



Inter-annual variation of physiological traits between urban and forest great tits

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ABSTRACT

Urbanization is characterized by rapid environmental changes such as an increase in building surface, in pollution, or a decrease in invertebrate abundance. For many bird species, morphological and physiological differences have been observed between urban and rural individuals that seem to reflect a negative impact of urban life on the health and fitness of individuals. Studies on passerine birds also showed important differences between populations and species in their responses to the urban environment. We propose to test physiological differences between urban and forest individuals over 3 years to understand whether the observed patterns are constant or subject to variations across years. For this purpose, we assessed the health parameters of adults and fledgling of great tits, *Parus major*, living in an urban and in a forest site in the Eurometropole of Strasbourg, for three years. Bird health was estimated with morphological parameters (body condition and size) and also with physiological parameters (oxidative status and telomere length). Our results showed lower body condition of urban fledglings regardless of the year, but no site effects on telomere length. On the contrary, for adult breeders, urban individuals had longer telomeres than forest ones except for one year which coincide with bad weather conditions during reproduction where no difference was detected. Urban birds also had higher antioxidant capacity whatever the years. These results suggest that cities act as a filter in which only good quality individuals survive and achieve successful reproduction regardless of year, whereas in the forest the selection occurs only during harsh weather years.

1. Introduction

The growth of the world's population has led to a significant increase in urbanized habitats at the expense of natural habitats. This rapid urbanization has led to a modification of biotic and abiotic factors, forcing species to adapt rapidly to their new environment (Marzluff, 2001). Thus, factors such as habitat fragmentation, chemical and noise pollution, artificial lighting, and changes in interspecific relationships have contributed to a restructuring of animal community, particularly for birds (Gil and Brumm, 2013). Some species adapted successfully to this urban environment and have seen their populations persist or increase in cities. These species bring together the “urban exploiters,” which are the most adapted species that are dependent on the urban environment,

and the “urban adapters,” which take advantage of the urban environment but reside there optionally. Other species, known as “urban avoiders,” have completely disappeared from cities, leading to a decline in biodiversity in cities. To understand how species adapt to the urban environment and how this environment impacts the survival and fitness of birds, it is essential to study the underlying mechanisms at the individual scale.

Body condition (Meillère et al., 2015, 2017; Seress et al., 2012; Liker et al., 2008), telomeres (Biard et al., 2017; Grunst et al., 2020; Ibáñez-Álamo et al., 2018; Salmón et al., 2016; Salmón et al., 2017), and oxidative status (Herrera-Dueñas et al., 2017; Isaksson, 2015, 2010; Stauffer et al., 2017) are good proxies of birds' health and survival and are frequently used to investigate the underlying effects of urbanization

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on birds' physiology. Body condition reflects the energetic and nutritional status of individuals and is dependent on the quantity and quality of food available in the environment. The influence of this parameter on the reproductive success of adults or the survival of fledglings has been widely demonstrated (Chastel et al., 1995; Milenkaya et al., 2015). Telomeres are non-coding sequences at the end of chromosomes that maintain genetic integrity during cell divisions and which are involved in the mechanism of cells senescence and the aging of organisms. Telomeres shorten with age in numerous species (e.g. Hall et al., 2004) and the attrition rate affects the lifespan of individuals (Haussmann et al., 2005; Haussmann and Mauck, 2008). Telomere attrition also appears to be accelerated by environmental conditions (Chatelain et al., 2020) such as the proximity of roads (Grunst et al., 2020) and metal exposure (Saulnier et al., 2020; Stauffer et al., 2017), but also by intrinsic parameters such as high oxidative stress (Epel et al., 2004). Telomere length is therefore also representative of how individuals can cope with intrinsic and extrinsic constraints and can be considered as a good proxy of individual quality (Pauliny et al., 2006; Blackburn and Epel, 2012). In the same way, the oxidative status represents the balance between the production of reactive oxygen species (ROS) and antioxidants. Chronic exposure to chemicals, pollutants such as polycyclic aromatic hydrocarbons, polychlorinated biphenyls and trace metals, and other stressors such as light and noise pollution, have been shown to increase ROS production (Herrera-Dueñas et al., 2017; Isaksson, 2015, 2010; Stauffer et al., 2017). Non-neutralized ROS can cause oxidative stress, lead to macromolecular damages such as lipid peroxidation, protein carboxylation and DNA damage, and potentially affect the health of birds. To maintain oxidative balance and counteract ROS, birds have endogenous and exogenous antioxidants. Endogenous antioxidants are low molecular weight proteins as glutathione and antioxidant enzymes as the superoxide oxide dismutase (SOD) or catalase (CAT). Exogenous antioxidants correspond to antioxidants provided by food such as vitamins or carotenoids and are generally in low concentration in cities (Isaksson and Andersson, 2007).

