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Longitudinally monitored lifetime changes in blood heavy metal concentrations and their health effects in urban birds

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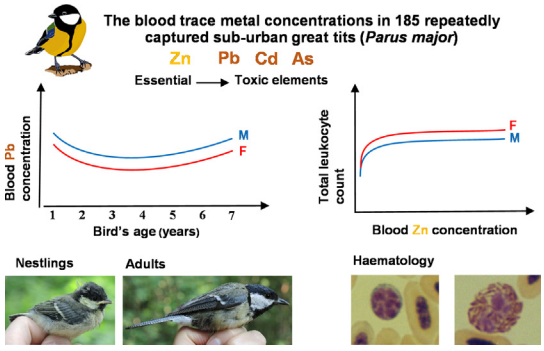
HIGHLIGHTS

- Unknown precise age may preclude interpretation of avian biomonitoring data.
- Great tit nestlings showed higher Pb blood levels than adults.
- Blood heavy metal concentrations were not linked to the erythrocyte counts.
- Total leukocyte count increased with increasing Pb, Cd and Zn blood concentrations.
- Precise age data are not necessary for blood heavy metal biomonitoring in adults.

GRAPHICAL ABSTRACT



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ABSTRACT

Urban heavy metal pollution can impair the health of humans and other organisms inhabiting cities. While birds are suggested as one of the appropriate bioindicators for essential and non-essential trace element monitoring, the process of particular elements' accumulation in blood and its possible adverse health effects during ageing of individuals remain unexplored. We have investigated lifetime changes in blood lead (Pb), cadmium (Cd), arsenic (As) and zinc (Zn) concentrations and searched for links to health-related traits in sub-urban free-living great tit (*Parus major*) population monitored over a long period of time. The blood As concentrations were under the limit of detection in most samples. The blood Pb levels showed a non-linear relationship to individuals age, where the highest Pb concentrations were measured in nestlings and in a very small group of highly senescent birds (over 7 years old), while no clear trend was observed for the majority of the adult age stages. No age-related patterns were found for blood Cd or Zn concentrations. The positive relationship between date of capture and blood Cd and Zn levels may reflect seasonal changes in diet composition. We did not reveal any anaemia-like conditions (decreased total erythrocyte count or increased immature erythrocyte count) in relation to blood heavy metal concentrations in the investigated birds. Total leukocyte counts, heterophil/lymphocyte (H/L) ratio and total heterophil and lymphocyte counts increased with increasing Pb, Cd and Zn concentrations in

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blood. This study demonstrates the suitability of avian blood for actual heavy metal spatial and temporal biomonitoring even in situations when the precise age of the individuals remains unknown.

1. Introduction

Globally, various animal species colonise urban habitats where they face novel environmental threats (Francis and Chadwick, 2012; Isaksson, 2015). Among these, anthropogenic heavy metal pollution has received special attention due to its possible toxic effects on humans and wildlife and non-biodegradable nature (in this study we define heavy metals as elements with specific density >5 μg/m^3, including essential trace elements and metalloids; Assi et al., 2016; Jaishankar et al., 2014; Järup, 2003; WHO, 2007). Although intensive research in avian ecotoxicology has focused especially on industrial or post-industrial environments (Berglund et al., 2012; Dauwe et al., 2006; Fritsch et al., 2012; Migula et al., 2000), growing attention is also being paid to urban habitats lacking any particular point source of heavy metal pollution (Bailly et al., 2017; Bauerová et al., 2017; Frantz et al., 2012; Roux and Marra, 2007; Scheifler et al., 2006). Once released by combustion processes, heavy metals most often contaminate the bodies of living organisms through the food chain (Mann et al., 2011), resulting in only slow improvement of heavy metal biocontamination (i.e. contamination of animal biota, here specifically of avian biota) after significant emissions decrease (Berglund et al., 2012; Berglund and Nyholm, 2011; Eeva and Lehikoinen, 2015).

Some of these elements, including zinc (Zn), copper (Cu) and iron (Fe), play important roles in animal metabolism and therefore are, at low concentrations, essential (Goyer et al., 2004; Sharma and Agrawal, 2005). However, if accumulated at higher concentrations they may have toxic effects (Sharma and Agrawal, 2005; Tchounwou et al., 2012). By contrast, non-essential heavy metals such as lead (Pb), cadmium (Cd) and arsenic (As) have been proven to be highly toxic, with detrimental effects observed even in very low doses (Goyer et al., 2004; Järup, 2003). After entering the organism, toxic heavy metals replace the original essential metals and cause metabolic failures (Jan et al., 2015; Järup, 2003; Tchounwou et al., 2012). In addition, these elements have the ability to generate free radicals that increase oxidative stress, thus damage to the cellular structures (Flora et al., 2008; Jaishankar et al., 2014; Stohs and Bagchi, 1995).

Evidence from humans- and laboratory-animals studies suggests that individual heavy metals interfere with distinct physiological pathways. Pb enters the bloodstream and soft tissues rapidly and is slowly redistributed into the bones later (WHO, 1995). By contrast, Cd, which is one of the most toxic metals detectable in human bodies, rapidly enters the liver where it binds to metallothionein (replacing essential Zn) causing hepatotoxicity (Jaishankar et al., 2014; Järup, 2003). Eventually, Cd accumulates mostly in the kidneys, although partial accumulation in the bones (similar to Pb) is also known (Burger, 2008). Highly toxic metalloid As is deposited primarily in the skin and skin derivatives (in birds' claws, feathers and beaks; WHO, 2007), while interfering with most organ systems (cardiovascular, nervous, gastrointestinal and respiratory; Tchounwou et al., 2012). Finally, Zn is essential in a large number of different metalloproteins (Zargar et al., 2015). Absorbed Zn is stored in the muscles, bones, liver, pancreas, kidneys and other organs and excessive Zn is typically excreted in the faeces (WHO, 2001). Assessment of trace element concentrations in most tissues does not allow to estimate the period of time over which the elements were accumulated and differentiation of current contamination from the past one. In mobile organisms this impairs relating information on element concentration with the locality of exposure. Blood could serve as a tissue for recent contamination estimate.

