Associations of urban environmental pollution with health-related physiological traits in a free-living bird species

Petra Bauerová [a,b], Jitka Vinklerová [a], Jakub Hraníček [c], Vojtěch Čorba [a], Libor Vojtek [d], Jana Svobodová [b], Michal Vinkler [a,*]

[a] Charles University, Faculty of Science, Department of Zoology, Viničná 7, 128 44 Prague 2, Czech Republic, EU
[b] Czech University of Life Sciences Prague, Faculty of Environmental Sciences, Department of Ecology, Kamýcká 1176, 165 21 Prague 6, Czech Republic, EU
[c] Charles University, Faculty of Science, Department of Analytical Chemistry, Hlavova 8, 128 43 Prague 2, Czech Republic, EU
[d] Masaryk University, Faculty of Science, Department of Experimental Biology, Kotlářská 2, 611 37 Brno, Czech Republic, EU

HIGHLIGHTS

- Cities varying in air pollution can vary in organism heavy metal contamination.
- Across regions heavy metal contamination in the great tits was linked to health.
- Higher blood contamination was associated with lower H/L in blood.
- Increased blood contamination was related to decreased erythrocyte counts.
- Urban pollution may affect physiology of synanthropic free-living organisms.

GRAPHICAL ABSTRACT
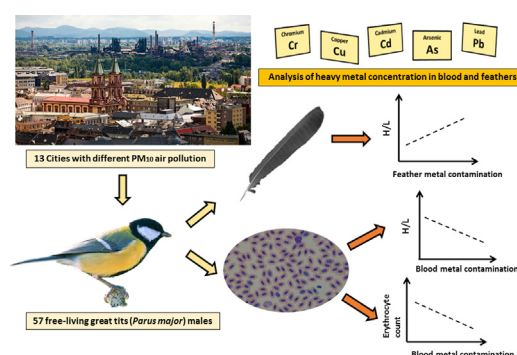
ARTICLE INFO

ABSTRACT

Urban environmental pollution results in contamination of the tissues of synanthropic organisms by toxic trace elements with potential impacts on human health. Passerine birds may serve as convenient indicators of such contamination. In this study we investigated the effect of blood and plumage contamination with heavy metals (lead Pb, cadmium Cd, copper Cu, chromium Cr) and arsenic metalloid (As) on condition, health and ornamental colour in free-living great tit (*Parus major*) males from 13 cities across the Czech Republic (EU), mist netted during the early breeding season (April–May). Our results showed a significant association of heavy metal tissue contamination with immune function, namely leukocyte composition in the avian blood circulation. High heavy metal contamination in bird feathers was linked to a high heterophil/lymphocyte (H/L) ratio, indicating long-term stress in individuals inhabiting heavily polluted environments. In contrast, males with higher concentrations of heavy metals in blood had a lower H/L ratio, assumingly due to the direct toxicity of heavy metals in certain cell types. This is also supported by traits indicative of anaemia-like haemolytic conditions (decreased absolute erythrocyte count) and increased haematopoiesis (a tendency for increased frequencies of immature erythrocytes). We did not find any association of heavy metal contamination with the bacteriolytic activity of plasma complement, feather growth or ornamentation (black breast stripe area and yellow colouration). There was no significant relationship between heavy metal contamination in blood or feathers and PM$_{10}$ pollution at the study sites. Our correlational study is the first to show on a large geographic scale that despite strict European air pollution regulations and regular monitoring that have allowed general improvements in

* Corresponding author at: Department of Zoology, Faculty of Science, Charles University, Viničná 7, 128 44 Prague 2, Czech Republic, EU.
E-mail address: michal.vinkler@natur.cuni.cz (M. Vinkler).

atmospheric contamination, non-degradable heavy metals persistently contaminate animal blood and feathers in anthropogenic environments at levels that may have subclinical yet physiological effects with varied influence on health.

1. Introduction

Metals are released into urban environments through atmospheric particulate matter pollution (including $PM_{10} < 10$ µm particles) and waste water, both commonly related to industrial production. Given the impacts of pollution on health of living organisms (Jarup, 2003; WHO, 2007) the potential environmental risks associated with industrial development must be considered. Heavy metals and metalloids (for simplicity considered as heavy metals in this article) are especially dangerous due to their persistence, high mobility and ability to accumulate in human and animal tissues (Mora, 2003; Walker et al., 2012). The world-wide understanding of the severity of this issue (WHO, 2000) has led most countries to establish measures reducing particulate matter and heavy metal emissions (WHO, 2006a; WHO, 2006b). In the Czech Republic the Clean Air Act No. 201/2012 Coll. (reflecting present EU directives) sets hygienic limits for PM_{10} air pollution and some metals (e.g., arsenic (As), cadmium (Cd), nickel (Ni) and lead (Pb); also EEA, 2015). For other metals (e.g. chromium (Cr), copper (Cu) and mercury (Hg)) no legal measures have yet been set, even though their ecological importance in the environment is significant.

It has been shown that simple contamination of the environment with heavy metals may not reliably reflect their absorption rates and physiological effects in living organisms (Scheifler et al., 2006). Therefore, biological indicators of such a relationship are needed to monitor environmental quality (Burger and Gochfeld, 2001). Avian tissue contamination has been frequently considered a valuable and cost-effective bioindicator of environmental pollution (Burger and Gochfeld, 2001). Unlike most other tissues such as lungs, kidneys or liver (Cui et al., 2016), feathers (Bianchi et al., 2008; Dauwe et al., 2002a; Dauwe et al., 2000; Markowski et al., 2013) and avian blood (Carneiro et al., 2015; Coeurdassier et al., 2012; Costa et al., 2014; Geens et al., 2010) may serve as non-destructive and easy-to-obtain biological materials for such monitoring. Heavy metal contamination in feathers is assumed to be representative of intra-annual exposure (Dauwe et al., 2002a). It has been documented that heavy metals deposited into tail feathers (rectrices) reflect the levels in key internal tissues resulting from long-term accumulation (Dauwe et al., 2002b). In contrast, blood is a transport medium and accumulation of heavy metals there is unlikely. Thus, blood contamination reflects immediate exposure (Geens et al., 2010; Scheifler et al., 2006), and there is typically no correlation between blood and feather heavy metal contents (Dauwe et al., 2005).

