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Multi-Element Analysis of Blood Samples in a Passerine Species: Excesses and Deficiencies of Trace Elements in an Urbanization Study

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Urbanization is a growing phenomenon characterized by a complete restructuring of natural areas. In urban bird populations, a reduced offspring survival and body condition and an overall lower breeding success are often observed compared to populations inhabiting more natural habitats. Higher pollution levels and poorer quality of natural resources in cities are two environmental factors frequently mentioned in the literature to explain the differences between urban and non-urban populations. Pollution and poor quality of food may lead to an excess of non-essential elements such as Pb or Cd or to deficiencies of essential elements such as Cu or Zn, which may explain some of the impacts, notably on immunity, observed in urbanization studies. The present study compared the breeding parameters, brood body mass and condition, and haptoglobin levels, a marker of inflammatory immunity, in two urban and two forest populations of Great tits in Eastern France, together with a multi-element analysis (25 non-essential and essential trace elements) of blood samples from 13-day-old nestlings from the four populations. The concentration of NO_2, a gaseous pollutant typical of urban pollution, was also measured. The NO_2 concentrations were significantly higher in the urban areas, but no association with biological variables was detected. Non-essential metals were undetectable in the plasma of the birds from both habitats, except Pb, whose concentrations, however, did not differ between the urban and forest birds. A positive relationship was found between the plasmatic richness in essential elements (as assessed from the coordinates of the first axis of a PCA including 12 elements) and the average brood body mass and condition. We suggest that lower quality resources or/and a higher metabolic demand may be a causal mechanism for the reduced body condition often observed in urban bird nestlings. Finally, our exploratory study could promote more mechanistic experiments (e.g., supplementation) to explain the negative effect of urban conditions on bird populations.

Keywords: urban ecology, pollution, *Parus major*, body condition, NO_2

INTRODUCTION

Urbanization is increasing, and 54% of the world population now lives in urban areas (United Nations, 2014). Urban habitats have been colonized by many wild species, including birds (McKinney and Lockwood, 1999), which can reach high densities (Chace and Walsh, 2006). Many studies have suggested that urban conditions exert strong constraints on bird fitness (Chamberlain et al., 2009; Vaugoyeau et al., 2016). A number of breeding parameters are affected, with the most consistent patterns being earlier laying dates, lower clutch size, lower nestling weight or condition, and an overall lower productivity per nesting attempt in urban areas (Chamberlain et al., 2009) even if inter specific variations between those life history traits and the intensity of urbanization have recently been demonstrated (Vaugoyeau et al., 2016). Other individual parameters can be modified in urban bird populations compared to rural ones. For example, urban birds are often—but not always—smaller and in poorer condition (Liker et al., 2008; Evans et al., 2009a) and show higher oxidative stress and antioxidant activity than birds from more natural habitats (Isaksson et al., 2005).

Urban and natural environments differ strongly in many factors (Grimm et al., 2008). Temperature, predator and parasite communities, artificial noise and light, availability and/or quality of resources, and pollution are among important features that contrast between cities and natural habitat (Collier, 2006; Evans et al., 2009b; Slabbekoorn, 2013). Cities are frequently described as resource-poor habitats for birds during the breeding season, and several works have shown that prey required for chicks are less abundant, smaller and poorer in key nutrients for growth and survival, such as carotenoids (Isaksson and Andersson, 2007; Seress et al., 2012). Those descriptive studies provided some data that allowed the design of experimental supplementation aimed at testing whether the quantity (energy intake) or the quality of resources (e.g., carotenoids) could explain the contrast of breeding performance between urban and non-urban populations (Peach et al., 2014; Giraudeau et al., 2015). Several other key resources, such as essential elements, could be involved in the differences of growth, body condition, and/or breeding performance between rural and urban nestlings. For instance, calcium (Ca) is an essential micronutrient during egg formation and skeletal development of nestlings (Patten, 2007; Reynolds and Perrins, 2010). Supplementation experiments in wild populations of Great tits (*Parus major*) reported that Ca-supplemented birds produced larger brood and raised more nestlings of better body condition compared to control groups (Tilgar and Reynolds, 2005; Espín et al., 2016a). Other essential elements such as zinc (Zn) or copper (Cu) are also of great interest in the bird physiology and behavior. They affect enzymatic activity and antioxidant defenses (Powell, 2000; Sahin et al., 2005) and play an important role in embryo development and nestling growth (Park et al., 2004). Their concentration can exceptionally raise toxicity thresholds (Sundaresan et al., 2008), but, more likely for vertebrates that are very tolerant to these elements, they may impact physiological performances if their concentrations do not meet homeostatic requirements (deficiencies) (Keen et al., 1998). Despite the crucial role of essential elements in nestling growth, and in bird physiology more generally, very few studies have focused on the potential deficiencies in these elements in urbanization studies. Therefore, quantifying these elements in birds might allow the discrimination of urban and rural populations and the identification of deficiencies in urban individuals.