Since in the modern globalised world the human diet is typically devoid of most of its local specificity, human-orientated research cannot bring much understanding to the spatial and temporal patterns in heavy metal contamination of different biological systems. Therefore, the use of appropriate bioindicators (i.e. indicators of biota contamination, not necessarily proportionally reflecting abiotic environment) is essential. Environmental quality assessment studies have adopted several different bioindicators for different trophic levels, such as mosses and higher plants (Nickel et al., 2018; Sawidis et al., 2011), small mammals (Tête et al., 2015; Wijnhoven et al., 2007) and free-living birds (Chatelain et al., 2014; Eens et al., 1999; Frantz et al., 2012; Scheifler et al., 2006). Especially the avian models have proven particularly useful thanks to their omnipresence and frequently generalist foraging strategies (Burger and Gochfeld, 2004; Furness, 1993; Pollack et al., 2017). Urban environmental pollution can affect the overall health and welfare of free-living birds both clinically and sub-clinically (Bailly et al., 2016; Bauerová et al., 2017; McClelland et al., 2019; Meillère et al., 2016). While there are several studies focusing on particular physiological effects of different heavy metals in wild birds (e.g. Fair and Ricklefs, 2002; Ferreyra et al., 2015; Holladay et al., 2012; Koivula and Eeva, 2010), the effect of lifetime changes in blood heavy metal concentrations on health-related traits remains mostly neglected in birds. From the perspective of haematology one of the most frequently observed health symptoms of sub-lethal doses of toxic elements is anaemia resulting from increased erythrocyte mortality. Furthermore increased total white blood cell counts (TWBC), often accompanied by increased heterophils (heterophilia) or decreased lymphocytes (lymphopenia; together causing increased H/L ratio) indicating possible acute or chronic toxicosis, long-term stress or inflammation (Campbell and Ellis, 2007; Jones, 2015).

The practical use of avian bioindicators may be confounded by the effects of individual traits associated with heavy metal contamination. While age-independent heavy metal contamination in avian blood can be predicted, given the role of blood as a transportation medium interconnecting tissues (WHO, 1995), without relevant evidence this presumption is unjustified. Haematopoiesis in the bone marrow could serve as an endogenous source of some heavy metals, such as Pb or Cd. Thus, the blood Pb and Cd levels could indicate both current and long-term exposure (WHO, 1995). As described in humans (Flora et al., 2012) bones can contribute around 40–70% of Pb released into blood in adults. However, the degree of redistribution depends on several factors, including also individual age and these processes are presently unknown in birds. Since in the field birds cannot be often precisely aged based on plumage characteristics (Svensson, 1984), age effects may bias the monitoring results. Dealing with this issue by non-longitudinal comparing broad categories of juveniles (either collected during the nesting period or during their 1st year of age) and adults (over the 1st year of age; Berglund et al., 2011; Carvalho et al., 2013; Coeurdassier et al., 2012; Fritsch et al., 2012; Janssens et al., 2001; Meillère et al., 2016; Van Wyk et al., 2001) most studies adopt an only too general age classification that is unable to cover ontogenetic changes in adults. Surprisingly, almost nothing is presently known about the process of heavy metals accumulation in free-living birds throughout their lifetime. Although named as one of the pitfalls of current research (Fritsch et al., 2019), this drawback partially reflects the fact that internal organs cannot be repeatedly sampled from the same individuals, precluding for many tissues (except blood and feathers) longitudinal studies.

Using unique long-term (2006–2018) monitoring data on repeatedly captured free-living great tits (*Parus major*; a common species used for pollutant biomonitoring; Bauerová et al., 2017; Berglund et al., 2011; Eeva et al., 2003) we have tested two main hypotheses:

1) the hypothesis of increasing heavy metal concentrations in blood during the life of an animal and 2) the hypothesis of negative effect of this contamination on health. To the first hypothesis we had two alternative predictions: i) based on the bi-directional exchange of some metals between tissues and blood, accumulation of selected heavy metals (the toxic elements Pb and Cd, unlike As and the essential Zn) throughout the life can be monitored in blood, or ii) in blood that is a transportation medium no accumulation occurs allowing the use of blood for measuring the actual biocontamination levels. To the second hypothesis we predicted to observe associations between the lifetime changes in heavy metal blood concentrations and health-related haematological traits.

2. Materials and methods

2.1. Field procedures

During the years 2006–2018 the total number of 374 blood samples from 185 repeatedly captured birds were collected within a nest-box-breeding study population of free-living great tits inhabiting a suburban forest fragment of Čimický háj and Ďáblický háj in Prague (50°8′7.186″ N, 14°27′57.422″E, Czech Republic, EU; birds were re-captured minimally two times, on average three times, maximally five times; for a histogram of re-capturing frequencies see Fig. S1, for the number of samples in particular years see Fig. S2 in the electronic supplementary material (ESM)).The dendrological composition of the forest that is being managed in an environmentally-friendly manner under a Forest Stewardship Council (FSC) certificate – a mostly natural representation of deciduous trees is supplemented locally with coniferous species. The forest area is situated in the vicinity of a small military airport, Kbely, from which complete information was available on total daily precipitation, average temperatures, humidity and sunshine duration. Birds capturing and blood sampling was performed mostly during the early breeding season (April–May); only 7 samples (from 4 repeatedly captured animals) were collected during the winter season (January–February). The nests were checked regularly to estimate the breeding onset (in total 265 nest boxes were installed within the study area), the nestlings were ringed and sampled at the age of 15 days in the nest boxes, while the adults were mist netted in their breeding territory. The minimum known age of the birds was determined and controlled according to inter-annual monitoring (the nestlings are further referred to as birds in their 1st year of age, the minimum age of the adults at their first capture was assessed on the basis of plumage traits as either the 2nd year or older; Svensson, 1984). For a histogram of age classes with sex-specific distribution see Fig. S3 in ESM. In all of the birds a blood sample (100–150 μL) was taken from the jugular vein using a heparinised syringe within 15 min after the capture. Then, the birds were weighed with digital scales (accuracy 0.02 g; Pesola AG, Baar, Switzerland, type PPS200) and their tarsus lengths were measured with a digital calliper (accuracy to 0.01 mm; Kinex, Prague, Czech Republic) to estimate size-standardised weight (calculated as weight/tarsus length; hereafter called as body mass). Each firstly captured individual was ringed with a standard steel ring of the Czech Bird Ringing Centre (National Museum, Prague). Finally, all birds were released within 30 min after capture. A small part of each collected blood sample was used immediately for haematological analysis and the rest was stored in a microtube with 96% ethanol and later deep frozen (−80 °C) for subsequent heavy metal analysis.

