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Carotenoid- but not melanin-based plumage coloration is negatively related to metal exposure and proximity to the road in an urban songbird

1 **Carotenoid- but not melanin-based plumage coloration is negatively related to metal exposure and**

2 **proximity to the road in an urban songbird**

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4 **Coloration and anthropogenic disturbance**

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6 **Melissa L. Grunst[1*], Andrea S. Grunst[1], Rianne Pinxten[1,2], Lieven Bervoets[3], Marcel Eens[1]**

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8 **[1]Department of Biology, Behavioural Ecology and Ecophysiology Group, University of Antwerp,**

9 **2610 Wilrijk, Belgium**

10 **[2]Faculty of Social Sciences, Didactica Research group, University of Antwerp, 2000 Antwerp,**

11 **Belgium**

12 **[3] Department of Biology, Systemic Physiological and Ecotoxicological Research Group, University**

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15 **[*]Corresponding author. Email: melissa.grunst@uantwerpen.be, Telephone: +32 (0)466 16 65 74**

16

17 **ABSTRACT**

18 Rapid urbanization is a global phenomenon that is increasingly exposing organisms to novel stressors.

19 These novel stressors can affect diverse aspects of organismal function, including development of

20 condition-dependent ornaments, which play critical roles in social and sexual selection. We investigated

21 the relationship between metal pollution, proximity to roads, and carotenoid- and melanin-based plumage

22 coloration in a common songbird, the great tit (*Parus major*). We studied populations located across a

23 well-characterized metal pollution gradient and surrounded by roadway networks. Metal exposure and

24 road-associated pollution could reduce carotenoid-based pigmentation by inducing oxidative stress or

25 affecting habitat quality, but metals could also enhance melanin-based pigmentation, through effects on

26 melanogenesis and testosterone concentrations. Using a large sample size (N >500), we found that birds

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27 residing close to a point source for metals had reduced ultraviolet chroma, a component of carotenoid-

28 based pigmentation. Moreover, birds with high feather metal concentrations had lower carotenoid

29 chroma, hue, and ultraviolet chroma, with effects modified by age class. Birds residing closer to roads

30 also had lower carotenoid chroma and hue. Melanin-based pigmentation showed high between-year

31 repeatability, and no association with anthropogenic pollution. Results suggest that carotenoid-, but not

32 melanin-, based pigmentation is negatively affected by multiple anthropogenic stressors. We are the first

33 to demonstrate a negative association between roads and a plumage-based signaling trait, which could

34 have important implications for sexual signaling dynamics in urban landscapes.

35

36 **Capsule**: In a common urban song bird, carotenoid-, but not melanin-based plumage pigmentation was

37 negatively associated with individual feather metal concentrations and proximity to the road.

38

39 **Keywords**: carotenoid-based pigmentation; melanin-based pigmentation; metal pollution; roadways;

40 anthropogenic disturbance

41

42 **INTRODUCTION**

43 Urbanization is rapidly increasing globally, leading to habitat loss, fragmentation, and declines in

44 biodiversity (Seto et al., 2012; Oliveira et al., 2017; Marzloff, 2017), and introducing novel stressors,

45 including chemical (Eens et al., 1999; Zhang et al., 2015; Markiewicz et al., 2017), light (Gaston et al.,

46 2013) and noise pollution (McClure et al., 2013), and direct human disturbance (Møller, 2008; Clucas and

47 Marzluff, 2012). Anthropogenic stressors disrupt multiple aspects of biological function, and potentially

48 affect expression of condition-dependent sexually-selected traits, which signal individual quality to

49 competitors and prospective mates (Hill, 1991; Hamilton and Zuk, 1982; Andersson, 1994; Dauwe and

50 Eens, 2008; Geens et al., 2009; Giraudeau et al., 2015a, b). Indeed, sexually-selected plumage

51 pigmentation is reduced in some populations of successful urban-dwelling birds as compared to rural

52 counterparts (Hõrak et al., 2000; Giraudeau et al., 2015a; Biard et al., 2017; Sumasgutner et al., 2018), but

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53   the underlying causation remains unclear. Moreover, urban disturbance factors can also enhance

54   signaling traits, with implications for the information content of these signals. For instance, fruit-bearing

55   exotic shrubs in urban areas increase access to carotenoids in northern cardinals (*Cardinalis cardinalis*),

56   leading to a disconnect between body condition and carotenoid-based coloration (Jones et al., 2010).

57   Studies that consider multiple disturbance factors are needed to clarify the complexities of anthropogenic

58   impacts on sexual signaling traits, and to elucidate the potential for fitness ramifications.