The reported effects of the urban environment on these physiological parameters (body condition, oxidative stress and telomeres) in bird populations vary greatly between studies (reviewed in Sepp et al., 2017). For example, a study on adult great tits (*Parus major*) found longer telomeres in urban birds than in rural ones (Salmón et al., 2017), whereas another study on adult blackbirds (*Turdus merula*) showed the opposite pattern, with shorter telomeres for urban birds (Ibáñez-Álamo et al., 2018). Similarly, many studies in birds have found neutral or low effects of urbanization on oxidative status (Giraudeau and McGraw, 2014; Salmón, Stroh, et al. 2018; Isaksson, 2010) or body condition (Meillère et al., 2015; Bókonyi et al., 2012) compared to rural habitats, whereas some other studies have shown oxidative stress (Giraudeau et al., 2015; Herrera-Dueñas et al., 2014) or nutritional stress (Liker et al., 2008) associated with urban environments. Some of these physiological differences found between studies are associated with divergences in reaction norm and adaptation of species to environmental conditions (Salmón, Stroh, et al. 2018), but also to differences in the intensity of stressors to which individuals are exposed (Biard et al., 2017; Meillère et al., 2015; Herrera-Dueñas et al., 2014), particularly in the cases of chemical pollution or low vegetation cover surface. In addition, urban characteristics such as prey abundance or availability can change according to years and the consequences are observed as variation of nestling body size and body condition in great tits between years (Caizergues et al., 2021). Low temperature or many days of rain can explain this low prey availability (Dawson and Bortolotti, 2000; Avery and Krebs, 2008), emphasizing the impact of the lower quantity of invertebrates in town (Chamberlain et al., 2009). These conditions result in a decrease of daily activity in adult great tits (Schlicht and Kempenaers, 2020), increase of energy expenditure for foraging (Cox et al., 2019) and changes in thermoregulation in nestling of other birds species (Krijgsveld et al., 2003). Studying the effects of these annual variations in the context of urbanization is interesting because the urban

environment can exacerbate the effects of poor weather conditions. Thus, we expect more pronounced differences between the urban and rural environments in more challenging years with respect to weather conditions such as cold and rain events.

In this study, we aimed to evaluate the effects of the urban environment on physiological parameters of birds using proxies for health/quality of birds during several years to highlight interannual variation of these parameters in response to changes in the environmental constraints during reproduction (e.g. changes in weather conditions). A second aim was to compare these effects on the physiology of adult breeders vs fledglings. For this purpose, we followed an urban and a forest populations of great tits (*Parus major*) in the Eurometropole of Strasbourg (Alsace, France) for 3 years (2015, 2016, and 2018) and we recorded morphological, oxidative status, and telomere length measurements for both adults and fledglings. In a previous study (Saulnier et al., 2022), the environmental differences (pollution, vegetation cover, and food availability) between the two sites were also characterized, as well as the weather conditions during breeding (the average temperature, the average daily temperature range, the average daily rainfall rate and the number of rainy days). In this previous study, we highlighted a lower reproductive success (clutch size, number of fledglings) in the urban environment, whatever the year, due to a lower prey availability (Saulnier et al., 2022). Considering these previous results, we expected urban birds to be in worse conditions than rural birds. More precisely, we expected higher ROS and thus higher production of reactive oxygen metabolites (ROM), lower antioxidant capacity, and shorter telomere length in urban birds compared to forest ones. We expected similar results for both adults and fledglings. We also predicted that these urban and forest differences would appear to be more pronounced in year which was characterized by unfavorable weather conditions as previously seen for reproductive success (Saulnier et al., 2022).

2. Material and methods

2.1. Site and animal sampling

The study was performed in 2015, 2016, and 2018 on two populations of great tits (*Parus major*) living in artificial nestboxes in an urban site and a forest site of the Eurometropole of Strasbourg (France). The urban site (urban) consists of several spots in the town of Strasbourg such as urban parks, tree-lined walkways and roads and in the residential area of La Robertsau. The forest site (forest) corresponds to an alluvial forest in the vicinity of the small town of La Wantzenau at 20 km North of Strasbourg city center (48.647962, 7.833667). More details about nestboxes occupation and concentration of air pollutants and food availability along this urbanization gradient were provided in a previous article (Saulnier et al., 2022). Briefly, results showed no difference in terms of pollutants signature between sites but prey availability (caterpillars and spiders) was significantly lower in the city than in the forest. Forest site was mostly characterized by high vegetation around nest boxes, whereas urban site is mostly characterized by non-vegetal and low-vegetal surfaces (Saulnier et al., 2022).

The great tit is a common ubiquitous species living in both urbanized and natural environments across the Eurasian continent. This species nests easily in artificial nestboxes, which makes it a good model species for studies on the effects of urbanization on birds. In addition, adults of both sexes actively feed the nestlings, so that males and females can be caught with the same technique and in the same proportion. We monitored the reproduction of great tits during the first clutch, between the end of March and May of each year. For each occupied nestbox, we recorded the hatching date (± 1 day), the clutch size, and the fledgling number (14–18 days old) with regular visits to the nestboxes.

Adults were caught in the nestbox using a trapdoor when nestlings were between 8 and 15 days old to avoid nest desertion (which is likely to happen if nests are disturbed before hatchlings are 8 days old) or precocious fledgling (after 15 days old). We captured 45 adults and 212

fledglings in 2015, 35 adults and 200 fledglings in 2016 and 48 adults and 214 fledglings in 2018. Fledglings were caught in the nest when they were 14–18 days old (see Table A.1 of supplementary for headcount for each age group). At capture, each bird (adults and fledglings) was ringed and weighed with a precision scale (± 0.1 g). Adults were sexed based on the size of the black tie (larger for males) and the presence of the brood patch for females. For adults and six fledglings per nest (the two oldest, two intermediates, and the two youngest), the tarsus length and the head-beak length were also measured with a caliper (± 0.01 mm) and the wing length with a ruler (± 0.1 mm). These morphometric measures were used to estimate the body size and the body condition of fledglings (see “statistical analysis” part for more details). Morphometric measures were performed by several persons, but concordance tests were conducted to ensure the repeatability of measurements between them and both measured birds in urban and forest sites. We also collected 50 μ L of blood by skin-puncture in the brachial vein with heparinized capillary for physiological analyses. Samples were kept in ice during fieldwork waiting for centrifugation at 4000 g for 10 min at 4 °C. Plasma and erythrocytes were separated in two distinct tubes before storage at -80 °C until lab analyses.

