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Proximity to roads, but not exposure to metal pollution, is associated with accelerated developmental telomere shortening in nestling great tits

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1 **Proximity to roads, but not exposure to metal pollution, is associated with accelerated**  
2 **developmental telomere shortening in nestling great tits**

3

4 **Telomere shortening patterns in urban nestlings**

5

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16

17 **Abstract**

18 Comprehensively understanding the factors affecting physiology and fitness in urban wildlife requires  
19 concurrently considering multiple stressors. To this end, we simultaneously assessed how metal pollution  
20 and proximity to roads affect body condition and telomere shortening between days 8 and 15 of age in  
21 nestling great tits (*Parus major*), a common urban bird. We employed a repeated-measures sampling  
22 design to compare telomere shortening and body condition between nestlings from four urban study sites  
23 south of Antwerp, Belgium, which are located at different distances from a metal pollution point source.  
24 In addition, we explored associations between metal exposure and telomere dynamics on the individual  
25 level by measuring blood concentrations of five metals/metalloids, of which lead, copper and zinc were  
26 present at concentrations above the limit of detection. To assess whether roadway-associated stressors

27 (e.g. noise and air pollution) might affect nestling condition and telomere shortening, we measured the  
28 proximity of nest boxes to roads. Metal exposure was not associated with nestling telomere length or  
29 body condition, despite elevated blood lead concentrations close to a metal pollution source (mean  $\pm$  SE =  
30  $0.270 \pm 0.095$   $\mu$ g/g wet weight at the most polluted study site), suggesting that nestlings may have some  
31 capacity to detoxify metals. However, nestlings from nest boxes near roads exhibited more telomere  
32 shortening between days 8 and 15 of age, and shorter telomeres at day 15. Nestlings in poorer condition  
33 also had shorter telomeres, but proximity to the road was unrelated to body condition. Thus, nutritional  
34 stress is unlikely to mediate the relationship between proximity to roads and telomere length. Rather,  
35 proximity to roads could have affected telomere shortening by exposing nestlings to air or noise pollution.  
36 Our study highlights that traffic-related pollution, which is implicated in human health problems, might  
37 also affect urban wildlife.

38

39 **Keywords: metal pollution; urban ecology, roads; telomeres; developmental stress; biomolecular**  
40 **aging**

41

42 **Capsule:** Exposure to metals, particularly lead, was not related to telomere dynamics in nestlings,  
43 whereas proximity to roads was associated with an increase in developmental telomere shortening.

44

## 45 **Introduction**

46 Urbanization exposes wildlife to an array of novel stressors which threaten to overwhelm organismal  
47 coping mechanisms, including ecosystem restructuring (e.g. novel predators and competitors) and  
48 multiple types pollution (Sih et al., 2011; Swaddle et al., 2015). Chemical contaminants are often an  
49 especially serious problem in urban areas, where industrial activities and contamination from historical  
50 pollution elevate exposure levels (Elliot et al., 2015; Kristensen et al., 2017). Moreover, particularly  
51 along roadways, urban wildlife is also exposed to air and noise pollution from vehicular traffic (Barber et

52 al., 2010; Sanderfoot and Holloway, 2017), and artificial light at night (Swaddle et al., 2015; Raap et al.,  
53 2017), all of which can negatively affect physiology, behavioral patterns and health status (Kight and  
54 Swaddle, 2011; Van Kempen et al., 2012; Swaddle et al., 2015; Bauerová et al., 2017). Thus, developing  
55 a comprehensive understanding of the factors affecting physiology and fitness in urban wildlife requires  
56 concurrently considering multiple stressors. To this end, we simultaneously investigated the effects of a  
57 metal pollution point source and proximity to roads on body condition and telomere dynamics in nestling  
58 urban birds. Importantly, nestlings might be particularly sensitive to urban stressors due to  
59 underdeveloped physiological coping mechanisms, and the sensitivity of developmental trajectories to  
60 stress exposure (Meillère et al., 2015; Beaugeard et al., 2018).

61 Metal pollution and proximity to roads both have demonstrated negative effects on wildlife. Metals  
62 are persistent inorganic pollutants that remain in the environment long after remedial policies have been  
63 adopted to reduce emissions (Kristensen et al., 2017), and can have serious biological effects including  
64 altering gene expression (Tchounwou et al., 2012), inducing oxidative stress (Ercal et al., 2001; Rainio  
65 and Eeva, 2010), and disrupting behavior (Gorissen et al., 2005; Grunst et al., 2018; Grunst et al., 2019a)  
66 and reproductive success (Janssens et al., 2003). Moreover, a substantial amount of research has  
67 documented declines in avian biodiversity and reproductive success near roads, which might reflect  
68 negative effects of noise, light, or air pollution, edge effects, or mortality caused by collisions (Fahrig and  
69 Rytwinski, 2009; Summers et al., 2011; Swaddle et al., 2015; Raap et al., 2017). Past research has indeed  
70 demonstrated negative behavioral and physiological effects of both noise (Brumm, 2004; Injaian et al.,  
71 2018; Kleist et al., 2018) and light pollution (Swaddle et al., 2015) on birds, and urban air pollution has  
72 been shown to cause health problems ranging from cardiovascular disease (Hoffmann et al., 2007) to  
73 cognitive aging (Tzivian et al., 2015) in humans. However, whether metal pollution and dense road  
74 networks cause developmental stress in urban wildlife, and mechanisms underlying potential  
75 developmental effects, remain poorly elucidated.

76 Accelerated telomere shortening is an underexplored avenue whereby metal pollution, and roadway-  
77 associated stressors, could affect life-history trajectories, and effects on telomeres could be particularly

78 pronounced during development. Telomeres are complexes of nucleotides and proteins that protect the  
79 ends of chromosomes from degradation, and progressively shorten with age (Blackburn, 1991) and stress  
80 exposure (Ercal et al., 2001; Epel et al., 2004; Boonekamp et al., 2014; Herborn et al., 2014). Telomere  
81 shortening can be particularly rapid in developing organisms, due to fast growth rates and underdeveloped  
82 physiological coping mechanisms (Boonekamp et al., 2014, 2017; Herborn et al., 2014; Nettle et al.,  
83 2013, 2015). Moreover, increases in telomere shortening during development can have life-long  
84 implications for physiology, behavior and fitness (Hausmann et al., 2012; Monaghan and Hausmann,  
85 2006; Boonekamp et al., 2014; Monaghan, 2014; Reichert et al., 2014; Bateson et al., 2015). Indeed,  
86 premature telomere shortening can lead to genomic instability, disease (Hausmann et al., 2005;  
87 Monaghan and Hausmann, 2006), and reduced longevity (Salomons et al., 2009; Heidinger et al., 2012;  
88 Bize et al., 2009; Barrett et al., 2013).

