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► **To cite this version:**

Agnès Saulnier, Josefa Bleu, Anne Boos, Maurice Millet, Sandrine Zahn, et al.. Inter-annual variation of physiological traits between urban and forest great tits. *Comparative Biochemistry and Physiology - Part A: Molecular and Integrative Physiology*, 2023, 279, pp.111385. 10.1016/j.cbpa.2023.111385 . hal-04379565

HAL Id: hal-04379565

<https://hal.science/hal-04379565v1>

Submitted on 8 Jan 2024

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

Agnès Saulnier*¹, Josefa Bleu^{1,2}, Anne Boos^{1,2}, Maurice Millet^{2,3}, Sandrine Zahn^{1,2}, Pascale Ronot^{1,2}, Islah El Masoudi¹, Emilio R. Rojas^{1,4}, Pierre Uhlrich¹, Mirella Del Nero^{1,2}, Sylvie Massemin^{1,2}

*Corresponding author: agnes.saulnier1@gmail.com

¹ Université de Strasbourg, CNRS, IPHC UMR 7178, 23 rue du Loess, F-67000 Strasbourg, France

² ZAEU, Maison Interuniversitaire des Sciences de l'Homme - Alsace (MISHA), 5, allée du Général Rouvillois, CS 50008, 67083 Strasbourg cedex , France

³ Université de Strasbourg, CNRS, ICPEES 7515, F-67087 Strasbourg Cedex 3, France

⁴ Wildstat, 43 rue de la Hoube, 67280 Urmatt, France

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

26 urban fledglings regardless of the year, but no site effects on telomere length. On the contrary,
27 for adult breeders, urban individuals had longer telomeres than forest ones except for one year
28 which coincide with bad weather conditions during reproduction where no difference was
29 detected. Urban birds also had higher antioxidant capacity whatever the years. These results
30 suggest that cities act as a filter in which only good quality individuals survive and achieve
31 successful reproduction regardless of year, whereas in the forest the selection occurs only
32 during harsh weather years.

33 **Keywords: urbanization, oxidative stress, telomere, antioxidant concentration**

34

35 Introduction

36 The growth of the world's population has led to a significant increase in urbanized habitats at
37 the expense of natural habitats. This rapid urbanization has led to a modification of biotic and
38 abiotic factors, forcing species to adapt rapidly to their new environment (Marzluff 2001). Thus,
39 factors such as habitat fragmentation, chemical and noise pollution, artificial lighting, and
40 changes in interspecific relationships have contributed to a restructuring of animal community,
41 particularly for birds (Gil and Brumm 2013). Some species adapted successfully to this urban
42 environment and have seen their populations persist or increase in cities. These species bring
43 together the “urban exploiters,” which are the most adapted species that are dependent on the
44 urban environment, and the “urban adapters,” which take advantage of the urban environment
45 but reside there optionally. Other species, known as "urban avoiders," have completely
46 disappeared from cities, leading to a decline in biodiversity in cities. To understand how species
47 adapt to the urban environment and how this environment impacts the survival and fitness of
48 birds, it is essential to study the underlying mechanisms at the individual scale.

49 Body condition (Meillère et al. 2015, 2017; Seress et al. 2012; Liker et al. 2008), telomeres
50 (Biard et al. 2017; Grunst et al. 2020; Ibáñez-Álamo et al. 2018; Salmón et al. 2016; Salmón et
51 al. 2017), and oxidative status (Herrera-Dueñas et al., 2017; Isaksson, 2015, 2010; Stauffer et
52 al., 2017) are good proxies of birds' health and survival and are frequently used to investigate
53 the underlying effects of urbanization on birds' physiology. Body condition reflects the
54 energetic and nutritional status of individuals and is dependent on the quantity and quality of
55 food available in the environment. The influence of this parameter on the reproductive success
56 of adults or the survival of fledglings has been widely demonstrated (Chastel, Weimerskirch,
57 and Jouventin 1995; Milenkaya et al. 2015). Telomeres are non-coding sequences at the end of
58 chromosomes that maintain genetic integrity during cell divisions and which are involved in
59 the mechanism of cells senescence and the aging of organisms. Telomeres shorten with age in