2.2. Measures of physiological parameters

We measured three physiological parameters in the blood of birds: the reactive oxygen metabolites (ROM), the total antioxidant capacity (OXY) in plasma, and the relative telomere length (RTL) in erythrocytes. Means and Standard Deviation (SD) of all parameters are provided in appendix B of supplementary materials.

The concentration of ROM (as hydroperoxides) in the birds' plasma was measured using the diacron- reactive oxygen metabolite test (d-ROM test, Diacron, Italy, [Costantini, 2016](#)), adapted to 96 well plates. In each well, 4 μ L of plasma is added to 200 μ L of the reaction mix, containing acid buffer and chromogenic substance. The plate was incubated at 37 °C for 75 min. The absorbance of samples at 510 nm was measured using an Infinite® 200 PRO microplate reader (Tecan). The absorbance was converted to mg of H_2O_2 /dL (based on Carratelli Units) using the absorbance of a standard sample of known concentration following the kit instruction. A total of 397 samples were measured in duplicates.

The OXY was measured using the OXY-Adsorbent Test (Diacron, Italy) adapted to 96 wells plate. In each well, 1:100 diluted plasma was mixed with the reaction mix provided in the kit. The plates were incubated for 10 min at 37 °C. The absorbance of samples at 510 nm was measured using an Infinite® 200 PRO microplate reader (Tecan). The measured absorbance is inversely proportional to the concentration of OXY in the sample. The OXY concentration of the sample (in μ mol HClO/ml) was obtained using the absorbance of a standard sample with a known oxidizing power following kit instructions. A total of 529 samples were measured in duplicates.

For both ROM and OXY, all samples were run in duplicates and a control sample (goose plasma) was run in each plate to control the inter-plate variations. Adult and fledgling samples were measured in the same plates in a randomized way. The mean coefficient of intra- and inter-plate variation was 4.47% and 9.48% respectively for ROM and 3.68% and 7.92% respectively for OXY.

Relative telomere length (RTL) was measured in erythrocytes using a method previously described by [Criscuolo et al. \(2009\)](#). Erythrocyte DNA was extracted on silica-membrane columns using the kit Nucleo-Spin® Blood Quick Pure (Macherey-Nagel®), and the concentration and purity were checked using the Nanodrop 1000 (Thermo scientific®), a microvolume spectrometer. The RTL of each sample was measured with a quantitative polymerization chain reaction (qPCR) in 96 wells plate. The RTL is a ratio (T/S) between telomere repeat copy number (T) to control gene copy number (S) expressed in the same way for each bird. The samples mean Cq of T and mean Cq of S (number of cycles necessary to detect amplified DNA above basal signal) were used as a reference in the calculation of RTL with a ratio (T/S) arbitrary set to 1 (see [Criscuolo](#)

[et al., 2009](#) for calculation). The *Parus major* zinc finger protein (ZENK) gene (GenBank: EF568148) was used as the control gene. Sequences of used primers are provided in appendix C of supplementary material. In both 2015 and 2016, the final dilution of primer was 500 nM for the control gene and 100 nM for telomeres. The protocol in 2018 was modified and the final dilution was 500 nM for the control gene and 200 nM for telomeres. Finally, amplification cycle was performed with a final volume of 10 μ L per well with 2 ng of DNA (2 μ L), and 8 μ L of reaction mix containing a GoTaq® qPCR (Promega, Madison, USA). For the control gene, amplification started with 2 min at 95 °C followed by 40 cycles of 15 s at 95 °C, 30 s at 56 °C, and 1 min 30 s at 72 °C. The average efficiency of amplification for the control gene and telomere was 99.8% and 100% respectively in 2015–2016; and 95.8% and 98.9% in 2018. Mean intra-individual quantification cycle Cq; number of cycles necessary to detect amplified DNA above basal signal) variation was 0.91% and 1.39% for the control and the telomere assay in 2015–2016 and 1.03% and 2.06% for control and telomere assay in 2018. Mean inter-run Cq variation was 0.75% and 1.28% for control and telomere genes respectively in 2015–2016 and 0.67% and 1.16% in 2018. A total of 436 samples were measured (281 for 2015–2016 and 155 for 2018).

2.3. Statistics analyses

All statistical analyses were performed with R software (version 4.1.0) ([R Development Core Team, 2021](#)). The significance threshold was set to $\alpha = 0.05\%$. For all analyses, the hatching date was expressed in relative day from March 1.

Tarsus, wing, and head-beak lengths were used to estimate a body size index. More precisely, the body size index was estimated as the value of each individual on the first dimension (PC1) of a principal component analysis (PCA) including tarsus length, wing length, and head-beak length (variables were scaled before analysis). This index was calculated separately for adults and nestlings. PC1 explained 60.66% of body size variation for adult birds and 73.73% for fledglings. Body condition was obtained as the residuals of the regression between body mass and body size index (adults: $R^2 = 0.44$, $p < 0.001$; fledglings: $R^2 = 0.47$, $p < 0.001$).