2.2. Haematological analysis

The haematological analysis was performed using methodology previously described elsewhere (Bauerová et al., 2017; Svobodová et al., 2018; Vinkler et al., 2010). In brief, 15 μL of blood was diluted in 2985 μL of Natt and Herrick's solution, stored in a cooling bag and analysed the same day for the total red blood cell count (TRBC) and total white blood cell count (TWBC) under a light microscope with 40× objective magnification (Olympus Corporation, Tokyo, Japan, type CX-31). Using a Bürker's counting chamber we manually quantified the leukocytes in 100 large squares (in a total representation of 10 mm^3 of blood diluted 1:200) to calculate the TWBC. The TRBC was based on the quantification of erythrocytes in two digital images of 6 squares (representation of 0.048 mm^3 of diluted blood) in automated counting software (Štěpka, 2013).

Two blood smears were prepared for the differential leukocyte and erythrocyte analysis. Unfixed dried blood smears were stained with Wright-Giemsa Modified stain (product no. WG128, Sigma-Aldrich, St. Louis, MO, USA) and analysed under a microscope with a 100× objective magnification. The frequencies of lymphocytes, heterophils, basophils, eosinophils and monocytes were counted in a total sample of approximately 120 leukocytes. Since heterophils and lymphocytes were the most abundant leukocyte types, the H/L ratio was calculated as a measure of long-term physiological stress (Davis et al., 2008). The total lymphocyte count (TLC) and the total heterophil count (THC) were determined by recalculating the cell-type frequencies to the TWBC. The immature erythrocyte count (IEC) was determined as a frequency of immature red blood cells (%) obtained from 5 digital images of randomly chosen monolayer fields (a sample of approximately 2000–4000 cells). For all individuals the particular procedures were always performed only by a single person to ensure minimum variation over the measurements.

2.3. Analysis of heavy metal content in dried blood

Before analysis, all whole blood samples were first dried and weighed on analytical scales (accuracy to 0.01 mg; Sartorius, Goettingen, Germany, type R160P). The weight of the samples ranged between 0.5 and 17.0 mg. In glass vials, the samples were mixed with 0.5 mL of concentrated nitric acid (HNO_3, Merck, Suprapur, Germany), 0.2 mL of 30% hydrogen peroxide (H_2O_2, p.a., Analytika, Czech Republic) and 0.3 mL of deionised water (DIW, <0.2 μS/cm, Ultrapur, Watrex, USA) and digested in a microwave digestion system, UltraWAVE (Milestone, Sorisole, Italy). A three-step programme recommended by the manufacturer was followed in this order: 5-min ramp to a temperature of 150 °C (800 W), 10-min ramp to 170 °C (1500 W) and 10-min ramp to 190 °C (1500 W). The initial pressure inside the digestion chamber was 30 bar. After digestion, the solutions were quantitatively transferred into 15 mL polypropylene vials and diluted to 5 mL volume before analysis.

Inductively Coupled Plasma Mass Spectrometry (ICP-MS) measurements were carried out using an Agilent 7700× inductively coupled plasma mass spectrometer equipped with a MicroMist concentric nebulizer, High Matrix Interface and ASX-500 autosampler (Agilent Technologies, Santa Clara, CA, USA). Zn and As were measured at m/z 66 and 75, respectively, in a helium collision mode (4.1 mL min^{-1} He) in a collision cell. Cd and Pb were measured at m/z 111 and 206 + 207 + 208, respectively, in a no gas mode. Prior to nebulization, the sample/standard was on-line mixed with a solution containing 100 μg/L germanium (Ge), 50 μg/L yttrium (Y), 20 μg/L rhodium (Rh) and 10 μg/L bismuth (Bi) in 1% HNO_3, which were used as internal standards to correct for sensitivity drifts. Element quantification was performed using a matrix matched six-point external calibration. The results were processed using Agilent Mass Hunter software.

The measured concentrations of heavy metals are presented in μg/g dry weight. The accuracy of determination was verified by the measurement of two certified reference materials: Seronorm™ Trace Elements Whole Blood 210205 L-2 (LOT 1406264; Labmark, Prague, Czech Republic) and IAEA-A-13 Trace Elements in Freeze Dried Animal Blood (International Atomic Energy Agency, Vienna, Austria). All the determined values in Seronorm™ agreed well with the certified values (Table S1 in ESM). For IAEA-A-13, there is only a certified value for Zn and an information value for Pb: both these values agreed well with

the determined values (Table S1). These results suggest good accuracy of the employed methodology for the analysis of metals in dried blood samples in great tits. The limits of detection (LOD) for Zn, Cd, Pb and As were controlled by the digestion blanks. Numerically, the limits corresponded to 8 ng for Zn, 0.008 ng for Cd, 0.1 ng for Pb and 0.15 ng for As in the liquid samples (5 mL). As the amounts of the dried blood sample taken for digestion and analysis differed significantly (0.5–17 mg), the LOD in μg/g of dried sample had to be recalculated for each sample using a sample-specific dilution factor. The dilution factors (volume of the sample taken for ICP-MS analysis/sample weight taken for digestion) were in the range of 300–10000. The measurement error determined as the average coefficient of variation (standard deviation of 5 measured replicates relative to the sample mean) estimated in a subsample of 59 individuals was <2.17% for Zn, 3.37% for Cd and 6.16% for Pb.