59   We investigated the relationship between two anthropogenic disturbance factors, metal pollution and

60   proximity to roads, and plumage coloration in a common urban-dwelling songbird, the great tit (*Parus*

61   *major*). Metal exposure might have particularly strong effects on sexual ornaments because many metals

62   are powerful oxidative agents that contribute to oxidative stress (Ercal et al., 2001; Geens et al., 2009;

63   Koivula and Eeva, 2010). Oxidative stress occurs when pro-oxidant production overwhelms antioxidant

64   defenses, and has been implicated as a mechanism underlying life-history tradeoffs and sexual signaling

65   dynamics (Monaghan et al., 2009; Garratt and Brooks, 2012). Metals, and other pollutants, also affect the

66   integrity of biological communities, and contribute to food shortages and declines in health, with negative

67   effects on sexual ornaments (Eeva et al., 1998, 2003, 2008; Koivula et al., 2011). However, metal

68   exposure might also enhance expression of some ornaments, especially melanin-based pigmentation

69   (Dauwe and Eens, 2008; Chatelain et al., 2014; Chatelain et al., 2016; see below).

70   Roadways appear innocuous as compared to major sources of metal pollution. However, roads are

71   associated with edge effects, artificial light (Gaston et al., 2013; Raap et al., 2017), and noise (McClure et

72   al., 2013) and air pollution (Zhang et al., 2015; Brunekreef et al., 2017; Markiewicz et al., 2017). Roads

73   might also introduce metals into the environment (Zhang et al., 2015). Roadway-associated stressors

74   have wide-reaching deleterious effects on aquatic and terrestrial communities (Trombulak and Frissell,

75   2001; Goodwin and Shriver, 2010; Kociolek et al., 2011) and significantly affect the physiological

76   condition of individuals (Crino et al., 2011; Zhang and Batterman, 2013). Artificial light and noise along

77   roads have been implicated in affecting the timing and acoustical properties of birdsong (Brumm, 2004;

78   Kempenaers et al., 2010; Nemeth et al., 2013). However, studies assessing how proximity to roadways is

3

79   associated with the quality of plumage coloration are lacking. This is a significant oversight, given that

80   song and plumage characteristics in birds likely combine to affect mate choice dynamics.

81   Vertebrates, including great tits, utilize two prominent classes of pigmentation that act as sexual and

82   social signals, and which may be differentially affected by exposure to anthropogenic pollutants:

83   carotenoid- and melanin-based pigmentation (McGraw, 2006a, b). Carotenoids cannot be synthesized de-

84   novo by animals, and must be obtained from food (McGraw, 2006a; Isaksson and Andersson, 2007).

85   Consequently, carotenoid-based pigmentation can reflect foraging efficiency or availability of carotenoid-

86   rich food sources (Eeva et al., 1998; McGraw, 2006a; Biard et al., 2017). Carotenoids may also serve as

87   antioxidants (Alonso-Alvarez et al., 2004, 2008), although this function has been debated (Costantini and

88   Møller, 2008). If combating free radicles trades off against deposition of carotenoids in the plumage,

89   suppressed expression of carotenoid-based ornaments may reflect oxidative stress, which can be elevated

90   in polluted areas, as indeed suggested by some past research (Dauwe and Eens, 2008; Geens et al., 2009).

91   Melanin-based pigmentation can be synthesized by animals and has been proposed to be less condition-

92   dependent, and more genetically controlled, than carotenoid-based pigmentation (Roulin et al., 1998;

93   McGraw, 2006b; Roulin, 2016). However, melanin-based pigmentation is costly to produce, such that

94   only individuals in good body condition may be capable of producing dark, or large, melanin-based

95   pigment patches (Griffith et al., 2006; McGraw, 2006b; Hegyi et al., 2007, 2008; Roulin, 2016).

96   Consequently, declines in health status associated with pollution exposure could negatively affect the

97   expression of melanin-based traits (Pacyna et al., 2018). Moreover, melanin can sequester metal ions,

98   facilitating detoxification and favoring melanic phenotypes in polluted areas (Dauwe et al., 2003;

99   Chatelain et al., 2014, 2016; Pacyna et al., 2018). Certain metals may also increase circulating

100   testosterone, which could positively affect melanic traits, and metals such as calcium and zinc may serve

101   as micronutrients that support melanogenesis (Laskey and Phelps, 1991; Dauwe and Eens, 2008). Thus,

102   individuals contaminated with metals could express enhanced melanin-based signals, and increased

103   mating success.

104   We examined how exposure to metal pollution and proximity to roads are related to carotenoid- and

105   melanin-based pigmentation by sampling great tits from five populations located across a metal pollution

106   gradient in Antwerp, Belgium. In past studies, we found that great tits at sites closer to a metal pollution

107   point source have reduced carotenoid- and increased melanin-based pigmentation (Dauwe and Eens,

108   2008; Geens et al., 2009). Here, we used a large sample of blood and feather metal concentrations to

109   analyze how individual levels of metal exposure are associated with pigmentation. Moreover, we

110   simultaneously assessed the relationship between proximity to the road and pigmentation, and using birds

111   captured across multiple years, determined the repeatability of pigmentation traits. Repeatability offers

112   insight into the extent to which traits are stable attributes of individuals, which might have a genetic basis

113   (Dingemanse et al., 2002).   In a world increasingly urbanized and bisected by roads, our study offers

114   valuable insight into how complex pollution regimes affect animal populations, and may help guide

115   conservation policies.