Differences of morphological (body size index and body condition) and physiological parameters (ROM, OXY, RTL) were tested using linear models (function lm) for adults and linear mixed models (function lmer of “lme4” package ([Bates et al., 2015](#))) for fledglings and type III ANOVA. We used mixed models for fledglings to take into account the non-independence of fledglings from the same nest. The normality and the homoscedasticity of the residuals were checked using Kolmogorov-Smirnov test and Levène test respectively. To respect these conditions, the RTL was log-transformed. Moreover, to compare yearly RTL measurements in spite of the dilution volume change in the protocol, we applied a transformation to the data to obtain Z-scores (function “scale” in R) following the protocol described by [Verhulst \(2020\)](#). The data were centered and reduced for each year around a mean equal to 0 and a standard deviation equal to 1, thus allowing comparison of the differences in distributions between sites per year without assay-dependent variations. The transformation was applied to the log-transformed data.

In models, we tested the effect of sites (urban and forest), year (as a categorical variable), and the interaction between site and year. We also included the following covariates: (i) hatching date (to take into account the timing of the breeding season), (ii) fledgling number (to take into account the investment in reproduction for adults and competition for young), and (iii) body condition (for models on physiological parameters only). For adult birds, we also added the effect of sex and the interaction between site (urban or forest) and sex. The interaction between sex and year was not included due to an important collinearity (Variance Inflation Factor > 10, [O'Brien, 2007](#)). For fledglings, models also included the age and the brood identity as additive random effects and the body size as a covariate (for models on physiological parameters only), taking into account the birds' growth during the studied period.

Non-significant interactions in adult and fledgling models were sequentially removed from final models. Estimates of all statistical models are provided in appendix D of supplementary material.

3. Results

3.1. Body size and condition

The body size of adults was significantly different between sites and sexes (Table 1). Urban breeders were significantly smaller than forest ones (Tukey, $p = 0.002$) and males were significantly taller than females (Tukey, $p < 0.001$). A significant positive correlation between body size and the fledgling number was also observed: smaller birds had fewer fledglings than taller ones. The body size of fledglings was significantly different between sites depending on the year (the site and year interaction was significant, as shown in Table 2 and Fig. 1). Urban fledglings were smaller than forest fledglings in 2018 (Tukey, $p < 0.001$), and no site differences were observed in 2015 (Tukey, $p = 0.99$) nor in 2016 (Tukey, $p = 0.14$). Urban fledglings were smaller in 2018 than in 2015 (Tukey, $p = 0.004$) and 2016 ($p < 0.001$). However, forest fledglings were taller in 2018 than in 2016 (Tukey, $p = 0.02$). For body condition, none of the explanatory variables was significant in adult birds (Table 1), whereas the body condition of fledglings varied significantly between urban and forest sites (Table 2), with a poorer condition in urban fledglings (Tukey, $p = 0.004$). The fledgling body condition was also negatively correlated with the number of fledglings in each brood (Table 2, see also Table D.2 of supplementary material).

3.2. Oxidative stress

We considered plasmatic ROM and OXY concentrations to evaluate oxidative status. Both adults and fledglings showed a significant interaction between site and year for ROM plasmatic concentrations (Tables 1 and 2, Fig. 2). For adults, ROM concentrations were higher for urban breeders than forest breeders in 2018 (Tukey, $p = 0.014$) but not in 2015 (Tukey, $p = 0.88$) nor 2016 (Tukey, $p = 0.26$). Also, urban breeder ROM concentrations were higher in 2018 than in 2016 (Tukey, $p = 0.012$) but were similar in 2018 and 2015 (Tukey, $p = 0.13$). Plasmatic ROM concentration was also negatively correlated with the hatching date with later breeders having lower ROM concentrations (see also Table D.1 of supplementary materials). The plasmatic ROM concentration of fledglings were lower for urban birds than forest birds in 2016 (Tukey, $p = 0.009$) but not in 2015 (Tukey, $p = 0.49$) nor 2018 (Tukey, $p = 0.64$). As observed for adults, the urban fledglings ROM concentration were higher in 2018 than 2016 (Tukey, $p = 0.003$) but not in 2015 (Tukey, $p = 0.25$). However, forest fledglings had higher ROM concentrations in 2018 than in 2015 (Tukey, $p = 0.01$) but not in 2016 (Tukey, $p = 0.40$). ROM concentration was significantly positively correlated to the body condition of fledglings (Table 2, see also Table D.2 of supplementary materials). The OXY concentration of adult birds differed significantly between sites and years (Table 1 and Fig. 2).

Urban breeders had higher OXY concentration than forest breeders (Tukey, $p = 0.007$) and concentrations in 2016 were significantly lower than those observed in 2015 (Tukey, $p < 0.001$) and 2018 (Tukey, $p = 0.004$). For fledglings, a significant year difference was also observed (Table 2 and Fig. 2) with the same pattern (Tukey, all $p < 0.013$). The lowest concentration was observed in 2016 and the highest concentration in 2015. A positive correlation was also observed with body size (Table 2), meaning that taller fledglings had higher levels of OXY.

3.3. Telomere length

RTL of adult birds was significantly explained by the interaction between sites and years (Table 1, Fig. 3). In 2015 and 2018, urban birds had longer RTL than forest birds (Tukey, $p_{2015} = 0.02$, and $p_{2018} = 0.045$), but no difference was observed in 2016 (Tukey, $p = 0.15$). Difference between years were observed in forest area and RTL appeared to be longer in 2016 compared to 2018 (Tukey, $p = 0.04$). The RTL was also negatively correlated to the hatching date (Table 1). Birds breeding later had shorter telomeres. However, for fledglings, none of the tested variables was significant (Table 2, see also Table D.1 of supplementary).

