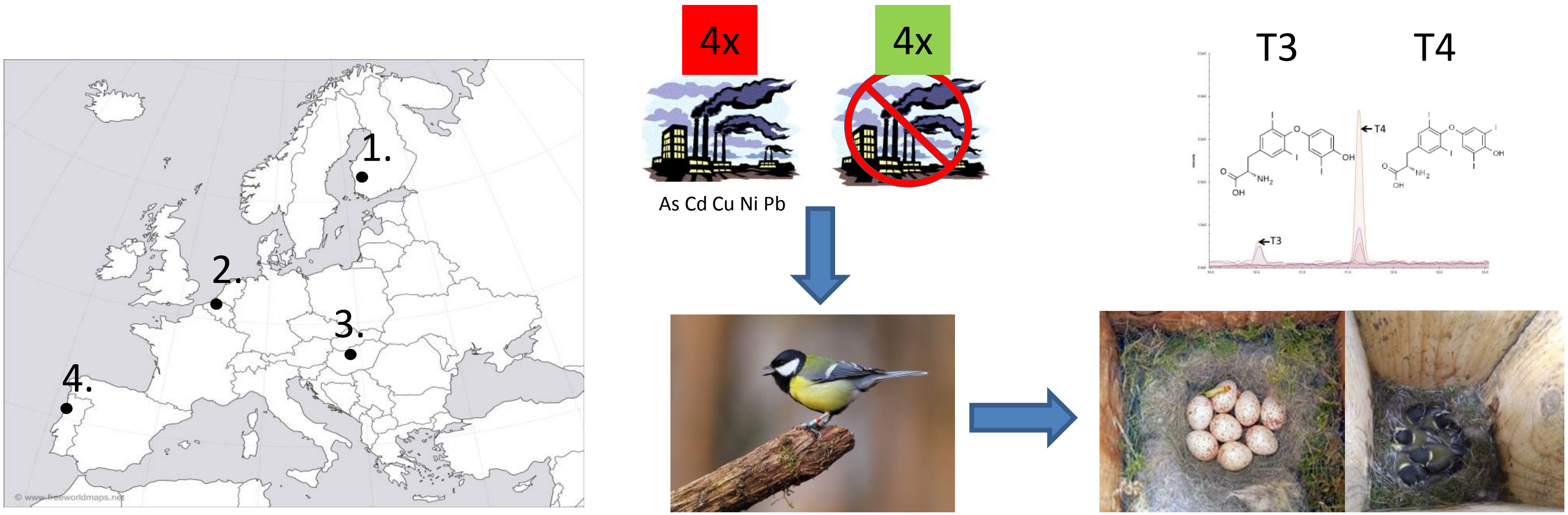
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Transgenerational endocrine disruption: does elemental pollution affect egg or nestling thyroid hormone levels in a wild songbird?

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Abstract

Endocrine disrupting chemicals (EDCs) include a wide array of pollutants, such as some metals and other toxic elements, which may cause changes in hormonal homeostasis. In addition to affecting physiology of individuals directly, EDCs may alter the transfer of maternal hormones to offspring, i.e. causing transgenerational endocrine disruption. However, such effects have been rarely studied, especially in wild populations. We studied the associations between environmental elemental pollution (As, Cd, Cu, Ni, Pb) and maternally-derived egg thyroid hormones (THs) as well as nestling THs in great tits (*Parus major*) using extensive sampling of four pairs of polluted and reference populations across Europe (Finland, Belgium, Hungary, Portugal). Previous studies in these populations showed that breeding success, nestling growth and adult and nestling physiology were altered in polluted zones compared to reference zones. We sampled non-incubated eggs to measure maternally-derived egg THs, measured nestling plasma THs and used nestling faeces for assessing local elemental exposure. We also studied whether the effect of elemental pollution on endocrine traits is dependent on calcium (Ca) availability (faecal Ca as a proxy) as low Ca increases toxicity of some elements. Birds in the polluted zones were exposed to markedly higher levels of toxic elements than in reference zones at the populations in Finland, Belgium and Hungary. In contrast to our predictions, we did not find any associations between overall elemental pollution, or individual element concentrations and egg TH and nestling plasma TH levels. However, we found some indication that the effect of metals (Cd and Cu) on egg THs is dependent on Ca availability. In summary, our results suggest that elemental pollution at the studied populations is unlikely to cause overall TH disruption and affect breeding via altered egg or nestling TH levels with the current elemental pollution loads. Associations with Ca availability should be further studied.

Keywords: endocrine disruption, elemental pollution, tri-iodothyronine, prohormone thyroxine, great tits, transgenerational effects, wild bird populations

Introduction

Endocrine disrupting chemicals (EDCs) include a wide array of pollutants, such as organophosphates, -chlorines and –bromines, some metals and other toxic elements, which may cause changes in the hormonal homeostasis, for example steroid, estradiol or thyroid hormones (Matthiessen et al. 2018, Norris & Carr 2006). However, the effect of pollutants may not be only restricted to adults given that various pollutants can have transgenerational effects via direct maternal transfer of chemicals through placenta or into eggs (Colborn et al. 1993; Dauwe et al. 2005; Marshall & Uller 2007; Ruuskanen et al. 2014). EDCs transferred to eggs and embryos can have various detrimental consequences on offspring development, physiology and even survival (Colborn et al. 1993, León-Olea et al. 2014). Pollutant-associated alteration of various aspects of female physiology may further affect for example gene expression via DNA methylation patterns, or alter the transfer of essential micro- and macronutrients to eggs and embryos, potentially causing transgenerational effects (Espín et al. 2016, Hargitai et al. 2016, Skinner et al. 2010, Windsor et al. 2018).

Moreover, disruption in female hormonal status via EDCs may alter the transfer of maternal hormones to offspring: this phenomenon is called transgenerational endocrine disruption. Hormones transferred from the mother to embryos and eggs are known to profoundly influence offspring development, physiology, morphology, behavior and even survival across taxa (Dantzer et al. 2013, McCormick 1999, Ruuskanen 2015, Ruuskanen & Hsu 2018, Uller et al. 2007, von Engelhardt & Groothuis 2011). Thus, alteration of the early-life hormonal environment via maternal exposure to EDCs, i.e. transgenerational endocrine disruption, could have detrimental consequences on offspring development and phenotype. The potential for transgenerational endocrine disruption depends on the interdependence of plasma hormone levels and hormones transferred to eggs and embryos (Groothuis & Schwabl

2008, Ruuskanen & Hsu 2018). Metals such as cadmium (Cd) have been been found affect the production of human placental hormones (leptin and progesterone) (Stasenko et al. 2010). In a rare example from a wild reptile population, eggs from polluted populations (incl. organochlorines, metals, agricultural runoff) had lower progesterone and estradiol levels than reference populations (Hamlin et al. 2010). However such effects of EDCs on maternally-derived hormone levels in the egg and embryo have rarely been studied.

Thyroid hormones (THs; prohormone thyroxine T4, and biologically active tri-iodothyronine, T3) are a key class of hormones that control and regulate vital biological processes such as thermogenesis, growth, and metamorphosis (Norris & Carr 2013). Plasma TH levels are determined by production/secretion from the thyroid gland, conversion of T4 to T3 in tissues by deiodinase enzymes as well as TH degradation (McNabb 2007). Recent studies suggest that maternal THs transferred to eggs and embryos are important for offspring development across vertebrates and can also affect offspring TH axis function (Brown et al. 2014, Hsu et al. 2017, Patel et al. 2011, Ruuskanen et al. 2016a, Ruuskanen & Hsu 2018, Vulsma et al. 1989). Some elements, for example, cadmium (Cd), lead (Pb), chromium (Cr), copper (Cu) and arsenic (As) have been shown to disrupt TH homeostasis via binding to receptor thiol groups and disturbing TH signalling (Norris & Carr 2006, Sun et al. 2016). Negative relationships have been reported between Pb exposure and plasma TH levels in many epidemiological and animal studies (Rana 2014). Cd and As toxicity has been repeatedly found to decrease serum T4 levels in captive model species (Sun et al. 2016). In a recent experimental study in zebrafish (*Danio rerio*), chronic maternal exposure to Pb at an environmentally relevant range of concentrations decreased egg T3 and T4, along with similar decreases in female plasma TH levels (Chen et al. 2017). However, to our knowledge the effects of TH disrupting agents on maternally-derived TH levels in the eggs have not been explored in other vertebrates, including in birds. Surprisingly, even the direct effects of dietary elemental pollution on circulating TH levels in nestling and adult birds in wild populations are poorly studied (Baos et al. 2006).