60 numerous species (e.g. Hall et al. 2004) and the attrition rate affects the lifespan of individuals
61 (Hausmann, Winkler, and Vleck 2005; Hausmann and Mauck 2008). Telomere attrition also
62 appears to be accelerated by environmental conditions (Chatelain, Drobniak, and Szulkin 2020)
63 such as the proximity of roads (Grunst et al. 2020) and metal exposure (Saulnier et al. 2020;
64 Stauffer et al. 2017), but also by intrinsic parameters such as high oxidative stress (Epel et al.
65 2004). Telomere length is therefore also representative of how individuals can cope with
66 intrinsic and extrinsic constraints and can be considered as a good proxy of individual quality
67 (Pauliny et al. 2006; Blackburn and Epel 2012). In the same way, the oxidative status represents
68 the balance between the production of reactive oxygen species (ROS) and antioxidants. Chronic
69 exposure to chemicals, pollutants such as polycyclic aromatic hydrocarbons, polychlorinated
70 biphenyls and trace metals, and other stressors such as light and noise pollution, have been
71 shown to increase ROS production (Herrera-Dueñas et al. 2017; Isaksson 2015, 2010; Stauffer
72 et al. 2017). Non-neutralized ROS can cause oxidative stress, lead to macromolecular damages
73 such as lipid peroxidation, protein carboxylation and DNA damage, and potentially affect the
74 health of birds. To maintain oxidative balance and counteract ROS, birds have endogenous and
75 exogenous antioxidants. Endogenous antioxidants are low molecular weight proteins as
76 glutathione and antioxidant enzymes as the superoxide oxide dismutase (SOD) or catalase
77 (CAT). Exogenous antioxidants correspond to antioxidants provided by food such as vitamins
78 or carotenoids and are generally in low concentration in cities (Isaksson and Andersson 2007).

79 The reported effects of the urban environment on these physiological parameters (body
80 condition, oxidative stress and telomeres) in bird populations vary greatly between studies
81 (reviewed in Sepp et al. 2018). For example, a study on adult great tits (*Parus major*) found
82 longer telomeres in urban birds than in rural ones (Salmón et al. 2017), whereas another study
83 on adult blackbirds (*Turdus merula*) showed the opposite pattern, with shorter telomeres for
84 urban birds (Ibáñez-Álamo et al. 2018). Similarly, many studies in birds have found neutral or

85 low effects of urbanization on oxidative status (Giraudeau and McGraw 2014; Salmón, Stroh,
86 et al. 2018; Isaksson 2010) or body condition (Meillère et al. 2015; Bókonyi et al. 2012)
87 compared to rural habitats, whereas some other studies have shown oxidative stress (Giraudeau
88 et al. 2015; Herrera-Dueñas et al. 2014) or nutritional stress (Liker et al. 2008) associated with
89 urban environments. Some of these physiological differences found between studies are
90 associated with divergences in reaction norm and adaptation of species to environmental
91 conditions (Salmón, Stroh, et al. 2018), but also to differences in the intensity of stressors to
92 which individuals are exposed (Biard et al. 2017; Meillère et al. 2015; Herrera-Dueñas et al.
93 2014), particularly in the cases of chemical pollution or low vegetation cover surface. In
94 addition, urban characteristics such as prey abundance or availability can change according to
95 years and the consequences are observed as variation of nestling body size and body condition
96 in great tits between years (Caizergues et al. 2021). Low temperature or many days of rain can
97 explain this low prey availability (Dawson and Bortolotti 2000; Avery and Krebs 2008),
98 emphasizing the impact of the lower quantity of invertebrates in town (Chamberlain et al. 2009).
99 These conditions result in a decrease of daily activity in adult great tits (Schlicht and
100 Kempnaers 2020), increase of energy expenditure for foraging (Cox et al. 2019) and changes
101 in thermoregulation in nestling of other birds species (Krijgsveld, Visser, and Daan 2003).
102 Studying the effects of these annual variations in the context of urbanization is interesting
103 because the urban environment can exacerbate the effects of poor weather conditions. Thus, we
104 expect more pronounced differences between the urban and rural environments in more
105 challenging years with respect to weather conditions such as cold and rain events.

106 In this study, we aimed to evaluate the effects of the urban environment on physiological
107 parameters of birds using proxies for health/quality of birds during several years to highlight
108 interannual variation of these parameters in response to changes in the environmental
109 constraints during reproduction (e.g. changes in weather conditions). A second aim was to

110 compare these effects on the physiology of adult breeders vs fledglings. For this purpose, we
111 followed an urban and a forest populations of great tits (*Parus major*) in the Eurometropole of
112 Strasbourg (Alsace, France) for 3 years (2015, 2016, and 2018) and we recorded morphological,
113 oxidative status, and telomere length measurements for both adults and fledglings. In a previous
114 study (Saulnier et al., 2022), the environmental differences (pollution, vegetation cover, and
115 food availability) between the two sites were also characterized, as well as the weather
116 conditions during breeding (the average temperature, the average daily temperature range, the
117 average daily rainfall rate and the number of rainy days). In this previous study, we highlighted
118 a lower reproductive success (clutch size, number of fledglings) in the urban environment,
119 whatever the year, due to a lower prey availability (Saulnier et al 2022). Considering these
120 previous results, we expected urban birds to be in worse conditions than rural birds. More
121 precisely, we expected higher ROS and thus higher production of reactive oxygen metabolites
122 (ROM), lower antioxidant capacity, and shorter telomere length in urban birds compared to
123 forest ones. We expected similar results for both adults and fledglings. We also predicted that
124 these urban and forest differences would appear to be more pronounced in year which was
125 characterized by unfavorable weather conditions as previously seen for reproductive success
126 (Saulnier et al. 2022).