In wild animals, toxic metals may have direct measurable negative physiological effects even in low sublethal concentrations (Geens et al., 2010; Hawley et al., 2009; Janssens et al., 2003). Effects on bird physiology (Aggarwal et al., 2008), reproduction (Belskii et al., 2005; Eeva et al., 2009; Eeva and Lehikoinen, 2015), diet composition (Eeva et al., 1998; Geens et al., 2009) and nutritional condition (Blanco et al., 2004; Eeva et al., 1998) have been reported. Pb, Cd and Cu in particular have also been reported to directly alter antioxidant capacity (Geens et al., 2009), immunity and health in free-living birds (Blanco et al., 2004; Fair and Myers, 2002; Geens et al., 2010; Snoeijs et al., 2004). Heavy-metal-induced changes in haematological parameters associated with erythrocytes (haematocrit, erythrocyte count and haemoglobin content) reflect anaemia resulting from intoxication (Geens et al., 2010; Papanikolaou et al., 2005). Since health, antioxidant capacity as well as immune function can be related to avian ornament expression (Vinkler and Albrecht, 2010), several authors (e.g. Dauwe and Eens,

2008 and Eeva et al., 1998) have suggested the possibility of decreased carotenoid-based ornamentation in birds at heavily polluted sites. In contrast, melanin-based ornamentation was found to be increased in adult great tits from highly-polluted sites (Dauwe and Eens, 2008).

The heavy metal burden in birds is higher in cities than in the countryside (Hargitai et al., 2016), as has been confirmed in blackbirds (*Turdus merula*; Meillere et al., 2016; Scheifler et al., 2006), and great (*Parus major*) and blue tits (*Cyanistes caeruleus*; Eens et al., 1999). However, even cities themselves may differ markedly in their levels of heavy metal contamination (CHMI, 2009). It has been previously shown that amounts of heavy metals in great and blue tit tissues decrease with the distance from the source of pollution (Dauwe et al., 2002b). We may predict, therefore, that urban habitats differing in sources of pollution will also differ in their levels of contamination. However, no comparison of avian tissue heavy-metal contamination across a large-scale geographic area has yet been done to show the effects of varying pollution levels on animal health-related physiological traits.

In this study, we hypothesised that differences in avian heavy metal contamination in urban free-living populations would manifest in variation in their health-related traits. We predicted that birds from more polluted cities would show impaired condition and health compared to birds from cleaner cities. To test this hypothesis we compared heavy metal (As, Cu, Cd, Pb, and Cr) concentrations in the blood and feathers of 57 great tit males originating from 13 different city regions varying in air pollution. Contrary to most other studies dealing with this issue, we did not focus on any particular candidate source of environmental contamination, but rather performed a large-area survey, with study sites distributed all over the area of interest, in our case the territory of the Czech Republic, EU. Study localities were chosen according to their annual average concentrations of PM_{10} (ranging from 27 µg/m³, to 65 µg/m³, Fig. 1, (CHMI, 2010), for closer details see Table 1). We used principal component analysis (PCA) of heavy metal contamination to reveal general effects of all heavy metals on individual health- and condition-related traits, namely fat deposition index, ptilochronologically-measured feather growth rate, leukocyte and erythrocyte levels in blood circulation, differential leukocyte count, immature erythrocyte frequencies, complement activity in plasma, and carotenoid- and melanin-based ornamentation.

2. Methods and materials

2.1. Field procedures

During the early breeding season (April–May) 2010 great tit adult males ($N = 57$) attracted by a song record were mist netted during the morning hours at 13 different urban areas in the Czech Republic, EU (map, Fig. 1 and Supplement 2). These areas were selected based on their PM_{10} air pollution levels in the previous year (the annual average of year 2009; data from the Czech Hydrometeorological Institute, CHMI, 2010), comprising putatively highly polluted sites ($PM_{10} \geq 40$ µg/m³, $n = 5$) as well as putatively lowly polluted sites ($PM_{10} < 40$ µg/m³, $n = 8$), and reflecting the distribution of the most important urban centres in the Czech Republic (see Table 1 for additional details). The mist-netting sites were always located within a radius of 2 km from a CHMI automatic air pollution monitoring station (see Table 1 and Supplement 2 for a detailed description), always in the same habitat type (deciduous forest with no major roads or industrial buildings in their vicinity). In most locations the sample sizes of
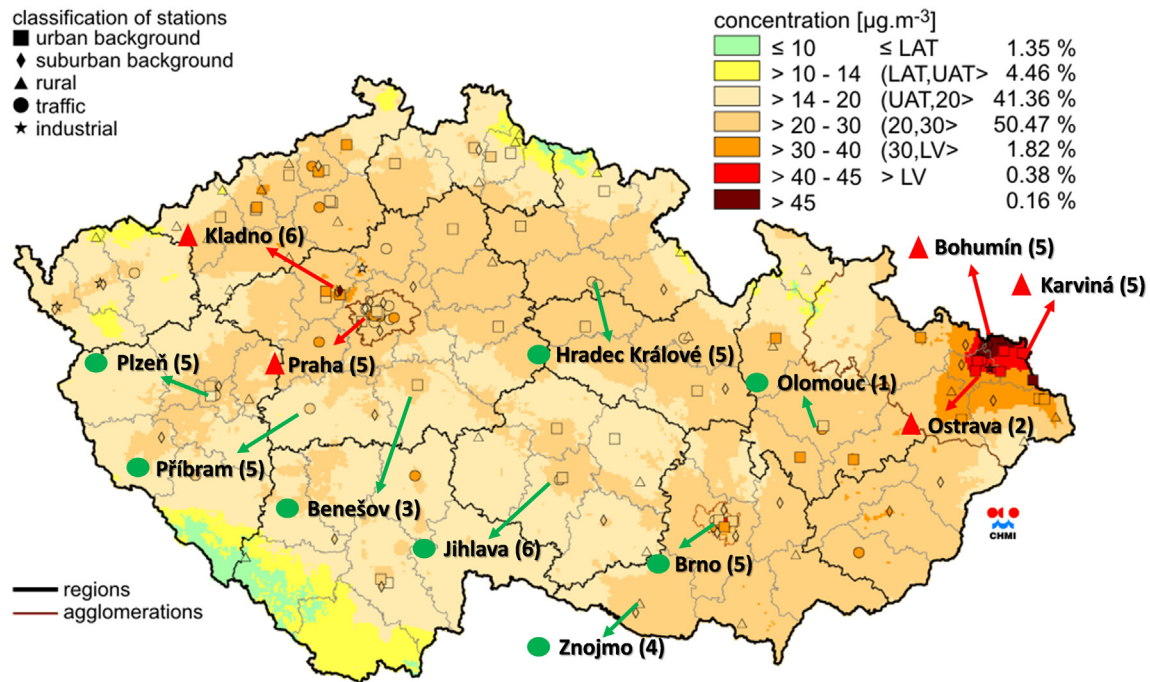
Fig. 1. Pollution distribution in the Czech Republic (annual average concentration of PM10 in year 2009). The geographical distribution of the cities where great tits were sampled in 2010 is indicated by red triangles (locations with PM10 > 40 μg/m³) or green circles (locations with PM10 ≤ 40 μg/m³). Numbers of individuals mist-netted per location are given in parentheses. Classification of the measuring station is indicated by the symbol at the beginning of each arrow (legend included in the figure; map source CHMI, 2010). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