116

117   **METHODS**

118   **Study system**: Our populations are located along a pollution gradient arising from the Umicore smelter

119   and metal refinery facility in Hoboken (southwest of Antwerp), which is a major point source for metals,

120   including lead (Pb), cadmium (Cd) and arsenic (As) (Eens et al., 1999; Janssens et al., 2002; Van Parys et

121   al., 2008; Vermeulen et al., 2015). Metal deposition is high near the smelter, sometimes exceeding

122   emission limits and exponentially decreases with distance, with the fastest decrease in contamination

123   levels occurring in close proximity to the smelter (VMM, 2016). The populations are located 0–600 m

124   (site UM), 2500 m (F7), 4000 m (F6), 5000 m (F5) and 8500 m (F4) from Umicore (Grunst et al., 2018,

125   2019a, b). Within study sites, nest boxes also differ in proximity to the road (Grunst et al., 2019a).

126   Locations of nest boxes were previously measured with a GPS and mapped into Google Earth (version

127   7.1.8.3036). Thus, we measured the distance of each nest box from the road and from the UM smelter

128   facility using Google Earth's vector measurement tool. The Behavioral Ecology and Ecophysiology

129   Group at the University of Antwerp has studied these populations since 1999, and has documented

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130   diverse effects of metal pollution on plumage coloration (Dauwe and Eens, 2008; Geens et al., 2009),

131   reproductive success (Janssens et al., 2003a), body condition (Janssens et al., 2003b), immunity

132   (Vermeulen et al., 2015), and behavior (Grunst et al., 2018, 2019a, b). Metal levels in great tits are

133   among the highest reported in songbirds (Janssens et al., 2002; Dauwe et al., 2002, 2004, 2005). All sites

134   contain >30 nest boxes, allowing large sample sizes, and are similar in habitat type and nest box density

135   (Eens et al., 1999; Janssens et al., 2002; Geens et al., 2009).

136

137   **Field sampling**: We captured most birds by checking nest boxes at night (574/624 observations, some

138   birds captured multiple times) from November to March, 2017 and 2018. We took standard body

139   measurements, and calculated body condition as the residuals of a regression predicting body mass ($\pm$

140   0.01 g) from tarsus length ($\pm$ 0.01 mm) (Schulte-Hostedde et al., 2005). We obtained ~200 µl of blood

141   from the brachial vein using 25-gauge needles and microcuvettes (N total (UM, F7, F6, F5, F4): 2017:

142   276 (75, 58, 82, 40, 21); 2018: 199 (48, 61, 28, 27, 35); both years: 87), removed the left, second-to-

143   outermost tail feather for use in determining feather metal concentrations (N total (UM, F7, F6, F5, F4):

144   2017 only: 250 (62, 48, 82, 25, 33)), and collected 10-20 breast feathers for use in measuring carotenoid-

145   based coloration (N total (UM, F7, F6, F5, F4): 2017: 295(76, 58, 82, 40, 39); 2018: 328(49, 63, 153, 27,

146   36); both years: 101). To measure the size of the melanin-based breast patch, we obtained three pictures

147   of each individual's breast using a Nikon Coolpix S65100 digital camera held 20 cm above the bird (N

148   total (UM, F7, F6, F5, F4): 2017: 290 (75, 57, 81, 40, 37); 2018: 298(48, 50, 144, 27, 29); both years:

149   90). We held the bird firmly by the legs and beak, and extended on its back against a one-half centimeter

150   grid (Fig. S1). Metal measurements were not obtained from all blood and feather samples, and contour

151   feathers and photographs were missing for some individuals. Thus, we report sample sizes associated

152   with all statistical tests.

153

154   **Measuring sexual coloration**: We used an Ocean Optics USB4000 spectrophotometer with a deuterium-

155   halogen light source (DH-2000-BAL; Ocean Optic; the Netherlands) and Spectra Suite software to

156   measure carotenoid reflectance spectra. We fitted the tip of the spectrophotometer probe with a rubber

157   sheath to exclude external light and standardize the measurement distance. We overlapped 10 breast

158   feathers on a black felt background (Quesada and Senar, 2006; Dauwe and Eens, 2008; Grunst et al.,

159   2014a, b, c), and obtained and averaged three spectra measurements per individual. Reflectance readings

160   were taken at 0.2 nm intervals between 300 and 700 nm. We held the spectrophotometer probe at an

161   angle of 90° when illuminating feathers, repositioned the probe between measurements, and standardized

162   measurements with white and black reflectance readings. Carotenoid pigmentation displays a bimodal

163   reflectance spectrum, with reflectance peaks for ultraviolet and yellow (or red) light (Andersson and

164   Prager, 2006). The carotenoid coloration of great tits derives predominantly from lutein, with some

165   contribution of zeaxanthin (Partali et al., 1987). To describe variance in carotenoid reflectance spectra,

166   we calculated carotenoid chroma, ultraviolet (UV) chroma, average reflectance, and the slope of the