127 **Material and methods**

128 *Site and animal sampling*

129 The study was performed in 2015, 2016, and 2018 on two populations of great tits (*Parus*
130 *major*) living in artificial nestboxes in an urban site and a forest site of the Eurometropole of
131 Strasbourg (France). The urban site (urban) consists of several spots in the town of Strasbourg
132 such as urban parks, tree-lined walkways and roads and in the residential area of La Robertsau.
133 The forest site (forest) corresponds to an alluvial forest in the vicinity of the small town of La
134 Wantzenau at 20 km North of Strasbourg city center (48.647962, 7.833667). More details about

135 nestboxes occupation and concentration of air pollutants and food availability along this
136 urbanization gradient were provided in a previous article (Saulnier et al., 2022). Briefly, results
137 showed no difference in terms of pollutants signature between sites but prey availability
138 (caterpillars and spiders) was significantly lower in the city than in the forest. Forest site was
139 mostly characterized by high vegetation around nest boxes, whereas urban site is mostly
140 characterized by non-vegetal and low-vegetal surfaces (Saulnier et al 2022).

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

149 Adults were caught in the nestbox using a trapdoor when nestlings were between 8 to 15 days
150 old to avoid nest desertion (which is likely to happen if nests are disturbed before hatchlings
151 are 8 days old) or precocious fledgling (after 15 days old). We captured 45 adults and 212
152 fledglings in 2015, 35 adults and 200 fledglings in 2016 and 48 adults and 214 fledglings in
153 2018. Fledglings were caught in the nest when they were 14-18 days old (see Table A.1 of
154 supplementary for headcount for each age group). At capture, each bird (adults and fledglings)
155 was ringed and weighed with a precision scale (± 0.1 g). Adults were sexed based on the size of
156 the black tie (larger for males) and the presence of the brood patch for females. For adults and
157 six fledglings per nest (the two oldest, two intermediates, and the two youngest), the tarsus
158 length and the head-beak length were also measured with a caliper (± 0.01 mm) and the wing
159 length with a ruler (± 0.1 mm). These morphometric measures were used to estimate the body

160 size and the body condition of fledglings (see “statistical analysis” part for more details).
161 Morphometric measures were performed by several persons, but concordance tests were
162 conducted to ensure the repeatability of measurements between them and both measured birds
163 in urban and forest sites. We also collected 50µL of blood by skin-puncture in the brachial vein
164 with heparinized capillary for physiological analyses. Samples were kept in ice during
165 fieldwork waiting for centrifugation at 4000g for 10 minutes at 4°C. Plasma and erythrocytes
166 were separated in two distinct tubes before storage at -80°C until lab analyses.

167 *Measures of physiological parameters*

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

172 The concentration of ROM (as hydroperoxides) in the birds’ plasma was measured using the
173 diacron- reactive oxygen metabolite test (d-ROM test, Diacron, Italy, Costantini. 2016),
174 adapted to 96 well plates. In each well, 4 µL of plasma is added to 200 µL of the reaction mix,
175 containing acid buffer and chromogenic substance. The plate was incubated at 37°C for 75 min.
176 The absorbance of samples at 510 nm was measured using an Infinite® 200 PRO microplate
177 reader (Tecan). The absorbance was converted to mg of H₂O₂/dL (based on Carrateli Units)
178 using the absorbance of a standard sample of known concentration following the kit instruction.
179 A total of 397 samples were measured in duplicates.

180 The OXY was measured using the OXY-Adsorbent Test (Diacron, Italy) adapted to 96 wells
181 plate. In each well, 1:100 diluted plasma was mixed with the reaction mix provided in the kit.
182 The plates were incubated for 10min at 37°C. The absorbance of samples at 510 nm was
183 measured using an Infinite® 200 PRO microplate reader (Tecan). The measured absorbance is
184 inversely proportional to the concentration of OXY in the sample. The OXY concentration of

185 the sample (in $\mu\text{mol HClO/ml}$) was obtained using the absorbance of a standard sample with a
186 known oxidizing power following kit instructions. A total of 529 samples were measured in
187 duplicates.