89 Past studies in birds suggest that the urban environment can accelerate developmental telomere  
90 shortening (Salmón et al., 2016), but which urban stressors are responsible for this effect remains unclear.  
91 Despite evidence from humans that pollutants (Zhang et al., 2013; Wong et al., 2014) including metals  
92 (Pawlas et al., 2015; Zota et al., 2015) can accelerate telomere shortening, evidence from wildlife is  
93 scarce, with only a handful of studies having addressed this issue (Blévin et al., 2016; Sletten et al., 2016;  
94 Salmón et al., 2016; Stauffer et al., 2017). Moreover, air and noise pollution have also been linked to  
95 telomere shortening in humans (Hoxha et al., 2009; Zhao et al., 2018). However, evidence for such  
96 effects in wildlife is rare, although experimental elevation of traffic noise has been demonstrated to  
97 shorten telomeres in nestlings (Meillère et al., 2015; Dorado-Correa et al., 2018). To our knowledge, how  
98 proximity to roads is related to developmental telomere shortening has not been investigated in wildlife.

99 We explored whether metal pollution and proximity roads are associated with accelerated rates of  
100 developmental telomere shortening in free-living populations of urban great tits. Great tits breed in nest  
101 boxes that shield adults and nestlings from artificial lighting (Raap et al., 2018). Thus, light pollution  
102 along roadways is unlikely to directly affect nestlings, and in a previous study, we indeed found no  
103 statistically significant effects of the artificial light environment on nestling physiology (Casasole et al.,

104 2017; Raap et al., 2017). However, noise and air pollution along roadways could directly affect  
105 nestlings, or edge effects on habitat quality (potentially mediated by artificial lighting) could lead to  
106 changes in resource availability and nestling condition. Indeed, we previously found elevated  
107 haptoglobin levels in great tit nestlings reared on noisy territories, suggesting that the noise environment  
108 can affect nestling physiology (Raap et al., 2017). In this study, we measured telomere length at two time  
109 points across the nestling period, at day 8 and 15, to monitor patterns of telomere shortening. Moreover,  
110 we measured concentrations of five metals/metalloids in the blood to explore the link between individual  
111 levels of metal exposure and telomere shortening, and monitored nestling body condition using  
112 morphological measurements. Our study grants new insights into the factors affecting developmental  
113 stress levels in urban environments.

114

## 115 **Methods**

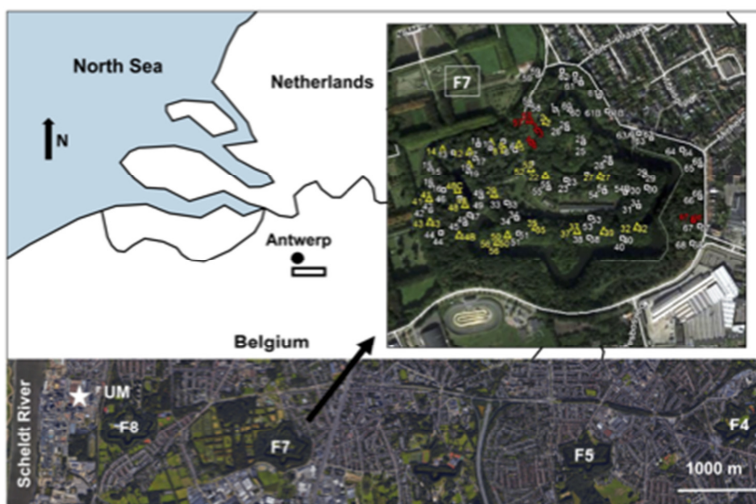
### 116 *Study system*

117 We studied four populations of great tits in the south of the city of Antwerp, Belgium. These populations  
118 are located at different distances from the Umicore smelter and metal refinery facility in Hoboken, which  
119 is a major point source for metal pollution, especially lead (Pb), cadmium (Cd) and arsenic (As) (Eens et  
120 al., 1999; Janssens et al., 2001, 2003; Grunst et al. 2018, 2019a, b). Metal deposition is high near the  
121 smelter, sometimes exceeding emission limits (or target values) set by Flemish and European authorities  
122 (VMM, 2016), and exponentially decreases with distance. The study populations are located 0–600 m  
123 (site UM), 2500 m (F7), 4000 m (F6), 5000 m (F5) and 8500 m (F4) from Umicore (Fig. 1), and are thus  
124 exposed to different levels of metal pollution. As in Grunst et al., (2018, 2019a, b), we grouped two  
125 previously separated study populations (UM and F8; see Eens et al., 1999; Janssens et al., 2001) into one  
126 site (referred to hereafter as UM), because these two sites are immediately adjacent, with some nest boxes  
127 at F8 being as close to sources of metal pollution (waste piles) as some nest boxes at UM (Fig. 1). We  
128 have studied these populations since 1999, and have accumulated substantial evidence that metal

129 pollution from Umicore causes high levels of metal exposure in great tits and other organisms, with  
130 implication for physiology, behavior and fitness (Janssens et al., 2003; Gorissen et al., 2005; Van Parys et  
131 al., 2008; Vermeulen et al., 2015). All study sites contain >30 nest boxes, allowing large sample sizes.

132 Furthermore, the study sites are all located within the urban matrix. However, within each study site,  
133 some nest boxes are in the immediate vicinity of roads, whereas others are located farther away (Grunst et  
134 al. 2019b; Fig. 1). The roads that past by study areas are all type 3 or type 4 roads, as classified by the  
135 European Open Transport Map (<http://opentransportmap.info>), and thus have similar traffic volumes.  
136 Type 4 roads are residential streets, whereas type 3 roads connect residential streets to larger  
137 thoroughfares.

