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**Urban metal pollution explains variation in reproductive outputs
in great tits and blue tits**

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Abstract

It is regularly reported that avian reproductive outputs are reduced in urban areas, yet the underlying reasons for discrepancies between urban and natural habitats are to date poorly explained. To address this knowledge gap, we tested whether the reproductive outputs of wild great tit (*Parus major*) and blue tit (*Cyanistes caeruleus*) populations in Warsaw (Poland) correlated with the concentrations of six main metallic/metalloid trace elements (MTEs; copper, zinc, lead, cadmium, arsenic and mercury) in three types of biological material pertaining to avian reproduction: nestling feathers, nest material and nestling droppings. For the first time, our study highlights consistent negative effects of copper and arsenic concentrations in nestling feathers on fledging success and nestling mass in both great tits and blue tits. Fledging success was also negatively correlated with cadmium and lead concentrations in nestling droppings. Importantly, while the relative proportions of each MTE were equivalent between the three biological materials, reproductive success correlated better with MTE concentrations in nestling feathers than in the two other materials; this result suggests that MTE absorption would explain part of the variation in individual fitness and emphasises the relevance of using nestling feathers for investigating the effects of MTE exposure on nestlings of hole-nesting birds. Altogether, our results suggest that urban MTE pollution likely contributes to the differences in reproductive outputs observed between tit populations living in urban and rural environments.

Keywords: heavy metals, chemical pollution, birds, reproduction, urbanisation, population productivity

1. Introduction

Bird populations inhabiting urban landscapes almost universally exhibit lower reproductive outputs than populations in more natural environments (reviewed in Chamberlain et al., 2009; see also Bailly et al., 2016; Biard et al., 2017; de Satgé et al., 2019; Seress et al., 2018, 2012). However, the underlying mechanisms responsible for reproduction impairment in urban areas remain unclear (Ouyang et al., 2018). Nestling mass, growth and survival of great tit and blue tit nestlings correlate negatively with road proximity and impervious surface area in Warsaw, Poland (Corsini et al., 2020, 2017). The replacement of vegetation with impervious surfaces limits vegetative cover, which is likely to limit food availability throughout urbanised areas (Pollock et al., 2017). At the same time, the presence of impervious surfaces and roads increases human activities such as car driving, directly responsible for the emission of metallic trace elements (MTEs; also called “heavy metals” although they include all the toxic metallic elements whatever their atomic mass) (Bai et al., 2017; Trombulak and Frissell, 2000). While MTE pollution is known to impair reproduction in birds nesting close to metallurgic smelters (Dauwe et al., 2005; Eeva et al., 2009; Janssens et al., 2003; Stauffer et al., 2016), knowledge of the extent to which MTE pollution affects the fitness of urban wildlife is scarce (but see Bailly et al., 2017; Chatelain et al., 2016; Espín et al., 2020; Fritsch et al., 2019).

MTEs such as lead, cadmium or zinc, while naturally present in the environment, are mainly emitted by anthropogenic activities involving fossil fuel combustion or the use of some anti-corrosive paints or alloys (Nriagu, 1979), and are especially abundant in the atmosphere and soil along roads (Trombulak and Frissell, 2000). As such, the concentrations of several MTEs (*e.g.* lead, cadmium, copper, zinc, arsenic, iron, etc.) are usually higher in birds from urban

areas than from rural environments (*e.g.* Bichet et al., 2013; de la Casa-Resino et al., 2014; Orłowski et al., 2014). Lead, mercury, cadmium and arsenic pollution is considered as a public health concern (Tchounwou et al., 2012). Other MTEs such as zinc and iron are essential: they play an important role in biological systems when absorbed at low concentrations, but may be toxic at higher levels (*e.g.* regarding zinc; Bozym et al., 2010; Greenberg and Briemberg, 2004; Prasad, 2009). In wild bird populations, breeding pairs nesting close to a smelter, where large quantities of MTEs are emitted, were reported to have a reduced clutch size, hatching and fledging success (Dauwe et al., 2005; Eeva et al., 2009; Janssens et al., 2003). In addition, the nestlings of great tits hatching close to a smelter exhibited shorter telomeres (Stauffer et al., 2016). The role of telomeres is to protect the genetic information. When reaching a lower size threshold, telomeres trigger cell division arrest and may induce cell apoptosis. Given their starting sizes, telomeres may then define cell lifespan and, as a consequence, organism survival (Cawthon et al., 2003; Hausmann et al., 2005; Salmón et al., 2017; Wilbourn et al., 2018). All in all, while it is now established that MTE exposure next to smelters engenders considerable physiological costs, overall increasing long-term fitness prospects in great tit nestlings and fledglings, similar insight into urban-driven MTE exposure is lacking (but see Bailly et al., 2017; Chatelain et al., 2016; Espín et al., 2020; Fritsch et al., 2019).

Explicit testing of the effects of MTE exposure on the physiology and concomitant fitness of juvenile organisms developing in the urban space requires reliable assessment of MTE exposure in nestlings. However, there is considerable variability in the reporting of MTE concentrations from study to study: indeed, MTE concentrations have been measured either in nestling blood (*e.g.* Dolan et al., 2017; Vermeulen et al., 2015), organs (*e.g.* Berglund et al., 2011; Swaileh and Sansur, 2006), droppings (*e.g.* Berglund et al., 2011; Rainio et al., 2013) or

feathers (*e.g.* Dolan et al., 2017; Rubio et al., 2016). Measuring MTEs in organs necessitates euthanasia, which is not an option in many wild bird populations and is not appropriate to the study of nestling growth and survival. On the other hand, the concentrations of several MTEs are very low in the blood, which can prevent MTE detection, and does not always accurately reflect individual exposure to MTEs (Dolan et al., 2017). Importantly, previous studies showed that MTE concentrations in nestling droppings vary between sites depending on their distance to a smelter (*e.g.* Dauwe et al., 2004; Janssens et al., 2002; Rainio et al., 2013) and correlate with MTE concentrations in caterpillars – their main food source (Dauwe et al., 2004). Nonetheless, MTE concentrations in droppings only reflect MTEs that were ingested and that did not pass into the blood stream. Because MTE gastrointestinal absorption rate varies between individuals (Diamond et al., 1998; Whitehead et al., 1996), it is possible that MTEs in droppings do not reflect MTE organismal burden (*i.e.* the amount of MTEs in the body). This amount might be better estimated by MTEs in nestling feathers, that is MTEs that were transferred from the blood stream to the feathers during feather growth (Burger, 1993) – *i.e.* during ca. 20 days in the case of tail feathers (De La Hera et al., 2011). Previous studies showed that MTE concentrations in nestling feathers also vary depending on the distance of the nest to a smelter (*e.g.* Berglund et al., 2011; Janssens et al., 2002). An experimental approach in blue tit nestlings further confirmed that lead exposure resulted in higher lead concentrations in the feathers (Markowski et al., 2013). Logistical and ethical arguments may also need to be taken into account, as measuring MTEs in nestling droppings and feathers necessitates to handle the individuals, which is known to induce a stress response (Le Maho et al., 1992). Consequently, measuring MTEs in a nestling's proximate environment, such as the nest, might be an alternative, non-invasive method. Testing how MTE concentrations in different biological materials correlate with avian health and fitness proxies is thus essential to identify the biomonitoring material(s) that estimate MTE exposure best.

