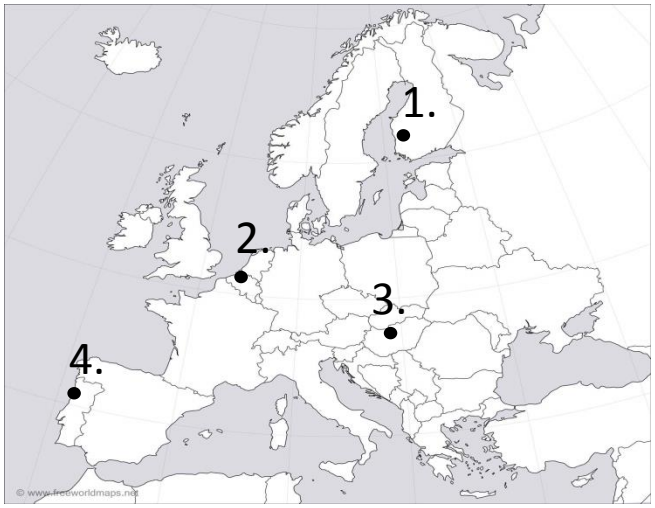
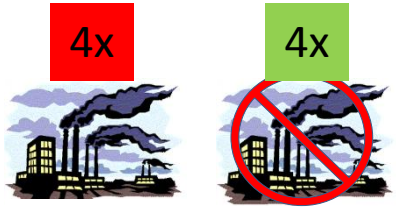
Transgenerational endocrine disruption : does elemental pollution affect egg or nestling thyroid hormone levels in a wild songbird?





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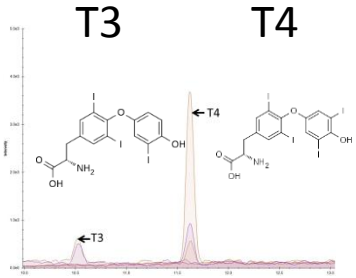

Transgenerational endocrine disruption: does elemental pollution affect egg or nestling thyroid hormone levels in a wild songbird?

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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

68 2008, Ruuskanen & Hsu 2018). Metals such as cadmium (Cd) have been been found affect
69 the production of human placental hormones (leptin and progesterone) (Stasenko et al. 2010).
70 In a rare example from a wild reptile population, eggs from polluted populations (incl.
71 organochlorines, metals, agricultural runoff) had lower progesterone and estradiol levels than
72 reference populations (Hamlin et al. 2010). However such effects of EDCs on maternally-
73 derived hormone levels in the egg and embryo have rarely been studied.

74 Thyroid hormones (THs; prohormone thyroxine T4, and biologically active tri-
75 iodothyronine, T3) are a key class of hormones that control and regulate vital biological
76 processes such as thermogenesis, growth, and metamorphosis (Norris & Carr 2013). Plasma
77 TH levels are determined by production/secretion from the thyroid gland, conversion of T4 to
78 T3 in tissues by deiodinase enzymes as well as TH degradation (McNabb 2007). Recent
79 studies suggest that maternal THs transferred to eggs and embryos are important for offspring
80 development across vertebrates and can also affect offspring TH axis function (Brown et al.
81 2014, Hsu et al. 2017, Patel et al. 2011, Ruuskanen et al. 2016a, Ruuskanen & Hsu 2018,
82 Vulsma et al. 1989). Some elements, for example, cadmium (Cd), lead (Pb), chromium (Cr),
83 copper (Cu) and arsenic (As) have been shown to disrupt TH homeostasis via binding to
84 receptor thiol groups and disturbing TH signalling (Norris & Carr 2006, Sun et al. 2016).
85 Negative relationships have been reported between Pb exposure and plasma TH levels in
86 many epidemiological and animal studies (Rana 2014). Cd and As toxicity has been
87 repeatedly found to decrease serum T4 levels in captive model species (Sun et al. 2016). In a
88 recent experimental study in zebrafish (*Danio rerio*), chronic maternal exposure to Pb at an
89 environmentally relevant range of concentrations decreased egg T3 and T4, along with
90 similar decreases in female plasma TH levels (Chen et al. 2017). However, to our knowledge
91 the effects of TH disrupting agents on maternally-derived TH levels in the eggs have not been
92 explored in other vertebrates, including in birds. Surprisingly, even the direct effects of

93 dietary elemental pollution on circulating TH levels in nestling and adult birds in wild
94 populations are poorly studied (Baos et al. 2006).