4. Discussion

This study aims to compare the physiology of urban and forest birds for several years to understand the impact of the urban environment on bird health and the interannual variation of traits in the different environments. We found inter-annual variation of some parameters between sites for both adults and fledglings. Some results are in opposition to our initial hypotheses.

No inter-annual variations were observed for adult morphological parameters (body size and body condition). Urban birds were consistently smaller than rural birds. Other studies have observed a smaller size of urban birds in the same species (Caizergues et al., 2018), and also in other species such as house sparrows (Meillère et al., 2015; Seress et al., 2012) and blackbirds (Evans et al., 2009). An adaptive response to urban environments is the hypothesis proposed to explain this urban small size (Meillère et al., 2015). A smaller size offers better mobility, is more effective at avoiding predators (Møller, 2008; Liker et al., 2008), and reduces food requirements, which can be advantageous in an environment with fewer prey (Gardner et al., 2011). Moreover, the body size divergence between urban versus forest was not observed for fledglings in 2015 and 2016, suggesting that the lower size of urban breeders found in this study would result from a selection of smaller individuals after fledgling (selection on survival or access to reproduction). Some studies revealed a lower size of fledglings in urban settings compared to forest great tits (Bailly et al., 2016; Biard et al., 2017; Corsini et al., 2020; Caizergues et al., 2021), blue tits (Corsini et al., 2020), or house sparrows (Meillère et al., 2015). However, it seems that these differences are correlated to the degree of urbanization to which the birds are exposed and are more pronounced in the largest cities (Biard et al., 2017; Meillère et al., 2015, 2017). Additionally, we found a

Table 1

Results of statistical analyses of morphological and physiological parameters of breeding adults between urban and forest sites during three years.

	Body size ($n = 128$)			Body condition ($n = 126$)			ROM ($n = 98$)			OXY ($n = 111$)			RTL ($n = 120$)		
	<i>F</i>	<i>df</i>	<i>p</i>	<i>F</i>	<i>df</i>	<i>p</i>	<i>F</i>	<i>df</i>	<i>p</i>	<i>F</i>	<i>df</i>	<i>p</i>	<i>F</i>	<i>df</i>	<i>p</i>
Site	9.91	1, 121	0.002	2.47	1, 119	0.12	11.50	1, 88	<0.001	7.34	1, 103	0.008	7.07	1, 110	0.001
Sex	84.22	1, 121	<0.001	0.38	1, 119	0.54	0.45	1, 88	0.51	0.06	1, 103	0.81	1.43	1, 110	0.23
Year	0.88	2, 121	0.42	0.97	2, 119	0.38	5.32	2, 88	0.006	12.38	2, 103	<0.001	2.40	2, 110	0.10
Hatching date	0.17	1, 121	0.68	0.04	1, 119	0.84	16.11	1, 88	<0.001	0.48	1, 103	0.49	9.44	1, 110	0.003
Fledgling number	5.79	1, 121	0.018	2.17	1, 119	0.14	0.004	1, 88	0.95	0.66	1, 103	0.42	3.23	1, 110	0.08
Body condition	0.06	1, 88	0.80	2.32	1, 103	0.13	0.02	1, 110	0.90
Site*Year	3.93	2, 88	0.02	5.21	2, 110	0.007
Site*Sex

Note. ROM corresponds to plasmatic concentration of reactive oxygen metabolites; OXY corresponds to plasmatic total antioxidant capacity and RTL corresponds to relative telomere length. RTL was transformed as Z-scores due to protocol modifications (see Material and methods for more details).

Table 2

Results of statistical analyses of morphological and physiological parameters of nestlings between urban and forest sites during three years.

	Body size (n = 625)			Body condition (n = 625)			ROM (n = 299)			OXY (n = 418)			RTL (n = 314)		
	F	df	p	F	df	p	F	df	p	F	df	p	F	df	p
Site	1.67	1, 614	0.19	8.77	1, 616	0.004	1.80	1, 288	0.17	2.02	1, 407	0.16	3.20	1, 303	0.08
Year	9.24	2, 614	<0.001	1.31	2, 616	0.27	5.73	2, 288	0.005	36.57	2, 407	<0.001	0.17	2, 303	0.85
Hatching date	3.15	1, 614	0.08	3.36	2, 617	0.07	0.000	1, 288	0.99	0.28	1, 407	0.60	0.01	1, 303	0.93
Fledgling number	3.60	1, 614	0.06	11.61	2, 618	0.001	0.34	1, 288	0.56	0.50	1, 407	0.48	0.07	1, 303	0.78
Body size	0.66	1, 288	0.42	4.41	1, 407	0.04	0.49	1, 303	0.49
Body condition	12.71	1, 288	<0.001	2.63	1, 407	0.11	0.06	1, 303	0.80
Site*Year	13.35	2, 614	<0.001	3.23	2, 888	0.04

Note. ROM corresponds to plasmatic concentration of reactive oxygen metabolites; OXY corresponds to plasmatic total antioxidant capacity and RTL corresponds to relative telomere length. RTL was transformed as Z-scores due to protocol modifications (see Material and methods for more details).