167   reflectance curve between 450 and 520 nm (see Table S1 for formulas). Carotenoid chroma reflects

168   saturation of coloration in yellow, visible wavelengths, and feather carotenoid content. UV chroma

169   reflects saturation of coloration around the UV peak of reflectance, and is affected by carotenoid content

170   and feather microstructure. Average reflectance is a measurement of overall feather brightness, and the

171   slope of the reflectance curve between 450 and 520 nm is a measurement of hue (i.e. more orange versus

172   yellow) (Saks et al., 2003; Andersson and Prager, 2006; Shawkey et al., 2006; Peters et al., 2007; Biard et

173   al., 2017). Although the variables describing carotenoid-based coloration variables are somewhat

174   intercorrelated, we analyzed variation in these variables separately, because they can convey distinct

175   information about individuals (Galván, 2010; Jacot et al., 2010). Measurement repeatability, was

176   moderate to high for carotenoid chroma ($r \pm$ SE [95% CI] = 0.744 $\pm$ 0.015 [0.713, 0.769], $p < 0.001$), UV

177   chroma ($r = 0.788 \pm 0.013$ [0.759, 0.810], $p < 0.001$), average reflectance ($r = 0.526 \pm 0.022$ [0.481,

178   0.567], $p < 0.001$), and hue ($r = 0.786 \pm 0.013$ [0.761, 0.811], $p < 0.001$)

179   We used the threshold color plugin in Image J (Parker et al., 2003; Grunst et al., 2014a, b) to measure

180   the area of the melanin-based breast stripe in a rectangle extending 4 cm from the lower side of the breast

181   stripe and encompassing the entire width (Fig. S1; similar to Nicolaus et al., 2016). We calculated

182   melanin coverage in three photographs per individual, and averaged measurements to obtain a final value.

183   Repeatability of this method was high ($r \pm$ SE [95% CI] = 0.963 ± 0.002 [0.958, 0.968], p < 0.001).

184

185   **Preparation of blood and feather samples**: We centrifuged blood to separate plasma from red blood

186   cells (RBCs), measured the wet weight of RBCs (±0.0001 g; mean ± SE: 20.82 ± 0.358 mg), and stored

187   RBCs at -80°C within 2 h. RBCs were later dried in an oven at 60°C for 24 h, cooled in a desiccator, and

188   weighted using a precision scale (Sartorius SE2 Ultra-micro (d=0.001 mg), Sartorius, 37075 Göttingen,

189   Germany; mean ± SE: 7.861 ± 0.227 mg). We digested RBCs using a 5:1 mixture (500 µL and 100 µL)

190   of HNO_3 (69%) and H_2O_2 (30%) using a microwave destruction procedure. After digestion, the solution

191   was weighed to accurately determine the volume, and diluted to 3-6% acid (final volume of 6 mL) using

192   deionized water (Milli-Q 185, Ultrapore USA).

193   We stored feathers in paper envelops in a dry and dark location. We removed external bound metals

194   by moving the feather through deionized water (MilliQ 185, Ultrapore USA) and acetone alternately three

195   times with a pincer. We ended the washing by spraying with deionized water while holding the feather

196   vertical to facilitate removal of external contamination in a consistent fashion. For processing, we placed

197   feathers individually in clean vials. As for blood, feathers were dried in an oven at 60°C for 24 h, cooled

198   in a desiccator, and weighed on a precision scale (mean ± SE: 7.542 ± 0.084 mg). Feathers were digested

199   using the procedure described for blood, and diluted to a final volume of 4 mL using deionized water.

200

201   **Metal measurements**: We measured concentrations of five metals (Pb, Cd, Cu, As, Zn) in blood and

202   feather samples using a coupled plasma-mass spectrometer (7700× ICP-MS, Agilent Technologies, Santa

203   Clara, CA, USA) (De Wit and Blust 1998; Vermeulen et al. 2015) (De Wit and Blust, 1998; Vermeulen et

204 al., 2015). For each batch of samples, we included 3-6 reference samples and 3-6 blanks. For blood, we

205 used bovine liver as reference material (Reference material 185R, Community Bureau of Reference,

206 Institute for Reference Material, B-2440 Geel, Belgium). For feathers, we used human hair (Reference

207 material 397, Community Bureau of Reference, Institute for Reference Materials and Measurements, B-

208 2440 Geel, Belgium) and mussel tissue (Standard reference material 2976, US department of Commerce,

209 Gaithersburg, MD 20899) as reference materials. Recovery levels averaged 106 to 133% for the blood

210 assay. For the feather assay, recovery levels were 79 -110 % for hair and 97 - 112 % for mussel tissue.

211   When the metal concentration fell below the limit of detection (LOD; 0.1 µg/L), we used LOD/2 in

212 further calculations (Custer et al., 2000; Bervoets et al., 2004). We calculated blood metal concentrations

213 (µg/g) on a fresh weight basis to facilitate comparability to Vermeulen et al., 2015, and because

214 microvials in which blood samples were stored might have changed weight in the oven, introducing

215 uncertainty into dry weights. For feathers, we expressed concentrations (µg/g) on a dry weight basis.