Independently of the type of biomonitoring material selected (*e.g.* feathers, nest material, droppings), it is essential to consider bird simultaneous exposure to all or at least the most abundant MTEs. Indeed, MTEs can have additive, synergetic or antagonistic effects (Wu et al., 2016). For instance, the exposure to a mixture of MTEs can have deleterious effects, even though the concentrations of each individual MTE are below the toxicity threshold. On the contrary, the toxicity of some MTEs can be lowered when in a mixture; for example, zinc reduces the absorption and retention of ingested lead (Cerklewski and Forbes, 1976; Chatelain et al., 2016; El-Gazzar et al., 1978); this can result in individuals not suffering from any adverse effect of lead exposure, unlike their counterparts exposed to lead only (Chatelain et al., 2016). While, so far, attention has virtually been on individual MTE toxicity (but see Bailly et al., 2017; Saulnier, 2020), it is only by taking into consideration MTE mixture that we will be able to quantify the extent to which MTE pollution affects wildlife.

Characterizing the effects of MTE exposure, assessed in different types of biological material, on the reproductive output of wild populations is a promising avenue to gain novel insight into urbanisation effects on urban avian biology and population dynamics. Here, we measured the link between MTE levels and the reproductive outputs of two common passerine birds nesting in a large range of urban MTE pollution in Warsaw, Poland. Our study aimed to test the following hypotheses:

- i) MTE exposure is comparable in nestlings of two common hole-nesting birds – blue tits and great tits.
- ii) there is a consistent link between urban MTE exposure and reproductive outputs in those two species.
- iii) relevant, accurate and non-invasive biomonitoring tools can be established to reliably assess MTE exposure in nestlings of hole-nesting birds.

To do so, we measured six of the most common MTEs – copper, zinc, lead, arsenic (a metalloid), cadmium and mercury – in three different types of material: nestling feathers, nest material and nestling droppings. A Principal Component Analysis on MTE concentrations was computed to assess whether and how reproductive outputs varies with bird overall exposure to the MTE mixture in their environment, in two distinct passerine species; these were then compared with analyses of reproductive outputs where each MTE was tested separately. Reproductive success was modelled as fledging success (both fledgling number and percentage), as well as nestling mass and telomere length at 15 days.

2. Methods

2.1. Study sites and bird sampling

The reproduction of wild populations of great tits *Parus major* and blue tits *Cyanistes caeruleus* was monitored from April to July in 2016 and 2017 using artificial woodcrete Schwegler nestboxes located in Warsaw and its vicinity (Poland). More specifically, the nestboxes were located in three sites characterised by different overall land use (an urban park and an urban forest located in Warsaw, and a rural forest located close to Palmiry ca. 20 km away from Warsaw city centre; see Corsini et al., 2017 for details on the field sites). These sites also displayed high intra-site heterogeneity in terms of MTE pollution levels: each of the MTEs measured in the nest material showed a large range of concentrations (Figure 1), reflecting the fact that the nestboxes were set in locations where MTE pollution levels varied in a continuous manner. We successfully monitored 144 nests in a gradient of urban MTE pollution – specifically, 71 great tit nests and 73 blue tit nests. During nest building and laying, nests were visited every week to assess clutch size and hatching date. When eggs were found for the first time in the nest, four pinches of moss or soft material were collected from

the nest cup and stored in plastic bags for MTE analyses. After hatching, the nests were visited when nestlings were fifteen days old to assess brood size and to weigh the nestlings. From each fifteen-day-old nestling, we collected a blood sample for subsequent DNA extraction and, for MTE analyses, the second left rectrix (*i.e.* the second tail feather from the left) and a dropping. Fifteen-day-old nestlings were also ringed. All nests were also visited 23 to 25 days after hatching to count the number of nestlings that successfully left the nest. Fledging success was calculated as the number of fledglings and as the percentage of fledglings: the number of fledglings over the total number of eggs laid during the breeding attempt (*i.e.* final clutch size).

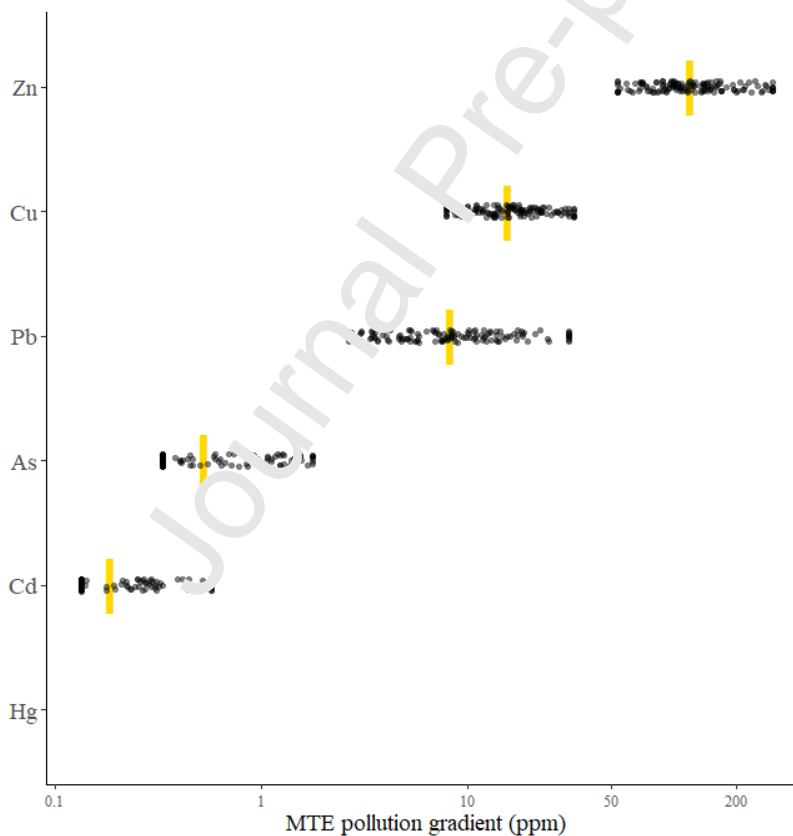


Figure 1. Zinc (Zn), copper (Cu), lead (Pb), arsenic (As), cadmium (Cd) and mercury (Hg) concentrations in the nest material (in ppm) in great tit and blue tit nests (pooled dataset). The mean concentrations are displayed in yellow. The X axis follows a logarithmic scale but the

values correspond to MTE raw concentrations. Mercury concentrations were below quantification limit (< 0.064 ppb).

2.2. MTE quantitative analysis

In feathers - Feathers were prepared for MTE analyses using a protocol adapted from Chatelain et al. (2016). The following MTEs were quantified: lead, zinc, copper, cadmium, arsenic, mercury. Briefly, nestling feathers from the same brood were pooled and washed alternatively with 0.25 M NaOH solution and ultrapure water (Milli-Q purified, Merck KGaA, Darmstadt, Germany) to remove external contamination, then dried 12 h at 50 °C to dry mass. Feathers were digested in 1 mL of HNO₃ 30% for 24h at 80°C. *In droppings* – From 1 to 3 droppings were selected per brood (depending on the collection success and brood size). We failed to collect any dropping in 17 out of 143 investigated broods (10 and 7 great and blue tit broods, respectively). Each dropping was homogenized, and c.55 mg were digested simultaneously in 1 mL of HNO₃ 65% and 0.5 mL of HF 40% for 24h at 80°C. *In nest materials* – Around 15 mg of a representative mix of the different materials composing the nest were successively digested in 1 mL of HNO₃ 65%, 1mL of H₂O₂ and 0.5 mL of HF 40% for 24h at 80°C at each step.