Similarly, the impact of pollution has received surprisingly little attention in urbanization studies, although cities are characterized by the circulation of many different pollutants in air, water, and soils. The air pollutants that draw the most concerns for ecosystem and human health include particulate matter (PM), ozone (O_3), carbon monoxide (CO), sulfur dioxide (SO_2), nitrogen dioxide (NO_2), lead (Pb), volatile organic compounds (VOC), and polycyclic aromatic hydrocarbons (PAHs) (Mayer, 1999; Berback et al., 2001; Marr et al., 2004). Very few prior studies were dedicated to the effects of air pollutants on birds in the context of urbanization, while studies in (generally heavily) industrially contaminated sites are numerous. To our knowledge, only one study found that reduced reproductive success of House sparrows (*Passer domesticus*) along an urban gradient was associated with air pollution from traffic as assessed by NO_2 concentrations in the air (Peach et al., 2008). Studies dealing with pollution by non-essential metal elements are more numerous, but most of them only report biomonitoring data. For instance, studies found higher concentrations of Pb in urban birds than in their rural counterparts with individuals exhibiting blood concentrations higher than the benchmark value related to subclinical and physiological effects in birds (Scheifler et al., 2006; Roux and Marra, 2007). Clearly, descriptive studies assessing elements in rural and urban birds may provide data about possible excesses of non-essential and potentially toxic elements such as Pb, arsenic (As), cadmium (Cd), or mercury (Hg) in urban birds compared to rural individuals, and may facilitate the design of more mechanistic experiments about the impacts of urbanization on birds.

Pollutants may interact with the immune system depending on the nature of the contamination (element or molecule involved, chronic vs. acute, high vs. low dose) with consequences on host immune profile. Components of inflammatory response showed both down-regulated or up-regulated activities in vertebrates exposed to contaminants (Tersago et al., 2004; Schiraldi and Monestier, 2009) with expected consequences on host-parasite interactions and host fitness (Sorci et al., 2013). However, how urban pollution may affect host immunity has been neglected, and the assessment of markers such as inflammatory markers can provide information on immune status of urban individuals. Recently, we observed contrasts in the production of haptoglobin, an acute protein associated to the inflammatory process, between urban and forest nestlings (Bailly et al., 2016a). Therefore, it could be of interest to explore how this inflammatory marker is affected by urban pollution.

In this study, we measured the concentrations of 25 non-essential and essential trace elements (TEs) in nestling plasma together with the breeding parameters, nestling body condition, and the baseline plasma levels of an inflammatory marker (haptoglobin) in Great tits from two medium-sized cities of Eastern France (Besançon and Dijon) and two forests from the surroundings of each city (Forêt de Chaux and Forêt d'Auxonne, respectively). The atmospheric concentrations of NO_2, one of the main traffic-related pollutants and precursor forming photochemical smog (Han and Naeher, 2006), were also measured with passive air samplers in the studied areas. We hypothesized (i) contrasts between urban and rural populations in circulating TEs (with lower levels of essential elements and higher levels of non-essential elements in urban birds), breeding parameters (clutch size, hatching success, nestling survival, brood body mass and condition) and immunological marker (baseline level of circulating haptoglobin) with harsher conditions for urban birds, and (ii) correlations suggesting negative effects of non-essential TEs and positive effects of essential TEs. Comparison with rural organisms may bring crucial insights about the mechanisms underlying the urbanization process and open the route for more mechanistic and experimental approaches to test them.

MATERIALS AND METHODS

Study Sites

Two cities (Besançon, 47°25 N, 6°03 E; Dijon, 47°32 N, 5°02 E) and two forests (Forêt de Chaux, 47°09 N, 5°68 E; Forêt d'Auxonne, 47°10 N, 5°26 E) were studied in two regions of Eastern France, Franche-Comté, and Burgundy. Besançon and Dijon are two middle-sized cities (176,764 and 244,577 inhabitants in 2008, respectively, and 432.3 and 219.3 km^2, respectively) whose economies are dominated by tertiary activities (http://www.insee.fr). None of those cities have important industrial sources of metals like metallurgic plants for instance. The Forêt d'Auxonne and the Forêt de Chaux are deciduous forests of 7,800 and 20,493 ha, respectively. The most abundant tree species are oaks (*Quercus petrae* and *Q. robur*), beechwood (*Fagus sylvatica*) and charm (*Carpinus* sp.). Nest boxes were installed in medium- (50 years old) to old- (100 years old) growth stands at least 1 km from the edge of the forests. Each site received approximatively 150 artificial nestboxes (Nest box 1B with protective front panel, 12 cm diameter and 20 cm height, Schwegler, Germany). The nest boxes were hung from 0.5 m long steel tubes to protect the nests from predation. The nest boxes in the cities were dispersed in parks, squares, and along streets, with each patch containing one to 25 nest boxes separated by at least 50 m from each other. The vegetation of most of the area of the parks has been formed artificially. The tree cover is patchy with tree-free areas mainly covered by grass. The tree patches are composed of deciduous and coniferous species of different stands, including both native and exotic species. Each study site covers an average area measuring 5.6 km long and 2.8 km wide. In the forests, the nest boxes were placed along pathways to facilitate access, with the same minimal distance between two nest boxes as in the cities.