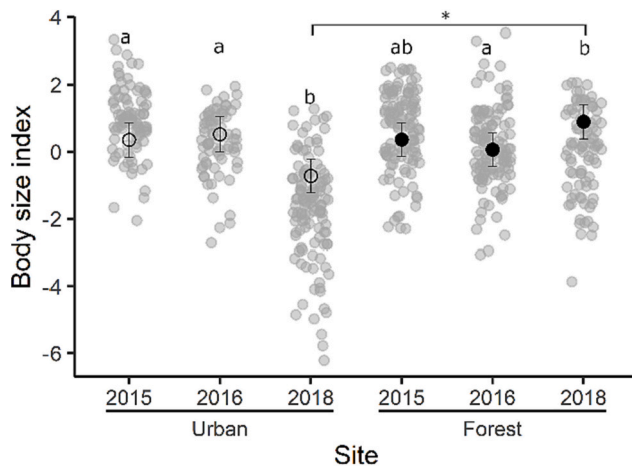


Fig. 1. Body size difference of great tit fledglings between urban and forest sites during three years. Differences between years are represented with different letters for a given site and differences between sites were represented with a bar for a given year. Mean predicted values of the statistical model presented in Table 2 are represented by circles and standard errors are represented by bars.

smaller size of urban fledglings in 2018. Variation of body size across the years is also observed in another study on the same species that has been associated with a probable fluctuation of food quantity (Caizergues et al., 2021). Furthermore, fledglings showed a lower body condition in cities, as found in the literature (Biard et al., 2017; Bailly et al., 2016; Demeyrier et al., 2016). This is probably the consequence of lower availability of arthropods (caterpillars and spiders) observed in the urban site in Strasbourg (Saulnier et al., 2022) and in other cities (Peach et al., 2015; Jones and Leather, 2012). The body condition of fledglings was also negatively correlated to the fledgling number whatever the site and year. In an enlarged brood, competition increases between nestlings, and the provisioning capacity of the breeder is not sufficient to cover the food requirement of all nestlings (Demeyrier et al., 2016), leading to the decrease of lipid reserves in fledglings (Hörak et al., 2000; Burness et al., 2000). In contrast, no site differences of body condition were observed for adults, as also reported in several other studies on great tits (Sepp et al., 2017). The similarity of body condition in breeders suggests that only birds with sufficient body condition can succeed at reproduction, and that birds with low body condition avoid or initiate and fail reproduction.

In this study, all physiological parameters in adults varied between years, and some also varied between sites. A higher plasmatic ROM concentration was measured in urban breeders in 2018, suggesting that the urban environment can be considered a stressful environment. This result is consistent with our prediction and elevation of hydroperoxide production suggest a higher oxidative stress in urban area similarly to other studies (Herrera-Dueñas et al., 2014; Giraudeau and McGraw,

2014). In addition, we found consistently higher plasma OXY concentrations for urban breeders compared to forest breeders. This same result has already been shown in the same species (Salmón, Stroh, et al. 2018), although in most studies the opposite result was observed in the great tits (Hörak et al., 2004; Isaksson et al., 2007) and house sparrows (Herrera-Dueñas et al., 2014, 2017). Considering that higher OXY concentration is correlated with increasing levels of corticosterone, higher plasma OXY in urban areas can result in metabolic response to environmental constraints (Cohen et al., 2008). Isaksson (2020) suggest that different variations of oxidative markers depends on the intensity of stressors to which individuals are confronted. Observation of individuals with high plasmatic antioxidant concentrations and few oxidative damage (as measured with ROM concentrations) might indicate that levels of urbanization are relatively low compared to detoxification capacity of birds (Isaksson, 2020) and that increase of antioxidant capacity is sufficient to counteract oxidative damage. Interestingly, annual variations were observed for both ROM (only in the urban site) and OXY concentrations (same pattern in both sites), but these markers do not vary in the same direction for a given year. Environmental parameters can variate from one year to another as well as levels of stressor to which individuals are confronted (for example levels of pollutants (Lévy et al., 2018) or prey availability (Wawrzyniak et al., 2020)). Higher ROM concentrations and low OXY concentrations can result of acute stress related to an increase of stress factor in the environment. Lower OXY concentrations result from the depletion of dietary antioxidants (carotenoids or vitamin E), and the variation of ROM between years could reflect to some extent variations in exogenous antioxidant availability. At the same time endogenous anti-oxidant molecules as glutathione or antioxidant enzymes (SOD or the CAT) can act in the process against ROS (Costantini and Verhulst, 2009) and have to be considered in future analyses to understand accurately the variation of oxidative stress. However, no difference of these antioxidant enzymes activities is detected along an urbanization gradient in the same species (Salmón et al., 2018a, 2018b).

Similar inter-annual ROM and OXY trends were observed in adults and fledglings. But contrary to adults, no sites differences were observed for fledglings OXY concentrations. Other studies showed mitigate effect of urbanization on nestling oxidative status (Isaksson et al., 2005; Salmón, Watson, et al. 2018). However, our study highlights the influence of growth and body condition on oxidative status at fledge. A positive correlation between body condition and ROM concentration was indeed found. The body condition of a nestling is partly related to its lipid reserve (Ardia and Clotfelter, 2007). However, there is a positive correlation between lipid peroxidation and plasmatic lipid content (Pérez-Rodríguez et al., 2015). Higher levels of circulating lipids lead to peroxidation increasing and higher ROM concentration. Furthermore, OXY was also positively correlated with body size. It is possible that taller individuals had a higher metabolism, indeed it has been shown in coal tits (*Periparus ater*) that nestlings with a higher metabolism had higher OXY than the others (Stier et al., 2014).