The product of digestion was transferred into plastic tubes and ultrapure water was added to reach a final 1% acid concentration. Total content of lead (Pb; average of Pb 206, Pb 207 and Pb 208 isotope concentrations), zinc (Zn; average of Zn 66 and Zn 68 isotope concentrations), copper (Cu; average of Cu 63 and Cu 65 isotope concentrations), cadmium (Cd; Cd 111 concentrations), arsenic (As; As 75 concentrations) and mercury (Hg; average of Hg 200 and Hg 202 isotope concentrations) were determined using an inductively coupled plasma mass spectrometer (NexION 300D ICP Mass Spectrometer, Perkin Elmer SCIEX, USA). A conventional Mainhardt nebulizer and a quartz cyclonic spray chamber were used

for sample introduction. Each isotope was measured three times and each sample was analysed two times. For concentrations above LOQ, measurements with RSD above 10% were excluded; details on the quality controls are provided in Appendix A. All measurements were performed at the Biological and Chemical Research Centre (NCBCh, University of Warsaw, Poland).

2.3. Telomere length assay

In each brood, telomere length was assessed in three randomly chosen chicks. DNA was extracted using the DNeasy 96 Blood & Tissue kit (Qiagen, Venlo, Netherland). Telomere length measurements were performed using the qPCR method originally developed for human samples (Cawthon, 2002) and further adapted to birds (Criscuolo et al., 2009). This method assesses average relative telomere length in a given DNA sample: telomere length (T) is typically compared to that of a single copy gene (S) by performing two different qPCR reactions, one using telomere primers and the other with single-copy gene primers. All individual T/S ratios are corrected to the efficiencies of the amplification reactions (Pfaffl, 2001) and are expressed relatively to a golden sample standard (with a TS value of 1), randomly chosen and repeated over the qPCR runs (Criscuolo et al., 2009). qPCR measurements were conducted separately for great tits and blue tits and normalised by a different golden sample standard. Therefore, relative telomere length should not be directly compared between species. Details on quality controls are provided in Appendix A.

2.4. Statistical analyses

Statistical analyses were performed using R software (version 3.5.1) (R Core Team, 2018). Data were corrected to consider concentrations below quantification limits and possible spurious outliers; the details about those corrections are provided in Appendix B. Importantly,

less than 5% of Cd in nestling feathers and Hg in nestling droppings and in the nest material were above the quantification limit; those MTEs were consequently excluded from all the analyses. All models were run at the brood level; for this purpose, we calculated the median per brood for MTE concentrations in nestling droppings, nestling mass and telomere length. While those parameters differed within broods, they significantly differed between nests (*i.e.* the inter-nest variability was significantly higher than the intra-brood variability; see Appendix B for details).


2.4.1. MTE exposure in great and blue tits

Differences in MTE concentrations between great tit and blue tit nestling feathers, nest material and nestling droppings were tested using linear mixed-effects models with MTE concentrations (*i.e.* Cu, Zn, Pb, As, Cd and Hg) as the dependent variable and the species, the biological material and their interaction as the explanatory variables. The nesting site (*i.e.* urban park, urban forest or rural forest) and the year (*i.e.* 2016 or 2017) were added as random intercepts. Correlations of MTE concentrations between nestling feathers, the nest material and nestling droppings were tested using Pearson's correlations.

2.4.2. Principal component analyses on MTEs

Pearson's correlations between MTE concentrations in great tits and blue tits, and in each of the three biological materials were calculated separately using the 'ggpairs' function of the 'GGally' package in R: in both species, MTE concentrations in the nest material were all positively correlated, though not always significantly so; overall, correlations were stronger in great tits than in blue tits (KMO = 0.70 and 0.46, respectively). Similarly, MTEs in nestling droppings were mostly positively correlated. In nestling feathers, most correlations between MTEs were weak ($r < 0.5$) and non-significant; they were alternatively positive or negative

depending on the MTEs and the species. All the correlations and their level of significance are presented in Appendix C. We ran Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy and Bartlett's test of sphericity on MTE correlation matrices, separately in nestling feathers, the nest material and nestling droppings in great tits and in blue tits. In agreement with the results of the Pearson's correlations, both tests indicated significant correlations between MTE concentrations within biological material and species (see Appendix C for details about MTE correlation matrices, the Kaiser-Meyer-Olkin and the Bartlett's sphericity statistics). Therefore, we performed Principal Component Analyses on MTE concentrations (PCA; `ade4` package in R), separately for great tits and blue tits and for each biological material (*i.e.* nestling feathers, nest material and nestling droppings). Principal components were retained based on the Kaiser-Guttman criterion (eigenvalue > 1 , which in our case is equivalent to a percentage of explained variance $> 20\%$): in both great tits and blue tits and for each biological material, the first two components were retained in the PCAs. PCAs explained between 55.9 and 79.6% of the variation in MTE concentrations. Correlations between the principal components and the concentrations of the different MTEs were tested using Pearson's correlations; they are presented in Table 1: each principal component correlated strongly ($|r| \geq 0.5$) with two to five MTEs. Hereafter, the principal components calculated from MTE concentrations in nestling feathers, the nest material and nestling droppings are named PC x feathers, PC x nest material and PC x droppings, respectively, with x the number of the component.

		Nestling feathers		Nest material		Nestling droppings	
		PC1	PC2	PC1	PC2	PC1	PC2
	<i>Var explained</i>	29.8%	26.1%	57.2%	20.3%	42.9%	29.3%
	Cu	0.13	0.88	-0.90	0.24	-0.77	-0.39
	Zn	0.69	-0.24	-0.58	0.71	-0.80	-0.18
	Pb	-0.43	0.09	-0.80	-0.40	-0.54	0.69
	As	0.72	0.48	-0.84	0.02	-0.66	-0.45
	Hg	-0.53	0.49				


	Cd			-0.61	-0.54	-0.46	0.78
	Main explanatory MTEs	+ Zn As - Hg	+ Cu (As, Hg)	- Cu Zn Pb As Cd	+ Zn	- Cu Zn Pb As (Cd)	+ Pb Cd
	<i>Var explained</i>	34.5%	25.6%	41.6%	27.0%	43.9%	35.7%
	Cu	-0.08	0.78	-0.75	0.47	0.08	-0.91
	Zn	-0.69	-0.08	-0.56	0.74	-0.69	-0.57
	Pb	0.73	0.17	-0.71	-0.60	-0.89	0.31
	As	-0.84	0.21	-0.72	-0.46	-0.32	-0.73
	Hg	-0.08	-0.77				
	Cd			-0.43	-0.03	-0.91	0.29
	Main explanatory MTEs	- Zn As + Pb	+ Cu - Hg	- Cu Zn Pb As	+ Zn (Cu) - Pb (As)	- Zn Pb Cd	- Cu Zn As

Table 1. Variance explained by each principal component (PC ; in %), separately in great tits (top) and blue tits (bottom) and for each biological material (*i.e.* nestling feathers, nest material and nestling droppings) and Pearson's correlation between each principal component and MTE levels (*i.e.* Cu, Zn, Pb, As, Hg and Cd). The main ($|r| \geq 0.5$) explanatory MTEs are summarized: “-” and “+”, reflecting a negative and a positive correlation, respectively.