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

193 Relative telomere length (RTL) was measured in erythrocytes using a method previously
194 described by Criscuolo et al. (2009). Erythrocyte DNA was extracted on silica-membrane
195 columns using the kit NucleoSpin[®] Blood Quick Pure (Macherey-Nagel[®]), and the
196 concentration and purity were checked using the Nanodrop 1000 (Thermo scientific[®]), a
197 microvolume spectrometer. The RTL of each sample was measured with a quantitative
198 polymerization chain reaction (qPCR) in 96 wells plate. The RTL is a ratio (T/S) between
199 telomere repeat copy number (T) to control gene copy number (S) expressed in the same way
200 for each bird. The samples mean C_q of T and mean C_q of S (number of cycles necessary to
201 detect amplified DNA above basal signal) were used as a reference in the calculation of RTL
202 with a ratio (T/S) arbitrary set to 1 (see Criscuolo et al. 2009 for calculation). The *Parus major*
203 zinc finger protein (ZENK) gene (GenBank: EF568148) was used as the control gene.
204 Sequences of used primers are provided in appendix C of supplementary material. In both 2015
205 and 2016, the final dilution of primer was 500nM for the control gene and 100nM for telomeres.
206 The protocol in 2018 was modified and the final dilution was 500nM for the control gene and
207 200nM for telomeres. Finally, amplification cycle was performed with a final volume of 10 μL
208 per well with 2ng of DNA (2 μL), and 8 μL of reaction mix containing a GoTaq[®] qPCR
209 (Promega, Madison, USA). For the control gene, amplification started with 2 min at 95°C

210 followed by 40 cycles of 15 sec at 95°C, 30 sec at 56°C, and 1 min 30 sec at 72°C. The average
211 efficiency of amplification for the control gene and telomere was 99.8% and 100 % respectively
212 in 2015-2016; and 95.8% and 98.9% in 2018. Mean intra-individual quantification cycle Cq;
213 number of cycles necessary to detect amplified DNA above basal signal) variation was 0.91%
214 and 1.39% for the control and the telomere assay in 2015-2016 and 1.03% and 2.06% for control
215 and telomere assay in 2018. Mean inter-run Cq variation was 0.75% and 1.28% for control and
216 telomere genes respectively in 2015-2016 and 0.67% and 1.16% in 2018. A total of 436 samples
217 were measured (281 for 2015-2016 and 155 for 2018).

218 *Statistics analyses*

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

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

229 Differences of morphological (body size index and body condition) and physiological
230 parameters (ROM, OXY, RTL) were tested using linear models (function `lm`) for adults and
231 linear mixed models (function `lmer` of “`lme4`” package (Bates et al. 2015)) for fledglings and
232 type III ANOVA. We used mixed models for fledglings to take into account the non-
233 independence of fledglings from the same nest. The normality and the homoscedasticity of the
234 residuals were checked using Kolmogorov-Smirnov test and Levène test respectively. To

235 respect these conditions, the RTL was log-transformed. Moreover, to compare yearly RTL
236 measurements in spite of the dilution volume change in the protocol, we applied a
237 transformation to the data to obtain Z-scores (function “scale” in R) following the protocol
238 described by Verhulst (2019). The data were centered and reduced for each year around a mean
239 equal to 0 and a standard deviation equal to 1, thus allowing comparison of the differences in
240 distributions between sites per year without assay-dependent variations. The transformation
241 was applied to the log-transformed data.

242 In models, we tested the effect of sites (urban and forest), year (as a categorical variable), and
243 the interaction between site and year. We also included the following covariates: (i) hatching
244 date (to take into account the timing of the breeding season), (ii) fledgling number (to take into
245 account the investment in reproduction for adults and competition for young), and (iii) body
246 condition (for models on physiological parameters only). For adult birds, we also added the
247 effect of sex and the interaction between site (urban or forest) and sex. The interaction between
248 sex and year was not included due to an important collinearity (Variance Inflation Factor > 10,
249 O’Brien 2007). For fledglings, models also included the age and the brood identity as additive
250 random effects and the body size as a covariate (for models on physiological parameters only),
251 taking into account the birds’ growth during the studied period. Non-significant interactions in
252 adult and fledgling models were sequentially removed from final models. Estimates of all
253 statistical models are provided in appendix D of supplementary material.

254 **Results**

255 **Body size and condition**

256 The body size of adults was significantly different between sites and sexes (Table 1). Urban
257 breeders were significantly smaller than forest ones (Tukey, $p = 0.002$) and males were
258 significantly taller than females (Tukey, $p < 0.001$). A significant positive correlation between

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

270 Oxidative stress

271 We considered plasmatic ROM and OXY concentrations to evaluate oxidative status. Both
272 adults and fledglings showed a significant interaction between site and year for ROM plasmatic
273 concentrations (Table 1 and 2, Fig. 2). For adults, ROM concentrations were higher for urban
274 breeders than forest breeders in 2018 (Tukey, $p = 0.014$) but not in 2015 (Tukey; $p = 0.88$) nor
275 2016 (Tukey, $p = 0.26$). Also, urban breeder ROM concentrations were higher in 2018 than in
276 2016 (Tukey, $p = 0.012$) but were similar in 2018 and 2015 (Tukey, $p = 0.13$). Plasmatic ROM
277 concentration was also negatively correlated with the hatching date with later breeders having
278 lower ROM concentrations (see also Table D.1 of supplementary materials). The plasmatic
279 ROM concentration of fledglings were lower for urban birds than forest birds in 2016 (Tukey,
280 $p = 0.009$) but not in 2015 (Tukey; $p = 0.49$) nor 2018 (Tukey, $p = 0.64$). As observed for adults,
281 the urban fledglings ROM concentration were higher in 2018 than 2016 (Tukey, $p = 0.003$) but
282 not in 2015 (Tukey, $p = 0.25$). However, forest fledglings had higher ROM concentrations in
283 2018 than in 2015 (Tukey, $p = 0.01$) but not in 2016 (Tukey, $p = 0.40$). ROM concentration was