birds were $n \geq 4$ per site, except Benešov, Ostrava and Olomouc (see Fig. 1 or Table 1). Note that our selection of the study sites was highly limited by the available number of distinct regions in the Czech Republic with high levels of air pollution. In all birds, a blood sample (ca. 150 μl) was taken from the brachial vein within 15 min of capture. Following haematological procedures included a blood smear preparation (ca. 5–10 μl), dilution for absolute haematological counting (15 μl), microcapillary centrifugation and haematocrit measurement (ca. 130 μl; see chapter 2.2 for details); plasma was frozen at −80 °C while the pellet was stored in ethanol at −20 °C. The ethanol-dried pellet was later used for analyses of the heavy metal content in the fixed blood cells (on average ca. 11 mg of dried blood; see Section 2.5). Then the birds were weighed with a digital scale (Pesola AG, Baar, Switzerland, type PPS200, accuracy 0.02 g) and their tarsus lengths (including the intertarsal joint) were measured with a digital calliper (Kinex, accuracy to 0.01 mm) as a general estimate of their size. The

age of the birds was estimated according to Jenni and Winkler (1994) and assigned to two categories: 1) birds in their second year of life and 2) older birds (for more details about the number of individuals in these age classes see Table 1). Then, standard digital images of breast ornaments were taken by an Epson Perfection V30 scanner (Seiko Epson Corporation, Japan; see Svobodová et al., 2013 for details), randomly taken samples of carotenoid and melanin-pigmented feathers were taken (ca. 20) and the second outermost rectrix from the left side (a homologous feather in all birds that moults after breeding, between August and October of the previous year, Jenni and Winkler, 1994) was collected for heavy metal analysis. A fat deposition index (hereafter termed fatness) was scored according to Pettersson and Hasselquist (1985). Finally, each individual was ringed with a standard steel ring of the Czech Bird Ringing Centre (National Museum Prague) and released within 30 min of capture. All birds were handled and measured by a single person (MV).

Table 1
Description of the sampling localities ($n_{locality} = 13$) of great tits from the Czech Republic, 2010 ($N = 57$). GPS = GPS coordinates, PM10 = annual average concentrations of PM10 (in μg/m³), n = number of birds captured per locality with the number of individuals in each age class in brackets (1st y = birds in their 1st year of life, ad = older birds), n-inhab. = number of city inhabitants, GDP = Gross Domestic Product (GDP in mil. CZK) given for the whole region (based on NUTS = Nomenclature of Units for Territorial Statistics). Localities are listed in order from the least polluted to the most polluted sites.

	GPS	PM10	n (1st y/ad)	n-inhab.[a]	Region (NUTS)	GDP[a]
Příbram	49°41′02.16″N, 14°01′19.18″E	27.22	5 (1/4)	34,068	Středočeský	380,158
Benešov	49°47′10.34″N, 14°41′51.06″E	27.64	3 (1/2)	16,343	Středočeský	380,158
Znojmo	48°51′27.24″N, 16°02′42.04″E	28.3	4 (1/3)	34,476	Jihomoravský	368,957
Olomouc	49°36′31.55″N, 17°15′26.74″E	28.46	1 (1/0)	100,233	Olomoucký	166,084
Plzeň	49°44′10.17″N, 13°25′01.82″E	29.86	5 (3/2)	168,808	Plzeňský	179,597
Hradec Kralové	50°11′42.52″N, 15°52′11.66″E	31.02	5 (2/3)	94,318	Královehradecký	164,478
Jihlava	49°23′21.48″N, 15°36′12.03″E	34.77	6 (1/5)	51,154	Vysočina	140,204
Brno	49°11′48.09″N, 16°35′43.92″E	37.79	5 (5/0)	371,371	Jihomoravský	368,957
Praha	50°03′49.86″N, 14°24′09.79″E	43.12	5 (4/1)	1,257,158	Praha	920,879
Karviná	49°52′26.86″N, 18°33′36.74″E	46.57	5 (5/0)	60,679	Moravskoslezský	351,484
Kladno	50°10′37.76″N, 14°11′58.71″E	49.17	6 (5/1)	69,938	Středočeský	380,158
Ostrava	49°48′01.88″N, 18°20′22.62″E	56.1	2 (2/0)	303,609	Moravskoslezský	351,484
Bohumín	49°53′39.96″N, 18°22′11.17″E	64.83	5 (3/2)	22,631	Moravskoslezský	351,484

[a] Data taken from the publicly available database of the Czech Statistical Office (CSO, 2017).

2.2. Haematological analysis

The haematological procedures followed the protocol previously published by Vinkler et al. (2010) and are described here only briefly. From each blood sample collected into a heparinised microcapillary, 15 µl of blood were transferred to 2985 µl of Natt and Herrick's solution and stored for several hours in a field refrigerator until the total red blood cell count (TRBC, i.e. number of erythrocytes per volume unit of blood), and the total white blood cell count (TWBC, i.e. number of leukocytes per volume unit of blood) was investigated using a Bürker's counting chamber (100 large squares for leukocytes were examined directly in the field and digital images of 20 rectangles were scanned for erythrocytes later in the lab using an automated counting system; Stepka, 2013). A blood smear was prepared from a blood droplet and the rest of the blood remaining in the microcapillary was centrifuged for 5 min at 11000 rpm (Nüve HN 075) to estimate haematocrit.

Blood smears (unfixed with methanol) were later stained in the lab with Wright-Giemsa Modified stain (product No. WG128, Sigma-Aldrich, St. Luis, MO, USA) and used to assess the differential leukocyte count and percentage of immature erythrocytes using a light microscope with 100× objective magnification (Olympus Corporation, Tokyo, Japan, type CX-31). The differential leukocyte count was based on a sample of ca. 120 leukocytes where five leukocyte categories were recognised: lymphocytes, monocytes, heterophils, basophils and eosinophils. We used heterophil and lymphocyte cell counts to calculate the heterophil:lymphocyte ratio (H/L), a commonly used indicator of long-term stress and health change (Davis et al., 2008). Given the very low frequency of eosinophils and monocytes (<2%), we excluded these leukocyte types from further analysis. The percentage of immature erythrocytes was estimated based on 5 photographed (Olympus, camera E-410) randomly chosen monolayer fields (ca. 2000–4000 cells). Copies of all images were transformed into a black and white 1 bit format and the total number of cells per image was automatically counted using the particle analyser in ImageJ software (Schneider et al., 2012). The original photographs were then used to manually count the immature erythrocytes in the images using the ImageJ cell counter. All cell counting was performed by one person only (smears, TRBC: PB, TWBC: MV) to minimise any potential variability among the measurements.