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

109 We studied the association between elemental pollution and egg TH and nestling
110 plasma TH levels in wild bird populations. The great tit (*Parus major*) was selected as our
111 study species as it is considered a good bioindicator of elemental pollution: it is a resident,
112 insectivorous species that occupies a mid-trophic position in the food chain, and forages in
113 small home ranges reflecting local contamination. We used extensive sampling across four
114 countries in Europe (Finland, Belgium, Hungary, Portugal): in each country data were
115 collected from both a polluted and a reference zone. These study populations show wide
116 variation in elemental pollution levels (Costa et al. 2012, Eeva & Lehikoinen 1996, Geens et
117 al. 2010, Hargitai et al. 2016). Previous studies from these populations showed that breeding

118 success, nestling growth, nestling and adult health (e.g. changes in haematological
119 parameters) and plumage carotenoid coloration were lower in polluted compared to reference
120 zones (Eeva et al. 2009, Eeva et al. 1998, Janssens et al. 2003, but see Costa et al. 2012).
121 Also egg quality, such as egg size and shell thickness (Eeva & Lehikoinen 1995) and
122 antioxidant composition of eggs (Espín et al. 2016, Hargitai et al. 2016), were altered in
123 polluted compared to reference zones. We sampled non-incubated eggs for maternally-
124 derived egg TH measurements, measured nestling plasma THs and used nestling faeces from
125 the same nests to assess dietary elemental exposure of arsenic (As), cadmium (Cd), copper
126 (Cu), nickel (Ni) and lead (Pb) in the four populations in polluted and reference zones. To
127 study the potential Ca-dependent effects of elemental toxicity on endocrine traits, we also
128 measured Ca levels in nestling faeces as a proxy for Ca availability.

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

135

136 **Methods**

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

142 Harjavalta (61°20'N, 22°10'E): the polluted zone is located at ca 1 km distance from a Cu-Ni
143 smelter with a reference zone at a distance of ca 7 km (Eeva & Lehikoinen 1996). The main
144 pollutants in Harjavalta are As, Cu, Ni, Pb and Zn (Eeva et al. 2012). In Belgium, the
145 populations are located in Antwerp (51°13'N, 4°24'E): the polluted zone is located next to a
146 non-ferrous metallurgical plant with a reference zone at a 6 km distance (Eens et al. 1999).
147 The main pollutants in Antwerp are As, Cd, Cu, Pb and Zn (Janssens et al. 2001). In
148 Hungary, the polluted site is an urban park in Budapest (47°28'N, 19°02'E) with a reference
149 zone at ca 27 km distance. The main pollutants in Budapest are As, Cu, Ni, Pb and Zn
150 (Hargitai et al. 2016). In Portugal the populations are located in Figueira da Foz (40°02′N,
151 8°52′W): the polluted zone is located at a 1 km distance from a pulp factory with a reference
152 zone 20 km away. The main pollutants in Figueira da Foz are As, Cd, Cu, Hg, Ni, Pb, Se and
153 Zn (Costa et al. 2012, Costa et al. 2011).

154 The nest boxes were checked periodically to monitor the development of nest building
155 and record the laying date (date of laying the 1st egg), clutch size, hatching date, brood size,
156 and number of fledglings. In total we monitored 153 great tit nests (50 in Belgium, 33 in
157 Finland, 38 in Hungary and 32 in Portugal), see final sample sizes in Fig 2. The 4th egg was
158 collected on the day of laying, replaced by a plasticine egg, and frozen at –20 °C for later TH
159 analyses (see details below). Faecal samples of nestlings were collected from 141 of the 153
160 nests for element analyses (see details below). Elemental concentrations in nestling faeces are
161 a common indicator for local pollution levels (Dauwe et al. 2004, Eeva et al. 2014, Espín et
162 al. 2016). Faecal calcium levels have been found to correlate with calcium availability in the
163 diet (estimated as amount of snail shells in the nest, their primary source of calcium) in
164 another similar-sized passerine, the pied flycatcher (*Ficedula hypoleuca*) in Harjavalta
165 (Finland) study area (Eeva and Lehikoinen 2004). Also in adults, elemental levels measured
166 during breeding reflect recent exposure (within 2 weeks), and thus very local pollution load at

167 the feeding range of the individual (Berglund et al. 2011). Unfortunately, the egg elemental
168 levels could not be directly measured due to resource constraints.

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

177 All samples were collected under appropriate licenses from local authorities in each
178 study population, as following: *Finland*: The experiment was conducted under licenses from
179 the Animal Experiment Committee of the State Provincial Office of Southern Finland
180 (license number ESAVI/11579/04.10.07/2014) and the Centre for Economic Development,
181 Transport and the Environment, ELY Centre Southwest Finland (license number
182 VARELY/593/2015). All applicable institutional and/or national guidelines for the care and
183 use of animals were followed. *Belgium:* The Flemish Agency 'Natuur en Bos' provided
184 permission for this study (ANB BL FF V16-00105-VB). *Hungary:* The Middle-Danube-
185 Valley Inspectorate for Environmental Protection, Nature Conservation and Water
186 Management (PE/EA1432-6/2016), the Pest County Government Office of the National Food
187 Chain Safety Office (PE/KTF 8988-5/2016) and the Mayor's Office of Budapest
188 (FPH061/1829-3/2016) provided permissions for this study. *Portugal*: All animals were
189 handled according to current Portuguese law and following the license number 217, issued by
190 ICNF – Institute for Nature Conservation and Forest.
191