2.4.3. MTE exposure and reproductive outputs

To investigate whether MTE exposure (*i.e.* the two MTE principal components per biological material explaining the largest variance) explain nestling mass, telomere length and fledging success, we built generalized linear mixed-effects models (glmer) and linear mixed-effects models (lmer) with either i) the median of nestling mass per brood at 15 days (Gaussian distribution), ii) the median of nestling telomere length per brood at 15 days (Gaussian distribution), iii) the number of fledglings (Poisson distribution) or iv) the percentage of fledglings (binomial distribution), as the dependant variable and the MTE principal components and their interaction as the explanatory variables. Importantly, because our study aimed at measuring the causal link between MTE exposure and reproductive success, the nesting site and the year were added as random intercepts. Models on the percentage and

number of fledglings also included the identity of the nest as a random intercept to correct for overdispersal (observation-level random effects; Harrison, 2014). In addition, because this study also aimed at evaluating the benefit of using a multivariate analysis approach when investigating the effects of MTE exposure on wildlife, we run similar models but with the concentration of each single MTE as explanatory variable. All models were performed separately in great tits and blue tits.

Lmer and glmer were fitted using the restricted maximum likelihood (REML) method and the Laplace approximation of the maximum likelihood method, respectively, using the 'lme4' package in R. For each model, we performed a backward stepwise selection using the AIC. A Type III Wald chisquare test Anova was used to determine the significance of the retained variables in the final models. When discrete explanatory variables were retained in the models, contrasts among groups were tested using least-square mean pairwise comparisons (contrast function of the 'lsmeans' package in R) (Lenth, 2016). When interactions between continuous explanatory variables were retained in the models, simple slope analyses using Johnson-Neyman intervals were performed ('sim_slopes' function of the 'jtools' package in R).

3. Results

3.1. MTE exposure in great tits and blue tits: comparison between biological materials

MTE signatures – the relative proportions of each MTE – were overall strikingly equivalent between both species and whatever the type of material it was extracted from (nestling feathers, nest material or nestling droppings (Figure 2; see also Appendix D for a summary of the raw data). At a finer level however, MTEs concentrations in nestling feathers slightly differed between the two species: Pb and As were higher in the feathers of blue tit nestlings

than great tit nestlings ($t=3.33$, $df=392$, $P=0.012$ and $t=3.96$, $df=376$, $P=0.001$, respectively). In contrast, Cd were higher in the droppings of nestling great tits than of nestling blue tits ($t=10.80$, $df=254$, $P<0.001$). MTE concentrations in the nest material were not significantly different between blue tits and great tits, suggesting that while physiological processing of MTEs may be species specific, environmental exposure is similar for both species. Importantly, some MTEs could not be quantified in some of the materials: Hg in the nest material and nestling droppings were below quantification limit while Hg were quantifiable in 32% of nestling feathers samples. On the contrary, Cd were below quantification limit and only 39% of Pb were above the quantification limit in nestling feathers. Details of MTE-specific variation between the three types of biological material is further presented in Appendix E. Finally, while the significant correlations in MTE concentration between the three biological materials were all positive, the results were not fully consistent between species (*i.e.* different correlations were significant in great tits and in blue tits) (Figure 3).

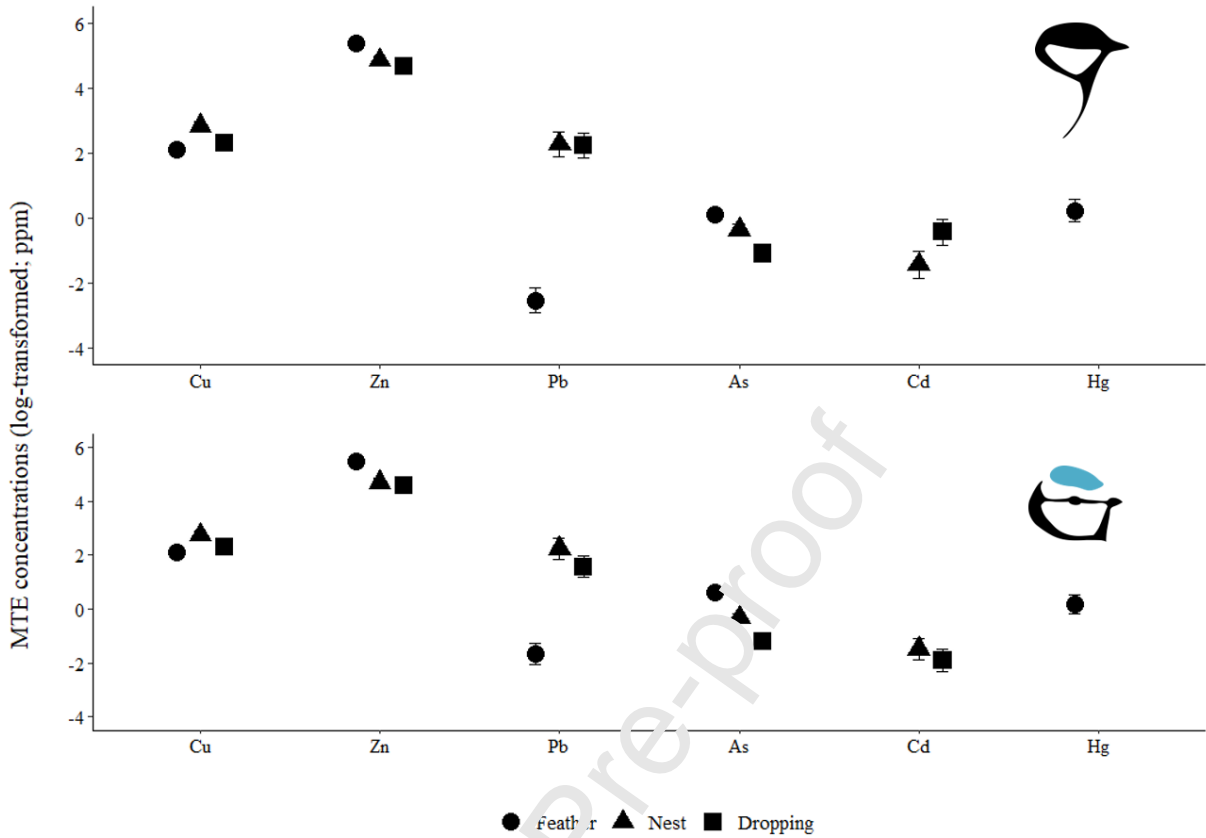


Figure 2. Mean \pm se Cu, Zn, Pb, As, Cd and Hg in nestling feathers, the nest material and nestling droppings in great tits (top panel) and blue tits (bottom panel). Means and standard errors were extracted from the ‘lsmean’ output of the full model with the species, the biological material and their interaction as explanatory variables; they account for the site and the year.

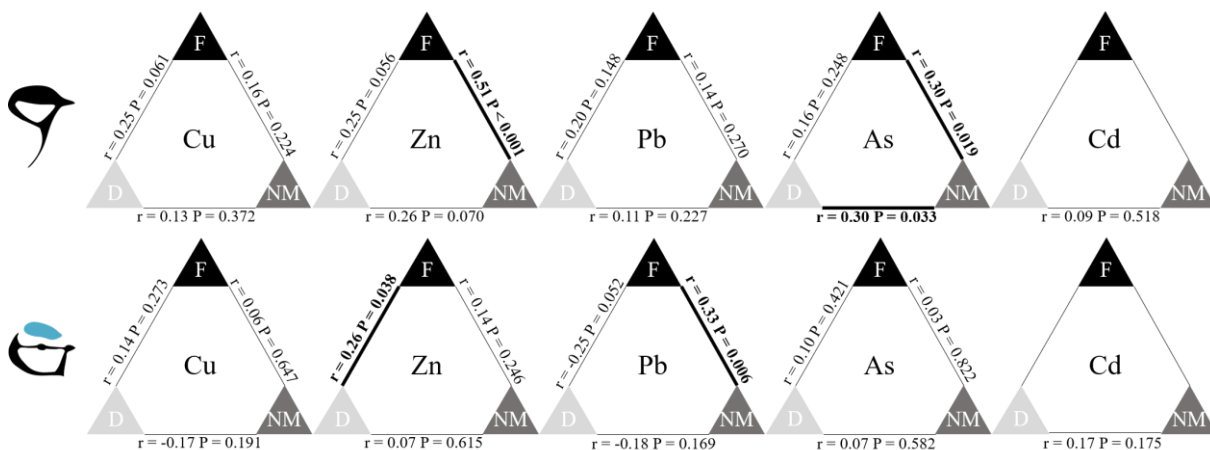


Figure 3. Correlations of MTE concentrations (*i.e.* Cu, Zn, Pb, As and Cd concentrations) between nestling feathers (F), the nest material (NM) and nestling droppings (D) of great tits (top) and blue tits (bottom). Significant correlations are highlighted in bold.