Toxicity of elements and their potential role as EDCs may further depend on calcium (Ca) availability: low Ca availability has been shown to increase absorption, accumulation and mobility of metals (Scheuhammer 1996). Due to structural similarity, elements such as Pb, can compete with Ca for its binding sites (in calcium channels, Ca-binding proteins and second messenger Ca receptors; Scheuhammer 1996, Goyer 1997). Experimental studies showed that dietary Ca availability affected especially the level of Pb-associated oxidative stress, immune function and brain monoamines (Espín et al. 2017, Prasanthi et al. 2010, Prasanthi et al. 2005, Snoeijs et al. 2005), but not corticosterone levels (Snoeijs et al. 2005). Ca ingestion and overall nutritional quality have also been found to be lower in polluted compared to unpolluted sites (e.g. Eeva et al. 1997, Eeva and Lehikoinen 1998, 2004, Jones & Paine 2006, Sillanpää et al. 2008), which could contribute to the effects of toxic elements on endocrine as well as other physiological traits. To our knowledge, Ca-dependent effects of pollutants on circulating THs or THs transferred to offspring have not been studied up to date.

We studied the association between elemental pollution and egg TH and nestling plasma TH levels in wild bird populations. The great tit (*Parus major*) was selected as our study species as it is considered a good bioindicator of elemental pollution: it is a resident, insectivorous species that occupies a mid-trophic position in the food chain, and forages in small home ranges reflecting local contamination. We used extensive sampling across four countries in Europe (Finland, Belgium, Hungary, Portugal): in each country data were collected from both a polluted and a reference zone. These study populations show wide variation in elemental pollution levels (Costa et al. 2012, Eeva & Lehikoinen 1996, Geens et al. 2010, Hargitai et al. 2016). Previous studies from these populations showed that breeding success, nestling growth, nestling and adult health (e.g. changes in haematological parameters) and plumage carotenoid coloration were lower in polluted compared to reference zones (Eeva et al. 2009, Eeva et al. 1998, Janssens et al. 2003, but see Costa et al. 2012). Also egg quality, such as egg size and shell thickness (Eeva & Lehikoinen 1995) and antioxidant composition of eggs (Espín et al. 2016, Hargitai et al. 2016), were altered in polluted compared to reference zones. We sampled non-incubated eggs for maternally-derived egg TH measurements, measured nestling plasma THs and used nestling faeces from the same nests to assess dietary elemental exposure of arsenic (As), cadmium (Cd), copper (Cu), nickel (Ni) and lead (Pb) in the four populations in polluted and reference zones. To study the potential Ca-dependent effects of elemental toxicity on endocrine traits, we also measured Ca levels in nestling faeces as a proxy for Ca availability.

We hypothesised that elemental pollution would decrease egg and nestling TH concentrations. Altered maternal TH transfer to eggs may have carry-over effects, modifying nestling TH axis function and thus nestling plasma TH levels. Alternatively, nestling TH function may be disrupted by maternally-derived toxic element load in the egg, or more directly due to nestling dietary exposure to elemental pollution. We further predicted that elemental pollution may have stronger negative effects on THs when Ca availability is poor.

Methods

The study was conducted in polluted environments (industrial/urban sites) and respective non-polluted reference areas in four European countries, i.e. Finland, Belgium, Hungary and Portugal, in populations of great tits using nest boxes in 2016. Thus, the study setup consists of four pairs of polluted and reference zones. In each country, polluted and reference zones were selected to represent similar habitats. In Finland the populations are located in

Harjavalta (61°20’N, 22°10’E): the polluted zone is located at ca 1 km distance from a Cu-Ni smelter with a reference zone at a distance of ca 7 km (Eeva & Lehikoinen 1996). The main pollutants in Harjavalta are As, Cu, Ni, Pb and Zn (Eeva et al. 2012). In Belgium, the populations are located in Antwerp (51°13’N, 4°24’E): the polluted zone is located next to a non-ferrous metallurgical plant with a reference zone at a 6 km distance (Eens et al. 1999). The main pollutants in Antwerp are As, Cd, Cu, Pb and Zn (Janssens et al. 2001). In Hungary, the polluted site is an urban park in Budapest (47°28’N, 19°02’E) with a reference zone at ca 27 km distance. The main pollutants in Budapest are As, Cu, Ni, Pb and Zn (Hargitai et al. 2016). In Portugal the populations are located in Figueira da Foz (40°02′N, 8°52′W): the polluted zone is located at a 1 km distance from a pulp factory with a reference zone 20 km away. The main pollutants in Figueira da Foz are As, Cd, Cu, Hg, Ni, Pb, Se and Zn (Costa et al. 2012, Costa et al. 2011).

The nest boxes were checked periodically to monitor the development of nest building and record the laying date (date of laying the 1st egg), clutch size, hatching date, brood size, and number of fledglings. In total we monitored 153 great tit nests (50 in Belgium, 33 in Finland, 38 in Hungary and 32 in Portugal), see final sample sizes in Fig 2. The 4th egg was collected on the day of laying, replaced by a plasticine egg, and frozen at –20 °C for later TH analyses (see details below). Faecal samples of nestlings were collected from 141 of the 153 nests for element analyses (see details below). Elemental concentrations in nestling faeces are a common indicator for local pollution levels (Dauwe et al. 2004, Eeva et al. 2014, Espín et al. 2016). Faecal calcium levels have been found to correlate with calcium availability in the diet (estimated as amount of snail shells in the nest, their primary source of calcium) in another similar-sized passerine, the pied flycatcher (*Ficedula hypoleuca*) in Harjavalta (Finland) study area (Eeva and Lehikoinen 2004). Also in adults, elemental levels measured during breeding reflect recent exposure (within 2 weeks), and thus very local pollution load at the feeding range of the individual (Berglund et al. 2011). Unfortunately, the egg elemental levels could not be directly measured due to resource constraints.

Nestling blood samples (ca 60 µl) were collected from 14-day old nestlings into heparinised capillaries from the brachial vein. One nestling per nest was randomly sampled. Nestling plasma THs were only analysed from the Belgian population due to resource constraints. This population shows the highest elemental pollution loads of the studied populations (Janssens et al. 2001 and results of this study). The sample size was 23 nests from the polluted zone and 18 nests from the reference zone. Blood samples were stored in a cooler and centrifuged (4400 g, 5 min) later each day to separate plasma and red blood cells. Samples were stored at –80°C until analysis.

All samples were collected under appropriate licenses from local authorities in each study population, as following: *Finland*: The experiment was conducted under licenses from the Animal Experiment Committee of the State Provincial Office of Southern Finland (license number ESAVI/11579/04.10.07/2014) and the Centre for Economic Development, Transport and the Environment, ELY Centre Southwest Finland (license number VARELY/593/2015). All applicable institutional and/or national guidelines for the care and use of animals were followed. *Belgium:* The Flemish Agency 'Natuur en Bos' provided permission for this study (ANB BL FF V16-00105-VB). *Hungary:* The Middle-Danube-Valley Inspectorate for Environmental Protection, Nature Conservation and Water Management (PE/EA1432-6/2016), the Pest County Government Office of the National Food Chain Safety Office (PE/KTF 8988-5/2016) and the Mayor's Office of Budapest (FPH061/1829-3/2016) provided permissions for this study. *Portugal*: All animals were handled according to current Portuguese law and following the license number 217, issued by ICNF – Institute for Nature Conservation and Forest.