2.4. Statistical analysis

All statistical procedures were conducted in R software 3.5.1 (R Development Core Team, 2018). Gaussian data distributions of the selected response variables were checked using the Shapiro-Wilk normality test. Given the non-Gaussian distribution of all heavy metal concentrations and most haematological parameters, the Spearman's rank correlation coefficient was used for testing correlations. To achieve model residual normality, the logarithmic transformation was applied to the response variables before model testing (except for the IEC, where the Box-Cox transformation with lambda −0.10 was used instead).

Despite intercorrelation, particular metals were tested separately in models regarding their different effects on body metabolism (Jaishankar et al., 2014; Järup, 2003; Tchounwou et al., 2012). The possible accumulation of heavy metals in the blood during the life of free-living birds was tested through analysis of deviance of the generalised linear mixed models (GLMMs, R package lme4; Bates et al., 2015), where concentrations of Pb/Cd/Zn in the blood were used as response variables and the age of the individuals (centred, in linear and quadratic form), sex, body mass, tarsus length and the date of capture converted to the Julian calendar (further referred to as the date) were as explanatory variables (fixed terms). The year of capture and individual ID were used in all the models as a variable with a random intercept effect, while age was used as a random slope effect within ID (age|ID) to allow for inter-individual variation in age-related changes (for full GLMMs models, see Table S2 in ESM). The Satterthwaite approximation method was used for F- and p-value estimation (R package lmerTest; Kuznetsova et al., 2017). For testing the relationship between lifetime changes of metal concentrations and particular haematological traits, the GLMMs were used with TRBC, IEC, TWBC, H/L, TLC and THC, respectively, serving as response variables and the age, sex, age:sex interaction, log transformed blood Pb/Cd/Zn concentrations, body mass, tarsus length and average air temperature 7 days before capturing (further referred to as the temperature) used as explanatory variables. Of the meteorological data available we used only the temperature (a potential stress factor linked to haematological traits) because of its statistically significant correlation with all other parameters, such as average air humidity ($r = -0.41$, $p < 0.01$), total precipitation ($r = 0.21$, $p < 0.01$) and average sunshine duration ($r = 0.66$, $p < 0.01$). The year of capture and individual ID were again used as variables with a random intercept effect, age was used as a random slope variable within ID (Table S2 in ESM).

Minimum adequate models (MAMs; here defined as models with all fixed terms significant at the level of $p \leq 0.05$ or with marginally insignificant terms at the level of $p < 0.10$) were selected by backward elimination of non-significant terms from the full models. All the steps of backward elimination in the models were verified by changes of deviance with an accompanied change in degrees of freedom (ANOVA) and Akaike information criterion (AIC) by using F statistics. Slopes and standard errors (SE) were calculated for all continuous variables within the MAMs. The figures were made using the R package visreg (Breheny and Burchett, 2017).

3. Results

The measured blood Pb, Cd and Zn concentrations were above the detection limit of the method adopted in most of the samples (3% below the LOD for Pb, 0.22% for Cd and 0.46% for Zn). Conversely, As was predominantly non-measurable (75% of the samples below the LOD), therefore it was excluded from further analyses. The concentrations of measurable heavy metals were in the following order: Zn > Pb > Cd (summary statistics in Table 1) and were significantly positively correlated with each other (see Table 2).

3.1. Changes in blood heavy metal concentrations related to age

We found only moderate evidence of heavy metal concentration changes in the blood during the individual lifetime. In the case of Pb, our results indicated a significant sex-specific quadratic relationship between the blood Pb concentrations and age (MAM 1 in Table 3, Fig. 1), where males had higher blood Pb levels than females and where in both cases the highest concentrations were measured in nestlings and in the oldest individuals. However, this relationship was no longer significant when the age category of nestlings (age = 1) was excluded from the tested dataset (see MAM 1a in Table S3 and Fig. S4 in ESM). Exclusion of highly senescent individuals (age = 7) did not change the trend significance (see MAM 1b in Table S3 and Fig. S5 in ESM). In the cases of blood Cd and Zn concentrations we did not detect any such associations (only a significant positive relationship with the date of capture was observed; MAM 2 and MAM 3 in Table 3, Figs. S6 and S7 in ESM).

3.2. Associations between blood heavy metal concentrations and haematological parameters

The summary statistics of all the haematological parameters analysed in this study and their correlations are listed in Table S4 and Table S5, respectively (both in ESM). Our results showed no relationship between blood Pb, Cd or Zn concentrations and TRBC or IEC in the

Table 1
Summary statistics of heavy metal concentrations (μg/g dry weight) in the blood of free-living re-captured great tits at different ages.

Heavy metal	Age	N_{obs}[a]	Mean ± SD	Range	CV[b]
Zn	1	72	185.69 ± 213.35	23.37–792.01	114.90
Cd		72	0.04 ± 0.06	0.00–0.39	158.67
Pb		67	1.47 ± 1.72	0.02–7.86	117.11
Zn	2	118	600.25 ± 892.72	11.59–4203.59	148.72
Cd		118	0.06 ± 0.08	0.00–0.36	134.45
Pb		114	1.44 ± 1.71	0.04–6.49	118.14
Zn	3	123	459.65 ± 659.91	11.54–3057.00	143.57
Cd		124	0.07 ± 0.09	0.00–0.47	129.33
Pb		123	1.28 ± 1.43	0.02–6.70	112.09
Zn	4	75	661.40 ± 674.28	17.36–2808.08	101.95
Cd		75	0.09 ± 0.11	0.00–0.47	116.10
Pb		73	2.10 ± 1.85	0.04–7.70	87.75
Zn	5	34	718.25 ± 965.67	19.72–4545.31	134.45
Cd		34	0.10 ± 0.11	0.01–0.39	114.04
Pb		34	1.48 ± 1.34	0.06–4.73	90.53
Zn	6	11	257.58 ± 419.61	15.68–1396.72	162.91
Cd		12	0.04 ± 0.07	0.01–0.244	176.12
Pb		12	1.83 ± 2.26	0.04–6.59	123.87
Zn	7	3	846.40 ± 353.98	599.47–1251.94	41.82
Cd		3	0.13 ± 0.10	0.012–0.20	79.28
Pb		3	4.30 ± 2.65	1.29–6.26	61.61

N_{TotObs} = 416 (number of total observations), N_{ind} = 177 (number of individuals).
[a] N_{obs} = number of observations.
[b] CV = coefficient of variation.