3.2. MTE exposure and reproductive outputs

3.2.1. Models on principal components

Importantly, the associations between MTE exposure and reproductive outputs that we measured and that are described hereafter were independent of the habitat type (*i.e.* urban park, urban forest and rural forests), which was added as random intercepts in the analyses. In great tits, fledging success (both defined as a number and a percentage of fledglings) and nestling mass decreased with increasing PC1 feathers (*i.e.* with increasing Zn and As but decreasing Hg) and with increasing PC2 feathers (*i.e.* with increasing Cu and, to a lower extent, As and Hg; Table 2a; Figures 4 and 5); in other words, fledging success and nestling mass were the lowest when Cu, As and Zn in nestling feathers were highest. Moreover, fledging success increased with increasing PC1 nest material (*i.e.* with decreasing Cu, Zn, Pb, As and Cd; Figure 4). Finally, fledging success also depended on the interaction between PC1 and PC2 droppings: it decreased with increasing PC2 droppings (*i.e.* when increasing Pb and Cd) and when PC1 droppings were decreasing (*i.e.* when increasing Cu, Zn, As, Pb and Cd; Figure 4); in other words, fledging success decreased with increasing Pb and Cd in droppings while the overall MTE concentrations were high. Telomere length variation was not explained by any of the MTE principal components (Table 2a).

In blue tits, fledging success decreased with increasing PC2 feathers (*i.e.* with increasing Cu but decreasing Hg; Table 2b; Figure 4). Moreover, fledging success and nestling mass increased with increasing PC1 feathers (*i.e.* with decreasing Zn, As but increasing Pb; Figures 4 and 5). The percentage of fledglings tended to increase with

increasing PC1 droppings (*i.e.* with decreasing Zn, Pb and Cd; Figure 4). Finally, telomere length decreased with increasing PC2 droppings (*i.e.* with decreasing Cu, Zn and As). PCx nest material were retained in none of the models.

3.2.2. Models on single MTEs

The results of the models on single MTEs were largely similar to the ones obtained through the multivariate approach (Table 2). In great tits, the number of fledglings decreased with increasing Cu and As in nestling feathers (Table 2a). Quite similarly, the percentage of fledglings decreased with increasing Cu, Zn and As in nestling feathers, and nestling mass decreased with increasing Cu, Zn and As in nestling feathers although not significantly so in the case of Hg. Quite similarly again, the number of fledglings decreased with increasing Cu and the percentage of fledglings decreased with increasing Cu and As in the nest material. Finally, fledging success decreased with increasing Cd in nestling droppings although not significantly so in the case of the number of fledglings. Telomere length variation was not explained by any of the variables.

In blue tits, as for great tits, fledging success decreased with increasing Cu and As in nestling feathers (Table 2b). The percentage of fledglings also increased with increasing Pb and Hg in nestling feathers. Quite similarly, nestling mass decreased with increasing Cu, Zn and As while it increased with increasing Pb in nestling feathers. The percentage of fledglings decreased with increasing Pb and As in nestling droppings. Finally, telomere length increased with increasing As in nestling droppings.

a)



		Nr fledglings	% fledglings	Mass	Telomere length
Nestling feathers	N	69	69	71	69
	PC1	– Chi ² =4.52, P=0.033	– Chi ² =14.37, P<0.001	– Chi ² =9.91, P=0.002, R ² =0.142	
	PC2	– Chi ² =7.64, P=0.006 R ² =0.136	– Chi ² =16.00, P<0.001 R ² =0.127	– Chi ² =8.84, P=0.003, R=0.113	
	Cu	– Chi ² =4.37, P=0.037, R ² =0.062	– Chi ² =11.76, P<0.001, R ² =0.070	– Chi ² =10.74, P=0.001, R ² =0.135	
	Zn		– Chi ² =4.14, P=0.042, R ² =0.025	– Chi ² =7.26, P=0.007, R ² =0.100	
	Pb			Chi ² =0.75, P=0.386	
	As	– Chi ² =10.05, P=0.002, R ² =0.131	– Chi ² =24574, P<0.001, R ² =0.119	– Chi ² =4.06, P=0.044, R ² =0.049	
Hg			Chi ² =1.64, P=0.200		
Nest mat.	N	59	59	61	59
	PC1	+ Chi ² =7.61, P=0.006, R ² =0.102	+ Chi ² =5.09, P=0.024, R ² =0.035	Chi ² =1.96, P=0.161	
	PC2			Chi ² =0.02, P=0.882,	
	Cu	– Chi ² =10.26, P=0.001, R ² =0.130	– Chi ² =6.39, P=0.012, R ² =0.043	Chi ² =2.93, P=0.087	
	Zn			Chi ² =0.95, P=0.329	
	Pb			Chi ² =1.46, P=0.226	
	As		– Chi ² =5.16, P=0.023, R ² =0.037	Chi ² =0.19, P=0.667	
Cd			Chi ² =0.18, P=0.672		
d	N	58	58	60	58

PC1	Chi²=2.81, P=0.094 + / PC2 > 0.45	Chi²=2.96, P=0.085 + / PC2 > 0.35	Chi ² =0.204, P=0.652	
PC2	Chi²=0.55, P=0.428 - / PC1 < -1.90	Chi²=0.83, P=0.362 - / PC1 < -0.93	Chi ² =1.34, P=0.246	
PC1 x PC2	Chi²=3.37, P=0.037 R²=0.122	Chi²=5.94, P=0.015 R²=0.057		
Cu	Chi ² =1.86, P=0.172	Chi ² =2.95, P=0.086	Chi ² =0.40, P=0.528	
Zn	Chi ² =0.02, P=0.886	Chi ² <0.01, P=0.974	Chi ² =0.79, P=0.373	
Pb	Chi ² =0.11, P=0.735	Chi ² =0.19, P=0.660	Chi ² =0.04, P=0.851	
As	Chi ² <0.01, P=0.985	Chi ² =0.15, P=0.698	Chi ² =1.04, P=0.307	
Cd	- Chi²=4.00, P=0.046, R²=0.056	- Chi²=8.03, P=0.005, R²=0.046	Chi ² =2.30, P=0.130	

b)