192 *Thyroid hormone analyses*

193 The egg and plasma samples were analysed for T3 and T4 at the University of Turku. LC-
194 MS/MS was conducted at the facilities of Turku Center for Biotechnology. In the egg
195 samples, yolk and albumen were separated after thawing. Yolk was weighed (0.01 g
196 accuracy) and mixed with milli-Q water (1:1) and vortexed thoroughly. T4 and T3 were
197 extracted from yolk and plasma following previously published methods (de Escobar et al.
198 1985, Ruuskanen et al. 2018). In short, yolk-water mixture (ca 150 mg of pure yolk) or
199 plasma (25 µl) was homogenized in methanol. As an internal recovery tracer, a known
200 amount of $^{13}C_{12}$-T4 (Larodan) was added to each sample. This allowed us to control for the
201 variation in recovery (i.e. extraction efficiency) for each sample. Chloroform was then added
202 and after centrifugation (15 min, 1900 g, +4°C), the supernatant was collected and the pellet
203 was re-extracted in a mixture of chloroform and methanol (2:1). Back-extraction into an
204 aqueous phase (0.05% $CaCl_2$) was followed by a re-extraction with a mixture of
205 chloroform:methanol: 0.05% $CaCl_2$ (3:49:48) and this phase was further purified on Bio-Rad
206 AG 1-X2 resin columns. The iodothyronines were eluted with 70% acetic acid, and
207 evaporated to dryness under vacuum overnight. Blanks (plain reagents without any sample)
208 were analysed in each extraction batch to detect any contamination. Yolk samples from
209 different populations were equally distributed across five extraction batches, and extraction
210 batch was used as a random intercept in the statistical models to control for any differences
211 among the batches. Nestling plasma THs were extracted in a single extraction batch. T3 and
212 T4 were quantified using a nanoflow liquid chromatography-mass spectrometry (nano-LC-
213 MS/MS) method, developed and validated in Ruuskanen et al. (2018). Briefly, before the
214 analysis, the dry samples were diluted in ammonium (NH_3). Internal standards $^{13}C_6$-T_3 and
215 $^{13}C_6$-T_4 (Sigma) were added to each sample to identify and quantify the THs. A triple
216 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).

241 *Statistical analysis*

242 Statistical analyses were performed with SAS 9.4 statistical package. Yolk T3 and T4
243 concentrations (pg/mg), T3 and T4 content (ng/yolk) and T3:T4 ratio were log-transformed to
244 reach normality. Also plasma T3 and T4 concentrations (pmol/ml) were log-transformed.
245 Both yolk TH content and concentration were analysed because both are important for
246 offspring development and endocrine disruption may differentially affect them. In turn,
247 altered T3:T4 ratio may reflect changes in the peripheral deiodination of T4 (i.e. conversion
248 of T4 to T3 in tissues by deiodinase enzymes, McNabb 2007). All element concentrations
249 from faecal samples were log-transformed to reach normality. In the element data, there were
250 24 values in As that were very close or below detection limit (16% of the data: 18 samples in
251 Hungary, 4 in Finland, 1 in Belgium, 1 in Portugal), 4 values for Cd (1 in each study
252 population) and 3 for Ni (all in Portugal). As suggested in the literature (Croghan & Egeghy
253 2003), we replaced these values with LOD/sqrt(2), where LOD refers to lowest detection
254 limit that was set to 0.05, to improve the distribution. This resulted in a normal distribution.

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

262 We then analysed the effect of general pollution load on egg THs using linear mixed
263 models (LMM). The fixed factors in these models included zone (polluted/reference), country
264 (Finland, Belgium, Hungary, Portugal) and their interaction. We included yolk TH analysis
265 batch as a random intercept to control for potential variation among the hormone extraction

266 batches (samples from all countries and populations were equally distributed across the
267 batches). Laying date (centred for each population to study at relative differences among
268 early and late breeders) and clutch size were included as covariates to control for potential
269 differences in individual quality, resource availability or reproductive investment.

270 We analysed the combined load of toxic elements by performing a principal
271 component analysis for the metals Cd, Ni, Cu, Pb and metalloid As (log-transformed and
272 LOD corrected values). PC1 fitted the data relatively well as the eigenvalue was 2.75, the
273 vector explained 55% of the variation. Loadings of all elements were positive (Pb = 0.71; As
274 = 0.81, Cd = 0.84, Cu = 0.61, Ni = 0.70). We then analysed the association between PC1 and
275 yolk T4 and T3 concentration and content using LMM. Given that the elemental toxicity is
276 often affected by Ca availability, we also included Ca concentration (log-transformed) and
277 the interaction between PC1 and Ca as fixed factors. Country and extraction batch were
278 included as random intercepts given the non-independence of data in each study population.
279 Population-centred laying date and clutch size were included as covariates. We found that in
280 Portugal, the elemental levels tended to be higher in reference than polluted zone. We thus
281 rerun all models excluding Portugal but as the results remained qualitatively the same, we
282 report analyses including all populations.