138 The location of nest boxes has been mapped using GPS. Thus, we measured the distance of each nest  
139 box to the nearest road using Google Earth (version 7.1.8.3036) measurement tools. In addition, as in  
140 Grunst et al. (2018, 2019b), we measured the distance of each nest box to the Umicore facility, as an  
141 indicator of metal exposure levels. Measurements were made to the closest edge of the Umicore  
142 complex, where the smelter and waste piles are located.



143  
144 \*Satellite imagery from Google Earth Pro (version 7.1.8.3036): ©2018 Google, buildings ©2009 Stad  
145 Antwerpen, height ©2004 AGIV. 51°09'05.95" N 4°28'11.19" E.

146 **Figure 1.** Location of study sites in relationship to (top left) Antwerp, Belgium, and (bottom) the  
147 Umicore smelter facility (white star), a major source of lead and cadmium emission. UM = Umicore  
148 study site, F8 = Fort 8, F7 = Fort 7, F5 = Fort 5, and F4 = Fort 4. For this 2018 study, UM and F8 were  
149 grouped into one study area, referred to as UM. The rectangle on the upper panel shows the location of  
150 the pollution gradient. The inset (top right) shows the location of nest boxes within F7, to illustrate that  
151 nest boxes are located at varying distances from roads, and thus experience different levels of noise and  
152 air pollution from traffic. Within all of the other study sites, nest boxes are also found at varying  
153 distances from the road.

154

#### 155 *Nest monitoring and sampling nestlings*

156 Beginning in mid-March 2017 and 2018, we checked nest boxes every other day to monitor nest building,  
157 egg laying, incubation, and hatching. We only monitored first clutches. On day 8 of the nestling period,  
158 we banded all nestlings with aluminum bands, and randomly selected 3-4 focal nestlings per nest to blood  
159 sample for telomere length determination. We collected small ~10-20  $\mu$ L blood samples via brachial  
160 venipuncture using 26-gauge needles and microcapillary tubes. Blood samples were immediately ejected  
161 into a microvial containing 500  $\mu$ L of glycerol buffer (50 mM Tris-Cl, 5 mM MgCl, 0.1 mM EDTA, 40%  
162 glycerol), and stored on ice in the field. Within 5 hours, samples were flash frozen in liquid nitrogen and  
163 then stored at -80°C. On day 15, we again collected blood samples from surviving focal nestlings for  
164 telomere analysis, and also took an additional ~150  $\mu$ L blood sample for measuring blood metal levels.  
165 Larger blood samples were stored on ice in the field, centrifuged to separate red blood cells (RBCs) from  
166 plasma after return to the laboratory, and stored at -80°C. Overall, data on telomere length derived from:  
167 N (measurements, nestlings, clutches)= total: 1140, 662, 194; F4:165, 100, 26; F5: 202, 123, 38; F7: 400,  
168 227, 66; and UM: 372, 212, 64. However, samples for measuring blood metal concentrations were  
169 collected only in 2017. Thus, for the analysis involving telomere length and blood metal levels, data



170 derived from: N (measurements, nestlings, clutches) = total: 483, 273, 89; F4: 92, 49, 20; F5: 65, 37, 13;  
171 F7: 180, 101, 33; UM: 146, 86, 26.

172 On both day 8 and 15, we also measured the mass ( $\pm 0.01$  g) and tarsus length ( $\pm 0.01$  mm) of nestlings  
173 and recorded brood size. We estimated nestling body condition using the residuals of a linear regression  
174 of body mass on tarsus length (Schulte-Hostedde et al., 2005). The analyses regarding nestling condition  
175 incorporated a slightly different group of nestlings than the telomere analysis (see results section for  
176 sample sizes).

177

#### 178 *DNA extraction*

179 We extracted DNA from 200  $\mu$ L of the mixture of glycerol buffer and whole blood using the Macherey-  
180 Nagel NucleoSpin® blood kit, and used a NanoDrop spectrometer to quantify the final concentration and  
181 purity of DNA samples. Samples were of high purity, as indicated by 260/280 ratios close to the  
182 recommended value of 1.8 ( $1.851 \pm 0.059$ ), and 260/230 ratios close to the recommended value of 2.1  
183 ( $2.720 \pm 0.744$ ) (Desjardins and Conklin, 2010). We diluted DNA samples to a concentration of 1 ng/ $\mu$ L  
184 for use in qPCR.

185

#### 186 *Measuring telomere length*

187 We determined telomere length using a relative real-time qPCR assay modified from Criscuolo et al.,  
188 2009, which measures telomere length relative to a single copy reference gene. We used glyceraldehyde-  
189 3-phosphate dehydrogenase (GAPDH) as our reference gene. We amplified GAPDH using primers  
190 specifically designed and previously utilized in the great tit, GAPDH F (5'-TGTGATTTTC  
191 AATGGTGACAGC-3') and GAPDH R (5'-AGCTTGACAAAATGGTCGTTC-3') (Atema et al., 2013),  
192 and telomere sequences using the primers Tel1b (5'CGGTTTGTGGTTTGGGTTTGGGTTTGGGTTT  
193 GGGTTTG GGT-3') and Tel2b (5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-  
194 3'), which amplify telomere sequences across avian species (Criscuolo et al., 2009). For both telomeres

195 and GAPDH, we ran 15  $\mu\text{L}$  qPCR reactions containing 7.5  $\mu\text{L}$  of FastStart Essential DNA Green Master  
196 (Roche Diagnostic Corporation, Indianapolis, IN). Telomere reactions contained 0.9  $\mu\text{L}$  each of forward  
197 and reverse primers at a concentration of 10  $\mu\text{M}$  (final concentration: 600 nM), 2.325  $\mu\text{L}$  of water, 0.375  
198  $\mu\text{L}$  of 100% DMSO (2.5% of total reaction volume), and 3  $\mu\text{L}$  of 1 ng/ $\mu\text{L}$  DNA. GAPDH reactions  
199 contained 0.3  $\mu\text{L}$  each of forward and reverse primers at a concentration of 10  $\mu\text{M}$  (final concentration:  
200 200 nM), 3.9  $\mu\text{L}$  of water, and 3.0  $\mu\text{L}$  of 1 ng/ $\mu\text{L}$  DNA.