216

217 **Statistical analyses**: We completed statistical analyses in R 3.4.1 (R Core Team, 2017). Feather metal

218 concentrations were highly correlated (*r* as high as 0.96). Thus, we performed a principal components

219 analysis (PCA; function prcomp) to describe variation in this data. Blood metal concentrations were not

220 highly correlated, so we used blood concentrations of each metal as predictor variables.

221   We examined how blood and feather metal levels differed among study sites and with distance from

222 the road using linear models for feathers (LMs, feather metals were only measured in 2017) and linear

223 mixed effect models for blood (LMMs; R package lme4; Bates et al., 2015). We included site and

224 distance to the road as fixed effect predictors, and age, sex and capture date as covariates. Individual ID

225 and year were entered as random effects in the LMMs. We performed separate models for each metal

226 detectable in the blood (log transformed), and feather metal PC1 and PC2. We used an equivalent

227 approach (LMMs) to examine how body condition varied between sites and with distance to the road.

228   We then used LMMs to explore whether carotenoid- (carotenoid and UV chroma squared) or melanin-

229 based coloration (square-root transformed) differed between study sites or with proximity to the road. We

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230   predicted the coloration variables from the two-way interactions between site, distance from the road, age

231   (yearling versus older), and sex, with capture date and body condition as covariates and individual ID and

232   year as random effects. We also constructed a model within site UM in which we predicted coloration

233   variables from the distance between individual nest boxes and the smelter. Within site UM, metal

234   exposure is highly dependent on proximity to the UM facility, whereas this is not the case at the other

235   sites, because emissions from the facility exponentially decrease with distance.

236   We constructed separate LMMs to predict coloration variables from individual blood and feather metal

237   concentrations. In the blood model, we tested the two-way interactions between the concentration of each

238   metal, age and sex, with capture date, body condition, and distance to the road as covariates. The feather

239   model was equivalent, but used feather metal PC1 and PC2. We included individual ID, year, and study

240   site as random effects in the blood model, and study site as a random effect in the feather model.

241   For LMMs, we estimated p-values based on Sattherwaithe approximations for degrees of freedom (R

242   package lmerTest; Kuznetsova et al., 2016). We used backwards stepwise reduction to remove non-

243   significant (α = 0.05) fixed effects. Posthoc comparisons were conducted via the Tukey method (R-

244   package lsmeans; Lenth, 2016). We also assessed across-year repeatability of coloration variables using

245   R package rptR, which estimates repeatability based on variance components extracted from mixed

246   effects models (Stoffel et al., 2017). We retained age and sex in the LMMs when calculating

247   repeatability.

248

249   **Ethical statement**: This study was approved by the ethical committee of the University of Antwerp (ID

250   2016-71) and conducted in accordance with Belgian and Flemish laws. We made all possible efforts to

251   minimize stress experienced by birds. The Belgian Royal Institute for Natural Sciences provided banding

252   licenses for authors and technical personnel.

253

254   **RESULTS**

255   **Variation in metal exposure among study sites and with distance from the road**

256 **Blood metal**: Blood Pb concentrations were higher at UM than at all other sites (Table 1a; $p < 0.001$),

257 higher at F6 than at other sites, excluding UM, (Table 1a; $p < 0.001$), higher at F4 than at F5 (Table 1; $p =$

258 0.002), and similar between F4 and F7 (Table 1a, $p = 0.391$). Blood Cu concentrations did not differ

259 between sites (Table 1a; $p > 0.15$). Blood Zn concentrations were lower at F6 than at all other sites

260 (Table 1a; $p < 0.001$). There were no other site differences (Table 1a; $p > 0.70$). Distance to the road was

261 not associated with blood metal levels, and there was no effect of age, sex, capture date, or interaction

262 terms ($p > 0.15$). Cadmium and As were not detectable in the blood (< 0.05 µg/g in almost all samples).

263 **Feather metal**: From the feather metal PCA, we extracted two principal components (PCs), which

264 explained 95.26% of the variance. All metals, especially Pb, Cd, Cu and As, loaded positively onto PC1

265 (Fig. S2; Table S2). Zinc loaded positively, whereas the other metals loaded negatively, onto PC2 (Fig.

266 S2; Table S2). PC2 had an eigenvalue < 1, but was retained to explain >85% of the variance, and to

267 include a PC with a strong association with Zn.

268   Feather Pb, Cd, Cu and As concentrations were higher at UM than at other sites (Table 1b). Feather

269 Pb, Cd and As were also elevated at F7, relative to at F4, F5, and F6. Feather Zn did not vary among sites

270 (Table 1b). Feather PC1 was higher at UM than at other sites ($p < 0.001$), but did not differ among other

271 sites. Feather PC2 did not differ between sites ($p > 0.050$).