Table 2
Correlation matrix of heavy metal concentrations in blood.[a]

Variable	Pb	Cd	Zn
Pb	1.00		
Cd	**0.74**	1.00	
Zn	**0.79**	**0.64**	1.00

[a] Spearman correlation coefficients (r_s) are shown, values highlighted in bold are statistically significant at $p = 0.05$ level. $N_{samples} = 416$.

peripheral blood of tits (MAMs 4–9 in Table 3, Figs. S8–S13 in ESM). On the contrary, a significantly positive relationship between blood concentrations of all heavy metals and TWBC was detected. The strongest positive trend was detected in the case of blood Zn levels (slope = 0.146, $p < 0.001$; MAM 10 in Table 3, Fig. 2), then in blood Cd levels (slope = 0.101, $p = 0.003$; MAM 11 in Table 3; Fig. S15 in ESM) and the weakest trend was found in the case of blood Pb levels (slope = 0.089, $p = 0.017$; MAM 12 in Table 3; Fig. S14 in ESM). The differential leukocyte count analysis showed the H/L ratio to be only marginally insignificantly associated with the blood Cd concentrations (slope = 0.095, $p = 0.068$; MAM 14 in Table 3; Fig. S17 in ESM), while in the case of blood Pb and Zn concentrations there was none relationship ($p = 0.36$ and 0.17, Figs. S16 and S18 in ESM, respectively). Furthermore, our results have shown slightly (yet significantly) increased THC for individuals with higher blood Cd and Zn concentrations (slope = 0.133, $p = 0.008$, MAM 17; slope = 0.153, $p = 0.003$, MAM 18, both in Table 3; Figs. S20 and S21 in ESM). Also, the increased TLC levels were significantly linked to the higher blood Pb and Zn concentrations (slope = 0.092, $p = 0.034$, MAM 19; slope = 0.117, $p = 0.011$, MAM 21 in Table 3; Figs. S22 and S24 in ESM). The relationship between the blood Cd concentrations and TLC was marginally insignificant ($p = 0.084$, MAM 20 in Table 3; Fig. S23 in ESM). An overview of the relationships between all analysed metals and individual haematological parameters is shown in Table 4.

4. Discussion

Although several studies have already confirmed that free-living birds can serve as useful bioindicators of environmental quality (Bauerová et al., 2017; Evers et al., 1998; Hargitai et al., 2016; Ruuskanen et al., 2014), the process of accumulation of different heavy metals during the avian lifetime has remained mostly unknown. Furthermore, the age-related effects of heavy metal concentration changes on individual health status have not yet been studied. In this correlative study we benefitted from the long-term ringing and longitudinal sampling effort in our free-living urban great tit population. The determination of the minimum age of each individual and statistical controlling for inter-annual variation allowed us to investigate the lifetime changes in blood heavy metal concentrations (Pb, Cd, As and Zn) and their associations with particular health-related haematological parameters.

Consistently with our previous study (Bauerová et al., 2017), the measured concentrations of heavy metals decreased in our samples from the essential Zn to Pb to the highly toxic Cd and As. This trend may not reflect only the environmental contamination patterns, but also bioaccumulation-associated heavy metal turnovers. We assume that a very short biological half-life of As in the blood (in humans, only from units to tens of hours; Lehmann et al., 2001) could be one of the reasons which prevented us from finding a reliable (measurable) levels of blood As contamination. The time dynamics and organ-specific deposition also need to be considered when interpreting our other findings. While a biological half-life of Pb in the blood is reported at about 1 month, in the bones (which remained unstudied in our research) it spans decades (in humans; Järup, 2003). The same is also true for Cd accumulation in the kidneys (Burger, 2008; Faroon et al., 2012). Although in this study we lack the holistic insight for a comparison of different tissues, because this approach would not allow repeated measures within the same individuals, the use of blood appear relevant for longitudinal biomonitoring from the perspective of practical application: firstly, blood is an easily accessible biological material frequently used for both metal biomonitoring and health assessment based on haematological and biochemical traits (Bailly et al., 2017; Bauerová et al., 2017; Coeurdassier et al., 2012; Fair and Ricklefs, 2002; Ferreyra et al., 2015); secondly, blood is the transport medium that allows to detect current contamination with heavy metals regardless of their later site of deposition (García-Fernández et al., 1996; Van Wyk et al., 2001); and thirdly, both Pb and Cd are also deposited in the bones (Deng et al., 2007; García-Fernández et al., 1996; Janaydeh et al., 2018), from where, through haematopoiesis these trace elements can be partly redistributed back into the bloodstream.

Although it is known that essential elements such as Ca or Zn can protect the body against the absorption of toxic metals (Pb or Cd; Chatelain et al., 2016; Goyer et al., 2004; Hogstad, 1996) we have not confirmed this sort of negative relationship in birds' blood. Similarly to some other studies in free-living birds, we have revealed only significantly positive correlations between Zn and Pb or Cd concentrations (Dauwe et al., 2002; Fritsch et al., 2012; Janssens et al., 2001; Migula et al., 2000).