201 We performed qPCR using a LightCycler<sup>®</sup>480 System (Roche). We ran telomere and GAPDH qPCR  
202 reactions on separate 96-well plates. Telomere thermocycling conditions were: 10 min preincubation at  
203 95°C, followed by 30 cycles of 15 sec at 95°C, 30 sec at 58°C and 30 sec at 72°C. GAPDH conditions  
204 were: 10 min preincubation at 95°C, followed by 40 cycles of 15 sec at 95°C, 20 sec at 60°C, and 20 sec  
205 at 72°C. We used a ramp speed of 4.4°C/sec, and followed amplification programs with high resolution  
206 melting curve analysis.

207 Each 96-well plate contained a serial dilution (12 ng, 6 ng, 3 ng, 1.5 ng, 0.75 ng, and 0.375 ng) of  
208 DNA, run in duplicate, which was used to determine and control for the qPCR's amplification efficiency.  
209 The coefficient of determination for standard curves was high, averaging  $99.2 \pm 0.001\%$  for GAPDH  
210 reactions and  $98.1 \pm 0.002\%$  for telomere reactions. Amplification efficiency averaged  $97.6 \pm 1.3\%$  for  
211 GAPDH reactions and  $97.2 \pm 0.73\%$  for telomere reactions. Each plate also contained a golden standard  
212 reference sample, derived by pooling DNA samples from multiple individuals. We ran all samples in  
213 duplicate and in the same position on the GAPDH and telomere reaction plates. Negative controls were  
214 included on every plate, and melting curve analysis confirmed amplification of a single product.

215 We used the following formula to calculate calibrator-normalized relative telomere length (RTL;  
216 amount of telomere sequence relative to GAPDH; T/S ratio):  $RTL = E_T^{CtT(C)-CtT(S)} * E_R^{CtR(S)-CtR(C)}$ .

217 In qPCR, the  $C_T$  (crossing threshold) is the number of amplification cycles needed for products to exceed  
218 a threshold florescent signal, and varies with the amount of target DNA sequence in the sample.  $E_T$  is the  
219 efficiency of the telomere qPCR reaction (e.g. 88% efficiency = 1.88),  $CtT(S)$  is the  $C_T$  of each sample,

220 and CtT(C) is the  $C_T$  of the calibrator (golden standard).  $E_R$  is the efficiency of the GAPDH qPCR  
221 reaction, CtR(S) is the  $C_T$  of each sample, and CtR(C) is the  $C_T$  of the calibrator (Pfaffl, 2001). The mean  
222 intra-plate coefficient of variation of  $C_T$  values was 1.19% and 0.46%, and the mean inter-plate  
223 coefficient of variation was 3.77% and 0.80%, for the telomere and GAPDH reactions, respectively. The  
224 intra-plate coefficient of variation averaged 11.84% and the interplate-assay coefficient of variation for  
225 RTL, calculated from duplicate samples run on each plate, averaged 14.20%. We calculated the inter-  
226 plate coefficient of variation for RTL based on the third standard, which contained the same amount of  
227 DNA (3 ng per reaction) as the samples for individual birds.

228

#### 229 *Genetic sexing*

230 Sex might influence telomere dynamics and body condition. Thus, we also determined the sex of  
231 nestlings using molecular methods. We determined nestling sex by using the CHD-1F and CHD-1R  
232 primers to amplify a region of the CHD gene that is located on the W and Z sex chromosomes  
233 (Fridolfsson and Ellegren, 1999; Lee et al., 2010). Females were identified by two bands and males by  
234 one band when products were visualized on an agarose gel.

235

#### 236 *Measuring metal exposure*

237 We determined the concentration of five metals/metalloids: lead (Pb), cadmium (Cd), copper (Cu),  
238 arsenic (As), and zinc (Zn), in red blood cells (RBCs). We measured concentrations in RBCs, rather than  
239 whole blood, for comparability with a previous study on nestlings in these populations (Vermeulen et al.  
240 2015), and to allow other measurements to be performed using plasma samples. After blood sampling  
241 nestlings, we centrifuged whole blood samples to separate plasma from cell fraction, and measured the  
242 mass of RBCs to the nearest 0.1 mg. RBCs were later dried in a desiccator, after which the dry weights of  
243 samples were measured using a precision scale (Sartorius SE2 Ultra-micro (d=0.001 mg), Sartorius,  
244 37075 Göttingen, Germany). We then digested RBCs using a 5:1 mixture of  $HNO_3$  (69%) and  $H_2O_2$  (30%)

245 using a microwave destruction procedure (De Wit and Blust 1998). After digestion, the solution  
246 containing RBCs was weighed and then diluted to a final volume of 6 mL using deionized water (Milli-Q  
247 185, Ultrapore USA) to obtain 3-6% acid. Metal concentrations were subsequently measured using a  
248 coupled plasma-mass spectrometer (7700× ICP-MS, Agilent Technologies, Santa Clara, CA, USA) (De  
249 Wit and Blust 1998; Vermeulen et al. 2015).

250 For each batch of 108-165 samples, we included 6 reference samples and 6 blanks. We used  
251 bovine liver as a reference material (Reference material 185R, Community Bureau of Reference,  
252 Institute for Reference Material, B-2440 Geel, Belgium). The metal concentrations measured for  
253 reference samples indicated an average recovery of 106 to 133%. For all of the  
254 metals/metalloids measured, except Zn, blood levels fell below the limit of detection (LOD,  
255 <0.10 µg/L) for some samples. In these cases, we used LOD/2 in further calculations (Bervoets  
256 et al., 2004; Custer et al., 2000). All metal concentrations were calculated in µg/g on a fresh  
257 weight basis, but using dry weights yielded qualitatively similar conclusions.

258

## 259 **Statistics**

260 *Variation in metal exposure:* We conducted all statistical analysis in R version 3.4.0 (R Core Team,  
261 2017). We first assessed whether the blood metal concentrations of nestlings differed among study sites,  
262 with distance to the road, or as a function of the distance of individual nest boxes from the Umicore  
263 industrial complex (within study sites). To this end, we performed a linear mixed effect model (LMM, R  
264 package lme4; Bates et al., 2015) with Satterthwaite approximations for degrees of freedom (R package  
265 lmerTest; Kuznetsova et al., 2016). We entered log-transformed blood metal concentrations, measured at  
266 day 15, as the dependent variable. As fixed effect predictors, we entered distance to the road and the  
267 interaction between study site and distance to UM. We explored this interaction because we hypothesized  
268 that, within study sites, the distance from the industrial complex would have the strongest effect on metal

269 exposure levels at site UM. We entered clutch ID as a random effect to account for the nonindependence  
270 of nestlings from the same clutch (up to 4 nestlings were sampled per brood). To directly compare blood  
271 metal concentrations among study sites, we then eliminated distance from Umicore and the interaction  
272 term from the model, and used R package lsmeans (Lenth, 2016) to perform posthoc Tukey tests.