For measuring the antibacterial activity of the plasma complement (in part of the sample, $N = 22$, see below) we used the bioluminescence-based method (for more details see Buchtikova et al., 2011). The viability of bioluminescent *Escherichia coli* K12 (genetic modification pEGFPluxABCDEamp; Atosuo et al., 2013) was measured (continuously for approx. 3 h at 37 °C) in a Chameleon V plate luminometer (Hidex, Turku, Finland), where the intensity of the emitted light corresponds to bacterial viability over time. Subsequently, the required time for killing 50% of the bacteria volume was determined. A shorter time, therefore, implies higher plasma complement bactericidal activity. This analysis required a minimum volume of 25 µl of pure plasma; in our case only 22 samples met this criterion. However, even in this reduced dataset there were samples present from most of the studied localities ($n_{locality} = 9$; 4 localities with $PM_{10} < 40$ µg/m^3, 5 localities ≥ 40 µg/m^3), and the number of individuals per locality were $n \geq 2$, except for Benešov and Plzeň ($n = 1$).

2.3. Ptilochronological analysis

To measure the differences between individual males in their nutritional condition over the moulting period, ptilochronological measurements of tail feather growth rates were performed according to Grubb (2006), with several modifications. The rectrices were scanned with a 50 mm scale using a scanner (Epson V30) in the grey scale reflex mode with 600dpi resolution. Digital images were adjusted in Corel Photo-Paint X3 (Corel Corporation, Ottawa, Canada) software by the function of Local Equalization (with parameters Width 100 and Height

100). Thereafter, the digital images were used to measure the total rectrix length and the mean width of the growth bars in ImageJ (Schindelin et al., 2015). To estimate the mean growth bar width, a segment of 10 growth bars with its centre located at 2/3 of the feather length was used.

2.4. Analysis of the feather ornamentation

Digital images of the great tit melanin breast ornaments (ventral side of the bird) were analysed using Adobe PHOTOSHOP CS.3 software version 10.0 (Adobe Systems, San Jose, CA, USA). First, all pictures were standardized in colouration according to a white, 50% grey and 100% black reference swatch (grey card GC 18 and colour & grey chart Q 14; Danes-Picta, Praha, Czech Republic) and rulers (1 mm = 8 pixels; Vinkler et al., 2012). Then, the area of the black melanin-pigmented breast stripe (further referred to as stripe area) was measured at 50 mm in length from the neck using the selection tool and area measuring function.

Saturation of the yellow carotenoid-based breast feather ornament (hereinafter termed yellow chroma) was measured using an Avaspec 2048 spectrometer with an Avalight XE light source (Avantes, Eerbeek, Netherlands) and the Avasoft 7.0 processing system (Avantes). In each individual we measured the colour of a layer of 10 sampled carotenoid-based feathers fixed on the surface of a slide with tape (according to Quesada and Senar, 2006) under standardized conditions (for details on the method, see Albrecht et al., 2009). To describe the inter-individual variation in colour we calculated the yellow chroma (difference between reflectance at 700 nm and reflectance at 450 nm, relative to reflectance at 700 nm; the interval for absorbance of carotenoid pigments is 450–700 nm) from the spectral data (Montgomerie, 2006).

2.5. Analysis of heavy metal content in blood and feathers

All tail feather samples were weighed with an accuracy ± 0.01 mg (Sartorius 2004 MP, Sartorius, USA; the dry feather mass ranged between 0.007 and 0.010 g). Although unwashed feathers exhibit ca. 40% external contamination with heavy metals (Scheifler et al., 2006), rinsing has been shown to be only partially effective for avoiding this contamination (Jaspers et al., 2004). Therefore, in this study we did not perform any rinsing in order to avoid potential artificial variation between samples due to variations in washing efficiency. The feathers were digested in 5 ml of 65% HNO_3 (Analpur, Analytika, Czech Republic) using a Digestion MDS-2000 microwave system with lined digestion vessels. Original solutions were transferred into 10 ml volumetric flasks and filled up to the mark with deionized water (<0.1 mS cm^{-1}; Milli-QPLUS, Millipore, USA). Given the potential difficulties in ensuring valid wet weight data (variation in blood plasma content), we also tested dried blood samples. The dried blood samples were weighed (weight ranged between 0.001 and 0.031 g) and then dissolved in 1.5 mL of 65% HNO_3 in a 40 °C water bath. Original solutions were transferred into 10 ml volumetric flasks and filled up to the mark with deionized water. Afterwards, for both types of samples (blood and feathers) Electrothermal Atomization – Atomic Absorption Spectrometry (ETA-AAS) was used to determine Pb, Cd, Cu, and Cr contents (sampling volume 20 µl), and Hydride Generation–Atomic Absorption Spectrometry (HG-AAS) was used to determine As content (sampling volume 500 µl). ETA-AAS measurements were carried out using a Model ContrAA 700 high-resolution continuum source atomic absorption spectrometer (Analytic Jena AG, Jena, Germany), equipped with a transversely heated graphite tube. The ContrAA 700 with a xenon short-arc lamp was operated with a nominal power of 300 W in hot-spot mode. The analytical lines at 283.306, 228.802, 324.754 and 357.869 nm were used for Pb, Cd, Cu and Cr, respectively. The chemical modifiers $NH_4H_2PO_4$ for Pb and Cd, $Pd/Mg(NO_3)_2$ for Cu and $Mg(NO_3)_2$ for Cr were used. HG-AAS measurements were carried out using the atomic absorption spectrometer UNICAM 939 Solaar AA with deuterium

correction (Unicam, UK) with the optimized Vapor Generation Accessory VGA-76 (Varian Techtron, Australia). The spectrometer was operated using predefined optimal settings. The Varian Se hollow cathode lamp was operated at 196.0 nm with 0.5 nm spectral band passes. A lamp current of 10 mA was used. All reagents and modifiers used were of analytical reagent grade or higher purity. Working standards were prepared by diluting standard 1.000 g L^{-1} stock solutions of As^{3+}, Cd^{2+}, Cr^{3+}, Cu^{2+}, and Pb^{2+} (Merck, Germany). All measurements were carried out in triplicates (results are presented as medians with relative standard deviations). Concentrations of individual metals were recalculated from micrograms per litre to micrograms per gram of dry blood samples or feathers samples, including all dilutions. Measured concentrations in all samples were for all elements higher than appropriate limits of detections, which are summarised with other analytical figures in Table S1. Measurement procedures included blank in each series of digestions, with concentration detected being always under the limit of detection for each element. The accuracy of the methods was verified using a sample spiked with Pb, Cd, Cu, Cr and As. The recovery range for these elements was between 93.2 and 102%. Certified material (IAEA-A-13 Animal Blood, Terrestrial Environment Laboratory, Vienna, Austria) was analysed for quality control. Recovered concentrations of the certified samples were within 8% of the certified values, which is an acceptable margin.