Reproductive Parameters and Blood Sampling

The reproduction of the Great tits was monitored from March to June 2013. Only the first breeding attempts were included in the study, second broods were not studied due to logistical constraints. The nests were inspected once a week from March 20th to record the laying date of the first egg. When more than one egg was found in the nest, the laying date of the first egg was back calculated, assuming that one egg was laid per day. Incubation was assumed to begin 1 day before clutch completion, and we did not observe asynchronous hatching (except sometimes for one egg). The nests were not visited again until the estimated hatching date (13 days after the beginning of incubation), and any eggs that had not hatched were then checked every 2 days until hatching. The number of nestlings was noted on day 1 (D1), 7 (D7), and 13 (D13) post-hatching. The body mass and tarsus length of the 13-day-old nestlings were measured using a Pesola spring balance (±0.05 g), and an electronic caliper (±0.1 mm). We defined clutch size as the number of eggs laid and hatching success was the proportion of eggs that hatched in the clutches where at least one egg hatched. We defined nestling survival as the proportion of hatched nestlings that were still alive on D13 in nests where at least one nestling survived until D13. When nestlings were 7 days old, they were fitted with individually numbered metal leg rings (Museum National d'Histoire Naturelle, CRBPO, Paris, France). Blood was sampled (50–100 μl) from one brachial vein of 13-day-old nestlings using sterile needles and heparinized capillary tubes. The blood samples were stored in a cool box until their return to the laboratory within 4 h of collection. Then, the blood was centrifuged (10 min, 4,000 rpm, 4°C), and the plasma was separated from the pellet and stored at −80°C until analysis.

Haptoglobin Assay

Haptoglobin (Hp) belongs to the category of acute phase proteins and is used as a marker to measure the intensity of the inflammatory response in vertebrates, including birds (Matson et al., 2006, 2012). The production of this protein by the liver is stimulated by activated inflammatory cytokines (Dobryszycka, 1997), so as the amount of plasma Hp increases, the inflammatory reaction becomes stronger. Here, we quantified the baseline level of this protein to assess the inflammatory status of the nestlings. Hp was measured in the plasma of 13-day-old nestlings using a commercial assay kit (TP-801, Tridelta Development LTD, Ireland). First, 7.5 μl of each sample were randomly aliquoted on a flat-bottomed, 96-well plate. Samples were distributed randomly among the plates. Standard curves were included (in duplicate) on each plate ($n = 16$) containing the plasma samples, and the curves consisted of dilutions of an initial standard of 2.5 mg/ml diluted five times to 0 mg/ml. One hundred microliters of a stock solution of hemoglobin (catalyser) and 140 μl of a stock solution of chromogen (coloration) were added to all wells. The plates were agitated to ensure the mixing of the samples with hemoglobin and chromogen and left to incubate for 5 min at room temperature. The absorbance of each well was read at 630 nm, and the intra-assay and inter-plate variations were, respectively, 2.4 and 2.99%. The samples above the detection threshold were diluted and assayed again.