The higher urban OXY concentration and the absence of ROM differences (except in 2018) between sites in adults can be related to the

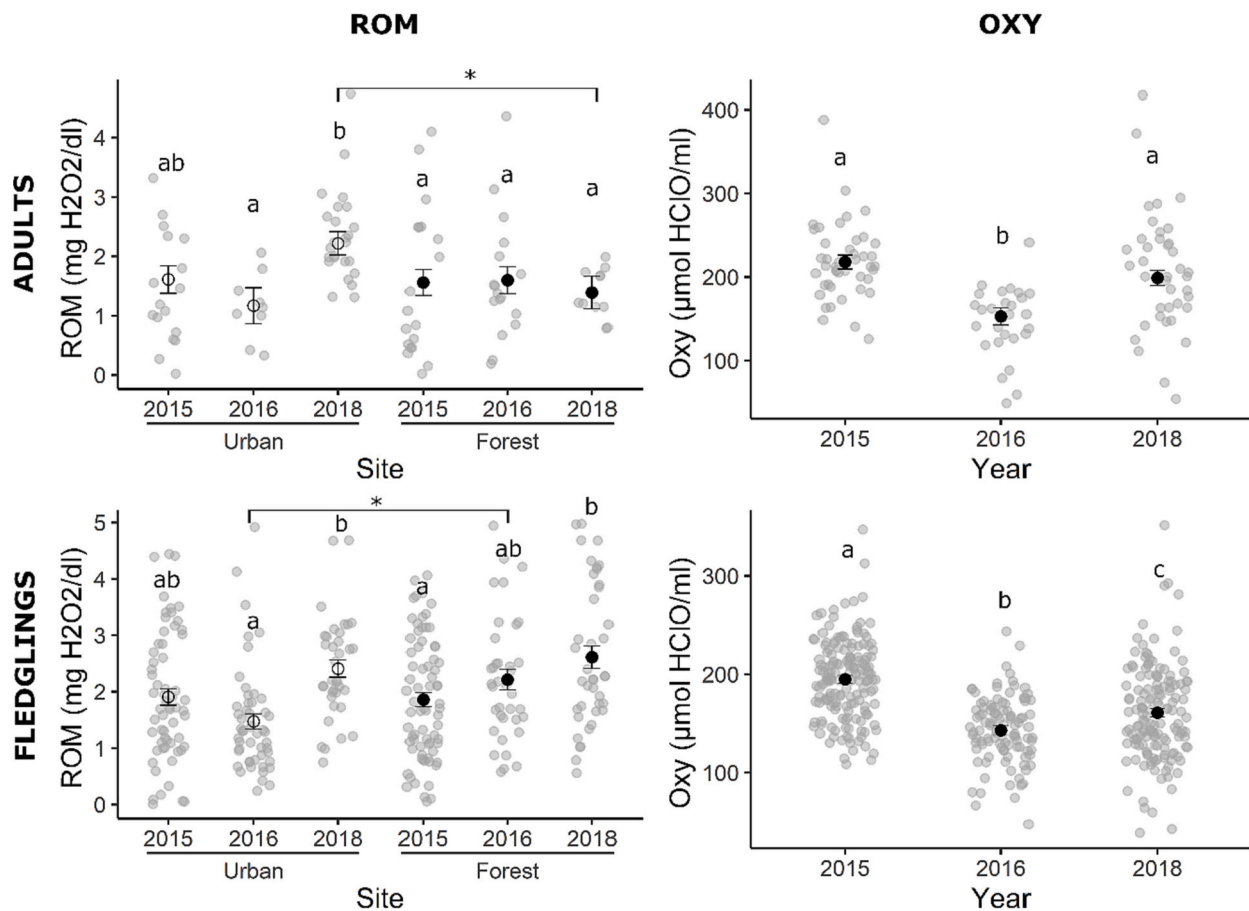


Fig. 2. Oxidative status difference of great tit breeders and fledglings between urban and forest sites during three years. Differences between years are represented with different letters for a given site and differences between sites were represented with a bar for a given year. Mean predicted values of the statistical model presented in Tables 1 and 2 are represented by circles and standard errors are represented by bars.

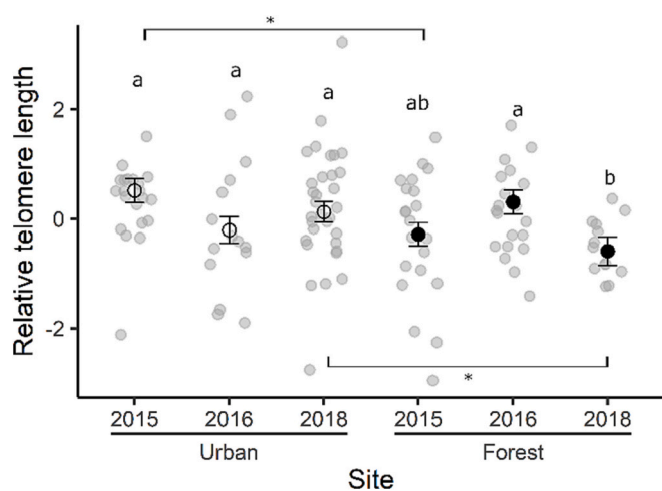


Fig. 3. Relative telomere length difference for great tit breeders between urban and forest sites for three years. Differences between years are represented with different letters for a given site and differences between sites were represented with a bar for a given year. Mean predicted values of the statistical model presented in Table 1 are represented by circles and standard errors are represented by bars.