2.6. Statistical analysis

Data distribution in the selected response variables was tested using the Shapiro-Wilk normality test. Due to the non-Gaussian distribution of some of the haematological and condition-related traits we used the Spearman's rank correlation coefficient for testing correlations. For models involving the non-Gaussian response variable stripe area we used logarithmic transformation to achieve normality. Two variables (fatness and TWBC) followed a Poisson data distribution. The values of heavy metal content (dry weight) in blood and feathers were transformed to a normal distribution by a Box-Cox transformation (Osborne, 2010). Because concentrations of heavy metals in blood were intercorrelated and the same pattern was (to lesser extent) observed in feathers (see Table S2), as well as to reduce the metal exposure variables to a single value, we performed principal component analysis (PCA, Frantz et al., 2012; Koivula et al., 2011) separately for blood and for feather samples. The main (first) components (PC1B for blood and PC1F for feathers) were used for all subsequent analyses.

We first tested the association between annual average concentration of PM$_{10}$ air pollution and PC1B and PC1F, respectively, with the locations ($n = 13$) as random factors. The PC1B and PC1F were used as response variables in Generalised Linear Mixed Models (GLMMs) and tested against annual average PM$_{10}$ concentrations at the localities (as explanatory variables) using Analysis of Covariance (ANCOVA). Given the non-normal distribution of PC1B, a logaritmic transformation was

performed (log(x + 2)). PC1F followed a normal distribution. For testing the relationship between selected health and condition-related traits (response variables: fatness, mean growth bar width, TWBC, H/L, TRBC, immature erythrocyte frequency, bacteriolytic activity of plasma complement, yellow chroma, and stripe area) and selected explanatory variables (body mass, tarsus length, age, PC1B and PC1F) we used GLMMs including locality as a variable with a random effect. Since the original data, however, showed a marked deviation of the most polluted locality (Bohumín) from other sites in the data set in the levels of environmental pollution (PM$_{10}$; Fig. 1, Table 1) as well in average blood contamination of the sampled individuals (Fig. S7, Table 2), in Supplement 1 we also show the results of analysis using untransformed heavy metal data without the random effect of locality (which blurs the effects of the outlying data points and localities). A minimum adequate model (MAM; model with all terms either significant, $p \leq 0.05$, or marginally non-significant, $p < 0.10$) was obtained through backward elimination of terms from the full model; i.e. we first fitted all the variables of interest in the full model to assess the model's residual deviance; next, based on the Akaike information criterion (AIC) we remove the least significant terms, and by using Analysis of Varinace (ANOVA) F statistics we tested whether omitting this term caused a significant increase in the deviance - if not, the term was deleted and the model was further reduced in the same way until the model (MAM) only contained significant terms. Candidate models were compared based on the change in deviance with the accompanied change in degrees of freedom (ANOVA) using F statistics. The statistical analysis was performed using R software v. 3.1.3 (R Core Team, 2016).

3. Results

3.1. PM$_{10}$ air pollution at the sampling sites and blood and feather metal concentrations in the examined birds

For all blood and feather samples analysed in this study ($N = 57$), the concentrations of all selected heavy metals (As, Cu, Cd, Pb and Cr) were above the detection limit of the method adopted (summary statistics for individual locations are listed in Tables 2 and 3). In blood, the metal concentrations followed the order Cu > Pb > Cd > Cr > As, while in feather samples it was Cu > Pb > Cr > As > Cd. Blood and feather metal concentrations were mostly uncorrelated (the only three significant correlations detected out of the 25 pairs tested were found between Pb in blood and feathers, and Cd in feathers and Cu in blood and Cr in blood, but all with R^2 < 0.1; Table S2 in Supplement 1). In contrast, concentrations of individual heavy metals within blood (10 out of 10 correlations significant with $r \geq 0.40$, R^2 on average = 0.35) and within feather samples (4 out of 10 correlations significant with $r >$ 0.30, R^2 on average = 0.19) were intercorrelated to each other (see Table S2 in Supplement 1). Therefore, we performed the PCA analysis, separately for blood and feathers (Table S3 in Supplement 1). The

Table 2
Average values of heavy metal concentrations (μg/g dry weight) in blood per locality ($n_{locality} = 13$) in great tits from the Czech Republic, 2010 ($N = 57$). SD = standard deviation, n = number of birds captured per locality. Values for the most polluted locality (based on PM$_{10}$) are highlighted in bold.

Locality	n	Pb Mean ± SD	Cd Mean ± SD	Cu Mean ± SD	Cr Mean ± SD	As Mean ± SD
Benešov	3	6.42 ± 3.95	0.63 ± 0.22	19.07 ± 10.93	0.91 ± 0.52	0.11 ± 0.02
Bohumín	**5**	**7.67 ± 4.33**	**1.96 ± 1.69**	**61.81 ± 36.11**	**2.28 ± 1.30**	**0.63 ± 0.58**
Brno	5	3.74 ± 2.85	0.36 ± 0.09	15.40 ± 11.91	0.33 ± 0.16	0.09 ± 0.03
Hradec Králové	5	5.41 ± 7.25	0.56 ± 0.24	15.87 ± 13.68	1.09 ± 0.82	0.17 ± 0.17
Jihlava	6	4.12 ± 3.48	0.58 ± 0.42	22.15 ± 17.58	0.44 ± 0.39	0.25 ± 0.18
Karviná	5	1.79 ± 0.92	0.93 ± 0.67	29.73 ± 18.81	0.58 ± 0.34	0.13 ± 0.09
Olomouc	1	2.04	0.80	27.02	0.42	0.20
Ostrava	2	5.09 ± 6.99	0.54 ± 0.22	23.01 ± 21.34	1.21 ± 0.87	0.13 ± 0.07
Plzeň	5	5.72 ± 3.14	0.48 ± 0.13	17.96 ± 12.82	0.95 ± 0.46	0.17 ± 0.18
Praha	5	4.76 ± 1.08	0.54 ± 0.16	10.20 ± 4.81	0.33 ± 0.21	0.11 ± 0.06
Příbram	5	2.68 ± 1.06	0.57 ± 0.24	16.90 ± 4.44	0.73 ± 0.45	0.14 ± 0.08
Kladno	6	5.35 ± 3.70	0.47 ± 0.16	7.91 ± 9.00	0.40 ± 0.40	0.09 ± 0.02
Znojmo	4	4.04 ± 2.16	0.68 ± 0.34	23.70 ± 11.34	0.36 ± 0.06	0.15 ± 0.04

Table 3
Average values of heavy metal concentrations (μg/g dry weight) in feathers per location ($n_{location} = 13$) in great tits from the Czech Republic, 2010 ($N = 57$). SD = standard deviation, n = number of birds captured per location. Values for the most polluted locality (based on PM_{10}) are highlighted in bold.