NO_2 Measurements

Passive NO_2 samplers were put on 6–58 nest boxes in the four study sites during the nestling stage (6 and 7 in the two forests, 38 in Dijon, and 58 in Besançon). Not all nestboxes were equipped for both financial and logistical reasons. The samplers were set up vertically on the middle of the tube from which the nest boxes were hung, allowing air to circulate freely around them. Passive samplers (Palmes et al., 1977) are calibrated tubes, 7 cm long, with an inside diameter of 1 cm, in which gases move only by molecular diffusion (Gradko International, Winchester, Great Britain). A triethanolamine solution, which was deposited on the grid at one end of the tube, fixed the NO_2. The other end of the tube remained open for gas diffusion during the sampling period. Each passive sampler allowed a measurement of the NO_2 pollution level over a period of 14 days, optimal duration of sampling (Bernard et al., 1997). At 21.1°C and at a pressure of 1 atmosphere, the diffusion coefficient for NO_2 is 0.154 cm^2/s, which means that the collection rate for NO_2 passive samplers could be calculated at 72 cm^3/h. Mean hourly concentration of NO_2 (expressed in $\mu g/m^3$; 1 ppb = 1.91 μg/m3 at 21.1°C) in the air sample was calculated on the basis of the amount of pollutant collected, exposure time, and gas collection rate in the passive sampler. Absorbed NO_2 was measured by spectrophotometry using a variant of the Griess-Saltzman method (Atkins, 1990). In an earlier study, NO_2 measurements with passive samplers were validated on chemiluminescence analysers, equipment used by the French network of air quality monitoring and advocated by European legislation (Bernard et al., 1998).

Multi-Element Analysis

The concentrations of 25 TEs [aluminum (Al), antimony (Sb), As, Cd, Ca, chromium (Cr), cobalt (Co), Cu, tin (Sn), iron (Fe), magnesium (Mg), manganese (Mn), Hg, molybdenum (Mo), phosphorus (P), Pb, potassium (K), nickel (Ni), selenium (Se), silicon (Si), sodium (Na), strontium (Sr), titanium (Ti), thallium (Tl), and Zn] were determined in 93 composite (one sample per family) plasma samples. Four samples had volumes lower than 50 μl and could not been analyzed. Eight samples had a volume ranging from 55 to 85 μl. For the other samples, 100 μl were used. The samples were acid digested in 700 μl of nitric acid (HNO_3, 65%, analytical quality, Optima) for half a day in open tubes under hood, and then for 60 h in closed tubes at 60°C in an oven. After digestion, samples were diluted to 30 ml by the addition of ultra-pure water (18.2 $M\Omega/cm^2$). Blanks (acid + ultra-pure water) and Certified Reference Materials (CRMs; lobster hepatopancreas, TORT-2, National Research Council Canada, and drinking water, ERM®-CA011b, European Reference Materials) were prepared and analyzed using the same methods as the samples. Elements were analyzed by Inductively Coupled Plasma—Atomic Emission Spectrometry (ICP-AES, ThermoFisher Scientific iCAP 6,000) or by Inductively Coupled Plasma—Mass Spectrometry (ICP-MS, ThermoFischer Scientific XSeries 2). Over the 25 elements measured, only 12 had a satisfactory proportion of values above quantification limits (Table S1), allowing for statistical analyses. For the values under quantification limits for these 12 elements, the value was replaced by the quantification limit divided by square root of two for statistical analysis (Helsel, 2010). Average recoveries of the CRMs ranged from 73 (for Fe) to 135% (for Pb) for the TORT-2, and from 78 (Fe) to 124% (for Se) for the ERM®-CA011b (Table S1). The concentrations of the elements in the plasma are expressed as μg/dl.

Statistical Analysis

Levels of atmospheric NO_2, plasmatic TEs concentrations, and biological parameters were compared among the four study sites using the non-parametric Kruskal–Wallis test because the distribution of most of the data were skewed. When a significant effect was detected, *post-hoc* Tukey HSD tests were applied to detect differences among sites. Urban vs. forest differences were analyzed using Wilcoxon Mann–Whitney test. All NO_2 data were used to compare NO_2 levels among the four study sites while only the data related to nestboxes hosting successful reproduction were used to study the relationships between NO_2 levels and biological parameters. Moreover, because NO_2 concentrations in the forests were, as expected, very low and exhibited low variations, only data from the two cities were analyzed to test the relationships between the NO_2 concentrations and biological parameters (clutch size, hatching success, nestling survival, brood body mass and condition, baseline level of circulating haptoglobin). Correlations among TEs concentrations and NO_2 levels were studied using Spearman's correlations due to skewed distribution. A Principal Component Analysis (PCA) was conducted to investigate the relationships among TEs concentrations. The first axis of the PCA (PC1) was used as a plasma synthetic index describing richness in the essential elements and was used for further analysis with trace elements (see Section Results). Because we have only one measure of NO_2 and TEs concentrations per brood, the average brood mass, tarsus length, and baseline levels of Hp per brood were used for further analysis. An average brood condition at D13 was estimated as the residuals of the linear regression between the log-transformed average brood mass and the log-transformed average brood tarsus. The correlations between richness in essential elements as assessed by PC1 and brood mass and condition were studied using Spearman's correlations because of the skewed distribution of the data.