Thyroid hormone analyses

The egg and plasma samples were analysed for T3 and T4 at the University of Turku. LC-MS/MS was conducted at the facilities of Turku Center for Biotechnology. In the egg samples, yolk and albumen were separated after thawing. Yolk was weighed (0.01 g accuracy) and mixed with milli-Q water (1:1) and vortexed thoroughly. T4 and T3 were extracted from yolk and plasma following previously published methods (de Escobar et al. 1985, Ruuskanen et al. 2018). In short, yolk-water mixture (ca 150 mg of pure yolk) or plasma (25 µl) was homogenized in methanol. As an internal recovery tracer, a known amount of $^{13}C_{12}$-T4 (Larodan) was added to each sample. This allowed us to control for the variation in recovery (i.e. extraction efficiency) for each sample. Chloroform was then added and after centrifugation (15 min, 1900 g, +4°C), the supernatant was collected and the pellet was re-extracted in a mixture of chloroform and methanol (2:1). Back-extraction into an aqueous phase (0.05% $CaCl_2$) was followed by a re-extraction with a mixture of chloroform:methanol: 0.05% $CaCl_2$ (3:49:48) and this phase was further purified on Bio-Rad AG 1-X2 resin columns. The iodothyronines were eluted with 70% acetic acid, and evaporated to dryness under vacuum overnight. Blanks (plain reagents without any sample) were analysed in each extraction batch to detect any contamination. Yolk samples from different populations were equally distributed across five extraction batches, and extraction batch was used as a random intercept in the statistical models to control for any differences among the batches. Nestling plasma THs were extracted in a single extraction batch. T3 and T4 were quantified using a nanoflow liquid chromatography-mass spectrometry (nano-LC-MS/MS) method, developed and validated in Ruuskanen et al. (2018). Briefly, before the analysis, the dry samples were diluted in ammonium (NH_3). Internal standards $^{13}C_6$-T_3 and $^{13}C_6$-T_4 (Sigma) were added to each sample to identify and quantify the THs. A triple quadrupole mass spectrometer (TSQ Vantage, Thermo Scientific, San Jose, CA) was used to analyse the samples. For the chromatographic separation of hormones, a nanoflow HPLC system Easy-nLC (Thermo Scientific) was applied. On-column quantification limits were 10.6 amol for T4 and 17.9 amol for T3. MS data was acquired automatically using Thermo Xcalibur software (Thermo Fisher Scientific) and analysed using Skyline (MacLean et al. 2010). For the analyses, peak area ratios of sample to internal standard were calculated. TH concentrations are expressed as pg/mg fresh yolk and as pmol/ml plasma.

Element analyses

Nestling faecal samples were used for all element analyses. Faecal samples were collected from nestlings when 7–9 days old, placed into Eppendorf tubes and frozen at −20°C. Samples of the same nest were combined to analyse brood level element concentrations. Samples were dried for 72 h at 45 °C and analysed at the University of Murcia, Spain. Before the analysis, the faecal samples were placed in digestion tubes with 4 ml of HNO_3 (70%) and 1 ml of H_2O_2 (33%) (Espín et al. 2016). After that, the samples were heated in a microwave and diluted in ultrapure water. The accuracy of the analysis was tested beforehand by determining the recovery of metals in a reference material (TORT-2, lobster hepatopancreas, National Research Council Canada). The recoveries of the metals from 15 replicates of the reference material were between 74 and 120 %. Also, a coefficient of variation (CV) was calculated to estimate repeatability and it was under 20 %. An inductively coupled plasma optical emission spectrometer (ICP-OES) was used to analyse the concentrations of As, Cd, Cu, Ni, Pb and Ca with a quantification limit of 1 ppm for Ca and 0.01 ppm for the others. Element concentrations were expressed as µg/g dry weight (d.w), except for Ca concentration as mg/g (d.w).

Statistical analysis

Statistical analyses were performed with SAS 9.4 statistical package. Yolk T3 and T4 concentrations (pg/mg), T3 and T4 content (ng/yolk) and T3:T4 ratio were log-transformed to reach normality. Also plasma T3 and T4 concentrations (pmol/ml) were log-transformed. Both yolk TH content and concentration were analysed because both are important for offspring development and endocrine disruption may differentially affect them. In turn, altered T3:T4 ratio may reflect changes in the peripheral deiodination of T4 (i.e. conversion of T4 to T3 in tissues by deiodinase enzymes, McNabb 2007). All element concentrations from faecal samples were log-transformed to reach normality. In the element data, there were 24 values in As that were very close or below detection limit (16% of the data: 18 samples in Hungary, 4 in Finland, 1 in Belgium, 1 in Portugal), 4 values for Cd (1 in each study population) and 3 for Ni (all in Portugal). As suggested in the literature (Croghan & Egeghy 2003), we replaced these values with LOD/sqrt(2), where LOD refers to lowest detection limit that was set to 0.05, to improve the distribution. This resulted in a normal distribution.

Differences among polluted and reference zones in the elemental concentrations were analysed using linear models (LM) with fixed factors zone (polluted/reference), country (Finland, Belgium, Hungary, Portugal) and their interaction. Pairwise comparisons *within* each country were conducted using Tukey post-hoc tests to study the differences among polluted and reference zones in a given country. One observation from polluted zone in Finland was excluded as an outlier, as it had extremely high values (10 to 100 times higher than in other samples) in most elements.

We then analysed the effect of general pollution load on egg THs using linear mixed models (LMM). The fixed factors in these models included zone (polluted/reference), country (Finland, Belgium, Hungary, Portugal) and their interaction. We included yolk TH analysis batch as a random intercept to control for potential variation among the hormone extraction batches (samples from all countries and populations were equally distributed across the batches). Laying date (centred for each population to study at relative differences among early and late breeders) and clutch size were included as covariates to control for potential differences in individual quality, resource availability or reproductive investment.

We analysed the combined load of toxic elements by performing a principal component analysis for the metals Cd, Ni, Cu, Pb and metalloid As (log-transformed and LOD corrected values). PC1 fitted the data relatively well as the eigenvalue was 2.75, the vector explained 55% of the variation. Loadings of all elements were positive (Pb = 0.71; As = 0.81, Cd = 0.84, Cu = 0.61, Ni = 0.70). We then analysed the association between PC1 and yolk T4 and T3 concentration and content using LMM. Given that the elemental toxicity is often affected by Ca availability, we also included Ca concentration (log-transformed) and the interaction between PC1 and Ca as fixed factors. Country and extraction batch were included as random intercepts given the non-independence of data in each study population. Population-centred laying date and clutch size were included as covariates. We found that in Portugal, the elemental levels tended to be higher in reference than polluted zone. We thus rerun all models excluding Portugal but as the results remained qualitatively the same, we report analyses including all populations.

Subsequently, we analysed the association between yolk THs and individual elements (As, Cd, Cu, Ni and Pb) and their interaction with Ca in separate models. The literature points especially to the specific TH-disrupting effects of As, Cd, Pb and to some extent Cu (Rana 2014, Sun et al. 2016). The models used were similar as for PC1 of elements (see above).

We studied the covariation between egg T4 and T3 and the potential differences in this covariation among polluted and reference zone, and in relation to total toxic element exposure (PC1). Such a difference in covariation might indicate altered thyroid function, either production/secretion or altered deiodination (conversion of T4 to T3 or to inactive forms such as T2) in tissues. We performed LMMs with egg T4 as the dependent and egg T3 as the independent factor, together with zone and their interaction, and PC1 and its interaction with T3. Country and extraction batch were included as random intercepts.

For analysing the associations between elemental pollution and nestling plasma T3 and T4 concentrations, a PC1 of element load was also constructed for the Belgian population (PC1 eigenvalue 3.68, explained 73% of the variation). The effect of pollution zone on nestling plasma THs was tested with linear models as samples originated only from one population. Body mass at the age of 14 days and laying date were included as covariates. Pearson correlations were used to analyse the associations between PC1, individual elements and nestling plasma THs.

Models were reduced by removing non-significant factors ($\alpha = 0.05$). Degrees of freedom were estimated with Kenward-Rogers estimation method. Zone, PC1 or element concentrations were retained in the models as these variables were of main interest. Removed fixed effects and covariates were re-introduced individually to the reduced model and statistics from the reintroductions are reported.

Results

Elemental pollution across polluted and reference zones

The results of the comparisons of element levels between polluted and reference zones across and within the four study populations are reported in Table 1. Elemental levels varied markedly across countries and showed different patterns across polluted and reference zones in different study populations (Table 1, country × zone interaction, $p < 0.001$). Arsenic concentrations were higher in polluted than reference zones in Finland, Belgium and Hungary

(Tukey post-hoc tests for polluted vs reference zone within a country, t–values >9.5, p <0.001) but not in Portugal. Cd and Pb concentrations were higher in polluted zones than reference zones in Finland and Belgium (Tukey post-hoc tests, t>5.1, p<0.001), but not in Hungary and Portugal. Cu concentrations were generally higher in polluted than reference zones across all countries (Table 1). Ni concentrations were higher in polluted than reference zones in Finland and Hungary (Tukey post-hoc tests, t>3.0, p<0.01), but not in Belgium and Portugal. Ca concentrations were higher in the polluted than reference zone in Hungary (t = 4.3, p<0.001), but did not differ among polluted and reference zones in the other populations (Table 1).

The PC1 of elements (As, Cd, Cu, Ni, Pb) showed different patterns across polluted and reference zones in different study populations (country × zone interaction $F_{3,137} = 30.66$, p<0.001, Fig 1): in Finland, Belgium and Hungary toxic element levels were higher in polluted compared to reference zones (Tukey post-hoc tests for polluted vs reference zone within a country, Belgium t = 10.8, p<0.001: Finland t = 7.6, p <0.001; Hungary t = 3.3, p = 0.03), while in Portugal a tendency for higher elemental pollution levels in the reference zone (t = −3.08, p = 0.054).