273

274 *Telomere dynamics and body condition:* To model telomere dynamics across the nestling period, we  
275 performed repeated measures LMMs using R packages lme4 and lmerTest. We first investigated whether  
276 telomere length across the nestling period differed among study sites or with proximity to the road. We  
277 entered log-transformed RTL as the dependent variable, with RTL at day 8 and day 15 entered for each  
278 individual. As fixed effect predictors, we entered study site, the distance of each nest box to the nearest  
279 road, nestling age (8 or 15 days), sex, brood size, nestling condition, and hatching date. To test whether  
280 telomere shortening between days 8 and 15 differed as a function of the distance to the road or study site,  
281 we entered two-way interactions between these predictor variables and nestling age. We entered nestling  
282 ID, clutch ID, nest box ID, year, and assay number as random effects. Within site UM, we constructed an  
283 equivalent model to explore whether distance to the industrial complex was related to telomere length.

284 We also explored whether individual levels of metal exposure (blood Pb, Cu, and Zn levels) were  
285 related to telomere length. Our sample size was reduced for this analysis, because nestling blood metal  
286 levels were measured only in 2017, and we did not obtain large blood samples from all individuals. We  
287 constructed a model in which we entered log-transformed RTL as the dependent variable, and blood metal  
288 levels as fixed effect predictors. This model included the same additional fixed and random effect  
289 predictors as listed above (with the exception of year), but used study site as a random effect.

290 We performed similar LMMs to assess how body condition (residual mass) varied between study sites  
291 and with metal exposure. We entered residual mass as the dependent variable, with mass at day 8 and 15  
292 entered for each individual. We entered nestling age, sex, brood size, hatching date, and study site as  
293 fixed effect predictors, and nest and individual identity as random effects. To test whether changes in  
294 nestling condition between days 8 and 15 differed as a function of the distance to the road or study site,

295 we entered two-way interactions between these predictor variables and age. In a second model, we  
296 entered individual blood metal levels as fixed effects, with study site used as a random rather than fixed  
297 effect, as done when considering telomere length.

298 We centered and standardized all continuous predictor variables, and report results (mean estimates  
299 (and SE) of regression coefficients and their statistical significance) based on global models in all cases.  
300 For models including study site, we used R package lsmeans to calculate least square means with 95%  
301 confidence intervals for each study site, and to perform post-hoc comparisons (Tukey method). The  
302 interaction between study site and age was excluded from the model when performing posthoc  
303 comparisons among sites. We found no support for an effect of nestling sex on telomere length. Thus,  
304 for models involving telomere length, we expanded our dataset to include 72 unsexed nestlings.

305

306 *Ethical statement:* This study was approved by the ethical committee of the University of Antwerp (ID  
307 2016-71) and conducted in accordance with Belgian and Flemish laws. The Belgian Royal Institute for  
308 Natural Sciences (Koninklijk Belgisch Instituut voor Natuurwetenschappen) provided banding licenses  
309 for authors and technical personnel.

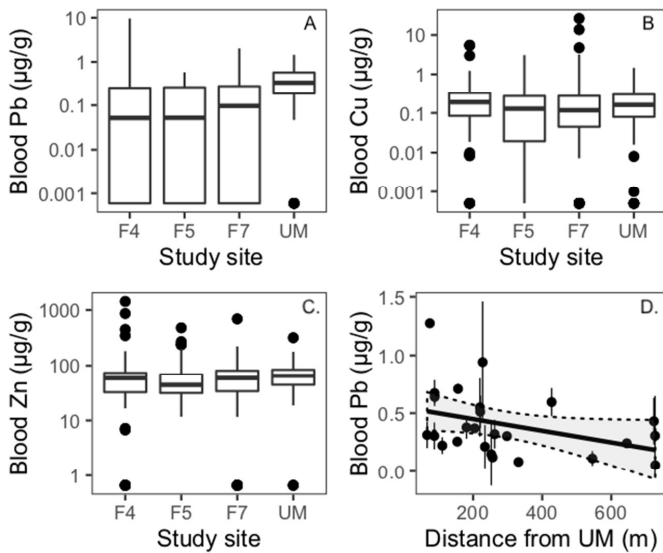
310

## 311 **Results**

### 312 *Patterns of metal exposure*

313 We found detectable levels of Pb, Cu, and Zn in the blood of great tit nestlings, but levels of Cd and As  
314 were below the LOD in almost all samples (>90%). Blood Pb and Cu levels ranged from the below the  
315 LOD to 9.62 µg/g and 26.28 µg/g, respectively, whereas blood Zn levels ranged from 0.656-1424 µg/g  
316 (see Fig. 2A-C for boxplots of blood metal levels across the study sites). Blood Pb concentrations of  
317 nestlings varied significantly among study sites ( $F_{3,81} = 5.143$ ,  $p = 0.002$ ), and there was also a significant  
318 interaction between the distance of nest boxes from the pollution source and study site in predicting blood  
319 Pb levels ( $F_{3,80} = 4.376$ ,  $p = 0.006$ ). This interaction reflected the fact that there was a negative  
320 relationship between the distance of nest boxes from the industrial complex and blood Pb concentrations

321 within site UM ( $\beta \pm SE = -0.027 \pm 0.008$ ,  $t_{86} = -3.379$ ,  $p = 0.001$ ; Fig. 2D), whereas this relationship was  
 322 non-significant at other study sites. Blood Pb concentrations were significantly higher at site UM than at  
 323 F7 ( $\beta \pm SE = 0.166 \pm 0.059$ ,  $t_{82} = 2.803$ ,  $p = 0.031$ ), F5 ( $\beta \pm SE = 0.205 \pm 0.074$ ,  $t_{85} = 2.740$ ,  $p = 0.036$ )  
 324 and F4 ( $\beta \pm SE = 0.253 \pm 0.071$ ,  $t_{82} = 3.547$ ,  $p = 0.003$ ), but did not differ significantly between F4, F5  
 325 and F7 (Fig. 2A). Thus, differences in the exposure of nestlings to Pb occur as a function of distance from  
 326 the pollution source within site UM, and on a broader spatial scale, between site UM and the other sites.