283 Subsequently, we analysed the association between yolk THs and individual elements
284 (As, Cd, Cu, Ni and Pb) and their interaction with Ca in separate models. The literature points
285 especially to the specific TH-disrupting effects of As, Cd, Pb and to some extent Cu (Rana
286 2014, Sun et al. 2016). The models used were similar as for PC1 of elements (see above).

287 We studied the covariation between egg T4 and T3 and the potential differences in
288 this covariation among polluted and reference zone, and in relation to total toxic element
289 exposure (PC1). Such a difference in covariation might indicate altered thyroid function,

290 either production/secretion or altered deiodination (conversion of T4 to T3 or to inactive
291 forms such as T2) in tissues. We performed LMMs with egg T4 as the dependent and egg T3
292 as the independent factor, together with zone and their interaction, and PC1 and its interaction
293 with T3. Country and extraction batch were included as random intercepts.

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

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

306

307 **Results**

308 *Elemental pollution across polluted and reference zones*

309 The results of the comparisons of element levels between polluted and reference zones across
310 and within the four study populations are reported in Table 1. Elemental levels varied
311 markedly across countries and showed different patterns across polluted and reference zones
312 in different study populations (Table 1, country × zone interaction, $p < 0.001$). Arsenic
313 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

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

376 In contrast to our predictions, we did not find any associations between overall
377 elemental pollution and egg T4 or T3 levels or nestling plasma TH levels. The lack of overall
378 association between toxic element exposure and THs is surprising because metals like Pb and
379 Cd have been found to affect plasma TH concentrations negatively in other taxa (Rana 2014),
380 in particular in other bird species (hen chicks and adult cockerels: e.g. Chaurasia et al. 1995,
381 Gupta & Kar 1999) as well as egg THs in fish (Chen et al. 2017). However, Baos et al.
382 (2006) did not find associations between toxic elements (Pb, Zn, Cu, Cd, As) and THs in
383 plasma of nestlings or adults of another wild bird population (white storks, *Ciconia ciconia*),
384 whereas steroid hormones were negatively correlated with elemental pollution levels. Finally,
385 our study did not investigate for example the effect of mercury (Hg) on THs, while it has

386 previously been linked with TH disruption in another passerine bird, the tree swallow
387 (*Tachycineta bicolor*) (Wada et al. 2009).

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

406 The lack of a general association between toxic elements and egg and nestling plasma
407 THs could be explained by several, mutually non-exclusive hypotheses: (1) the low exposure
408 load; (2) no effect of elemental pollution on female plasma THs, and thus no effect on
409 maternal transfer to the egg; (3) an effect of elemental pollution female plasma THs, but
410 compensatory TH transfer to eggs. Also, (4) species differences in sensitivity to toxic element

411 pollution could explain the contrasting results in our study compared to other studies (e.g.
412 Chaurasia et al. 1995, Gupta & Kar 1999, Baos et al. 2006).

413 First, the pollutant exposure levels across studies should be critically evaluated
414 (hypothesis 1). In our study, the levels of pollutants were markedly higher in polluted
415 compared to reference zones especially in Belgian and Finnish populations (As
416 concentrations were 10 times higher in polluted than reference zones, Cd 5–10 times higher,
417 Cu 2–5 times higher, Ni 2–15 times higher and Pb 5–10 times higher, respectively).
418 However, the levels measured in our study are somewhat lower than in previous studies from
419 the same populations, which reported detrimental effects on reproductive parameters and
420 female and chick physiology. For example Janssens et al. (2003, Table 1) reported for the
421 Belgian population (sampled in 1999) 5 times higher As concentrations, 10 times higher Ni
422 concentration and 2 times higher Cu concentrations (but similar or lower Cd and Pb),
423 compared to our data (sampled in 2016 from the exact same sites). In a previous experimental
424 study on metal-associated TH disruption in birds, Gupta & Kar (1999) dosed hen chicks daily
425 with 2.5µg Pb/ g tissue and found a decrease in plasma THs. Interestingly, in the study
426 populations in Finland and Belgium, estimated daily Pb intake ranged between 2.2−8.5 µg
427 Pb/g tissue (Eeva et al. 2014). Thus Pb exposure levels in our wild populations could be
428 rather similar as in experiments with captive chicks, while no association between elemental
429 levels and THs was found in our study. Therefore, the lack of effect of toxic elements on THs
430 may be not only due to low exposure levels, but potentially species differences (hypothesis
431 4).

432 Second, we did not measure female plasma TH levels in this study due to practical
433 limitations. It is thus possible that toxic elements may not have caused TH disruption in the
434 female circulation, leading to no transgenerational TH disruption (hypothesis 2). The fact that
435 nestling plasma TH was not associated with elemental pollution load supports this

436 hypothesis. Alternatively, female plasma TH levels may have been affected, but due
437 independent regulation of plasma and egg TH levels, females compensated by transferring
438 proportionally more THs into the eggs to avoid detrimental effects on offspring (hypothesis
439 3). This could lead to no differences in egg TH levels. The molecular transfer and regulatory
440 mechanisms of THs from circulation to egg yolk are currently not well understood
441 (Ruuskanen & Hsu 2018). Indirect evidence suggests somewhat contrasting patterns in
442 plasma and yolk THs (Hsu et al. 2016, Van Herck et al. 2013, Wilson & McNabb 1997). If
443 such regulatory mechanism(s) are present, an independent effect of endocrine disruption on
444 plasma THs but not egg THs is possible.