	Nr fledglings	% fledglings	Mass	Telomere length
Nesting feathers	N	73	73	72
	PC1	+ Chi²=3.94, P=0.047	+ Chi²=6.35, P=0.012	+ Chi²=20.12, P<0.001, R²=0.140
	PC2	- Chi²=9.48, P=0.002 R²=0.120	- Chi²=11.76, P<0.001 R²=0.098	
	Cu	- Chi²=6.95, P=0.008, R²=0.074	- Chi²=8.27, P=0.004, R²=0.051	- Chi²=6.07, P=0.014, R²=0.085
	Zn			- Chi²=7.89, P=0.005, R²=0.070
	Pb		+ Chi²=5.58, P=0.018, R²=0.037	+ Chi²=5.66, P=0.017, R²=0.037
	As	- Chi²=9.52, P=0.002, R²=0.093	- Chi²=9.28, P=0.002, R²=0.053	- Chi²=18.12, P<0.001, R²=0.140
Hg		+ Chi²=4.16, P=0.041, R²=0.031	Chi ² =1.07, P=0.301	
Nest mat.		70	70	69
	PC2			Chi ² =1.70, P=0.193
	Cu			Chi ² =0.33, P=0.566
	Zn			Chi ² <0.01, P=0.932
	Pb			Chi ² =1.06, P=0.304
	As			Chi ² =1.15, P=0.284
	Cd			Chi ² =0.13, P=0.722
Nesting droppings	N	66	66	64
	PC1	Chi ² =0.23, P=0.629	+ Chi²=3.78, P=0.052, R²=0.035	Chi ² =3.24, P=0.072
	PC2	Chi ² =1.29, P=0.257		- Chi²=5.71, P=0.017, R²=0.010
	Cu	Chi ² =0.59, P=0.441	Chi ² =0.71, P=0.400	Chi ² =2.51, P=0.113
	Zn	Chi ² =0.03, P=0.864	Chi ² =1.10, P=0.295	Chi ² =2.15, P=0.143
	Pb	Chi ² =1.97, P=0.160	- Chi²=4.86, P=0.028,	Chi ² =0.24, P=0.621
			Chi ² =2.29, P=0.130	

		R²=0.045		
As	Chi ² =2.98, P=0.084	<i>– Chi²=4.61, P=0.032,</i> R=0.024	Chi ² =1.44, P=0.230	+ Chi²=4.51, P=0.034, R=0.006
Cd	Chi ² =2.93, P=0.087	Chi ² =0.27, P=0.607	Chi ² <0.01, P=0.944	Chi ² =0.69, P=0.406

Table 2. Results of the best fitting statistical models testing the effects of MTE principal components and single MTEs on reproductive outputs in (a) great tits and (b) blue tits. In some cases, either the null model was the best fitting model or only one PC axis was retained in the model; these translated to empty cells in the tables. For clarity, explanatory variables that were retained in none of the models were removed from the tables. When the interaction between the two MTE principal components was retained, the lines for the simple effects show in italic the results of the simple slope analyses: | means “if”; – and + indicate a negative and a positive significant correlation, respectively. For each model, sample size is indicated as (N).

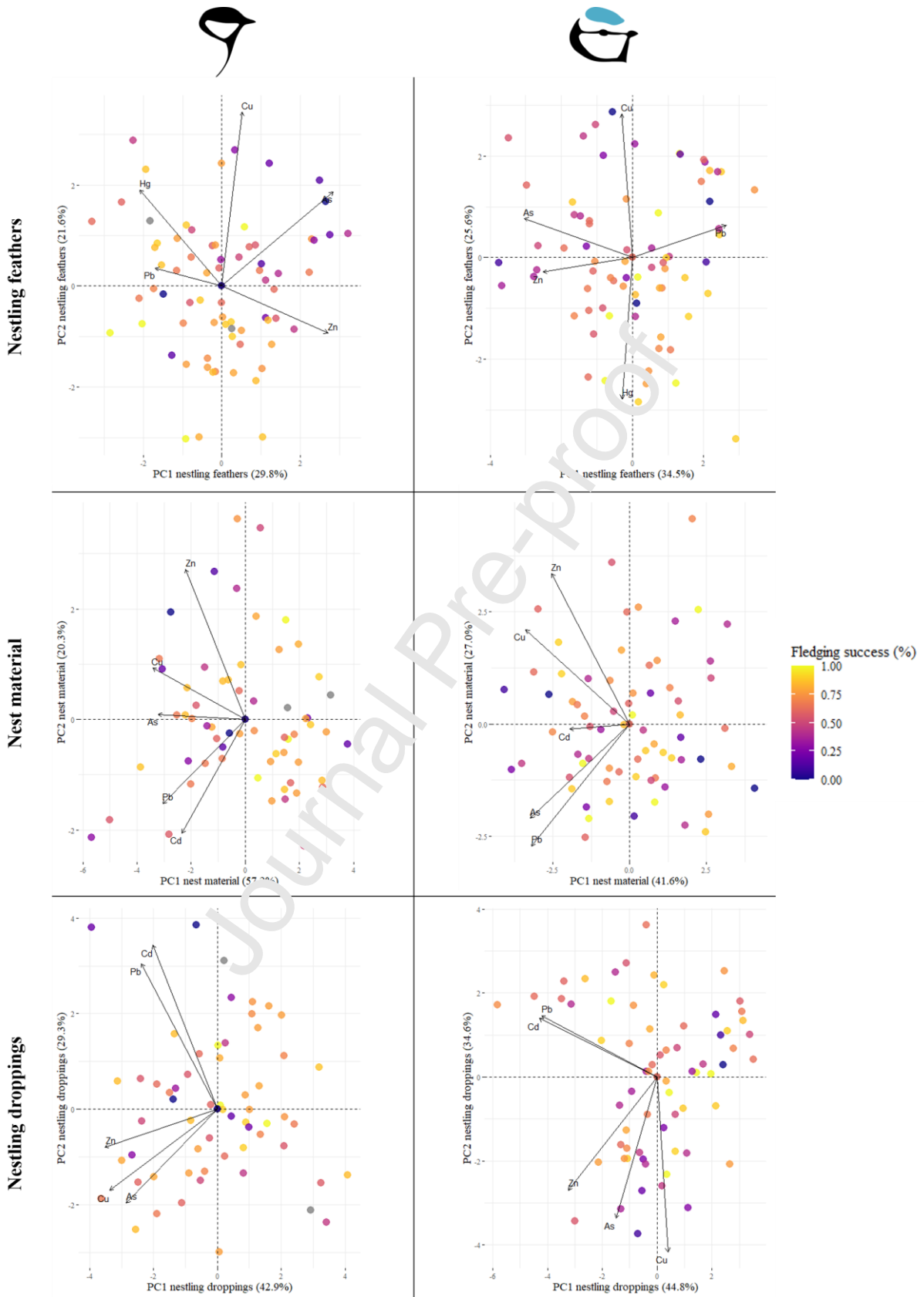


Figure 4. Fledgling success: comparison between biological materials - Principal component analysis on Cu, Zn, Pb, As, Cd and Hg concentrations in nestling feathers, the nest material and nestling droppings in great tits (left) and blue tits (right). The colour gradients represent the percentage of fledglings (*i.e.* the number of fledglings divided by final clutch size). In both great tits and blue tits, the percentage of fledglings decreased with increasing Cu, As and Zn in nestling feathers. In great tits, the percentage of fledglings also decreased with increasing Cu, Zn, Pb, As and Cd in the nest material and nestling droppings.