Association between egg thyroid hormones and elemental pollution

We did not find statistically significant differences in egg T3 or T4 concentration, total content or T3:T4 ratio between polluted and reference zones at any of the study populations (no statistically significant country × zone interaction nor main effect of zone, Table 2, Fig 2a, b). There was no statistically significant correlation between PC1 of elements and egg T3 or T4 concentration or content (Table 3, Fig 3a, b). Furthermore, the association between PC1 and egg THs was not dependent on the availability of Ca (Table 3). However, the association between Cd, Cu and egg T4 concentration was dependent on Ca availability: when faecal Ca concentrations were low, there was a positive correlation between egg T4 and Cd and egg T4 and Cu (in the lowest quartile, Ca values < 4.6 mg/kg; Cd vs egg T4: $r = 0.33$, $p = 0.05$; Cu vs egg T4: $r = 0.34$, $p = 0.048$, Fig 4a, Fig 5a), but no association was found when Ca levels were higher (Ca > 4.6 mg/kg, Cd vs egg T4: r –0.01 to –0.05, p>0.70; Cu vs egg T4: r –0.09 to 0.16; Table 4, Figs 4b–d, Figs 5b–d). Faecal As, Pb and Ni concentrations were not associated with egg TH concentrations or content, nor in interaction with Ca (Table 4).

Egg T3 concentration and content were negatively correlated with clutch size (estimate±SE: T3 concentration −0.0177 ± 0.009; T3 content −0.0242 ± 0.009, Table 3). Laying date was not associated with egg T3 or T4 concentration or content (Tables 2, 3). There was a positive correlation between egg T3 and T4 concentration and T3 and T4 content (estimate ± SE: hormone concentrations 0.37 ± 0.05; $F_{1,138} = 69.3$, $p < 0.001$, hormone contents 0.38 ± 0.04; $F_{1,133} = 71.1$, $p < 0.001$), but covariation between egg T3 and T4 did not differ between polluted and reference zones, nor in association with PC1 (F<0.12, p>0.48), suggesting no effect of elemental pollution on peripheral TH deiodination.

Association between nestling plasma thyroid hormones and elemental pollution

In Belgium, nestling plasma T3 or T4 concentrations did not differ between the polluted and reference zone (T3: $F = 0.06$, $p = 0.81$, T4: $F = 0.02$, $p = 0.88$, $N = 41$, see Fig 6). Nestling plasma T3 and T4 concentrations were further not associated with total elemental load (PC1 of elements vs T3: $r = –0.12$, $p = 0.43$; T4: $r = –0.05$, $p = 0.73$) or concentrations of individual elements (As, Cd, Cu, Ni and Pb; $–0.15 < r < 0.18$, $p > 0.34$).

Discussion

Birds at the polluted zones were exposed to markedly higher levels of toxic elements (As, Cd, Cu, Ni and Pb) than in reference zones at the study populations in Finland, Belgium and Hungary, but not in Portugal. These results are in accordance with previous studies from the study populations: As, Cd, Cu, Ni and Pb concentrations were reported higher in polluted than reference zones in great tit faeces/feathers in Belgium and in Finland while there was no difference in Ca across the zones (Eeva et al. 2009, Janssens et al. 2001). In Hungary, a previous study from the same population also reported higher As, Cu, Ni, Pb (but not Cd) and Ca in soil samples of urban (polluted) than a reference zone (Hargitai et al. 2016). Parallel to our results, in a previous study in the Portuguese populations, the analysed elements (Cd, Cu, Pb, with the exception of As) were not higher in the vicinity of a pulp-paper mill compared to a reference zone. The Portuguese reference zone is surrounded by agricultural fields, and thus pesticides and herbicides may explain somewhat elevated pollution load at the reference zone (Costa et al. 2012). However, mercury was higher in the polluted compared to the reference zone (Costa et al. 2012).

In contrast to our predictions, we did not find any associations between overall elemental pollution and egg T4 or T3 levels or nestling plasma TH levels. The lack of overall association between toxic element exposure and THs is surprising because metals like Pb and Cd have been found to affect plasma TH concentrations negatively in other taxa (Rana 2014), in particular in other bird species (hen chicks and adult cockerels: e.g. Chaurasia et al. 1995, Gupta & Kar 1999) as well as egg THs in fish (Chen et al. 2017). However, Baos et al. (2006) did not find associations between toxic elements (Pb, Zn, Cu, Cd, As) and THs in plasma of nestlings or adults of another wild bird population (white storks, *Ciconia ciconia*), whereas steroid hormones were negatively correlated with elemental pollution levels. Finally, our study did not investigate for example the effect of mercury (Hg) on THs, while it has previously been linked with TH disruption in another passerine bird, the tree swallow (*Tachycineta bicolor*) (Wada et al. 2009).

We found some support for the prediction that the effect of toxic elements on THs would be altered with low dietary Ca availability. At very low Ca levels, egg T4 concentrations increased with increasing Cd and Cu concentrations. The trend is contrary to expected as in previous studies increased metal and other pollutant exposure (As, Cd, Cr, Cu, Pb, Hg) was generally associated with decreased plasma THs (especially T3, but often also T4) across taxa (Rana 2014, Sun et al. 2016, Wada et al. 2009). However, in these studies Ca availability was not taken into account. We also have to note that we used faecal calcium as a proxy for calcium intake (i.e. availability in the diet), following Eeva et al. (2004) and Hargitai et al (2016). However, if faecal calcium concentration would be more influenced by intestinal calcium uptake, low faecal calcium concentrations would actually reflect high uptake and less metal-associated burden. The influence of calcium- and element-induced variation in egg THs on offspring development and fitness needs to be studied. Interestingly, in our previous study where egg TH levels were experimentally manipulated via injections of T4 and T3 into non-incubated eggs, the dose causing positive effects on growth (Ruuskanen et al. 2016a) was similar to the upper range of variation measured in the current study. This may suggest that metal-induced variation in egg THs in poor Ca conditions could be biologically relevant on offspring development and growth. Definitely, more studies on both THs vs Ca and Ca-modified toxic element vs TH interactions are needed.

The lack of a general association between toxic elements and egg and nestling plasma THs could be explained by several, mutually non-exclusive hypotheses: (1) the low exposure load; (2) no effect of elemental pollution on female plasma THs, and thus no effect on maternal transfer to the egg; (3) an effect of elemental pollution female plasma THs, but compensatory TH transfer to eggs. Also, (4) species differences in sensitivity to toxic element pollution could explain the contrasting results in our study compared to other studies (e.g. Chaurasia et al. 1995, Gupta & Kar 1999, Baos et al. 2006).

First, the pollutant exposure levels across studies should be critically evaluated (hypothesis 1). In our study, the levels of pollutants were markedly higher in polluted compared to reference zones especially in Belgian and Finnish populations (As concentrations were 10 times higher in polluted than reference zones, Cd 5–10 times higher, Cu 2–5 times higher, Ni 2–15 times higher and Pb 5–10 times higher, respectively). However, the levels measured in our study are somewhat lower than in previous studies from the same populations, which reported detrimental effects on reproductive parameters and female and chick physiology. For example Janssens et al. (2003, Table 1) reported for the Belgian population (sampled in 1999) 5 times higher As concentrations, 10 times higher Ni concentration and 2 times higher Cu concentrations (but similar or lower Cd and Pb), compared to our data (sampled in 2016 from the exact same sites). In a previous experimental study on metal-associated TH disruption in birds, Gupta & Kar (1999) dosed hen chicks daily with 2.5μg Pb/ g tissue and found a decrease in plasma THs. Interestingly, in the study populations in Finland and Belgium, estimated daily Pb intake ranged between 2.2−8.5 μg Pb/g tissue (Eeva et al. 2014). Thus Pb exposure levels in our wild populations could be rather similar as in experiments with captive chicks, while no association between elemental levels and THs was found in our study. Therefore, the lack of effect of toxic elements on THs may be not only due to low exposure levels, but potentially species differences (hypothesis 4).