327  
 328 **Figure 2.** Variation in blood metal concentrations of great tit nestlings from the four different study sites:  
 329 blood concentrations of (A) Pb, (B) Cu and (C) Zn, and (D) blood Pb concentrations according to the  
 330 distance of nest boxes from the pollution source within site UM. Note the log scale on the y-axis for  
 331 panels A-C. In the boxplots, whiskers extend from the first and third quartiles to the highest value within  
 332  $1.5 \times IQR$  (interquartile range) and points represent outliers. Within site UM (D), points represent the  
 333 mean value for each clutch, and error bars show 95% confidence limits.  $N$  (nestlings, clutches) = 273, 89;  
 334 F4: 49, 20; F5: 37, 13; F7: 101, 33; UM: 86, 26.

335 For Cu and Zn, there were no statistically significant differences in blood concentrations among the  
336 study sites (Fig. 2B, C;  $p > 0.110$ ), and the interaction between distance to the smelter and study site was  
337 also non-significant ( $p > 0.50$ ). See Online Supplement (Table S1) for mean Pb, Cu, and Zn  
338 concentrations at the different study sites. Distance to the road was not related to blood concentrations of  
339 any metal ( $p > 0.20$ ).

340

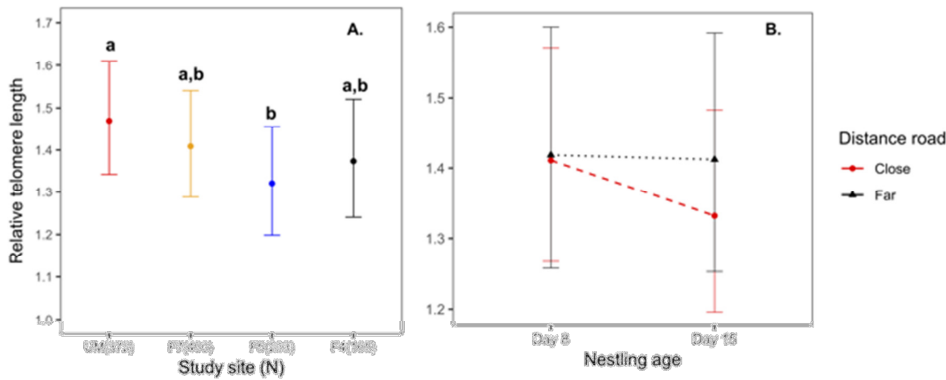
#### 341 *Telomere dynamics*

342 We found no evidence that high levels of metal pollution at site UM resulted in shorter telomere lengths  
343 in nestlings. The interaction between study site and age was statistically non-significant in the model  
344 predicting telomere length (overall  $F_{3,626} = 1.325$ ,  $p = 0.265$ ; see Table 1 for contrasts of each site to site  
345 UM), suggesting no substantial effect of study site on rates of telomere shortening between days 8 and 15  
346 of the nestling stage. Study site had a statistically significant effect on telomere length ( $F_{3,118} = 3.337$ ,  $p =$   
347  $0.022$ ; see Table 1 for contrasts of each site to site UM). However, the effect of study site was  
348 inconsistent with the hypothesized negative relationship between metal exposure and telomere length.  
349 Rather, posthoc comparisons indicated a significant difference in nestling telomere length only between  
350 sites UM and F5 ( $\beta \pm SE = 0.107 \pm 0.039$ ,  $t_{112} = 2.706$ ,  $p = 0.038$ ), with telomere length being slightly  
351 longer at UM (Fig. 3a). Other posthoc comparisons between sites were non-significant (Fig. 3a), and  
352 within site UM the distance of nest boxes from the Umicore industrial complex was not associated with  
353 telomere length ( $\beta \pm SE = 0.387 \pm 0.463$ ,  $t_{33} = 0.836$ ,  $p = 0.409$ ).

354 On the other hand, there was a statistically significant interaction between the distance of nest boxes  
355 from the road and nestling age in predicting telomere length (Table 1). This interaction indicated that  
356 distance from the road was positively related to nestling telomere length among 15-day old nestlings ( $\beta \pm$   
357  $SE = 0.047 \pm 0.018$ ,  $t_{156} = 2.523$ ,  $p = 0.012$ ,  $N = 583$ ), but not among 8-day old nestlings ( $\beta \pm SE = 0.001$   
358  $\pm 0.017$ ,  $t_{94} = 0.098$ ,  $p = 0.922$ ,  $N = 558$ ; Fig. 3b). In addition, nestlings in better body condition had  
359 longer telomeres, whereas the effects of brood size and hatching date were non-significant (Table 1).



360 When predicting RTL from blood metal concentrations, we also found no support for a relationship  
 361 between metal exposure levels and RTL ( $p > 0.15$  in all cases; see on Online Supplement Table S2 for  
 362 statistical model;  $N = 483$  observations, 273 nestlings, 89 nests).



363  
 364 **Figure 3.** Variation in relative telomere length (RTL) (a) among study sites, and (b) with distance from  
 365 the road and nestling age. Distance from the metal pollution source increases along the x-axis, from site  
 366 UM to F4, and the categories close and far from the road were created by splitting the dataset at the  
 367 median value of 91.41 m from the road. Error bars indicate 95% confidence intervals, and different letters  
 368 above bars indicate significant differences in telomere length between study sites. Plotted values are least  
 369 square means from LMMs.

370  
 371 **Table 1.** LMM predicting the telomere length of great tit nestlings at days 8 and 15 from study site,  
 372 distance to the road and covariates.