284 significantly positively correlated to the body condition of fledglings (Table 2, see also Table
285 D.2 of supplementary materials). The OXY concentration of adult birds differed significantly
286 between sites and years (Table 1 and Fig.2). Urban breeders had higher OXY concentration
287 than forest breeders (Tukey, $p=0.007$) and concentrations in 2016 were significantly lower than
288 those observed in 2015 (Tukey, $p<0.001$) and 2018 (Tukey, $p=0.004$). For fledglings, a
289 significant year difference was also observed (Table 2 and Fig.2) with the same pattern (Tukey,
290 all $p<0.013$). The lowest concentration was observed in 2016 and the highest concentration in
291 2015. A positive correlation was also observed with body size (Table 2), meaning that taller
292 fledglings had higher levels of OXY.

293 Telomere length

294 RTL of adult birds was significantly explained by the interaction between sites and years (Table
295 1, Fig. 3). In 2015 and 2018, urban birds had longer RTL than forest birds (Tukey, $p_{2015}=0.02$,
296 and $p_{2018}=0.045$), but no difference was observed in 2016 (Tukey, $p=0.15$). Difference between
297 years were observed in forest area and RTL appeared to be longer in 2016 compared to 2018
298 (Tukey, $p=0.04$). The RTL was also negatively correlated to the hatching date (Table 1). Birds
299 breeding later had shorter telomeres. However, for fledglings, none of the tested variables was
300 significant (Table 2, see also Table D.1 of supplementary).

301 Discussion

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

307 No inter-annual variations were observed for adult morphological parameters (body size and
308 body condition). Urban birds were consistently smaller than rural birds. Other studies have
309 observed a smaller size of urban birds in the same species (Caizergues, Grégoire, and
310 Charmantier 2018), and also in other species such as house sparrows (Meillère et al. 2015;
311 Seress et al. 2012) and blackbirds (Evans et al. 2009). An adaptive response to urban
312 environments is the hypothesis proposed to explain this urban small size (Meillère et al. 2015).
313 A smaller size offers better mobility, is more effective at avoiding predators (Møller 2008; Liker
314 et al. 2008), and reduces food requirements, which can be advantageous in an environment with
315 fewer prey (Gardner et al. 2011). Moreover, the body size divergence between urban versus
316 forest was not observed for fledglings in 2015 and 2016, suggesting that the lower size of urban
317 breeders found in this study would result from a selection of smaller individuals after fledgling
318 (selection on survival or access to reproduction). Some studies revealed a lower size of
319 fledglings in urban settings compared to forest great tits (Bailly et al. 2016; Biard et al. 2017;
320 Corsini et al. 2020; Caizergues et al. 2021), blue tits (Corsini et al. 2020), or house sparrows
321 (Meillère et al. 2015). However, it seems that these differences are correlated to the degree of
322 urbanization to which the birds are exposed and are more pronounced in the largest cities (Biard
323 et al. 2017; Meillère et al. 2015, 2017). Additionally, we found a smaller size of urban fledglings
324 in 2018. Variation of body size across the years is also observed in another study on the same
325 species that has been associated with a probable fluctuation of food quantity (Caizergues et al.
326 2021). Furthermore, fledglings showed a lower body condition in cities, as found in the
327 literature (Biard et al. 2017; Bailly et al. 2016; Demeyrier et al. 2016). This is probably the
328 consequence of lower availability of arthropods (caterpillars and spiders) observed in the urban
329 site in Strasbourg (Saulnier et al., 2022) and in other cities (Peach et al. 2015; Jones and Leather
330 2012). The body condition of fledglings was also negatively correlated to the fledgling number
331 whatever the site and year. In an enlarged brood, competition increases between nestlings, and

332 the provisioning capacity of the breeder is not sufficient to cover the food requirement of all
333 nestlings (Demeyrier et al. 2016), leading to the decrease of lipid reserves in fledglings (Hörak
334 et al. 2000; Burness et al. 2000). In contrast, no site differences of body condition were observed
335 for adults, as also reported in several other studies on great tits (Sepp et al. 2017). The similarity
336 of body condition in breeders suggests that only birds with sufficient body condition can
337 succeed at reproduction, and that birds with low body condition avoid or initiate and fail
338 reproduction.