Second, we did not measure female plasma TH levels in this study due to practical limitations. It is thus possible that toxic elements may not have caused TH disruption in the female circulation, leading to no transgenerational TH disruption (hypothesis 2). The fact that nestling plasma TH was not associated with elemental pollution load supports this hypothesis. Alternatively, female plasma TH levels may have been affected, but due independent regulation of plasma and egg TH levels, females compensated by transferring proportionally more THs into the eggs to avoid detrimental effects on offspring (hypothesis 3). This could lead to no differences in egg TH levels. The molecular transfer and regulatory mechanisms of THs from circulation to egg yolk are currently not well understood (Ruuskanen & Hsu 2018). Indirect evidence suggests somewhat contrasting patterns in plasma and yolk THs (Hsu et al. 2016, Van Herck et al. 2013, Wilson & McNabb 1997). If such regulatory mechanism(s) are present, an independent effect of endocrine disruption on plasma THs but not egg THs is possible.

Finally, it needs to be noted that species may differ in their sensitivity to elemental pollution (hypothesis 4). In a recent large-scale study comparing urbanized and rural sites in 199 populations across Europe it was concluded that urbanization decreased clutch size in collared and pied flycatchers (*Ficedula albicollis, F. hypoleuca*), but not in great tits and blue tits (*Cyanistes caeruelus*) (Vaugoyeau et al. 2016). Using pollution gradients, it was also reported that great tits respond less to pollution than other passerines (Eeva & Lehikoinen 2004), potentially due to species-specific differences in Ca-associated metal toxicity. Thus, great tits may be not especially sensitive to endocrine disruption caused by toxic elements. In summary, our results suggest that pollution at these populations is unlikely to cause transgenerational TH disruption or affect nestling plasma THs directly via dietary exposure to elemental pollution. Thus, maternally-deposited THs in eggs do not appear to be an additional mechanism that may cause detrimental effects on breeding birds in these populations, but the interactions with Ca should be further studied.

Interestingly, we found negative correlations between clutch size and egg THs. Given that the molecular structure of THs requires iodine, which organisms cannot produce themselves, females may face a trade-off between allocating THs (and associated iodine) to eggs versus themselves (Ruuskanen & Hsu 2018). This trade-off could be accentuated in large clutches, leading to decreased TH concentrations. Recent studies across vertebrates egg THs show substantial intra-specific variation both among and within females (Ruuskanen & Hsu 2018), which is associated with key environmental and ecological factors, such as food (Hsu et al. 2016) and temperature (Ruuskanen et al. 2016b), but previous studies did not reveal any association with clutch size. Together, these results suggest that egg THs can be an important plastic, hormonal mechanism underlying variation in offspring phenotype.

Conclusions

In our European-wide study on transgenerational endocrine disruption across four pairs of polluted and reference zones, we found that great tits at the polluted zones were exposed to markedly higher levels of toxic elements than in reference zones. However, in contrast to our expectations, we did not find any association between overall elemental pollution and egg TH levels or nestling TH levels at any of the populations. We found some indication (for Cd and Cu) that the effect of metals on egg THs is dependent on Ca availability. In summary, our results suggest that the elemental pollution experienced by these populations is unlikely to cause transgenerational TH disruption or disrupt nestling TH function with the current pollution load, but the interactions with Ca availability should be further studied. Thus, TH disruption may not be an additional mechanism that causes detrimental effects on breeding birds in the studied populations.

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Table 1. Faecal element concentrations (back-transformed marginal means with asymmetrical SEs) in polluted and reference zones of great tit nestlings at the four study locations and associated statistics from linear models. Element concentrations are presented in µg/g dry weight, except Ca in mg/g. Results are from GLMs where log-transformed values were used. Different letters ([a] and [b]) denote a statistically significant (Tukey post-hoc, p<0.05) differences between polluted and reference zones *within a country*. FI = Finland; BE = Belgium, HU = Hungary, PT = Portugal. Poll = polluted zone, Ref = reference zone. Arsenic (As), cadmium (Cd), copper (Cu), nickel (Ni), lead (Pb), calcium (Ca).

Population	As	Cd	Cu	Ni	Pb	Ca
FI Poll (N = 17)	7.25 (5.7-9.2)[a]	3.54 (2.8-4.4)[a]	145.6 (128.2-165.6)[a]	19.78 (16.5-23.7)[a]	2.86 (2.4-3.4)[a]	13.3 (11.0-16.1)[a]
FI Ref (N = 16)	0.22 (0.2-0.3)[b]	0.78 (0.6-1.0)[b]	63.5 (55.8-72.2)[b]	3.10 (2.6-3.7)[b]	1.22 (1.0-1.5)[b]	6.8 (5.5-8.2)[a]
BE Poll (N = 25)	13.68 (11.3-16.6)[a]	8.47 (7.1-10.1)[a]	66.3 (59.9-73.5)[a]	2.90 (2.5-3.3)[a]	61.77 (53.3-71.5)[a]	5.6 (4.8-6.5)[a]
BE Ref (N = 24)	0.99 (0.8-1.3)[b]	1.17(1.0-1.4)[b]	33.2(29.8-36.8)[b]	1.83 (1.6-2.1)[a]	6.52 (5.6-7.6)5[b]	5.1 (4.3-5.9)[a]
HU Poll (N = 15)	1.01 (0.8-1.3)[a]	0.58 (0.5-0.7)[a]	66.3 (58.1-75.7)[a]	3.67 (3.0-4.4)[a]	4.56 (3.9-5.6)[a]	12.3 (10.0-15.0)[a]
HU Ref (N = 16)	0.04 (0.03-0.05)[b]	0.69 (0.6-0.8)[a]	33.6 (30.0-37.5)[b]	1.36 (1.2-1.6)[b]	3.97 (3.4-4.7)[a]	4.0 (3.3-4.7)[b]
PT Poll (N =14)	0.39 (0.3-0.5)[a]	1.23 (1.0-1.5)[a]	107.0 (93.3-122.6)[a]	0.29 (0.2-0.4)[a]	0.65(0.5-0.8)[a]	11.2(9.1-13.7)[a]
PT Ref (N = 14)	1.03 (0.8-1.3)[a]	1.30 (1.0-1.6)[a]	76.1 (66.3-87.2)[a]	0.52 (0.4-0.6)[a]	0.95 (0.8-1.1)[a]	15.5 (12.6-19.1)[a]

	Fdf	p	*Fdf*	p	*Fdf*	p	*Fdf*	p	*Fdf*	p	*Fdf*	p
Pollution zone	$167.00_{1,137}$	<0.001	$30.03_{1,137}$	<0.001	$53.35_{1,137}$	<0.001	$31.13_{1,137}$	<0.001	$55.58_{1,137}$	<0.001	$9.06_{1,138}$	0.01
Country	$66.39_{3,137}$	<0.001	$23.78_{3,137}$	<0.001	$21.04_{3,137}$	<0.001	$87.35_{3,137}$	<0.001	$136.96_{3,137}$	<0.001	$9.16_{3,138}$	<0.001
Zone x country	$34.94_{3,137}$	<0.001	$14.77_{3,137}$	<0.001	$1.22_{3,137}$	0.43	$15.21_{3,137}$	<0.001	$23.99_{3,137}$	<0.001	$5.61_{3,138}$	0.01

Table 2. Linear models of the effects of zone (polluted or reference) and country on egg thyroid hormones. T4 = thyroxine, T3 = triiodothyronine. Reduced model is shown in bold. Statistics from other factors are from models where the factor was reintroduced to the reduced model. TH extraction batch was used as a random intercept. N = 141 for T3 and T4 concentrations and T3:T4 ratio, and N = 139 for T3 and T4 content.

Response	Zone		Country		Zone × country		Laying date		Clutch size	
	F_{ddf}	p	F_{ddf}	p	F_{ddf}	p	F_{ddf}	p	F_{ddf}	p
T4 conc (pg/mg)	**0.00_{136}**	**0.98**	0.76_{133}	0.51	0.60_{128}	0.61	1.59_{138}	0.21	0.99_{137}	0.32
T3 conc (pg/mg)	**0.63_{134}**	**0.42**	0.65_{129}	0.59	0.15_{126}	0.93	0.01_{136}	0.91	**4.80_{135}**	**0.03**
T4 cont (ng/yolk)	**0.04_{135}**	**0.84**	2.25_{134}	0.09	0.5_{131}	0.62	1.17_{134}	0.28	**3.98_{136}**	**0.048**
T3 cont (ng/yolk)	**1.23_{132}**	**0.28**	0.19_{128}	0.90	0.09_{124}	0.96	0.04_{134}	0.84	**7.9_{133}**	**0.009**
T3:T4 ratio	**0.16_{133}**	**0.69**	0.95_{131}	0.42	0.65_{126}	0.58	0.66_{135}	0.42	1.49_{124}	0.22

Table 3. Linear mixed models on the association between element concentrations (PC1 of As, Cd, Cu, Ni, Pb; measured from nestling faeces), calcium (Ca) concentration and their interaction on egg thyroid hormones (THs) in great tits. T4 = thyroxine, T3=triiodothyronine. Country and TH extraction batch were included as random intercepts. Reduced model is shown in bold. Statistics from the other factors are from models where the factor was reintroduced to the reduced model. N = 136 for T3 and T4 concentrations, and N = 134 for T3 and T4 content and T3:T4 ratio.