Location	n	Pb Mean ± SD	Cd Mean ± SD	Cu Mean ± SD	Cr Mean ± SD	As Mean ± SD
Benešov	3	6.60 ± 0.70	0.26 ± 0.06	40.04 ± 18.34	6.63 ± 4.46	1.51 ± 0.09
Bohumín	**5**	**7.11 ± 2.38**	**0.51 ± 0.07**	**24.17 ± 11.15**	**6.59 ± 4.72**	**1.25 ± 0.29**
Brno	5	6.07 ± 7.33	0.04 ± 0.03	24.13 ± 19.13	3.07 ± 1.09	0.91 ± 0.48
Hradec Králové	5	10.48 ± 7.94	0.42 ± 0.37	12.92 ± 2.86	3.89 ± 1.11	0.69 ± 0.38
Jihlava	6	4.58 ± 2.77	0.36 ± 0.27	8.57 ± 3.75	3.78 ± 1.36	0.77 ± 0.19
Karviná	5	8.71 ± 1.75	0.71 ± 0.78	10.95 ± 3.18	3.65 ± 2.04	1.19 ± 0.24
Olomouc	1	3.62	0.14	6.02	2.26	2.07
Ostrava	2	15.90 ± 2.73	0.50 ± 0.20	11.25 ± 7.73	2.80 ± 1.17	1.23 ± 0.04
Plzeň	5	3.85 ± 1.80	0.15 ± 0.10	9.43 ± 1.60	3.81 ± 1.53	1.36 ± 0.71
Praha	5	3.10 ± 1.72	0.20 ± 0.19	39.01 ± 47.01	4.48 ± 3.47	0.91 ± 0.21
Příbram	5	26.21 ± 21.91	0.59 ± 0.18	31.36 ± 11.55	6.42 ± 5.09	1.16 ± 0.39
Kladno	6	5.13 ± 8.23	0.07 ± 0.08	19.79 ± 29.60	3.93 ± 2.96	1.30 ± 0.32
Znojmo	4	6.22 ± 5.18	0.14 ± 0.05	8.50 ± 2.45	3.41 ± 3.48	1.07 ± 0.36

proportion of variation explained by PC1 and PC2 were 67.6% and 13.1% (blood) and 42.8% and 22.0% (feathers). Factor coordinates were all positive for PC1 and for blood ranged between 0.658 (Pb) to 0.914 (Cd; see Fig. S1) and for feathers 0.321 (As) to 0.794 (Pb; see Fig. S2). We did not find any relationship between PC1B or PC1F and annual average PM_{10} at the study sites (model 1: $p = 0.160$, model 2: $p = 0.684$; Table S4 in Supplement 1). Despite this lack of any clear general relationships, we found that the site with the highest PM_{10} (Bohumín) was also the site with the highest blood contamination levels in all individually analysed heavy metals examined in the great tits (Fig. S7; Tables 1 and 2; this trend was not detected for feathers, see Fig. S8; Table 3).

3.2. Effect of heavy metal contamination on condition-related traits

In our dataset the condition-related traits were only moderately intercorrelated, with two of 15 relationships significant: growth bar and tarsus length, and fatness and body mass (in both cases $R^2 > 0.15$; see Table S7 in Supplement 1). We did not find any significant associations between PC1B or PC1F and fatness or mean growth bar width ($p > 0.10$; see MAM 3 and MAM 4 in Table 4).

3.3. Effect of heavy metal contamination on haematological health-related traits

Summary statistics for all haematological health-related traits analysed are given in Table S8 in Supplement 1. Since haematocrit was significantly intercorrelated with TRBC ($p = 0.015$, $R^2 = 0.102$; Table S9 in Supplement 1), we retained only TRBC in further analyses (in all other pairs of haematological traits $p > 0.05$). No effect of PC1B or PC1F on TWBC was detected (MAM 5 in Table 4). In contrast to TWBC, the leukocyte differential count analysis revealed that the H/L

ratio is significantly related to both PC1B and PC1F (in both cases $p < 0.001$; note the contrasting slopes, Figs. 2, 3; MAM 6 in Table 4). Similar results were also obtained using analysis based on untransformed heavy metal data without the random effect of locality (Table S6, Figs. S3 and S4 in Supplement 1). Separate analyses including Pb and As instead of PC1B (Table S10) suggest a relationship between heterophil counts, and H/L and As contamination (Figs. S9 and S10). Furthermore, our results showed a significant decrease in TRBC with increasing PC1B ($p = 0.002$, MAM 7 in Table 4, Fig. 4). Again, analogous results were obtained using analysis based on untransformed heavy metal data without the random effect of locality (Table S6 and Fig. S5 in Supplement 1) and Pb data (Table S10; Fig. S11). This analysis (taking into account the effects of the outlying locality - Bohumín) also shows a link between increased PC1B and an increase in immature erythrocyte count ($p = 0.036$, MAM 8 in Table S6, Fig. S6), which, in contrast, remained undetected using the transformed heavy metal data in GLMM with locality as a random variable (MAM 8 in Table 4). We did not find any significant effect of PC1B or PC1F on plasma complement activity by any statistical approach adopted ($N = 22$, model 9 in Tables S4 and S5 in Supplement 1). For the blood parameters tested, body size (body mass or tarsus length), and the age of the birds were also important explanatory variables (Table 4).

3.4. Effect of heavy metal contamination on ornamental traits

The two ornamental traits analysed in this study (stripe area and yellow chroma) were not inter-correlated ($p > 0.70$; see Table S7 in Supplement 1). Neither of these two ornamental traits were associated with PC1B or PC1F (yellow chroma $p > 0.10$, model 10, Tables S4 and S5 in Supplement 1; stripe area $p > 0.10$, MAM 11 in Table 4 and Table S6).

Table 4
The Minimum adequate models (MAMs) based on great tit data from the Czech Republic, 2010 ($N = 57$). Locality used as a variable with random effect. Slope ± standard error (SE) values are only provided for individual continuous variables. Significant effects of heavy metal contamination are highlighted in bold.