339 In this study, all physiological parameters in adults varied between years, and some also varied
340 between sites. A higher plasmatic ROM concentration was measured in urban breeders in 2018,
341 suggesting that the urban environment can be considered a stressful environment. This result is
342 consistent with our prediction and elevation of hydroperoxide production suggest a higher
343 oxidative stress in urban area similarly to other studies (Herrera-Dueñas et al. 2014; Giraudeau
344 and McGraw 2014). In addition, we found consistently higher plasma OXY concentrations for
345 urban breeders compared to forest breeders. This same result has already been shown in the
346 same species (Salmón, Stroh, et al. 2018), although in most studies the opposite result was
347 observed in the great tits (Hörak et al. 2004; Isaksson et al. 2007) and house sparrows (Herrera-
348 Dueñas et al. 2014, 2017). Considering that higher OXY concentration is correlated with
349 increasing levels of corticosterone, higher plasma OXY in urban areas can result in metabolic
350 response to environmental constraints (Cohen, Hau, and Wikelski 2008). Isaksson (2020)
351 suggest that different variations of oxidative markers depends on the intensity of stressors to
352 which individuals are confronted. Observation of individuals with high plasmatic antioxidant
353 concentrations and few oxidative damage (as measured with ROM concentrations) might
354 indicate that levels of urbanization are relatively low compared to detoxification capacity of
355 birds (Isaksson 2020) and that increase of antioxidant capacity is sufficient to counteract
356 oxidative damage. Interestingly, annual variations were observed for both ROM (only in the

357 urban site) and OXY concentrations (same pattern in both sites), but these markers do not vary
358 in the same direction for a given year. Environmental parameters can variate from one year to
359 another as well as levels of stressor to which individuals are confronted (for example levels of
360 pollutants (Lévy et al. 2018) or prey availability (Wawrzyniak et al. 2020)). Higher ROM
361 concentrations and low OXY concentrations can result of acute stress related to an increase of
362 stress factor in the environment. Lower OXY concentrations result from the depletion of dietary
363 antioxidants (carotenoids or vitamin E), and the variation of ROM between years could reflect
364 to some extent variations in exogenous antioxidant availability. At the same time endogenous
365 anti-oxidant molecules as glutathione or antioxidant enzymes (SOD or the CAT) can act in the
366 process against ROS (Costantini and Verhulst 2009) and have to be considered in future
367 analyses to understand accurately the variation of oxidative stress. However, no difference of
368 these antioxidant enzymes activities is detected along an urbanization gradient in the same
369 species (Salmón et al. 2018).

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

382 The higher urban OXY concentration and the absence of ROM differences (except in 2018)
383 between sites in adults can be related to the absence of negative effect of the city on
384 physiological parameters even if reproduction is impacted (Saulnier et al. 2022). But this result
385 may also highlight a stronger selection of breeding individuals in the urban site, i.e. only the
386 best quality individuals, with high antioxidant defenses and high ability to neutralize oxidative
387 damage, could survive and succeed to reproduce. This hypothesis is supported by the results on
388 telomeres. Urban breeders had longer telomeres than forest breeders in 2015 and 2018. Higher
389 telomere length is correlated to higher survival of birds (Salmón et al. 2017; Hausmann,
390 Winkler, and Vleck 2005), a longer lifespan (Hausmann and Vleck 2002; Monaghan 2012),
391 and better fitness (Pauliny et al. 2006). Long telomeres in a constraining environment represent
392 a high investment in self-maintenance (Giraudeau, Angelier, and Sepp 2019). Telomere length
393 is negatively impacted by environmental stressors (Chatelain, Drobniak, and Szulkin 2020;
394 Grunst et al. 2020) or by intrinsic parameters such as high oxidative stress (Epel et al. 2004).
395 Thus, telomere length can be considered an indicator of bird quality in terms of health and
396 survival. The fact that fledglings have similar telomere length between urban and forest areas
397 and urban breeders have longer telomeres suggest that only good quality individuals are in the
398 capacity to allocate energy in both self-maintenance and reproduction and can survive and breed
399 in their constrained environment. Furthermore, forest sites present low environmental
400 constraints, individuals of lesser quality can also survive and successfully access reproduction,
401 lowering the RTL average of the captured adults. A part of these results is supported by the
402 work of Salmón et al. (2017), which shows that only urban nestlings with longer telomeres
403 survived and accessed to reproduction in their first year. However, the breeding selection occurs
404 also for forest birds under harsh weather constraints as in 2016, leading to an absence of
405 significant RTL difference between sites. Furthermore, since telomere size is correlated with
406 the age of individuals, we can hypothesize that longer telomeres of urban birds were due to the