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

458 Interestingly, we found negative correlations between clutch size and egg THs. Given
459 that the molecular structure of THs requires iodine, which organisms cannot produce
460 themselves, females may face a trade-off between allocating THs (and associated iodine) to

461 eggs versus themselves (Ruuskanen & Hsu 2018). This trade-off could be accentuated in
462 large clutches, leading to decreased TH concentrations. Recent studies across vertebrates egg
463 THs show substantial intra-specific variation both among and within females (Ruuskanen &
464 Hsu 2018), which is associated with key environmental and ecological factors, such as food
465 (Hsu et al. 2016) and temperature (Ruuskanen et al. 2016b), but previous studies did not
466 reveal any association with clutch size. Together, these results suggest that egg THs can be an
467 important plastic, hormonal mechanism underlying variation in offspring phenotype.

468 *Conclusions*

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

480

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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
	F*df* p	F*df* p	F*df* p	F*df* p	F*df* p	F*df* 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

693 Table 2. Linear models of the effects of zone (polluted or reference) and country on egg thyroid
694 hormones. T4 = thyroxine, T3 = triiodothyronine. Reduced model is shown in bold. Statistics
695 from other factors are from models where the factor was reintroduced to the reduced model. TH
696 extraction batch was used as a random intercept. N = 141 for T3 and T4 concentrations and
697 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

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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

718 **Figure legends**

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

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

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

730 Fig 4. Association between faecal cadmium (Cd) (log-transformed, µg/mg, dry weight) and egg
731 thyroxine (T4, pg/mg) in relation to calcium (Ca) availability (in faecal matter, classified in
732 quartiles): a) samples with lowest 25% of Ca concentrations, b) 25-50%; c) 50-75%, d) 75-
733 100%, i.e. samples with highest Ca concentrations. N = 35 per category.

734 Fig 5. Association between faecal copper (Cu) (log-transformed, µg/mg, dry weight) and egg
735 thyroxine (T4, pg/mg) in relation to calcium (Ca) availability (in faecal matter, classified in
736 quartiles): a) samples with lowest 25% of Ca concentrations, b) 25-50%; c) 50-75%, d) 75-
737 100%, i.e. samples with highest Ca concentrations. N = 35 per category.

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

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744 Fig 1.

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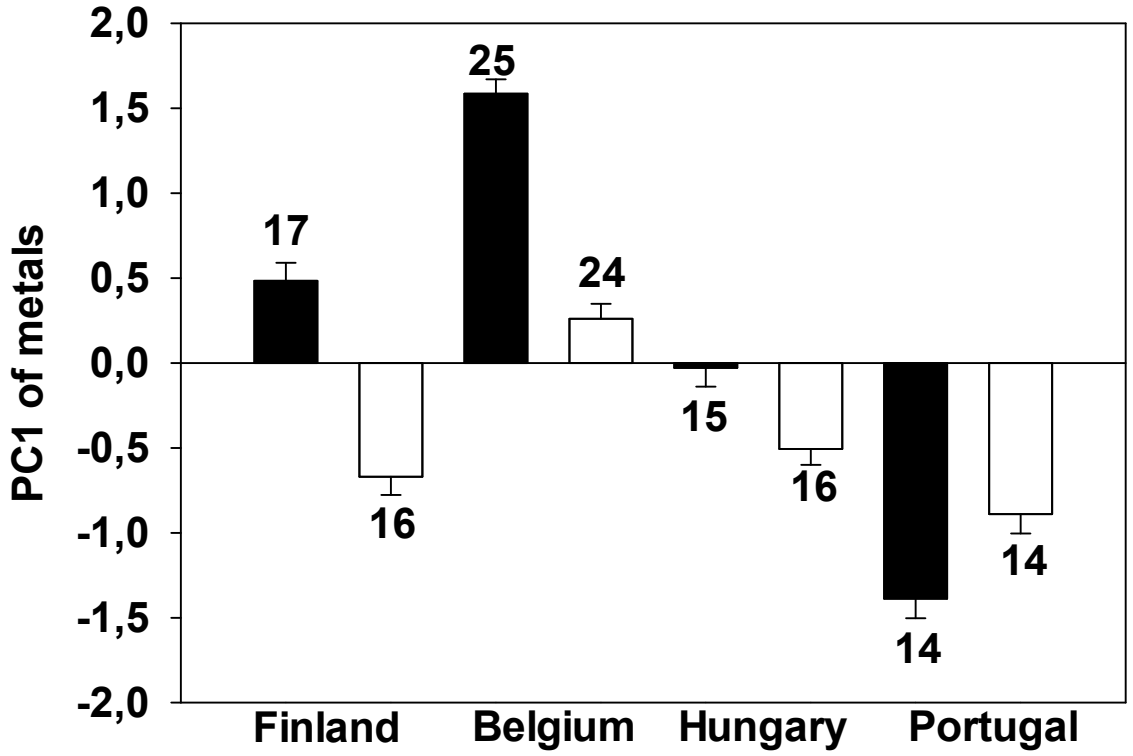