272   Birds residing farther from the road had lower feather Cu ($\beta = -0.092 \pm 0.024$, $t_{254} = -3.73$, $p < 0.001$)

273 and Zn ($\beta = -0.052 \pm 0.018$, $t_{254} = -2.85$, $p = 0.004$), and tended to have lower feather Pb ($\beta = -0.069 \pm$

274 0.037, $t_{254} = -1.84$, $p = 0.067$), and As ($\beta = -0.094 \pm 0.051$, $t_{254} = -1.83$, $p = 0.067$). There also tended to

275 be negative relationships between feather PC1 ($\beta = -0.155 \pm 0.082$, $t_{255} = -1.89$, $p = 0.060$) and PC2 ($\beta = -$

276 0.111 ± 0.060, $t_{127} = -1.84$, $p = 0.068$) and distance from the road.

277

278

11

279 **Table 1**. Blood metal and (a) feather metal (b) concentrations (µg/g; mean ± SE) from great tits residing

280 at study sites located across a pollution gradient in Antwerp, Belgium. Measurements were taken in 2017

281 (blood and feathers) and 2018 (blood only).

	Pb	Cu	Zn	Cd	As
(a) Blood metal concentrations					
UM	0.777 ± 0.214^a	0.014 ± 0.016^a	54.9 ± 6.10^a		
F7	$0.021 \pm 0.006^{c,d}$	0.009 ± 0.010^a	59.1 ± 9.66^a		
F6	0.161 ± 0.048^b	0.009 ± 0.010^a	53.2 ± 7.83^b		
F5	0.009 ± 0.003^d	0.022 ± 0.027^a	23.0 ± 2.85^a		
F4	0.037 ± 0.013^c	0.025 ± 0.030^a	47.3 ± 5.34^a		
(b) Feather metal concentrations					
UM	120 ± 8.47^a	28.8 ± 1.40^a	304 ± 10.9^a	10.2 ± 0.770^a	5.07 ± 0.510^a
F7	11.6 ± 0.890^b	9.46 ± 0.500^b	262 ± 10.2^b	1.12 ± 0.090^b	0.890 ± 0.090^b
F6	7.12 ± 0.430^c	8.51 ± 0.340^b	266 ± 7.84^b	0.670 ± 0.040^c	0.180 ± 0.010^d
F5	7.96 ± 0.820^c	8.14 ± 0.550^b	$271 \pm 13.5^{a,b}$	$0.540 \pm 0.060^{c,d}$	0.210 ± 0.030^d
F4	6.83 ± 0.640^c	9.08 ± 0.560^b	$275 \pm 12.4^{a,b}$	0.410 ± 0.040^d	0.360 ± 0.040^c

282 *Different letters following feather metal values indicate significant differences between sites.

283 N (blood, feathers) = (123, 62) at UM, (117, 48) at F7, (96, 82) at F6, (65, 25) at F5, (51, 33) at F4

284

285 **Variation in coloration across study sites and with distance from a road**

286 *Carotenoid-based coloration*: Carotenoid chroma ($F_{4, 458} = 0.414$, p = 0.799; Fig. 1a) and hue ($F_{4, 481} =$

287 2.075, p = 0.083; Fig. 1b) did not differ between study sites, but differences were present for UV chroma

288 ($F_{4, 452} = 4.487$, p = 0.001; Fig. 1c) and average reflectance ($F_{4, 462} = 8.28$, p < 0.001; Fig. 1d). Consistent

289 with an effect of metal exposure from the smelter, UV chroma was lower at UM than at F4, F5, and F7,

290 but not F6 (Fig. 1c; Table S3), and similar between other sites (p > 0.078). Within site UM, distance from

291 the smelter was positively related to UV chroma ($\beta = 0.019 \pm 0.009$, $t_{119} = 2.09$, p = 0.039, N = 125

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292 observations, 104 individuals). Site differences in average reflectance were inconsistent with an effect of

293 metal exposure from the smelter. Rather, birds at F6 had lower reflectance than birds at UM, F4, and F5,

294 and there were no other site differences (Fig. 1c; Table S4).

295 Birds found farther from the road had higher carotenoid chroma ($\beta = 0.067 \pm 0.006$, $t_{618} = 9.76$, $p <$

296 0.001; Fig. 2). In addition, males ($\beta = 0.022 \pm 0.008$, $t_{286} = 2.70$, $p = 0.007$, N = 335 observations, 284

297 individuals), but not females ($\beta = 0.0009 \pm 0.008$, $t_{259} = -0.111$, $p = 0.911$, N = 288 observations, 241

298 individuals), found farther from the road had higher carotenoid hue. Average reflectance was lower

299 among yearlings ($\beta = -1.15 \pm 0.377$, $t_{219} = -3.05$, $p < 0.001$, N = 228 birds), but not among older birds (β

300 $= -0.003 \pm 0.315$, $t_{376} = -0.01$, $p = 0. 992$, N = 385 observations, 316 birds), found farther from the road.