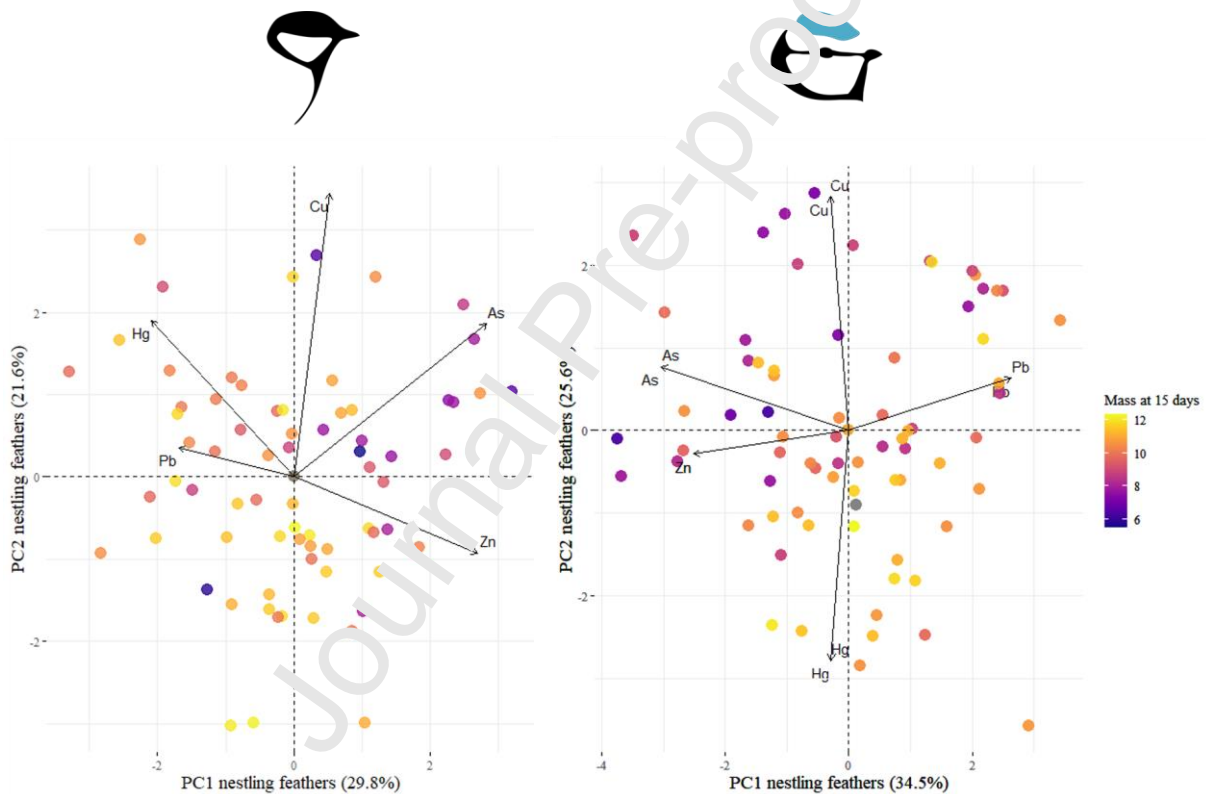


Figure 5. Nestling mass - Principal component analysis on Cu, Zn, Pb, As and Cd concentrations in nestling feathers, in great tits (left) and blue tits (right). The colour gradients represent nestling mass at 15 day. Nestling mass decreased with increasing Cu, and with increasing As and Zn in great tits and blue tits, respectively.

4. Discussion

In this study, great tit and blue tit nestlings were exposed to a gradient of MTE pollution measured in the capital city of Warsaw, Poland. MTE concentrations were particularly high relative to other published reports of populations of the same species nesting close to industrial sites. Two studies measured MTE concentrations in the feathers of great tit and blue tit nestlings within a transect from a metallurgic factory located in Antwerp (Belgium) to a few kilometres further; the factory is known to be responsible for lead, cadmium, arsenic, copper and zinc pollution (Dauwe et al., 2000; Janssens et al., 2002). Interestingly, when compared to the levels reported in Janssens et al. (2002), zinc concentrations in the feathers of great tit nestlings within Warsaw (502 ± 282 ppm) were 16 times higher than in the closest site to the metallurgic factory in Antwerp (between 100 and 400 m far from the factory). Similarly, copper (10.6 ± 1.4 ppm) and arsenic (2.4 ± 0.5 ppm) were more than 3 times higher in Warsaw than close to the metallurgic factory in Antwerp. Lead concentrations (2.9 ± 1.7 ppm) were 1.5 times lower in Warsaw than close to the metallurgic factory in Antwerp, but 5 times higher than the concentrations found in nestlings 400 to 600 m far from the factory (Janssens et al., 2002). Given that MTE pollution close to metallurgic factories are known to cause significant reproductive impairments in passerines (Dauwe et al., 2005; Eeva et al., 2009; Janssens et al., 2005), our results confirm the necessity to estimate MTE pollution threat on wildlife outside of industrial sites, specifically including urban environments (Nam and Lee, 2006).

4.1. MTE exposure in great tits and blue tits

Globally, great tits and blue tits were exposed to similar levels of MTE pollution (Figure 2). Indeed, MTE signature – the relative proportion of each MTE – were overall equivalent between both species and whatever the type of material it was extracted from (nestling

feathers, nest material, nestling droppings). When arranged from the most to the least concentrated, MTEs in the nest material are ordered as follows: zinc, copper, lead, arsenic, cadmium and mercury; these patterns differed only slightly in nestling feathers and droppings. Importantly, copper, zinc, lead, arsenic and cadmium concentrations in the nest material, *e.g.* nestling closest environment, were positively and often strongly correlated (*e.g.* $r > 0.50$ between copper and zinc, and lead and arsenic in both great tits and blue tits; see Appendix C). This suggests that broods were exposed to a similar mixture of MTEs but of various concentrations. Such correlations might result from MTEs sharing common emission sources, such as road traffic, residential heating, coal burning or industry (Duan and Tan, 2013).

4.2. MTE exposure and reproductive outputs

The exposure to different MTEs being sometimes correlated, it is difficult to disentangle the effects of each individual MTE on bird reproductive success. However, this study, by investigating the link between MTE concentrations and reproductive outputs across two species and measuring MTEs in several types of biological material (*i.e.* nestling feathers, the nest material and nestling droppings), allowed to identify the MTEs responsible for the strongest effects on reproductive success. Importantly, the associations between MTE exposure and reproductive outputs that we measured were independent of the habitat type (*i.e.* urban park, urban forest and rural forests), which was added as random intercepts in the analyses. Consequently, these associations likely result from direct effects of MTE exposure on bird reproduction rather than from the co-variation with other environmental factors that may vary between habitats (*e.g.* vegetation cover).

The most striking result of this study is the consistent negative association between copper and arsenic exposure and reproductive outputs whatever the species, the biological material

(although stronger associations were measured with MTEs in nestling feathers; see “4.3. Biomonitoring of MTE exposure”), the proxy for reproductive success (*i.e.* fledging success, fledgling mass but not telomere length), and the modelisation method (*i.e.* using principal components or single MTEs as explanatory variables). Interestingly, while Cu and As often covaried, their correlation was weak and non-significant in the feathers of blue tit nestlings. Yet, fledgling success and nestling mass were negatively associated with Cu and As in the feathers of blue tit nestlings, which suggests noxious effects of both MTEs. While the noxious effects of arsenic are consistent with the literature (reviewed in Hughes, 2002; Ratnaike, 2003), to the best of our knowledge, there was so far no evidence of copper toxicosis in wild bird populations (although it was measured in experimental studies; *e.g.* Almansour, 2006; Henderson and Winterfield, 1975). It likely results from the high concentrations of copper in urban environments compared to rural and even industrial areas (Costa et al., 2012; Dauwe et al., 2000; Janssens et al., 2002).