|	Slope ± SE	F	Df	p
MAM 3 Fatness ~ body mass	6.727 ± 3.042	4.891	1/55	0.028
MAM 4 Growth bar width ~ tarsus length	0.063 ± 0.031	4.483	1/55	0.044
MAM 5 TWBC ~ body mass	4.068 ± 2.091	5.171	1/55	0.050
MAM 6 H/L ~ PC1B + PC1F + age + body mass		72.561	4/56	≪0.001
PC1B	−0.080 ± 0.019	5.553	1/52	**<0.001**
PC1F	0.112 ± 0.025	20.801	1/52	**<0.001**
Age		23.732	1/52	≪0.001
Body mass	−4.483 ± 1.018	19.424	1/52	<0.001
MAM 7 TRBC ~ PC1B + age		13.120	2/56	0.001
PC1B	−0.139 ± 0.043	10.525	1/54	**0.002**
Age		3.577	1/54	0.078
MAM 8 Immature erythrocyte count ~ tarsus length	−0.959 ± 0.528	3.298	1/55	0.069
MAM 11 Stripe-area ~ tarsus length	0.024 ± 0.013	3.536	1/55	0.063
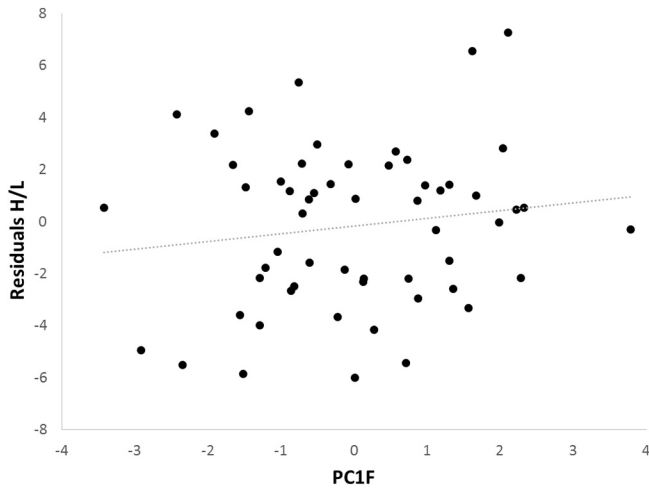
Fig. 2. Association between feather heavy metal contamination (PC1F) and heterophil/lymphocyte (H/L) ratio in urban great tits from the Czech Republic, 2010 ($N = 57$). On the Y axis, H/L ratio is shown as residuals from the MAM 6 (see in Table 4) excluding PC1F (X axis). Location used as a variable with random effect. $R^2 = 0.020$, $p < 0.001$.

Fig. 4. Association between blood heavy metal contamination (PC1B) and total erythrocyte count (TRBC; cells $\times 10^6$/µl) in urban great tits from the Czech Republic, 2010 ($N = 57$). On the Y axis, TRBC is shown as residuals from MAM 7 (see Table 4) excluding PC1B (X axis). Location used as a variable with random effect. $R^2 = 0.118$, $p = 0.002$.

4. Discussion

Although birds may serve as useful bioindicators of heavy metal pollution in anthropogenic environments, studies investigating large-scale geographic variation in heavy metal contamination of avian tissues are rare, and have so far focused only on eggshells (Ruuskanen et al., 2014). We performed a large-scale study examining associations between body condition and health-related physiological traits in great tits inhabiting urban areas with different levels of air pollution. To a certain extent, the blood and feather samples from all sites were contaminated with heavy metals, mainly with Cu and Pb (Cu being the only essential element of the metals analysed; Walker et al., 2012), but also with As, Cd and Cr in small amounts. Despite the generally low effects, the H/L ratio was significantly increased in birds suffering from higher feather heavy metal contamination (tested based on PC1F data), indicating their increased long-term stress. In contrast, increased blood heavy metal contamination (tested based on PC1B data) was significantly associated with a decreased H/L ratio, suggesting decreased heterophil counts in pollution-affected birds. In these birds, erythrocyte levels were also decreased and immature erythrocyte frequencies showed a tendency to be increased at the most polluted site (Bohumín),
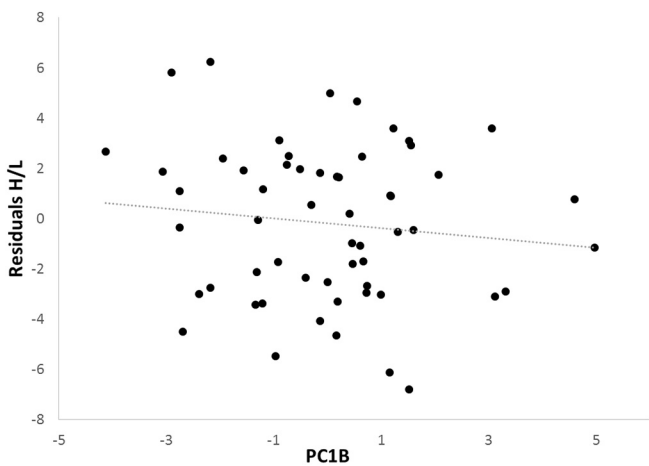

Fig. 3. Association between blood heavy metal contamination (PC1B) and heterophil/lymphocyte (H/L) ratio in urban great tits from the Czech Republic, 2010 ($N = 57$). On the Y axis, H/L ratio is shown as residuals from the MAM 6 (see in Table 4) excluding PC1B (X axis). Location used as a variable with random effect. $R^2 = 0.014$, $p < 0.001$.
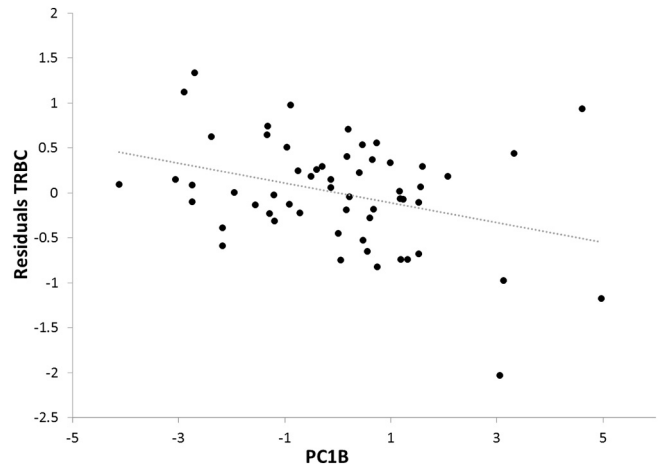
indicating anaemia-like haemolytic conditions and accelerated haematopoiesis. In contrast, we did not find any significant association between nutritional condition (fatness and mean growth bar width) and heavy metal contamination in blood or feathers. Similarly, the two ornamental traits analysed (stripe area and yellow chroma) were unrelated to heavy metal contamination.

In this study the subset of toxic heavy metals analysed (Pb, Cd, Cu, Cr, As) was similar to that in previous related studies (see e.g. Dauwe et al., 2002b; Eens et al., 1999). In general, the levels of heavy metal contamination were comparable to other studies on birds (Geens et al., 2010 on great tits; Meillere et al., 2016 on common blackbirds; Frantz et al., 2012 on pigeons Columba livia), although the dry weight values measured in this study are for methodological differences difficult to directly compare (the main aim of this study was to compare data from different localities sampled within this study). By far the highest levels of blood contamination within all heavy metals tested were detected in the samples from Bohumín, which was also the site where dust (PM_{10}) air pollution was the highest. The Bohumín site, located in the north-eastern part of the Czech Republic is a long-term strongly-polluted industrial locality (several iron works, steel works and metallurgical plants are located in the vicinity of the sampling site; CHMI, 2010). Similarly to Dauwe et al. (2005) and Scheifler et al. (2006), for most heavy metals we did not find any relationship between heavy metal content in blood and feathers. This difference may partially arise due to distinct contamination timing (virtually in all birds rectrices are changed during the post-breeding moult, Jenni and Winkler, 1994, while blood levels show the actual state at the time of sampling). However, the effect of exogenous contamination may be equally important. Although the feather tissue dies after the completion of feather growth (i.e. unable to actively accumulate heavy metals, apparently suggesting that the feathers contain information on heavy metal concentrations circulating in the blood at the time of moulting), heavy metal levels have been shown to further increase, possibly due to the deposition of dust and/or excretion of the uropygial gland (Jaspers et al., 2004; Scheifler et al., 2006). Therefore, heavy metal content in feathers reflects both endogenous and exogenous deposition, and blood and feather heavy metal concentrations may be considered distinct traits indicating different contamination features. While blood is a transport medium likely showing actual levels of heavy metal contamination, heavy metal concentrations in feathers reflect their general intra-annual accumulation since moulting (Dauwe et al., 2002b). In contrast to the lack of consistency between tissues, individual heavy metals within blood and within feathers were highly positively intercorrelated, suggesting that different heavy

metals were accumulated in both tissues in a similar way. It has been repeatedly shown that biolevels of various heavy metals tend to positively correlate one with each other (e.g. Eens et al., 1999; Frantz et al., 2012).