absence of negative effect of the city on physiological parameters even if reproduction is impacted (Saulnier et al., 2022). But this result may also highlight a stronger selection of breeding individuals in the urban site, i.

e. only the best quality individuals, with high antioxidant defenses and high ability to neutralize oxidative damage, could survive and succeed to reproduce. This hypothesis is supported by the results on telomeres. Urban breeders had longer telomeres than forest breeders in 2015 and 2018. Higher telomere length is correlated to higher survival of birds (Salmón et al., 2017; Haussmann et al., 2005), a longer lifespan (Haussmann and Vleck, 2002; Monaghan, 2012), and better fitness (Pauliny et al., 2006). Long telomeres in a constraining environment represent a high investment in self-maintenance (Giraudeau et al., 2019). Telomere length is negatively impacted by environmental stressors (Chatelain et al., 2020; Grunst et al., 2020) or by intrinsic parameters such as high oxidative stress (Epel et al., 2004). Thus, telomere length can be considered an indicator of bird quality in terms of health and survival. The fact that fledglings have similar telomere length between urban and forest areas and urban breeders have longer telomeres suggest that only good quality individuals are in the capacity to allocate energy in both self-maintenance and reproduction and can survive and breed in their constrained environment. Furthermore, forest sites present low environmental constraints, individuals of lesser quality can also survive and successfully access reproduction, lowering the RTL average of the captured adults. A part of these results is supported by the work of Salmón et al. (2017), which shows that only urban nestlings with longer telomeres survived and accessed to reproduction in their first year. However, the breeding selection occurs also for forest birds under harsh weather constraints as in 2016, leading to an absence of significant RTL difference between sites. Furthermore, since telomere size is correlated with the age of individuals, we can hypothesize that longer telomeres of urban birds were due to the strongest selection in favor of the younger

breeders, although survival is higher in urban than forest birds (Evans et al., 2009). Some results of 2018 provided in supplementary materials (see Table A.2 of appendix) revealed no difference between the sites in the proportion of young breeders (1 year old) and old breeders (> 1 year old). Other works did not find any relationship between age and telomeres in great tits (Salmón et al., 2017). However, we cannot exclude this hypothesis because the age repartition in the “old breeders” category is unknown.

RTL and ROM concentration were both negatively correlated with hatching date. This result means that late breeders had shorter telomeres but also lower ROM concentrations. Earlier breeders with longer telomeres is a result supported by other studies highlighting that earlier breeders are generally the most experimented breeders (Robertson and Rendell, 2001; Verhulst et al., 1995). However, the high ROM concentration suggests a cost of breeding too early in the breeding season, possibly due, in this context, to a mismatch between prey abundance and nestling energetic requirement. Prey abundance fluctuates during the season and a lag between prey availability and nestlings' food requirement can increase the energy expenditure required by adults to meet the needs of their chicks, which can increase stress in adults (Thomas et al., 2001; Chamberlain et al., 2009).

To conclude, the inter-annual variations in telomere length is due to a greater variance between forest individuals, as the urban environment seems to be more restrictive and selective. Our study seems to reveal that the urban environment acts as a filter where only individuals of good quality can survive and invest in their reproduction. Nevertheless, because we only captured adults when nestlings were between 8 and 15 days old, captured individuals do not necessarily reflect the state of health of all the urban individuals (breeders and non-breeders). Future studies could test this hypothesis by comparing the health parameters of successful and unsuccessful breeding birds. Moreover, the fact that we found in fledglings site differences only for some parameters (small body size and body condition in urban areas but not for physiological parameters) suggests that in Strasbourg, a medium-sized city, parental investment and reduced brood size limit the impact of environmental constraints during growth for fledglings compared to larger cities (Meillère et al., 2017). However, single-site based studies have some limits and results should be taken with caution (Szulkin et al., 2020). Even if our results echoed the litterature, it could be relevant to reiterate the experiment on several sites. Finally, this study highlights the importance of inter-annual studies to understand the impact of urbanization on birds, particularly to identify underlying selection mechanisms. As oxidative responses are dependent on the level of stressors for birds, it would be relevant to consider both temporal and spatial environmental changes that individuals are facing in city to understand the physiological responses of organisms under urban stressor, i.e.: phenotypic plasticity, adaptation or death of individuals.

Ethic approval

This work is in accordance with the French legislation concerning the capture and the biological sampling of wildlife. SM, JB and PU received ringing licenses and authorizations for blood sampling from the CRBPO (National Museum of Natural History) as part of a research program led by SM (PP N°673). The protocol has been approved by the French national ethical committee n°35 and approved by the French ministry of research (project APAFIS# 9160-2017030316182730 v2).

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CRedit authorship contribution statement

Agnès Saulnier: Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Josefa Bleu:** Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision. **Anne Boos:** Methodology, Validation, Investigation, Resources, Writing – review & editing. **Sandrine Zahn:** Methodology, Validation, Investigation, Resources, Writing – review & editing. **Pascale Ronot:** Methodology, Validation, Investigation, Writing – review & editing. **Islah El Masoudi:** Methodology, Validation, Investigation, Writing – review & editing. **Emilio R. Rojas:** Investigation, Data curation, Writing – review & editing. **Pierre Uhlrich:** Investigation, Data curation, Writing – review & editing. **Mirella Del Nero:** Writing – review & editing, Funding acquisition. **Sylvie Massemin:** Conceptualization, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

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.

Data availability

Data will be deposited on Mendeley data/indores upon acceptance of the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cbpa.2023.111385>.

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