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

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

423 To conclude, the inter-annual variations in telomere length is due to a greater variance between
424 forest individuals, as the urban environment seems to be more restrictive and selective. Our
425 study seems to reveal that the urban environment acts as a filter where only individuals of good
426 quality can survive and invest in their reproduction. Nevertheless, because we only captured
427 adults when nestlings were between 8 to 15 days old, captured individuals do not necessarily
428 reflect the state of health of all the urban individuals (breeders and non-breeders). Future studies
429 could test this hypothesis by comparing the health parameters of successful and unsuccessful
430 breeding birds. Moreover, the fact that we found in fledglings site differences only for some
431 parameters (small body size and body condition in urban areas but no for physiological

432 parameters) suggests that in Strasbourg, a medium-sized city, parental investment and reduced
433 brood size limit the impact of environmental constraints during growth for fledglings compared
434 to larger cities (Meillère et al. 2017). However, single-site based studies have some limits and
435 results should be taken with caution (Szulkin et al. 2020). Even if our results echoed the
436 literature, it could be relevant to reiterate the experiment on several sites. Finally, this study
437 highlights the importance of inter-annual studies to understand the impact of urbanization on
438 birds, particularly to identify underlying selection mechanisms. As oxidative responses are
439 dependent on the level of stressors for birds, it would be relevant to consider both temporal and
440 spatial environmental changes that individuals are facing in city to understand the physiological
441 responses of organisms under urban stressor, i.e. : phenotypic plasticity, adaptation or death of
442 individuals.

443 **Ethic approval**

444 This work is in accordance with the French legislation concerning the capture and the biological
445 sampling of wildlife. SM, JB and PU received ringing licenses and authorizations for blood
446 sampling from the CRBPO (National Museum of Natural History) as part of a research program
447 led by SM (PP N°673). The protocol has been approved by the French national ethical
448 committee n°35 and approved by the French ministry of research (project APAFIS# 9160-
449 2017030316182730 v2).

450 **Fundings**

451 This work was supported by ZAEU and by the “BIOECO-MET” project of the Program PEPs
452 FaiDoRa of CNRS. AS is also the recipient of a Ph.D. grant from the French Ministry of
453 Research.

454 **Data availability**

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

456 **Author's contribution**

457 **Agnès Saulnier:** Validation, Formal analysis, Investigation, Data curation, Writing- original
458 draft and Review & Editing, Visualization. **Josefa Bleu:** Validation, Formal analysis,
459 Investigation, Data curation, Writing- original draft and review & Editing, Visualization and
460 Supervision. **Anne Boos:** Methodology, Validation, Investigation, Resources, Writing- Review
461 & Editing. **Sandrine Zahn:** Methodology, Validation, Investigation, Resources, Writing-
462 Review & Editing. **Pascale Ronot:** Methodology, Validation, Investigation, Writing- Review
463 & Editing. **Islah El Masoudi:** Methodology, Validation, Investigation, Writing- Review &
464 Editing. **Emilio R. Rojas:** Investigation, Data curation, Writing- Review & Editing. **Pierre**
465 **Uhlrich:** Investigation, Data curation, Writing- Review & Editing. **Mirella Del Nero:** Writing-
466 Review & Editing, Funding acquisition. **Sylvie Massemin:** Conceptualization, Validation,
467 Formal analysis, Investigation, Resources, Data Curation, Writing - Original Draft and Review
468 & Editing, Visualization, Supervision, Project administration, Funding acquisition

469 **Acknowledgements :**

470 We would like to thank Caroline, Alice and all the students who assisted us in the field and
471 contributed to collect and analyze the data. We also thank the *Université de Strasbourg*, the
472 *Eurometropole of Strasbourg*, the associations *Société Régionale de Protection des Oiseaux*
473 (*S.R.P.O.*), *Campus Vert* and A. Gros for their authorization to install and monitor nestboxes
474 along the gradient.