Contrasting to our prediction on lifetime bioaccumulation of heavy metals detectable in blood, our results show only weak and non-linear age effect in blood heavy metal concentrations (significant in the case of Pb, but not in Cd or Zn). This relationship is apparently driven by the mild peaks in the cohort of nestlings (1st year of age) and highly senescent individuals (minimum known age of 7 years). Notably, if the nestling category was excluded from the dataset, the relationship between blood Pb concentrations and age would become insignificant. Thus, our results suggest only a very weak lifetime accumulation tendency for Pb in the blood of adult individuals living in an urban non-industrial environment. Since great tits represent omnivorous species feeding their nestlings with insects (Cramp et al., 1993), the bimodal pattern can be explained by increased intake through a metal-rich diet (namely caterpillars, spiders or beetles; Fritsch et al., 2012; Heikens et al., 2001) in nestlings on one hand (Eeva et al., 2005; Janssens et al., 2001), and by the impaired detoxification metabolism in aged individuals on the other. Furthermore, our results also suggest that Pb may be decontaminated from the blood of young individuals, while it can later accumulate in the bodies (possibly in bones) to get increasingly redistributed back into the bloodstream of the senescent ones. Here it is appropriate to highlight that given the low re-capture rate in nestlings the age recorded was the minimum-known age (i.e. some individuals might have been actually older than the recorded age, provided that they were captured the first time as full-grown adults). Although methodologically suboptimal, this shortcoming results from the biology of the studied species and could not be prevented. Since age was in this study controlled for the individual identity in the re-capture data, this should not bias our results. Although several non-longitudinal studies in free-living birds reported higher blood Pb concentrations in adults than in nestlings (Fritsch et al., 2012; Van Wyk et al., 2001), differential heavy metal retention or detoxification within differently aged adults has not been described before for Pb. The only study (according to our knowledge) focused on repeatedly measured adult individuals in free-living birds was Evers et al. (1998), which reported similar lack of relationship in case of blood Hg contamination and age in adult re-captured common loons (*Gavia immer*).

Contrary to Pb, we have not found any relationship between blood Cd or Zn concentrations and the age or sex of the individuals. The lack of an age-related pattern of Cd in the blood was also observed in other non-longitudinal studies on free-living birds (Coeurdassier et al., 2012; Fritsch et al., 2012), while in the case of Zn, the age-related pattern in blood concentrations was not expected since Zn is an essential element regulated by the homeostatic system (WHO, 2001; Zargar et al., 2015). This corresponds to our second prediction that blood as a

Table 3
Minimum adequate models (MAMs) obtained for the dataset of re-captured great tits.

Minimum adequate model[a]	Slope ± SE	F	Df	p	N_{obs}[b]/N_{ind}[c]
MAM 1 **Pb** ~ age + age^2 + sex		20.72	3/415	<0.001	416/177
Age	−0.054 ± 0.037	2.15	1/412	*0.144*	
Age^2	**0.077 ± 0.020**	**15.22**	**1/412**	**<0.001**	
Sex		4.98	1/412	0.026	
MAM 2 **Cd** ~ date	0.032 ± 0.004	57.13	1/410	<<0.001	412/175
MAM 3 **Zn** ~ date	0.031 ± 0.003	84.47	1/410	<<0.001	412/175
MAM 4 TRBC ~ age + temperature		7.61	2/262	0.022	263/114
Age	−0.019 ± 0.009	4.31	1/260	0.040	
Temperature	0.010 ± 0.005	4.19	1/260	0.042	
MAM 5 TRBC ~ age + temperature		7.57	2/261	0.023	262/112
Age	−0.018 ± 0.009	4.00	1/259	0.048	
Temperature	0.011 ± 0.005	4.33	1/259	0.039	
MAM 6 TRBC ~ age + temperature		8.31	2/266	0.016	267/115
Age	−0.019 ± 0.009	4.21	1/264	0.042	
Temperature	0.011 ± 0.005	5.08	1/264	0.025	
MAM 7 IEC ~ body mass	0.585 ± 0.325	3.24	1/317	*0.073*	319/138
MAM 8 IEC ~ body mass	0.640 ± 0.316	4.11	1/326	0.044	327/140
MAM 9 IEC ~ age + body mass		6.79	2/330	0.034	331/143
Age	−0.026 ± 0.015	2.92	1/328	*0.092*	
Body mass	0.642 ± 0.313	4.20	1/328	0.041	
MAM 10 TWBC ~ sex + Pb + body mass + temperature		22.85	4/294	<0.001	295/128
Sex		8.45	1/290	0.005	
Pb	**0.089 ± 0.034**	**7.04**	**1/290**	**0.017**	
Body mass	1.604 ± 0.695	5.33	1/290	0.022	
Temperature	0.040 ± 0.015	7.28	1/290	0.008	
MAM 11 TWBC ~ sex + Cd + body mass + temperature		27.22	4/298	<<0.001	299/128
Sex		5.94	1/294	0.016	
Cd	**0.101 ± 0.032**	**9.84**	**1/294**	**0.003**	
Body mass	1.281 ± 0.644	3.96	1/294	0.048	
Temperature	0.044 ± 0.015	9.35	1/294	0.003	
MAM 12 TWBC ~ sex + Zn + body mass + temperature		35.98	4/300	<<0.001	301/130
Sex		6.34	1/296	0.013	
Zn	**0.146 ± 0.018**	**63.46**	**1/296**	**<0.001**	
Body mass	1.627 ± 0.670	5.89	1/296	0.016	
Temperature	0.047 ± 0.012	15.27	1/296	<0.001	
MAM 13H/L ~ age + sex + temperature		32.95	3/327	<<0.001	328/141
Age	0.145 ± 0.041	12.42	1/324	0.001	
Sex		14.31	1/324	<0.001	
Temperature	−0.071 ± 0.021	11.45	1/324	0.001	
MAM 14H/L ~ age + Cd + sex + temperature		37.71	4/330	<<0.001	331/141
Age	0.159 ± 0.042	14.59	1/326	<0.001	
Cd	**0.095 ± 0.052**	**3.39**	**1/326**	***0.068***	
Sex		13.83	1/326	<0.001	
Temperature	−0.077 ± 0.021	12.86	1/326	<0.001	
MAM 15H/L ~ age + sex + temperature		35.45	3/331	<<0.001	332/144
Age	0.159 ± 0.041	15.22	1/328	<0.001	
Sex		14.04	1/328	<0.001	
Temperature	−0.073 ± 0.021	12.23	1/328	0.001	
MAM 16 THC ~ sex + body mass		19.57	2/293	<<0.001	294/127
Sex		12.49	1/291	<0.001	
Body mass	3.186 ± 1.044	9.31	1/291	0.003	
MAM 17 THC ~ sex + Cd + body mass		24.86	3/296	<<0.001	297/127
Sex		9.81	1/293	0.002	
Cd	**0.133 ± 0.049**	**7.25**	**1/293**	**0.008**	
Body mass	3.025 ± 1.012	8.94	1/293	0.003	
MAM 18 THC ~ sex + Zn + body mass		29.40	3/301	<<0.001	302/130
Sex		11.72	1/298	<0.001	
Zn	**0.153 ± 0.044**	**11.87**	**1/298**	**0.003**	
Body mass	3.001 ± 1.012	8.78	1/298	0.003	
MAM 19 TLC ~ age + Pb + temperature		23.95	3/293	<<0.001	294/127
Age	−0.094 ± 0.036	7.01	1/290	0.010	
Pb	**0.092 ± 0.040**	**5.23**	**1/290**	**0.034**	
Temperature	0.067 ± 0.018	14.53	1/290	<0.001	
MAM 20 TLC ~ age + Cd + temperature		25.06	3/296	<<0.001	297/127