Our results demonstrate associations between the heavy metal load and health-related physiological traits in free-living birds in urban areas. In particular, it appears that cumulative heavy metal contamination (PC1F in our study) may increase long-term stress (indicated by increased blood H/L) in birds. This relationship was earlier reported by Eeva et al. (2005) in pied flycatchers (*Ficedula hypoleuca*), where H/L ratios of nestlings were higher in a copper-polluted area than in a control area. The authors also observed a strong negative association between the H/L ratios of nestlings and their fledging success, indicating a survival effect resulting from heavy metal pollution. A similar effect of pollution on stress in urban birds has been reported by Meillere et al. (2016) in common blackbirds, where corticosterone levels in both juvenile and adult feathers positively correlated with Cd and Pb contamination of the feathers.

In contrast to feather contamination, blood heavy metal content was significantly negatively associated with the H/L ratio. Again, this was mainly due to the effect of the most contaminated samples. This result suggests differences between long-term and short-term exposure effects. Although non-migratory, great tits are vagrant (birds breeding in the Czech Republic may move as far as to central France or central Italy during their post-breeding period, Cepák et al., 2008). Therefore, different levels of heavy metals may be accumulated by individual birds during the breeding and non-breeding seasons. We suggest that the actual levels of pollution (indicated by blood contamination with heavy metals) may decrease the numbers of heterophils in blood circulation, also decreasing H/L, which might be caused by increased heterophil mortality. This has been previously observed in fish (Palikova et al., 2015) in response to low-level As intoxication. Also in our study the association between H/L and heavy metals in blood appears to be driven by As (Fig. S9) and there is a tendency for total heterophil count to decrease in response to increasing As contamination (Fig. S10). Since heterophils are among those cells with fast turnover in the blood (Tak et al., 2013), they may suffer more than the longer-lived lymphocytes from short-term fluctuations in heavy-metal-induced toxicity. Given the low statistical effects of the associations (despite the statistical significance of the trends), both relationships (increased H/L due to long-term stress and decreased H/L due to actual pollution) may co-occur in urban tit populations.

The indicated negative effects of heavy metals on avian health are further supported by the anaemia-like symptoms we detected in birds suffering from high blood heavy metal content. In these birds the total erythrocyte levels were decreased in blood circulation, while there was also a tendency for increased frequencies of immature erythrocytes in the most contaminated individuals (see Fig. S6). Previous studies have documented that the concentration of heavy metals in blood is mostly associated with their content in erythrocytes (Coeurdassier et al., 2012). It has been shown in wintering great tits that blood Pb contamination is negatively associated with blood haemoglobin concentrations, haematocrit, mean corpuscular volume and mean corpuscular haemoglobin (Geens et al., 2010). Although Geens et al. (2010) did not observe any association directly with the total red blood cell count, in this study we found a significant negative relationship between heavy metal levels (mainly Pb, Fig. S11) and the absolute erythrocyte count, likely resulting from increased heavy-metal-induced erythrocyte mortality in birds with high blood contamination levels. This heavy metal-associated decrease in erythrocyte levels may be linked to the increased frequency of immature erythrocytes in the most contaminated individuals. An increased frequency of immature erythrocytes in avian blood circulation has been suggested to reflect increased haematopoiesis (Vinkler et al., 2010), which is frequently associated with anaemia induced by environmental pollution (Belskii et al.,

2005; Yamato et al., 1996). The large variation observed in the associations between feather/blood heavy metal contamination and haematological parameters suggests that other important factors (not tested in this study) are involved, and indirect environmental impacts of pollution (e.g. lower quality of available food sources) may also explain the relationships revealed (Eeva et al., 1998).

In contrast to other studies (Dauwe and Eens, 2008; Eeva et al., 1998; Geens et al., 2009) we did not find any association between heavy metal pollution and ornamental trait expression (yellow chroma or breast stripe area). Given their role in oxidative balance maintenance (Tomášek et al., 2016), carotenoids in particular were predicted to show a trade-off between ornamentation and prevention against the negative effects of urban oxidative challenges. Heavy metal pollution has been reported to reduce carotenoid-based colouration in great tits (Geens et al., 2009; Eeva et al., 1998), but only in some studies (Koivula et al., 2011). The absence of any such relationship in our study may result from the fact that we did not focus on any particular effect of a single major source of heavy metal pollution, but rather investigated heavy metal contamination across cities with no extreme levels of pollution. This inconsistency in results suggests that the effects of heavy metal contamination on external phenotypic traits (such as ornamentation) is not general, but remains limited only to the most extreme cases of environmental pollution.

Taken altogether, this correlational study contributes to an understanding of the relationships between environmental pollution and the biology of organisms living in anthropogenic environments. We show that despite general improvements in the level of atmospheric contamination, non-degradable heavy metals persistently contaminate animal tissues in urban areas at levels having subclinical yet physiological effects, namely on blood cell levels. This supports other findings showing impaired physiological performance in urban birds (Bailly et al., 2016). We believe that these results highlight the necessity for ongoing environmental concern in urban areas even today.

Ethical approval

This research was approved by the Ethical Committee of the Institute of Vertebrate Biology, Czech Academy of Sciences (Permit No. 107/2009) as part of project P505/10/1871 and was conducted in accordance with the current laws of the Czech Republic and the EU.

Conflicts of interest

The authors declare they have no actual or potential competing financial interests.

follows: PB (30%) – haematological and ptilochronological analyses, statistical analysis and manuscript preparation, JV (10%) – field data collection, JH (10%) – heavy metal analysis, VČ (10%) – field data collection, LV (5%) – complement activity assay, JS (5%) – statistic leadership, MV (30%) – study design, field data collection and manuscript preparation. All authors contributed by their comments to the manuscript preparation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at 10.1016/j.scitotenv.2017.05.276. These data include Supplement 1 containing additional Tables and Figures to the article and Supplement 2 with a Google map showing the geographical distribution of the study sites.

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