In both species, lead (Pb) and cadmium (Cd) concentrations in nestling droppings were also associated with negative effects on fledging success. The concentrations of the two MTEs being strongly correlated ($r = 0.62$ and $r = 0.85$ in great tits and blue tits, respectively), it is not possible to disentangle their respective effects. Similarly, lead exposure was associated with reduced lifetime breeding success in wild black birds (Fritsch et al., 2019). While zinc is known to have protective effects against lead, cadmium, mercury, aluminium and copper toxicity on development (Chatelain et al., 2016; Herkovits and Alejandra Helguero, 1998), the present study did not highlight beneficial effects of zinc exposure on reproductive outputs. Indeed, the associations between zinc exposure and reproductive outputs were not consistent; together with the fact that Zn were always positively correlated with Cu and/or As, those results suggest that the associations we measured between zinc exposure and reproductive outputs were artefactual. This might result from zinc exposure

being particularly high in urban environments (Graganiello et al., 2001; Kekkonen et al., 2012). Finally, our study did not highlight any strong association between mercury exposure and reproductive outputs, likely because mercury concentrations were relatively low in our study (Ackerman and Eagles-Smith, 2009).

Interestingly, the multivariate approach detected slightly more associations between MTE exposure and reproductive success than the univariate approach. This suggests interactive effects between MTEs. More specifically, the link between MTE concentrations in the nest material (*i.e.* PC1 nest material) and fledging success and mass in great tits suggests additive or synergetic effects between some MTEs (among copper, zinc, lead, arsenic and cadmium). In the same way, the effects of lead, cadmium and arsenic mixture on rat cognition are stronger than the effects of each MTE inferred separately (Karri et al., 2016). On the other hand, our results did not highlight any antagonistic effects between MTEs. For instance, the protective effect of zinc against lead-induced reproductive impairments previously measured in the feral pigeon (Chatelain et al., 2015) was not observed in wild great tits and blue tits.

Importantly, while MTE exposure had clear effects on nestling weight and survival, its effect on telomere length was rather weak and inconsistent. Indeed, only MTE levels in droppings of nestling blue tits explained telomere length: telomere length was positively correlated with Cu, Zn and As in nestling droppings. This result is unexpected and contradicts the results of a recent experimental study on adult zebra finches (*Taeniopygia guttata*) that highlighted a negative associations between telomere length and cadmium, arsenic and lead concentrations in feathers (Saulnier, 2020). Further studies, including experimental studies controlling for MTEs and food intake, are necessary to accurately conclude on the link between MTE exposure and telomere length.

Nestling mass before fledging is associated with recruitment probability in great and blue tits (Magrath, 1991; Schwagmeyer and Mock, 2008). Therefore, our study suggests that nestling exposure to copper, arsenic, lead and cadmium, by decreasing fledging success and nestling mass before fledging, is likely to decrease chick and juvenile survival, and consequently - population productivity. The exposure to those MTEs, by increasing the costs of reproduction, may thus have long-term effects on population productivity. MTE exposure may therefore explain, in part at least, the differences in reproductive success observed between urban and rural environments (Chamberlain et al., 2009).

4.3. Biomonitoring of MTE exposure

Analysing three different types of material related to the breeding ecology of great tits and blue tits allows to infer the extent to which any of such material can be identified as a versatile, easily accessible biomonitoring tool of urban wildlife exposure to MTEs. Globally, MTE signatures were similar in nestling feathers, the nest material and nestling droppings in both bird species (Figure 2). Nonetheless, MTEs concentrations differed between the three biological materials. Importantly, cadmium, mercury and lead concentrations could not be measured in all of the three materials: while mercury could only be measured in nestling feathers, cadmium and, for some broods, lead could not be detected in this material.

Differences in MTE concentrations between the three biological materials may be explained by MTE-specific absorption rates or accumulation in organs and teguments (Diamond et al., 1998; Whitehead et al., 1996). For instance, lead absorption rate in zebra finches is less than 10% of the quantity of lead ingested (Dauwe et al., 2002) and, once passed the gastrointestinal barrier, lead rapidly accumulates in the soft tissues (Patrick, 2006). This would explain why MTE concentrations strongly covaried in the nest material and, to a lower extent, in nestling droppings, while their correlations were weak in nestling feathers

(Appendix C). This would also explain the lack of strong correlations of MTEs concentrations between the three biological materials (Figure 3). Importantly, this means that the amount of MTE that can interfere with physiological mechanisms may not strictly equal MTE concentrations in the environment.

Most importantly, reproductive success covaried more strongly with MTEs in nestling feathers than with MTEs in the nest material and nestling droppings; this was particularly evident in blue tits. Indeed, MTE concentrations in nestling feathers, that are MTEs that were transferred from the blood stream to the feathers during several days, is expected to reflect more accurately the amount of MTEs that are likely to interact with organic molecules and, therefore, to alter physiological processes. Similarly, in great egrets *Ardea alba*, mercury levels in nestling feathers were a better predictor of reproductive success than mercury levels in blood and eggs (Zabala et al., 2019).

To sum up, our study underlines the relevance of using nestling feathers for the biomonitoring of MTE absorption in wild birds. Nestling feathers are especially useful to measure mercury but also zinc, copper and arsenic exposure. However, this material can be less appropriate to measure lead and cadmium exposure in cases where the quantity of such feather material is small, for instance when studying smaller bird species such as great tits and blue tits.

4.4. Conclusion

Our study tested the effect of urban MTE pollution on the reproductive success of two common passerines. It highlighted the fact that i) great tit and blue tit exposure to MTEs are comparable. Importantly, ii) copper, arsenic, lead and cadmium have consistent negative effects on great tit and blue tit reproductive outputs. Finally, iii) MTEs in nestling feathers are good biomonitoring materials to assess MTE absorption in nestlings of hole-nesting birds,

even though lead and cadmium exposure might be underestimated. Overall, the present study identified that nest-specific variation in MTE pollution is a causal factor explaining reduced reproductive success in urban birds.

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6. Author Contributions

Marion Chatelain: Conceptualization, Investigation, Formal analysis, Writing - Original Draft, Writing - Review & Editing, Funding acquisition **Sylvie Massemin:** Validation, Writing - Review & Editing **Sandrine Zahn:** Investigation, Writing - Review & Editing **Eliza Kurek:** Investigation, Writing - Review & Editing **Ewa Bulska:** Validation, Writing -

Review & Editing **Marta Szulkin**: Conceptualization, Investigation, Writing - Review & Editing, Funding acquisition, Supervision.

7. Competing interests

The author(s) declare no competing interests.

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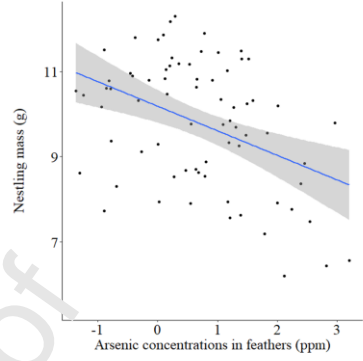
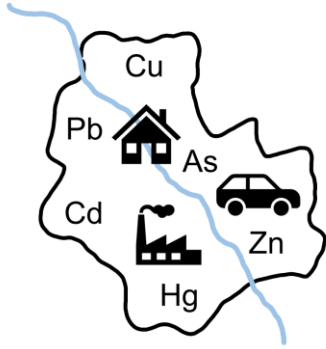
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Graphical abstract

Urban MTE pollution → MTE exposure in birds → Reproductive outputs



Journal Pre-proof

Highlights

- Urban bird populations exhibit lower reproductive outputs than rural populations.
- We measured MTEs in three biological materials: nestling feathers and droppings, and the nest material.
- We measured how MTEs exposure affects nestling survival, mass and telomere length.
- Cu, As, Pb and Cd negatively affect great tit and blue tit reproductive success.

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