Fixed effects	$\beta \pm SE$	Df	T	p (< t )
Intercept	0.325 $\pm$ 0.050	90.7	6.482	0.114
Site F4	-0.040 $\pm$ 0.020	130.3	-1.967	0.051
Site F5	-0.026 $\pm$ 0.012	126.3	-2.161	0.032
Site F7	0.004 $\pm$ 0.007	111.6	0.624	0.534
Distance road	0.030 $\pm$ 0.014	100.4	2.102	0.038
Nestling age	-0.015 $\pm$ 0.010	643.1	-1.495	0.135
Body condition	0.020 $\pm$ 0.009	805.3	2.238	0.025
Brood size	-0.015 $\pm$ 0.012	-1.212	-1.212	0.227

Date	0.0004 ± 0.022	1003	0.020	0.983
Site F4 × age	-0.023 ± 0.015	633.3	-1.501	0.133
Site F5 × age	0.010 ± 0.009	631.0	1.163	0.245
Site F7 × age	-0.003 ± 0.005	626.2	-0.630	0.528
Dist. road × age	0.023 ± 0.010	606.0	2.118	0.034
Random effects	Variance	SD	N	
Bird ID	0.011	0.108	662	
Clutch ID	0.004	0.067	194	
Nest box	0.002	0.048	141	
Assay	0.094	0.307	71	
Year	0.001	0.039	2	
Residual	0.092	0.304	1140	

373 N (observations, nestlings, clutches): total = 1140, 662, 194; F4 = 165, 100, 26; F5 = 202, 123, 38; F7 =  
374 400, 227, 66; UM = 372, 212, 64.

375

### 376 *Body condition*

377 Neither differences in exposure to metal pollution, as determined by study site (overall  $F_{3,144} = 1.070$ ,  $p =$   
378  $0.363$ ), nor the proximity of nest boxes to roads showed a statistically significant association with nestling  
379 body condition (Table 2). Moreover, there was not a statistically significant interaction between nestling  
380 age and study site in predicting nestling body condition (overall  $F_{3,918} = 1.672$ ,  $p = 0.171$ ). Rather, only  
381 nestling age and brood size explained a substantial amount of the variation in body condition (Table 2).  
382 Moreover, we did not find substantial support for a relationship between blood Pb, Cu, or Zn  
383 concentrations and nestling body condition ( $p > 0.15$ , see Online Supplement Table S3 for statistical  
384 model).

385

386 **Table 2.** LMM predicting the residual mass of great tit nestlings at days 8 and 15.

Fixed effects	$\beta \pm SE$	Df	t	$p(< t )$
Intercept	-0.010 ± 0.505	1.00	-0.022	0.985
Site F4	-0.012 ± 0.094	161.2	-1.312	0.191
Site F5	0.023 ± 0.055	150.1	0.424	0.672
Site F7	0.040 ± 0.030	135.3	1.333	0.184
Distance road	0.043 ± 0.065	117.1	0.665	0.507

Nestling age	0.070 ± 0.031	916.8	2.214	0.027
Brood size	-0.181 ± 0.053	233.0	-3.375	<0.001
Date	-0.001 ± 0.016	997.3	-0.034	0.973
Sex <sup>a</sup>	0.076 ± 0.064	1040	1.178	0.239
Site F4 × age	0.015 ± 0.047	914.6	0.325	0.745
Site F5 × age	-0.008 ± 0.029	928.1	-0.295	0.768
Site F7 × age	0.034 ± 0.016	913.1	2.129	0.033
Dist. road × age	-0.026 ± 0.033	907.8	-0.783	0.433
<b>Random effects</b>	<b>Variance</b>	<b>SD</b>	<b>N</b>	
Bird ID	< 0.001	< 0.001	575	
Clutch ID	0.265	0.515	188	
Nest box	0.103	0.322	139	
Year	0.500	0.707	2	
Residual	0.896	0.946	1081	

387 <sup>a</sup>Males contrasted to females

388 N (observations, birds, clutches): total = 1081, 575, 188; F4 = 152, 86, 24; F5 = 185, 104, 37; F7 = 379,  
389 198, 66; UM = 365, 192, 63.

390

### 391 **Discussion**

392 Little data is available to inform which stressors most strongly influence developmental stress levels in  
393 urban wildlife. In this study, we found no evidence that metal exposure affects developmental telomere  
394 dynamics in urban great tits, at least at the levels present in nestlings near the Umicore facility. On the  
395 other hand, proximity to roads was associated with an increase in the rate of developmental telomere  
396 shortening, suggesting an effect of roadway-associated stressors on telomere dynamics.

397 The finding that metal pollution from Umicore did not significantly affect nestling telomere shortening  
398 was contrary to our predictions, and to the results of some past studies. Past studies have documented  
399 that metal exposure can elevate oxidative stress (Ercal et al., 2001; Lopes et al., 2016), which could  
400 increase rates of telomere shortening (Haussmann et al., 2012; Stauffer et al., 2017). Indeed, a previous  
401 study in great tits documented shorter telomeres in nestlings from a metal-polluted area in Finland as  
402 compared to nestlings from an unpolluted zone (Stauffer et al., 2017). The discrepancy between this  
403 previous study and our own could reflect differences in the metal pollution regime (i.e. the types and

404 amounts of metal deposited). We cannot directly compare nestling metal exposure levels between the two  
405 studies because Stauffer et al., 2017, measured hepatic metal levels, whereas we measured blood levels.  
406 However, a recent study that measured fecal metal concentrations in both study populations found that  
407 nestlings from the UM population had higher fecal concentrations of Pb, Cd and As than the Finnish  
408 population, with Pb levels being over 20 times higher at UM (Pb levels were 61.77 µg/g dry weight at  
409 UM, versus 2.86 µg/g dry weight in Finland; Ruuskanen et al., 2019). On the other hand, Cu and Ni  
410 concentrations were higher in Finland than at UM (Ruuskanen et al., 2019). Thus, although differences in  
411 the metal pollution regimes do exist, these results suggest that low levels of metal exposure at site UM do  
412 not explain the discrepancy in results. An alternative possibility is that habitat quality is lower in Finland  
413 than in our study areas, such that nestlings in Finland are unable to combat effects of metal exposure on  
414 telomeres. However, we currently have no data regarding differences in habitat quality between the Finnish  
415 study area and our study sites. Thus, future research would be needed to substantiate this hypothesis.

416 Even if under some increased physiological stress due to metal exposure, as suggested by some of our  
417 past research (Vermeulen et al., 2015), nestlings exposed to metals from the Umicore facility might be  
418 able to avoid telomere damage through compensatory mechanisms, for instance by upregulating  
419 antioxidant defenses or telomerase activity. In support of this hypothesis, we previously found that the  
420 total antioxidant capacity of nestlings was higher at UM than at study sites farther from the metal  
421 pollution source (Geens et al., 2009). In addition, populations that have experienced long-term exposure  
422 to metal pollution might also undergo evolutionary changes enabling them to better cope with metal  
423 exposure (Reid et al., 2016; Andrew et al., 2019). Indeed, by scanning the genome for single nucleotide  
424 polymorphisms, a recent study on Australian house sparrows (*Passer domesticus*) found evidence that  
425 populations near long-term mining and smelting communities have genetically adapted to Pb exposure  
426 (Andrew et al., 2019). Also consistent with a capacity of nestlings to cope with metal exposure,  
427 Turzańska-Pietras et al., 2017, found no statistically significant effect of metal exposure on body  
428 condition in whitethroat (*Sylvia communis*) nestlings, and suggested that nestlings can detoxify metals.