Clutch size, hatching success, nestling survival, and brood mass, body condition, and Hp levels were analyzed using habitat and NO_2 levels or PC1 (as an estimate of the plasmatic richness in essential elements) as explanatory variables, and some co-variates when needed (see below). The variation in clutch size was analyzed using generalized linear models (GLMs) with Poisson error distributions (Zuur et al., 2009). For hatching success and nestling survival, GLMs with binomial distributions and logit link functions were used (Warton and Hui, 2011), and the first egg-laying date (standardized as the number of days between March 1st and the first egg-laying date) was added as a covariate (Cresswell and McCleery, 2003; Blondel, 2007). Because overdispersion was detected, the standard errors were corrected using a quasi-GLM model (Zuur et al., 2009). Linear models (LMs) were used for brood mass and body condition at D13 with the first egg-laying date and brood size included as covariates. Because body mass may affect nestling immunity (Alonso-Alvarez and Tella, 2001), it was included as a covariate for the Hp analysis.

Each analysis was performed using the full model with software R v.3.15.1 (R Development Core Team, 2014). Statistical significance was set at $p < 0.05$ for all results.

RESULTS

Reproduction Parameters and Immune Marker

Laying dates differed significantly among all groups except between Besançon and Dijon (KW $Chi^2 = 61.5$, df = 3, $p < 0.001$, Table S2) and urban Great tits laid earlier than forest individuals, in average of 4.3 days (W = 6699, $p < 0.001$). The number of eggs laid did not differ neither between the two cities nor between the two forests (KW $Chi^2 = 90.0$, df = 3, $p < 0.001$, Table S2). Urban Great tits, however, laid in average 2.7 eggs less than forest tits ($W = 8153$, $p < 0.001$). Nestlings were significantly heavier in the Forêt d'Auxonne than in the two cities but did not differ from the nestlings from the Forêt de Chaux (KW $Chi^2 = 43.3$, df = 3, $p < 0.001$, Table S2). Urban broods were significantly and in average of 1.8 g lighter than forest broods ($W = 2298$, $p < 0.001$). Nestling body condition was the highest in the Forêt d'Auxonne and did not differ between the two cities (KW $Chi^2 = 56.9$, df = 3, $p < 0.001$, Table S2). The condition was better for forest individuals than for urban ones ($W = 2312$, $p < 0.001$). Hatching success and nestling survival did not differ not among sites nor between the two habitats (Table S2). The baseline levels of circulating haptoglobin was higher in the Forêt de Chaux than in the other forest and in Dijon (KW $Chi^2 = 12.9$, df = 3, $p < 0.005$, Table S2). There was no difference of haptoglobin levels between urban and forest individuals ($W = 817$, $p = 0.3$).

Relationships between NO_2 and Biological Parameters

Nitrogen dioxide concentrations in those both middle-size urban areas were close to 20 $\mu g/m^3$ (mean ± standard deviation: 19.6 ± 4.6 and 23.4 ± 10.5 $\mu g/m^3$ in Dijon and Besançon, respectively, **Figure 1**) and those of forests were 7- to 10-fold lower (4.0 ± 0.9 and 1.8 ± 0.8 $\mu g/m^3$ in the Forêt d'Auxonne and the Forêt de Chaux, respectively). Nitrogen dioxide concentrations did not differ neither between the two cities nor between the two forests (KW $Chi^2 = 33.6$, df = 3, $p < 0.001$) while urban NO_2 concentrations were significantly higher than the ones measured in the forests ($W = 13$, $p < 0.001$). No significant correlation was detected between the NO_2 in urban areas and any of the biological parameters measured.

Relationships between Element Concentrations and Biological Parameters

Over the 25 elements analyzed in this study, 13 were detected in very few or even no birds, including the non-essential As, Cd, and Hg. Over the 12 remaining elements, Spearman's correlation showed significant but not very strong correlations ($r < 0.5$) among metals (Figure S1). The plasma concentrations of Ca ($W = 1297$, $p = 0.002$), Cu ($W = 1416$, $p < 0.001$), Fe ($W = 1301$, $p = 0.018$), P ($W = 1332$, $p < 0.001$), Si ($W = 1355$, $p < 0.001$), and Zn ($W = 1173$, $p = 0.045$) were lower in urban nestlings than in forest individuals, whereas the Mo concentrations were higher ($W = 86$, $p < 0.001$) in urban nestlings than in forest ones (Table S3). K, Mg, Na, Pb, and Se concentrations did not differ between the urban and forest populations of Great tits (*ps* > Table S3).