301 UV chroma did not vary with distance to the road ($\beta = 0.006 \pm 0.004$, $t_{507} = 1.48$, $p = 0.139$).

302 Carotenoid chroma, UV chroma and hue were higher in males and older birds (Tables S3, S5, S6),

303 whereas average reflectance was higher in males and yearlings (Table S4). Birds captured earlier in the

304 season had higher UV chroma and carotenoid hue (Tables S3, S6). Capture date had no effect on

305 carotenoid chroma ($\beta = -0.004 \pm 0.004$, $t_{612} = -1.20$, $p = 0.229$) or reflectance ($\beta = -0.106 \pm 0.254$, $t_{582} = -$

306 0.417, $p = 0.677$). Reflectance was positively related (Table S4), and hue was marginally positively

307 related ($\beta = 0.014 \pm 0.008$, $t_{586} = 1.87$, $p = 0.063$), to body condition. Carotenoid ($\beta = 0.002 \pm 0.005$, $t_{567} =$

308 0.36, $p = 0.721$) and UV chroma ($\beta = 0.001 \pm 0.005$, $t_{561} = 0.177$, $p = 0.860$) were unrelated to body

309 condition. Unless otherwise stated,

310 interaction terms had no effect on

311 pigmentation variables ($p > 0.10$).
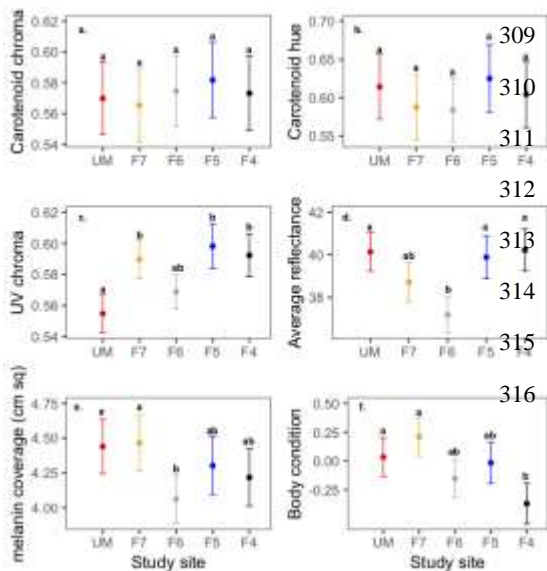
312 **Figure 1**. Variation in (a) carotenoid

313 chroma, (b) carotenoid hue, (c) UV

314 chroma, (d) average reflectance, (e)

315 melanin coverage and (f) body condition

316 across the study sites. Different letters

317   above bars denote significant differences. Values are least-square means adjusted for significant

318   covariates. Bars denote standard error. N(carotenoid, melanin, condition) = (125, 123, 124) at UM, (121,

319   107, 121) at F7, (236, 226, 227) at F6, (67, 67, 67) at F5, (75, 66, 75) at F4.

320
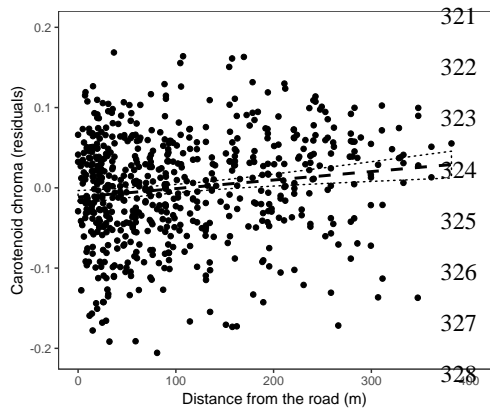
321 **Figure 2**. Carotenoid chroma increased with

322 distance from the road. Values for carotenoid

323 chroma are residuals adjusted for the effect of

324 significant covariates.

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329

330   *Melanin-based coloration*: Melanin coverage varied with study site ($F_{4, 456} = 5.198$, $p < 0.001$; Fig. 1e),

331   but not distance to the road ($\beta = -0.014 \pm 0.048$, $t_{524} = -1.17$, $p = 0.242$). Variation between sites was

332   inconsistent with an effect of smelter-related metal exposure. Rather, the only site differences were that

333   birds at F6 had lower melanin coverage than birds at UM and F7 (Fig. 1e; Table S7). Males and birds

334   captured later had larger breast stripes (Table S7). Melanin coverage was unrelated to age ($\beta = -0.027 \pm$

335   0.022, $t_{560} = -1.24$, $p = 0.215$), condition ($\beta = -0.013 \pm 0.013$, $t_{564} = -1.01$, $p = 0.315$), or interactions ($p >$

336   0.10).