Response	PC1 of elements		Ca		PC1×Ca		Laying date		Clutch size	
	F_{ddf}	p	F_{ddf}	p	F_{ddf}	p	F_{ddf}	p	F_{ddf}	p
T4 conc (pg/mg)	**0.00_{135}**	**0.95**	0.84_{134}	0.36	0.40_{132}	0.53	0.53_{132}	0.22	0.85_{133}	0.36
T3 conc (pg/mg)	**1.18_{132}**	**0.27**	0.00_{130}	0.97	0.21_{128}	0.65	0.11_{132}	0.74	**4.74_{131}**	**0.03**
T4 cont (ng/yolk)	**0.01_{25}**	**0.98**	0.65_{73}	0.42	0.08_{114}	0.77	1.23_{123}	0.22	2.66_{101}	0.10
T3 cont (ng/yolk)	**1.99_{131}**	**0.16**	0.19_{129}	0.66	0.03_{127}	0.86	0.04_{129}	0.84	**6.94_{130}**	**0.009**
T3:T4 ratio	**$1.37_{6.75}$**	**0.16**	$0.01_{82.4}$	0.90	0.18_{68}	0.67	0.51_{128}	0.47	1.23_{130}	0.26

Table 4. Linear mixed models on the association between arsenic (As), cadmium (Cd), copper (Cu), nickel (Ni), lead (Pb) and calcium (Ca) concentration and their interaction on egg thyroid hormones (THs) in great tits. Elements were measured from nestling faeces. T4 = thyroxine, T3 = triiodothyronine. Country and TH extraction batch were included as random intercepts. Reduced model is shown in bold. Statistics from the other factors are from models where the factor was reintroduced to the reduced model. Ndf=1. N = 136 for T3 and T4 concentrations, and N =134 for T3 and T4 content and T3:T4 ratio.

Response	As		Ca		As×Ca	
	F_{ddf}	*p*	F_{ddf}	*p*	F_{dfd}	*p*
T4 conc (pg/mg)	**0.13_{134}**	**0.71**	0.75_{133}	0.78	2.80_{130}	0.16
T3 conc (pg/mg)	**$0.05_{16.3}$**	**0.82**	$0.08_{50.7}$	0.77	$0.18_{81.8}$	0.67
T4 content (ng/yolk)	**$0.05_{51.5}$**	**0.91**	0.43_{91}	0.51	1.00_{118}	0.32
T3 content (ng/yolk)	**0.14_{130}**	**0.70**	0.20_{129}	0.88	0.08_{126}	0.78
	Cd		Ca		Cd×Ca	
	F_{ddf}	*p*	F_{ddf}	*p*	F_{ddf}	*p*
T4 conc (pg/mg)	**0.02_{132}**	**0.88**	**0.57_{132}**	**0.45**	**4.88_{132}**	**0.02**
T3 conc (pg/mg)	**0.17_{131}**	**0.67**	0.09_{62}	0.76	2.74_{128}	0.10
T4 content (ng/yolk)	**0.06_{81}**	**0.80**	0.46_{104}	0.51	2.29_{130}	0.13
T3 content (ng/yolk)	**0.12_{129}**	**0.73**	0.04_{129}	0.84	1.75_{129}	0.19
	Cu		Ca		Cu×Ca	
	F_{ddf}	*p*	Fd_{df}	*p*	F_{ddf}	*p*
T4 conc (pg/mg)	**$0.07_{34.2}$**	**0.79**	**$6.67_{98.2}$**	**0.01**	**$5.95_{79.7}$**	**0.017**
T3 conc (pg/mg)	**1.52_{128}**	**0.22**	**5.00_{128}**	**0.02**	**4.67_{127}**	**0.03**
T4 content (ng/yolk)	**$0.17_{97.3}$**	**0.67**	**4.31_{130}**	**0.03**	**3.91_{130}**	**0.05**
T3 content (ng/yolk)	**0.12_{129}**	**0.73**	0.04_{129}	0.84	3.68_{125}	0.06
	Ni		Ca		NI×Ca	
	F_{ddf}	*p*	F_{ddf}	*p*	F_{ddf}	p
T4 conc (pg/mg)	**0.27_{34}**	**0.60**	0.87_{133}	0.58	0.40_{131}	0.52
T3 conc (pg/mg)	**1.08_{131}**	**0.30**	0.17_{131}	0.68	2.30_{129}	0.13
T4 content (ng/yolk)	**0.00_{22}**	**0.94**	0.45_{102}	0.50	0.54_{130}	0.46
T3 content (ng/yolk)	**0.27_{130}**	**0.60**	0.85_{129}	0.29	2.53_{127}	0.11
	Pb		Ca		Pb×Ca	
	F_{ddf}	*p*	F_{ddf}	*p*	F_{ddf}	*p*
T4 conc (pg/mg)	**0.01134**	**0.94**	0.84133	0.35	0.01129	0.93
T3 conc (pg/mg)	**0.365.6**	**0.57**	0.20113	0.65	0.1798	0.62
T4 content (ng/yolk)	**0.1414.8**	**0.71**	0.48118	0.48	0.26124	0.61
T3 content (ng/yolk)	**0.70130**	**0.70**	0.09129	0.76	0.02125	0.88

Figure legends

Fig 1. Averages (±SE) of the 1st principal component of elements (As, Cd, Cu, Ni, Pb) across the four study populations in polluted (black bars), and reference (white bars) zones. Elements were analysed from great tit nestling (age of 7-9 days) faeces, one measurement for each brood. Sample size (number of nests) is indicated above the bars.

Fig 2. Yolk thyroxine, T4 (a) and triiodothyronine, T3 (b) concentrations (back-transformed marginal means ±SE, pg/mg) across four different great tit study populations (Finland, Belgium, Hungary, Portugal) in polluted (black circles) and reference (white circles) zones. Sample sizes are indicated above the bars.

Fig 3. Association between the first principal component (PC1) of elements (As, Cd, Ni, Cu, Pb), i.e. total element load, and egg (a) thyroxine (T4) and (b) triiodothyronine (T3) concentration (pg/mg) in great tits. N = 136 and 134 respectively

Fig 4. Association between faecal cadmium (Cd) (log-transformed, µg/mg, dry weight) and egg thyroxine (T4, pg/mg) in relation to calcium (Ca) availability (in faecal matter, classified in quartiles): a) samples with lowest 25% of Ca concentrations, b) 25-50%; c) 50-75%, d) 75-100%, i.e. samples with highest Ca concentrations. N = 35 per category.

Fig 5. Association between faecal copper (Cu) (log-transformed, µg/mg, dry weight) and egg thyroxine (T4, pg/mg) in relation to calcium (Ca) availability (in faecal matter, classified in quartiles): a) samples with lowest 25% of Ca concentrations, b) 25-50%; c) 50-75%, d) 75-100%, i.e. samples with highest Ca concentrations. N = 35 per category.

Fig 6. Thyroxine (T4) and triiodothyronine (T3) concentrations (average±SE, pmol/ml) in plasma of 14-day old great tit nestlings in polluted (black bars, N = 23) and reference (grey bars, N = 18) zones in the Belgian population.

Fig 1.