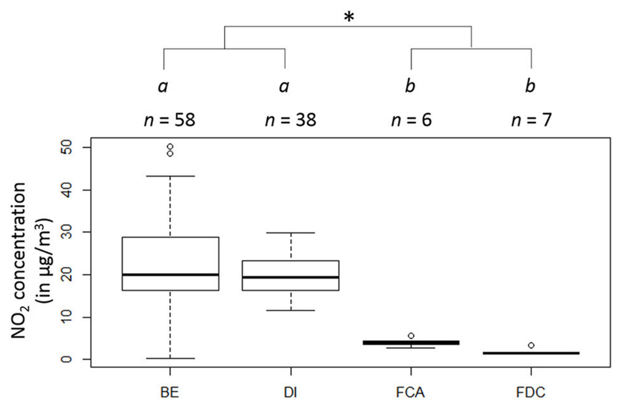

FIGURE 1 | Atmospheric concentrations of NO_2 ($\mu g/m^3$) in the different study sites: Besançon (BE), Dijon (DI), the Forêt d'Auxonne (FCA), and the Forêt de Chaux (FDC). Sites with different letters are significantly different. The asterisk indicates a difference between urban and forest levels.

PCA analysis found that 74.4% of the variation was driven by five elements (Ca, Na, Mg, P, and Si, Figure S2). The first and second axes of the PCA explained 27 and 18% of the variation, respectively. Based on the results of the PCA, we used PC1 as an index of the richness in essential elements, with negative values indicating high concentrations of essential elements, and positive values indicating low concentrations. The richness of the essential elements of Great tits did not differ neither between the two cities nor between the two forest sites, but was significantly higher in the forests than in the cities ($W = 265.5$, $p < 0.001$, **Figure 2**). Brood body mass and body condition were negatively correlated with PC1 (**Figure 3**, **Table 1**), therefore positively correlated to the richness in essential elements.

DISCUSSION

Our results showed that several components of Great tit reproductive output were negatively affected in the urban habitats. The clutch size, nestling body mass, and nestling body condition were lower in urban sites than in forests. However, we observed no difference in the circulating haptoglobin. The essential elements richness was lower in urban sites and, interestingly, it appeared to be positively correlated with the nestling mass and body condition. Our results are consistent with those reported in previous studies (Hõrak, 1993; Chamberlain et al., 2009; Bailly et al., 2016b) on the components of reproductive output, and suggest mechanisms to explain the negative effect of urbanization on the reproductive performance of Great tits.

Among ecological factors that might explain the observed contrast in clutch size, brood mass and condition, food availability has frequently been cited in the literature (see for instance Chamberlain et al., 2009). The quantity and the quality

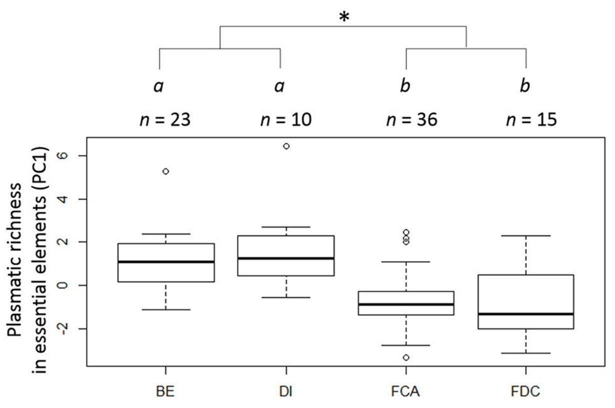

FIGURE 2 | Richness in essential elements (as assessed by the coordinates on the first axis of a PCA: PC1, with negative values indicating high concentrations of essential elements, and positive values indicating low concentrations) in the plasma of Great tit 13-day old nestlings from the four study sites: Besançon (BE), Dijon (DI), the Forêt d'Auxonne (FCA), and the Forêt de Chaux (FDC). Sites with different letters are significantly different. The asterisk indicates a difference between urban and forest individuals.

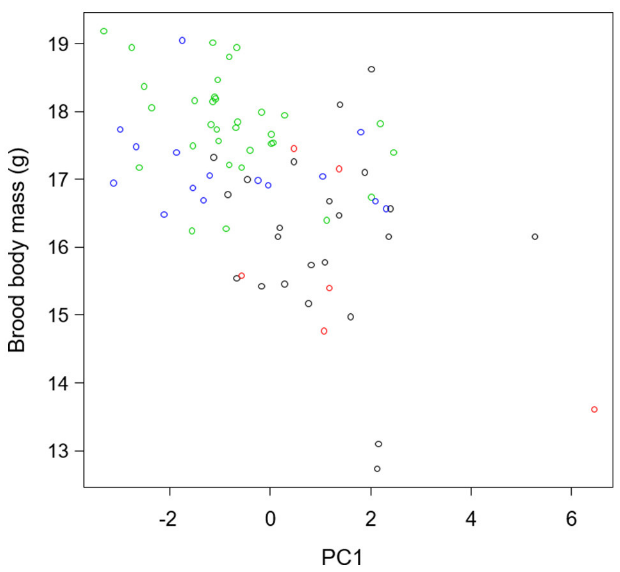

FIGURE 3 | Relationship between richness in essential elements (PC1) and brood body mass of 13-day old nestlings of Great tit. Black circles: Besançon, red circles: Dijon, green circles: Forêt d'Auxonne, blue circles: Forêt de Chaux.