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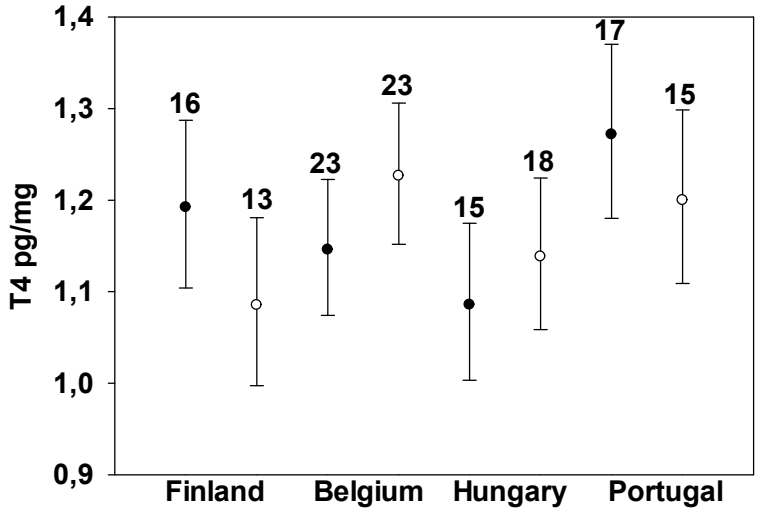
747

748 Fig 2.

749 a)



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752 b)

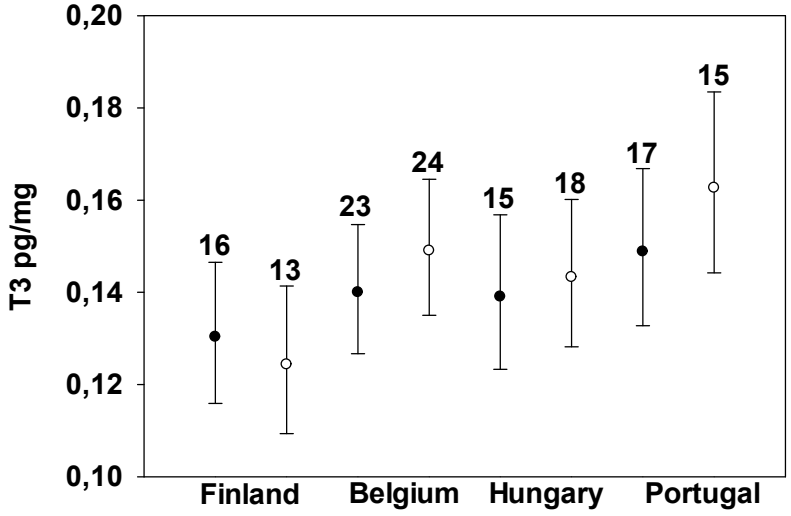

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Fig 3.

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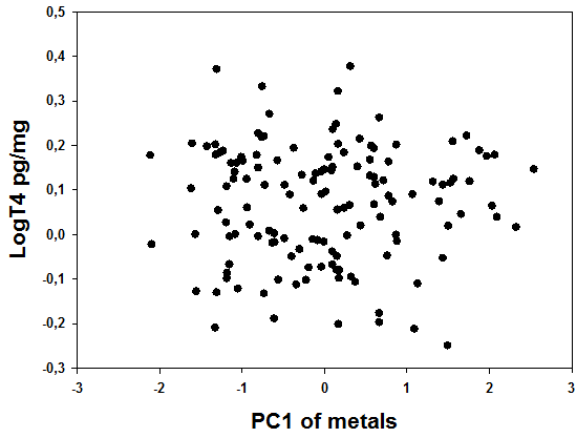
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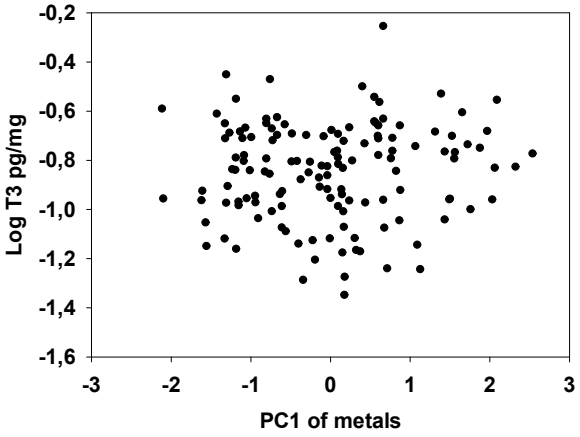
-0,2
-0,3
-3
-2
-1
0
1
2
3
PC1 of metals