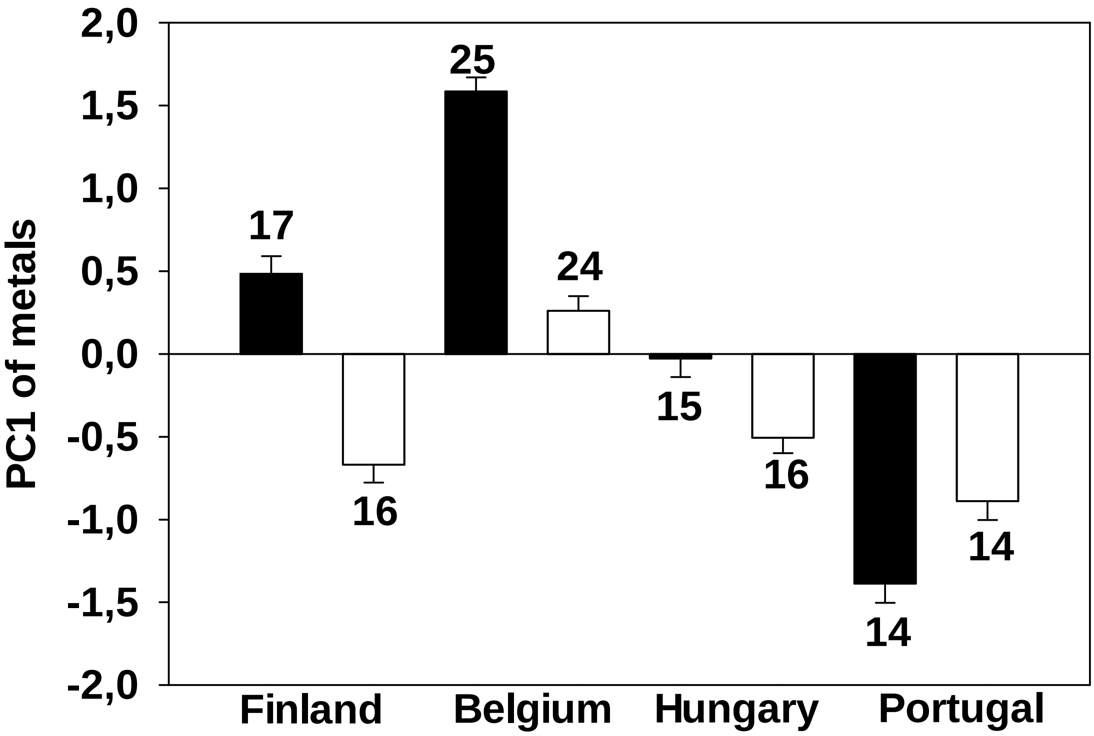
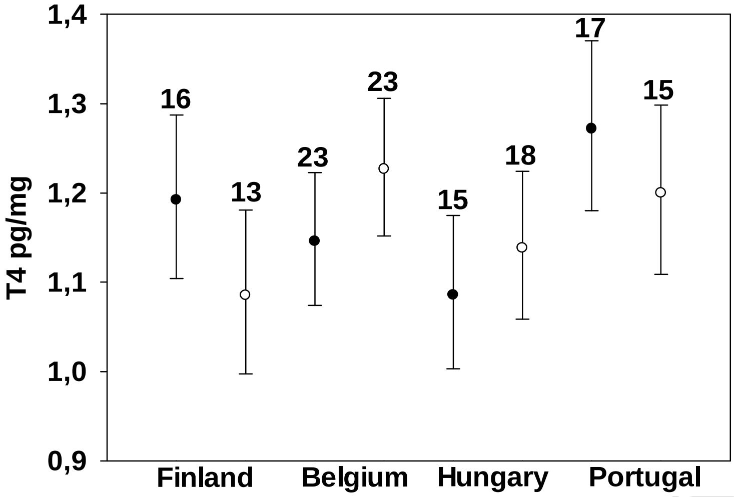

2,0
1,5
1,0
0,5
0,0
-0,5
-1,0
-1,5
-2,0
PC1 of metals
17
16
25
24
15
16
14
14
Finland
Belgium
Hungary
Portugal

Fig 2.

a)


1,4
1,3
1,2
1,1
1,0
0,9
T4 pg/mg
16
13
23
23
15
18
17
15
Finland
Belgium
Hungary
Portugal

b)

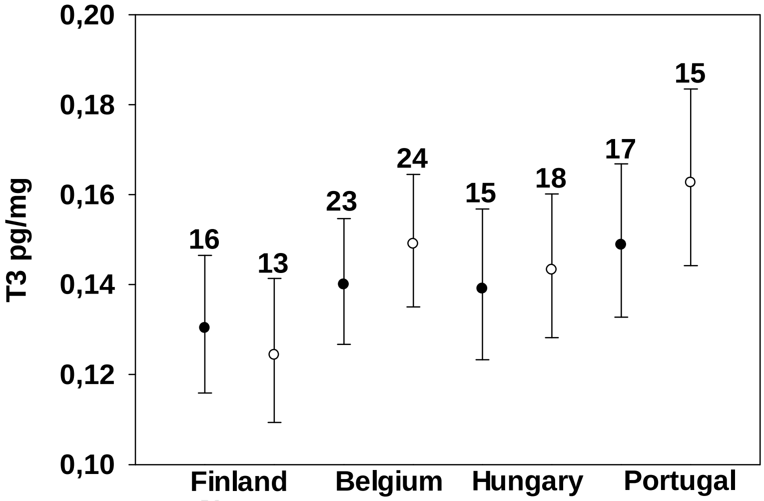
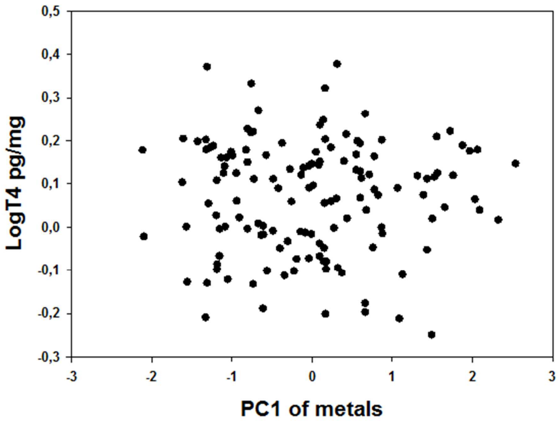
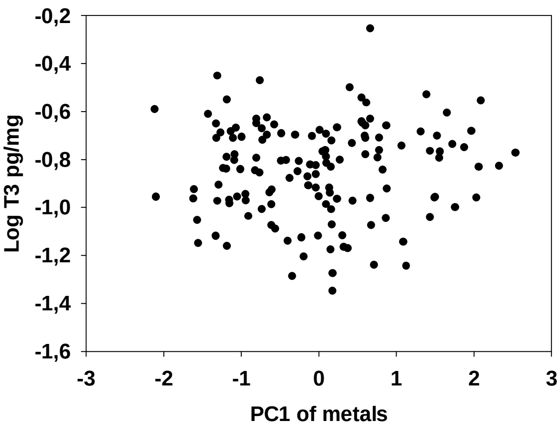

0,20
0,18
0,16
0,14
0,12
0,10
T3 pg/mg
16
13
23
24
15
18
17
15
Finland
Belgium
Hungary
Portugal

Fig 3.

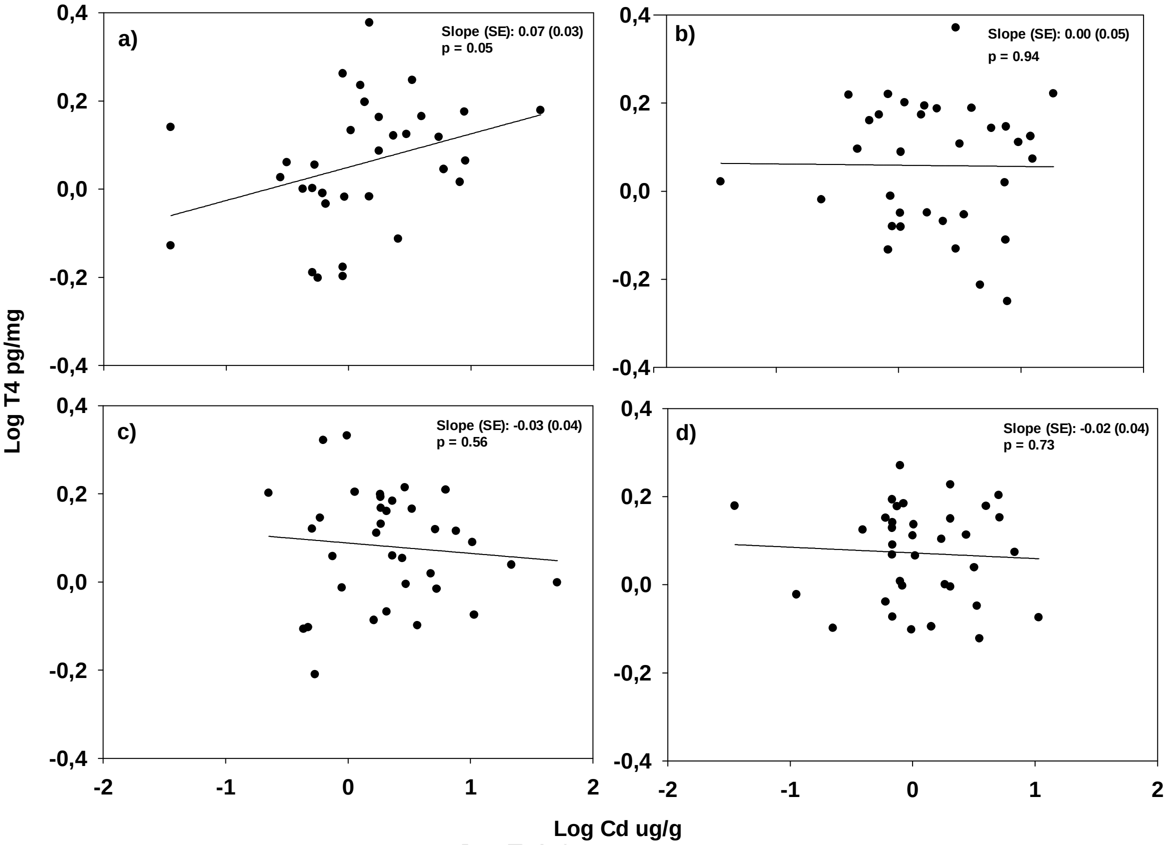
a)