of trophic resources strongly influence female investment in the clutch through its own condition (Marzal et al., 2005; Sofaer et al., 2012) as well as nestling growth and survival (Naef-Daenzer and Keller, 1999). The energetic requirement during growth is very high for tissue formation (for instance muscle, bone marrow) and the maintenance of those already established (Starck and Ricklefs, 1998). Several nutrients and elements are also essential for nestling development, and organisms have to obtain some of them from their food because they cannot

TABLE 1 | Relationship between richness in essential elements (as assessed by the coordinates on the first axis of a PCA: PC1) and average brood mass and body condition of 13-day old nestlings of Great tits according to their habitats (urban vs. forest).

	Brood mass			Brood condition		
	β	SE	*p*	β	SE	*p*
PC1	−0.18	0.07	0.012	−0.13	0.07	0.053
Habitat	−1.54	0.30	< 0.001	−1.65	0.29	< 0.001
First egg date	−0.03	0.02	0.11	−0.03	0.02	0.09
Brood size	−0.10	0.05	0.06	−0.13	0.05	0.01
R^2	0.43			0.45		
p	<0.001			<0.001		

Covariates, R^2 and significance of full models are also presented.

synthetize these elements. For instance, Isaksson and Andersson (2007) showed that carotenoids, crucial compounds for several functions, are less abundant in prey consumed by urban than by forest Great tits. Similarly, in the surroundings of a metal-processing factory in Harjavalta, SW Finland, both the intensity of the yellow color in the plumage of *P. major* nestlings and caterpillar abundance increased with increasing distant from the pollution source, suggesting a deficiency of carotenoids in birds breeding close to the factory (Eeva et al., 1998). Here, the richness of essential elements was positively correlated with average brood mass and body condition, suggesting that deficiency in essential elements may be one of the mechanisms underlying the lower body condition often observed in urban nestlings compared to forest ones. According to the PCA, the variability of the dataset is mainly driven by the concentrations of 5 elements, namely Ca, Na, Mg, P, and Si. Ca is particularly important for the structural strength of the avian skeleton, plays vital roles in many biochemical reactions and allows birds to lay large megalecithal eggs (Dacke, 2000). Because of its fundamental role in bird physiology, Ca has received relatively higher attention in environmental studies than the other elements studied here. Indeed, among the abundant literature dealing with the effects of acidification or naturally base-poor habitats on bird reproduction (see for instance Graveland et al., 1994; Graveland, 1996). Mänd et al. (2000) found a positive effect of Ca supplementation on the tarsus length and body mass in fledgling Great tits growing in base-poor pine forests. This positive effect was observed only in the most unfavorable year (in terms of weather conditions) over the 3 years studied (Mänd et al., 2000). A similar positive effect of Ca supplementation was observed in *P. major* nestlings in a Ca-poor area associated with metal pollution in Harjavalta, SW Finland (Espín et al., 2016a). Eeva and Lehikoinen (2004) found reduced eggshell thickness, egg size, and hatchability, and delayed ossification of nestling leg and wing bones in Pied flycatchers (*Ficedula hypoleuca*) living close to a copper smelter in Finland where Ca availability is poor (Eeva and Lehikoinen, 2004). However, this effect was not found in the control Great tits studied in the same area. In our study, Ca was one the main essential elements explaining the variability of the TEs dataset and circulated at lower levels in urban nestling plasma, suggesting that urban birds suffer from reduced Ca-rich resources compared to the forest populations. Phosphorus deficiencies can lead to poor bone mineralization or even abnormal bone development such as chondrodystrophy (deformity of the leg bones) (Driver et al., 2006), and Na is an essential nutrient known to influence several aspects of normal animal growth. Sodium deficiency has been shown to reduce growth and food consumption, impair feed conversion water intake, acid-base balance, and basal metabolism (Vieira et al., 2003). Magnesium and Si are also considered as bone minerals even if the role of Si in bone health is still unclear (Jugdaohsingh, 2007). We are not aware of any study reporting Mg, Na, P, or Si deficiency effects on wild birds, but our results showed their lower levels in urban nestling plasma (two of them, P and Si being significantly less concentrated in urban nestling plasma).