429       Whereas we found no evidence for an effect of metal pollution on nestling telomere dynamics, we did  
430 find an effect of proximity to roads. Specifically, nestlings from nest boxes near roads had shorter  
431 telomeres at day 15, but not day 8, of age. This suggests that nestlings from nest boxes near roads did not  
432 start development with short telomeres, for instance due to lower levels of antioxidants in eggs, or other  
433 maternal effects. Rather, in nestlings reared close to roads, faster telomere shortening appears to have  
434 occurred during the period of rapid growth between days 8 and 15.

435       The mechanism whereby proximity to roads would increase rates of developmental telomere  
436 shortening is unclear, because roads are associated with multiple stress factors, including edge effects on  
437 habitat quality, artificial light, noise pollution, and air pollution from vehicles. As previously mentioned,  
438 artificial light is unlikely to directly affect nestling great tits because nest boxes block light pollution  
439 (Raap et al., 2018). Moreover, we previously found no effect of the artificial light environment on  
440 nestling physiology (Casasole et al., 2017; Raap et al., 2017), and have also found that experimentally  
441 exposing nestlings to artificial light within the nest box does has no significant effect on telomere length  
442 (Grunst et al., 2019c). It nonetheless remains possible that artificial light along roadways could indirectly  
443 affect nestlings through an effect on adult provisioning behavior or resource availability. However,  
444 contrary to this hypothesis, proximity to roads was not associated with a decline in nestling condition,  
445 despite the effect on telomere length. This result suggests that nutritional stress due to poor habitat  
446 quality or low parental effort near roads is unlikely to explain the increased rate of telomere shortening in  
447 our study system. Rather, effects of exposure to traffic noise or air pollution might explain our results.  
448 Noise pollution (Meillère et al., 2015) and traffic-associated air pollutants such as nitrogen dioxide,  
449 carbon monoxide, black carbon, particulate matter, and polycyclic aromatic hydrocarbons (Hoxha et al.,  
450 2009; Pavanello et al., 2010) have all been associated with increased rates of telomere shortening. For  
451 instance, house sparrow nestlings experimentally exposed to traffic noise had shorter telomeres close to  
452 fledgling than did controls (Meillère et al., 2015), and exposure to air pollutants is associated with  
453 reduced telomere length in humans (Hoxha et al., 2009; Zhao et al., 2018). In line with our results, house  
454 sparrow nestlings exposed to noise pollution exhibited a decrease in telomere length in the absence of an

455 effect on body condition (Meillère et al., 2015). Air pollutants from vehicular traffic might also affect  
456 telomere length without affecting body condition if nestlings experience increases in corticosterone,  
457 oxidative stress, or respiratory problems, but are not under nutritional stress. Indeed, studies in humans  
458 have documented a wide range of health problems associated with proximity to roads and exposure to air  
459 pollution, for instance cardiovascular disease (Hoffmann et al., 2007), asthma (Morgenstern et al., 2008)  
460 and childhood cancer (Pearson et al., 2000). However, unidentified factors other than noise and air  
461 pollution could also contribute to our findings. Thus, future research will be needed to solidly determine  
462 which roadway-associated factors contribute to shortening of nestling telomere lengths.

463 High traffic volumes and a dense road network contribute to high levels of air pollution in Antwerp  
464 (Peters et al., 2014; Van Brusselen et al., 2016; Dons et al., 2018). Indeed, a recent analysis of imagery  
465 from the European Space Agency's Sentinel 5P satellite identified Antwerp as a hotspot for nitrogen  
466 dioxide pollution (Greenpeace, 2018). There has consequently been extensive concern over potential  
467 effects of air pollution on human health (Dons et al., 2018), but attention has not been focused on effects  
468 on wildlife. Although more data are needed to directly connect levels of air pollution with effects on  
469 nestlings, our results suggest that effects of poor air quality could extend to free-living animals,  
470 specifically developing nestlings. Our study sites are not located in the center of Antwerp city, but rather  
471 in an outlying suburban zone, where traffic volumes, noise pollution, and air pollution levels are still  
472 substantial, but are lower than in the city center (Van Brusselen et al., 2016). Thus, effects of roadway-  
473 associated stressors, such as air pollution and traffic noise, on urban wildlife might be even more  
474 pronounced in more highly urbanized zones.

475 In conclusion, whereas exposure to metal pollution had no statistically significant effect on telomere  
476 dynamics in nestling great tits in the present study, proximity to roads was associated with increased  
477 telomere shortening. However, higher levels of metal exposure, or metal pollution with a different  
478 composition, could increase developmental telomere shortening, as has indeed been reported by some  
479 past studies. More data are needed to disentangle the possible avenues whereby proximity to roads might  
480 affect telomere dynamics. However, body condition was not reduced near roads, suggesting that nestlings

481 were not under nutritional stress. Thus, the increase in telomere shortening near roads could be due to  
482 exposure to noise and air pollution, or to some other unidentified factor. Increased rates of telomere  
483 shortening during development reflect high levels of physiological stress, and can affect fitness in adults  
484 by leading to decreased survivorship and reproductive success (Heidinger et al., 2012; Barrett et al., 2013;  
485 Boonekamp et al., 2014). Therefore, increases in developmental telomere shortening near roads could  
486 have negative effects on individual fitness and population stability. Identifying the mechanisms whereby  
487 proximity to roads affects telomere dynamics and other aspects of health in wildlife should thus be a  
488 priority for future research.

489

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498

#### 499 **Data accessibility and Supplementary material**

500 Data associated with this paper are available in Dryad (<https://doi.org/10.5061/dryad.5x69p8d02>) and  
501 Supplementary Material (Tables S1-3) containing full statistical models for the relationship between  
502 blood metal levels, telomere length and nestling body condition is available online.

503

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