b)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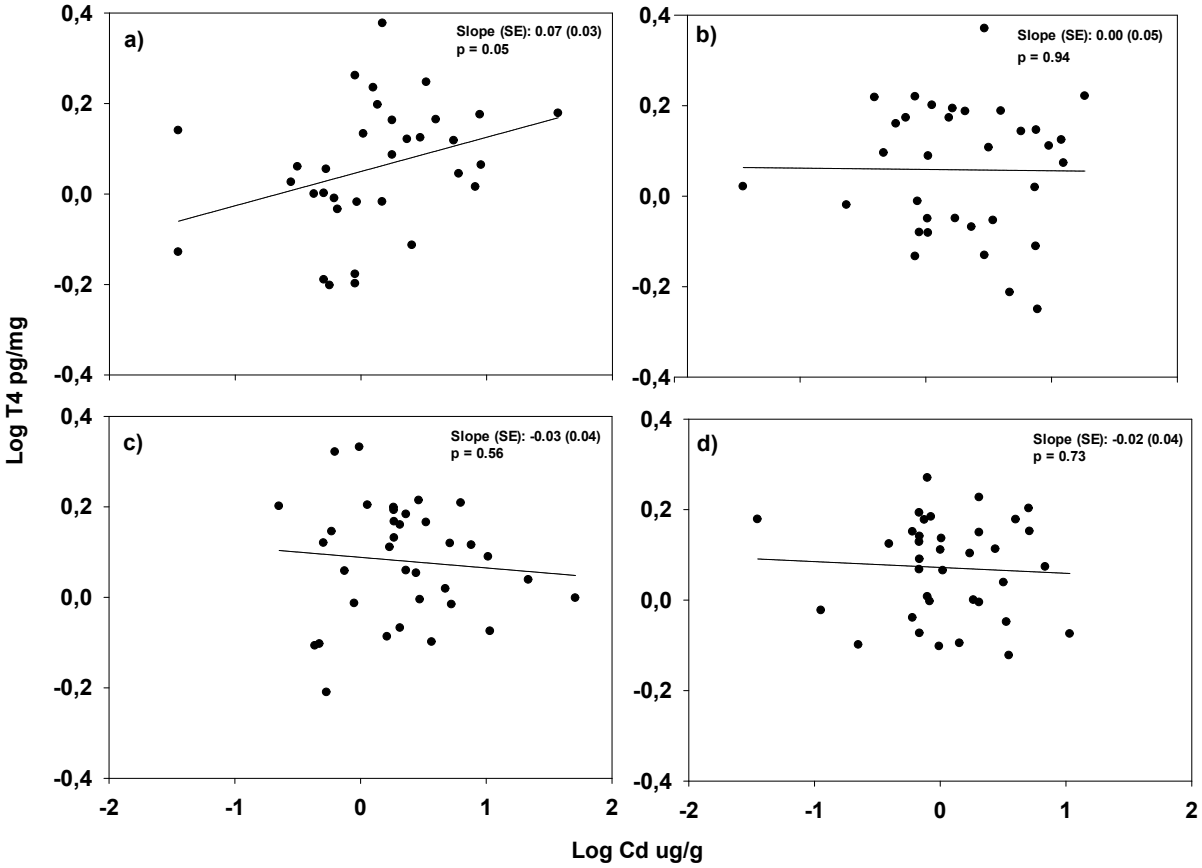
-2
-1
0
1
2
3
PC1 of metals

761 Fig 4.



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765 Fig 5.