Taken together, these results concerning so-called bone minerals, and the positive relationship between essential elements richness and body mass and condition, could explain the reduced growth found in urban nestlings. This may be linked to a lower quality, in terms of essential elements, of the resources that birds can forage in the cities compared to what is available in forest areas. An alternative hypothesis is that whatever the availability of these elements in urban habitat, the metabolic requirement of urban nestlings might be higher because of higher element consumption by organs. Indeed, stressful environmental conditions, as that prevailing in towns, are known to increase the organism demand for energetic and non-energetic resources.

Our study also shows that non-essential elements that are often considered as potentially toxic in urban or industrial areas are almost undetectable in the plasma of Great tits from the two middle size cities studied here. This may be linked to the fact that several metals have been shown to be mainly associated to erythrocytes rather than to plasma, as analyzed in the present work (Coeurdassier et al., 2012), but the lack of differences of the concentrations in the plasma of urban and forest birds suggests a low exposure to those elements in the two habitats. Pb, one of the non-essential elements for which there has been a great concern for wildlife conservation in urban and industrial areas, was detected in 78% of the broods studied but its concentrations did not differ between urban and forest birds. This suggests that this element, which has been banned from gasoline in most industrialized countries, tends to be less at risk for wildlife than it has been in the past for this category of middle-size and tertiary towns (see for instance Scheifler et al., 2006).

Atmospheric NO_2 is a pollutant that is particularly relevant in the context of urbanization as it is emitted from the burning of fossil fuels for traffic and heating. All the average NO_2 concentrations in the urban and forest study sites are below the thresholds fixed by French legislation (Code de l'environnement—Titre R22, 2013). They are also below the European hourly and annual average for urban background concentrations (European Environmental Agency, 2016), and below the values advised for health protection by the World Health Organization (2006). NO_2 concentrations in Besançon and Dijon are in accordance with atmospheric levels of NO_2 measured and modeled in middle-sized cities by Tenailleau et al. (2015). NO_2 levels in both of these middle-sized urban areas are low as compared to those observed in large cities (Lebret et al., 2000). Therefore, these values indicate that Besançon and Dijon are moderately polluted area.

Urban populations of Great tits were exposed to higher levels of atmospheric NO_2 than forest ones, but these concentrations were not linked with any component of the reproductive success measured in this study. This is not in agreement with the work of Peach et al. (2008) on the House sparrow, showing (for quite similar NO_2 concentrations ranging from 15 to 35 $\mu g/m^3$ in their study compared to 10–50 $\mu g/m^3$ in the present work) that the brood body condition of 2- to 6-day-old nestlings and the brood tarsus length and body mass of 10- to 12-day-old nestlings were strongly and negatively correlated to local summer NO_2 concentrations. The discrepancy between our study and this previous work may be due to a number of factors linked with environmental conditions, such as weather, and food availability, or with species-specific sensitivity to NO_2.

CONCLUSION

Overall, our study brings new data about plasmatic concentrations of various essential elements in birds from urban and forest habitats and their potential effects on nestling body mass and condition. In our opinion, this is a supplementary clue that suggests food availability and/or quality may indeed be a causal mechanism for reduced growth of nestling birds in urban areas, an issue that needs further investigation. More specifically, the simultaneous assessment of circulating essential and non-essential elements allows for (i) design supplementation experiments to test how the availability of specific essential elements or blends of elements explains the reduced performances of birds in urban habitat or/and (ii) identify non-essential elements that may alter biological functions in urban birds. Our case study suggests that supplementation experiments with Ca, Cu, Fe, P, or Si should be relevant as a next step to analyze the negative consequences of urban habitat on the reproduction of Great tits. Several Ca-supplementation experiments have already been performed, in particular in the surroundings of the Cu smelter of Harjavalta, SW Finland (Espín et al., 2016a,b), but to our knowledge not in a context of urbanization without heavy industrial pollution sources. However, even though the present study did not show an effect of low concentrations of NO_2 on any of the parameters studied and the concentrations of non-essential elements did not differ in the plasma of birds from the two habitats, the ecotoxicological dimension of the impacts of urbanization on birds deserves further attention because many common pollutants, and their interactions, have not yet been studied.

ETHICS STATEMENT

This study conforms to the legal requirements of France. The experiment has received the agreement of the Animal Care and Ethical Committee of the Université de Bourgogne, Dijon (protocol # 8112) and of the *Préfectures* from Côte d'Or, Doubs, and Jura (Arrêté # 448).

AUTHOR CONTRIBUTIONS

BF, RS, and JB conceived the design of the study. JB, BF, MS, VD, SG, DR, and RS conducted the field work. JB, NB, and NC conducted the lab analyses. JB, BF, and RS analyzed the data and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2017.00006/full#supplementary-material

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