Table 3 (*continued*)

Minimum adequate model[a]	Slope ± SE	F	Df	p	N_{obs}[b]/N_{ind}[c]
Age	−0.075 ± 0.034	4.69	1/293	0.033	
Cd	**0.068 ± 0.038**	**3.09**	**1/293**	***0.084***	
Temperature	0.075 ± 0.017	18.77	1/293	<<0.001	
MAM 21 TLC ~ age + Zn + temperature		27.16	3/301	<<0.001	302/130
Age	−0.084 ± 0.034	5.95	1/298	0.017	
Zn	**0.117 ± 0.033**	**13.04**	**1/298**	**0.011**	
Temperature	0.068 ± 0.016	17.27	1/298	<0.001	

[a] The year and individual ID were used as variables with a random intercept effect, the age of the individuals was used as a random slope within the ID (Age|ID). Slope ± SE values are only provided for continuous variables. Significant and marginally non-significant effects of heavy metal concentrations in the blood are highlighted in bold. Pb/Cd/Zn = heavy metal blood concentration (μg/g dry weight), date = day of capture, temperature = average air temperature 7 days before capturing, TRBC = total red blood cell count [cells × 10^{12}L], IEC = immature erythrocyte count [%], TWBC = total white blood cell count [cells × 10^{9}L], H/L = heterophil/lymphocyte ratio, THC = total heterophil count [cells × 10^{9}L], TLC = total lymphocyte count [cells × 10^{9}L].

[b] N_{obs} = number of observations.

[c] N_{ind} = number of individuals.

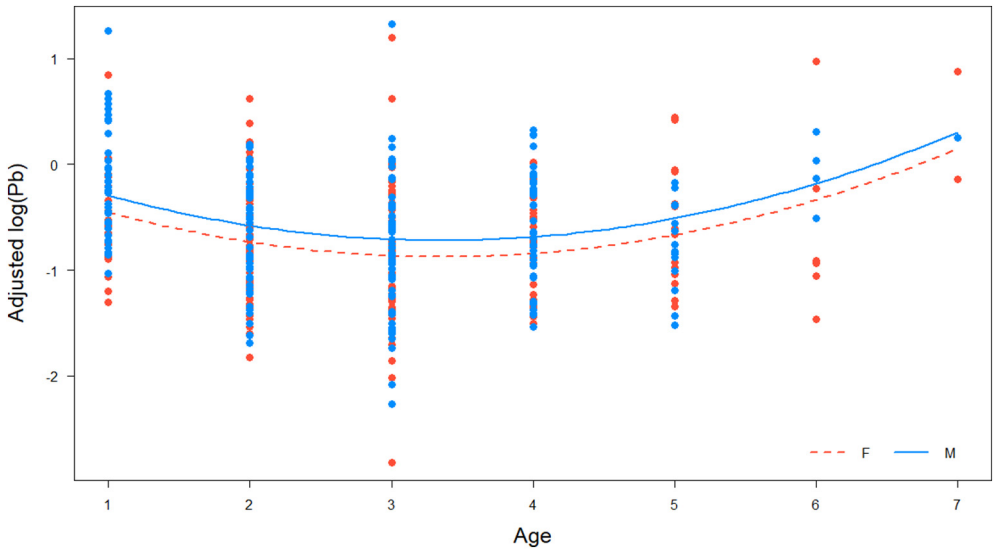

Fig. 1. The relationship between blood Pb concentration and age of great tit males (M, in blue) and females (F, in red; $N_{ind} = 177$, $N_{obs} = 416$). Blood Pb concentrations are shown on the y axis as adjusted value controlled for all significant effects of fixed variables in the MAM 1 model (Table 3), i.e. model residuals not including the random effects (done in R by package visreg; Breheny and Burchett, 2017).

transportation medium is not involved in accumulation of heavy metals, which allows use of blood for actual trace element biomonitoring. The only significant effect in the case of Cd and Zn blood concentrations was the date of capture: great tits mist netted in spring (during the breeding season) had significantly higher levels of Cd and Zn blood concentrations than the tits mist netted in winter. Although we are aware that we did not have sufficient sample of birds captured in winter ($n = 7$), we assume that these seasonal differences in blood Cd and Zn concentrations could reflect the changes in the tits' diet composition. While during the breeding season and summer this species is primarily fed on metal-rich insects, during the winter up to 90% of their diet consists of plant material and various seeds, which often have lower levels of heavy metal contamination (Eeva and Hasselquist, 2005; Fritsch et al., 2012; Ping et al., 2009). This highlights the need for accounting the seasonal effects in the biomonitoring studies.