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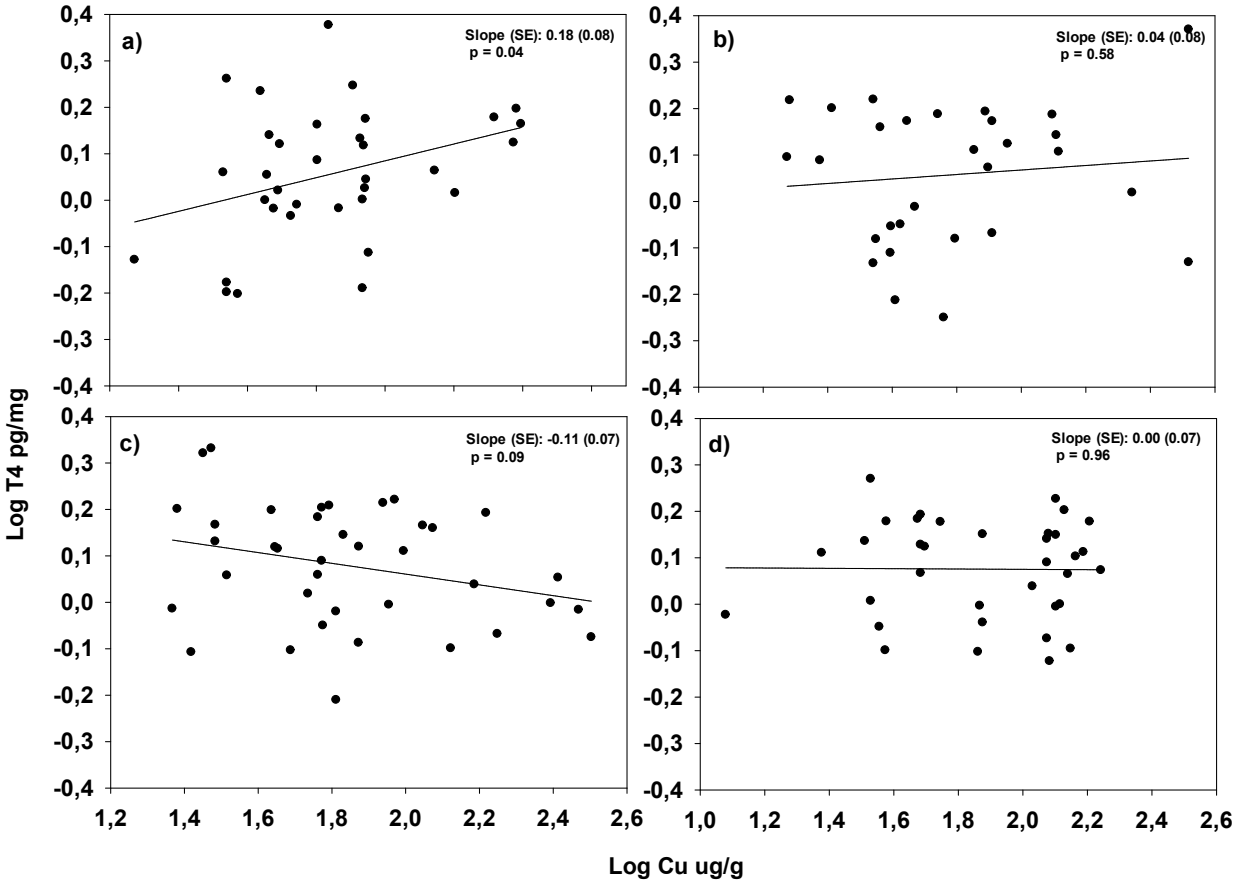

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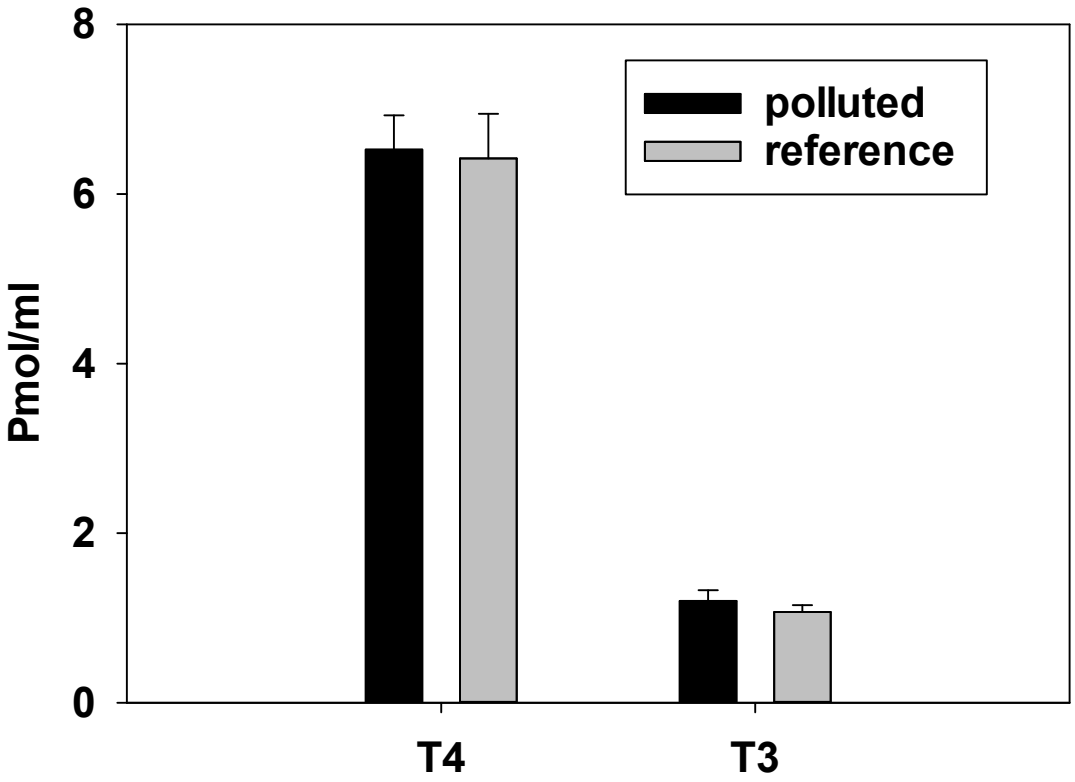
769 Fig 6.



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