LogT4 pg/mg
0,5
0,4
0,3
0,2
0,1
0,0
-0,1
-0,2
-0,3
-3
-2
-1
0
1
2
3
PC1 of metals

b)


Log T3 pg/mg
-0,2
-0,4
-0,6
-0,8
-1,0
-1,2
-1,4
-1,6
-3
-2
-1
0
1
2
3
PC1 of metals

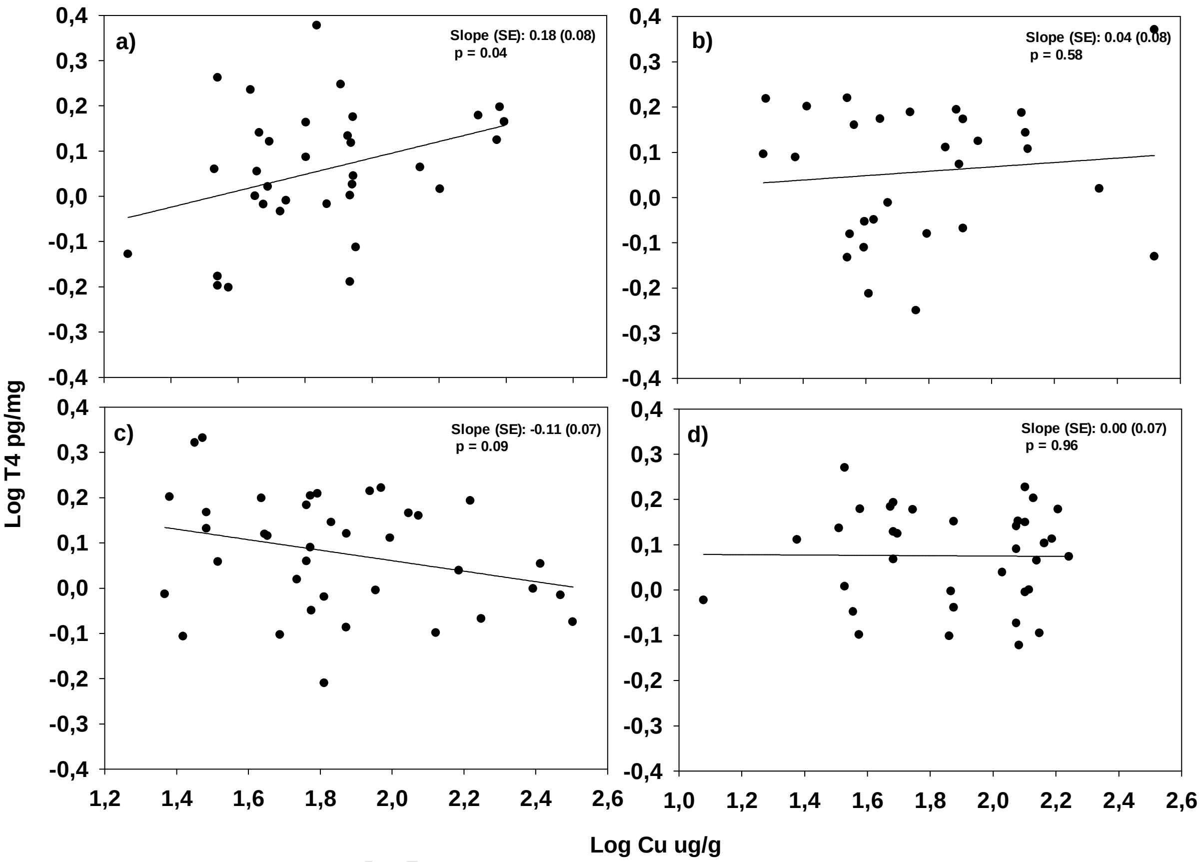
761 Fig 4.



762

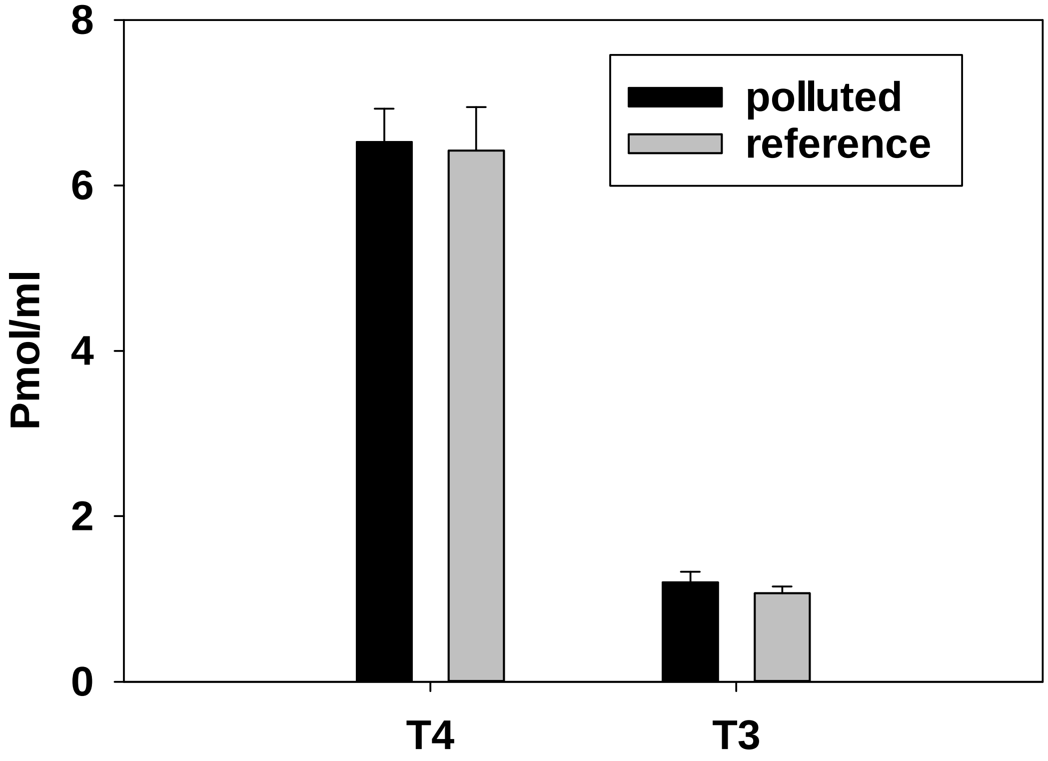
763

Fig 5.



a)
Slope (SE): 0.18 (0.08)
p = 0.04
b)
Slope (SE): 0.04 (0.08)
p = 0.58
c)
Slope (SE): -0.11 (0.07)
p = 0.09
d)
Slope (SE): 0.00 (0.07)
p = 0.96
Log T4 pg/mg
Log Cu ug/g
0,4
0,3
0,2
0,1
0,0
-0,1
-0,2
-0,3
-0,4
1,2
1,4
1,6
1,8
2,0
2,2
2,4
2,6
1,0

769 Fig 6.



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Highlights: Ruuskanen et al.

- We studied element-associated transgenerational endocrine disruption in wild populations
- We sampled four pairs of metal-polluted and reference sites across Europe
- Eggs of *Parus major* were analysed for maternal thyroid hormones, nestling plasma for thyroid hormones and nestling faeces for toxic elements
- We found no general association between toxic element exposure, egg and nestling plasma thyroid hormones
- The effect of cadmium and copper on egg thyroid hormones depended on calcium availability