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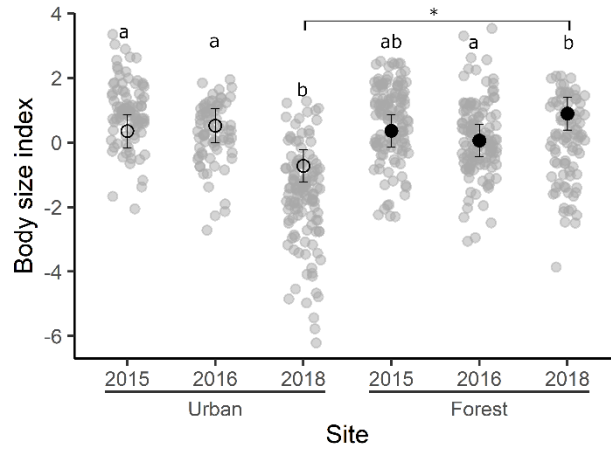
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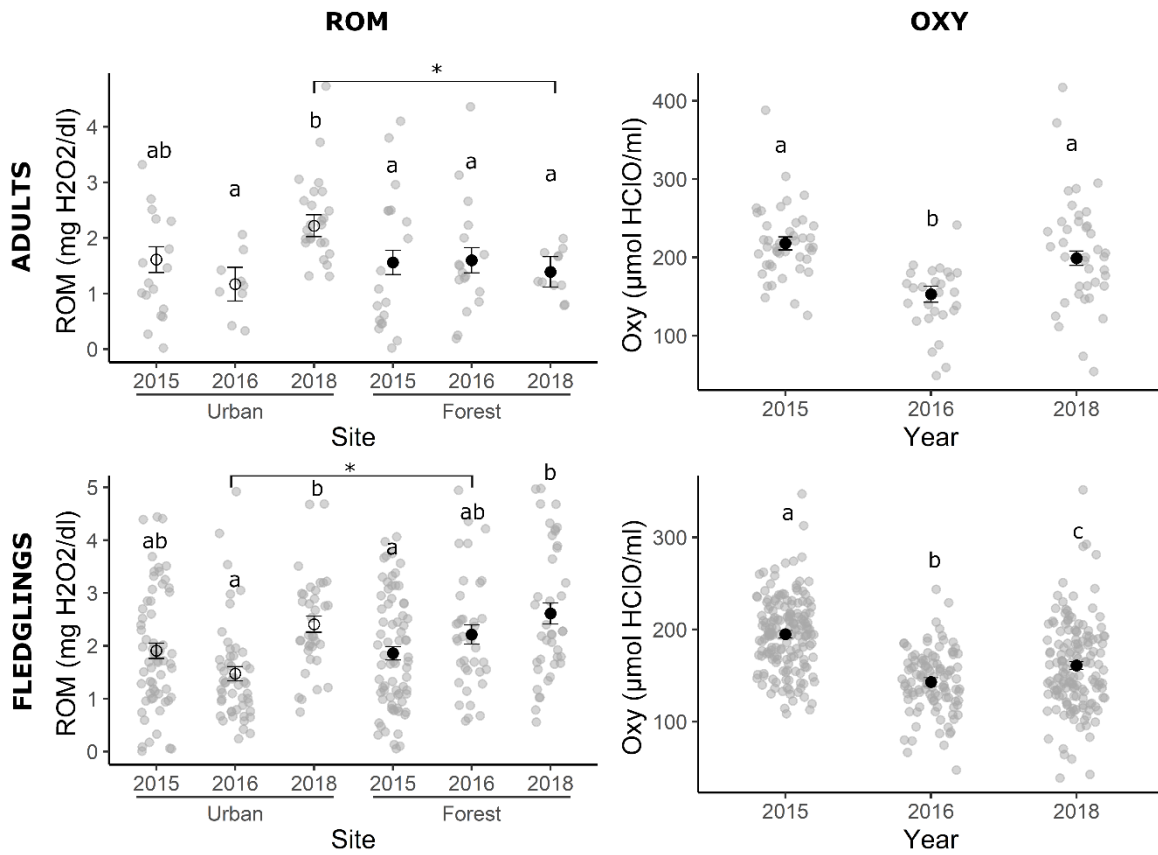
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737 Figure 1) Body size difference of great tit fledglings between urban and forest sites during three
 738 years. Differences between years are represented with different letters for a given site and
 739 differences between sites were represented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

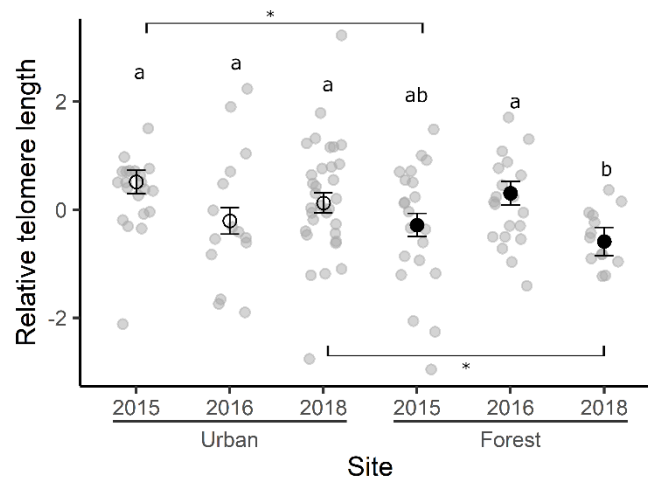
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745 Figure 2) Oxidative status difference of great tit breeders and fledglings between urban and
 746 forest sites during three years. Differences between years are represented with different letters
 747 for a given site and differences between sites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

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

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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 | .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) | | |
|------------------|-------------------|-----------|------------------|------------------------|-----------|--------------|-------------|-----------|------------------|-------------|-----------|------------------|-------------|-----------|----------|
| | <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 | 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)