Interestingly, contrary to our predictions based on our previous study in the same species (Bauerová et al., 2017), we found no anaemia-like conditions (decreased TRBC or increased IEC) in the individuals suffering from higher heavy metal contamination. This may be explained by much lower Pb and Cd blood levels detected in the present study that, unlike others, did not focus on industrial sites (Belskii et al., 2005; Geens et al., 2010). Despite this lack of a relationship to erythrocyte-associated haematological traits, in all of the investigated heavy metals we showed a significant positive trend with the absolute leukocyte numbers (TWBC). Apparently, both major leukocyte types increased with the higher metal levels, but the trend was slightly stronger for heterophils. This result is surprising, because toxic (Pb and Cd) and essential (Zn) elements showed the same trend and also because this trend was evidently not reflecting the increased H/L ratio indicating long-term stress (although there was a marginally non-significant tendency in this direction for Cd). Since the strongest relationship was detected with Zn, we suggest that this trend may be driven by a possible positive effect of the increased levels of this essential element on leukocyte proliferation (Chatelain et al., 2016). However, because a similar pattern was also observed for non-essential Cd and Pb, we cannot rule out even the alternative explanation through the negative health effects

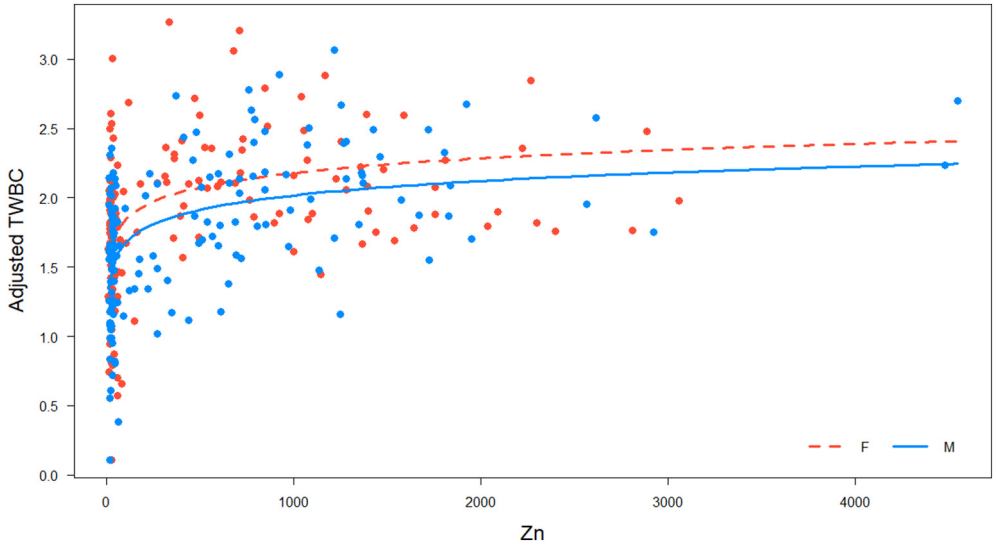

Fig. 2. Relationship between TWBC and blood Zn concentration (μg/g dry weight) of great tits, males (M, in blue) and females (F, in red; $N_{ind} = 130$, $N_{obs} = 301$). The TWBC is shown on the y axis as adjusted value controlled for all significant effects of fixed variables from MAM 12 (Table 3), i.e. including body mass and air temperature, but not including the random effects (done in R by package visreg; Breheny and Burchett, 2017).

Table 4
The summary table of slopes for the relationships[a] between blood heavy metal concentrations and particular haematological parameters.

Variable	Pb	Cd	Zn
TRBC	0 (MAM 4)	0 (MAM 5)	0 (MAM 6)
IEC	0 (MAM 7)	0 (MAM 8)	0 (MAM 9)
TWBC	+ **(MAM 10)**	+ **(MAM 11)**	+ **(MAM 12)**
H/L	0 (MAM 13)	+ *(MAM 14)*	0 (MAM 15)
THC	0 (MAM 16)	+ **(MAM 17)**	+ **(MAM 18)**
TLC	+ **(MAM 19)**	+ *(MAM 20)*	+ **(MAM 21)**

[a] (0) = no relationship, (+) = significant (bold) or marginally non-significant (italics) positive relationship. The reference to the particular MAMs in Table 3 is provided in brackets.

of toxic heavy metal peripheral blood contamination inducing leukocyte proliferation (Dumonceaux and Harrison, 1994; Jones, 2015). There are only a few studies focusing on associations between heavy metal body contamination and blood levels of different leukocyte types in wild birds (Bauerová et al., 2017; Fredricks et al., 2009). In the case of the TWBC neither of them showed a significant relationship with heavy metal blood content and in Bauerová et al. (2017) we revealed only negative effect of blood heavy metal contamination on the H/L ratio.

The results of this long-term monitoring field study can help to clarify the role of individual age in blood heavy metal contamination in free-living animals and the associations between lifetime contamination changes and the actual health-related condition. Taken altogether, we found only moderate evidence for nestling-driven age effect on Pb blood contamination with no clear trend observable in adults. No significant age-related trend was found in the cases of Cd and Zn. Thus, our research justifies the increasing preference for use of adult birds with unknown precise age for reliable indication of actual blood contamination levels during biomonitoring. These results are not biased by the effect of lifetime accumulation in body tissues and its possible redistribution back into the blood stream.

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Ethical approval

This research was approved by the Ethics Committees of the Institute of Vertebrate Biology, Czech Academy of Sciences and Charles University, Faculty of Science (Permits Nos. 107/2009, 09/2015 and 22003/ENV/16-1009/630/16) and was conducted in accordance with the current laws of the Czech Republic and the EU.

CRediT authorship contribution statement

Petra Bauerová: Methodology, Investigation, Formal analysis, Writing - original draft, Writing - review & editing.**Tereza Krajzingrová:**Investigation, Formal analysis, Writing - review & editing.**Martin Těšický:**Investigation, Writing - review & editing.**Hana Velová:**Investigation, Writing - review & editing.**Jakub Hraníček:**Formal analysis, Writing - review & editing.**Stanislav Musil:**Formal analysis, Writing - review & editing.**Jana Svobodová:**Investigation, Methodology, Writing - review & editing.**Tomáš Albrecht:**Investigation, Writing - review & editing.**Michal Vinkler:**Methodology, Investigation, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

The electronic supplementary material (ESM) contains results from full generalised linear mixed models, and all other supplementary tables and figures (5 tables and 24 figures). Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.138002.

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