337

338   **Variation in coloration with respect to individual-level metal measurements**

339   Blood metal levels were unrelated to pigmentation traits (Table S8), but carotenoid chroma, UV chroma

340   and hue were related feather metal concentrations (Fig. 3; Table S9). There was a negative relationship

341   between carotenoid chroma and hue and feather metal PC1 among older birds (carotenoid chroma: $\beta = -$

14

342   0.010 ± 0.004, $t_6 = -2.94$, p = 0.026, N = 190; hue: $\beta = -0.015 \pm 0.006$, $t_{19} = -2.33$, p = 0.030, N = 182; Fig

343   3a, c), but not yearlings (carotenoid chroma: $\beta = 0.005 \pm 0.004$, $t_9 = 1.14$, p = 0.282, N = 70; hue: $\beta =$

344   0.009 ± 0.008, $t_{63} = 1.24$, p = 0.220, N = 69; Fig. 3a, c). Conversely, there was a stronger negative

345   relationship with feather metal PC2 in yearlings (carotenoid chroma: $\beta = -0.048 \pm 0.014$, $t_{64} = -3.41$, p =

346   0.001; hue: $\beta = -0.081 \pm 0.024$, $t_{63} = -3.28$, p = 0.001; Fig. 3b, d) than in older birds (carotenoid chroma: β

347     $= -0.004 \pm 0.004$, $t_{184} = -0.734$, p = 0.464; hue: $\beta = -0.010 \pm 0.008$, $t_{176} = -1.24$, p = 0.216; Fig. 3b, d).

348   Bird captured farther from the road had higher carotenoid chroma and hue, and birds in better condition

349   had higher hue (Table S9). Birds with higher feather metal PC1 scores had lower UV chroma across age

350   classes (Fig. 3e). High feather metal PC2 scores were associated with lower UV chroma only in yearlings

351   ($\beta = -0.059 \pm 0.019$, $t_{66} = -3.08$, p = 0.003, N = 70), and not in older birds ($\beta = -0.004 \pm 0.005$, $t_{186} = -$

352   0.733, p = 0.464, N = 190) (Fig. 3f). We found no relationship between average reflectance or the area of

353   the melanin-based breast stripe and feather metal PC1 or PC2 (p > 0.20).

354

355   **Repeatability**: Carotenoid chroma (r $\pm$ SE [95% CI] = 0.048 ± 0.079 [0, 0.262], p = 0.341, $N = 626$

356   observations, 526 individuals) and UV chroma (r = 0.001 ± 0.063 [0, 0.212], p = 0.500) were not

357   repeatable. Average reflectance (r = 0.157 ± 0.093 [0, 0.348], p = 0.032) and carotenoid hue (r = $0.180 \pm$

358   0.090 [0.037, 0.383], p = 0.006) were moderately repeatable, and the area of the melanin-based breast

359   stripe was highly repeatable (r = 0.458 ± 0.077 [0.311, 0.605], p < 0.001, N = 590 observations, 499

360   birds).

361
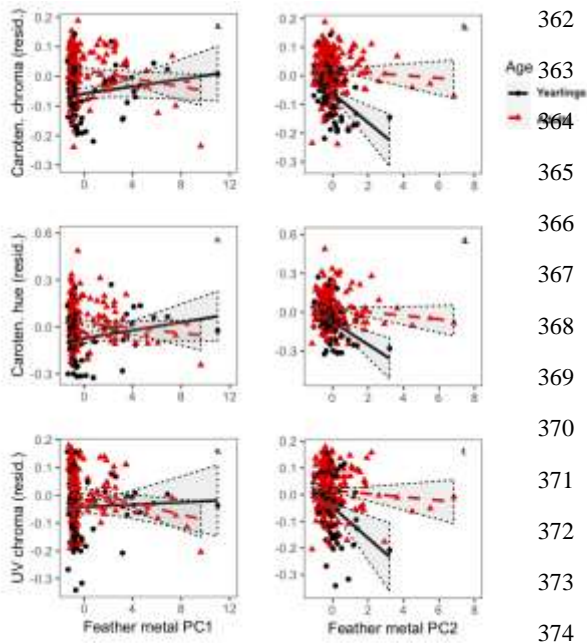
362 **Figure 3**. Age-specific
363 relationships between carotenoid
364 chroma (a, b), carotenoid hue (c, d),
365 and UV chroma (e, f) and feather
366 metal PC1 (a, c, e) and PC2 (b, d,
367 e). Values for color variables are
368 residuals adjusted for significant
369 covariates. Shaded regions show
370 95% confidence intervals.

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375 **Body condition**: Body condition differed among sites ($F_{4, 497} = 7.6$, $p < 0.001$; Fig. 1f), but in a way

376 inconsistent with an effect of metal exposure from the smelter. Birds at F4 were in poorer condition than

377 birds at UM, F7, and F6 and tended to be in poorer condition than birds at F5. Birds at F6 were in poorer

378 condition than at F7 (Fig. 1f; Table S8). Older birds and males were in better condition (Table S10), and

379 birds residing farther from the road tended to be in better condition ($\beta = 0.06 \pm 0.03$, $t_{554} = 1.9$, $p =$

380 0.064). Blood and feather metal concentrations were unrelated to body condition ($p > 0.20$; Table S10).

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383

384 **DISCUSSION**

385 Our analysis suggests that metal pollution and proximity to roads have negative effects on carotenoid-

386 based pigmentation, with potential ramifications for mate-choice dynamics. These results agree with past

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387 studies suggesting that carotenoid-based pigmentation is sensitive to environmental pollutants and

388 urbanization (Eeva et al., 1998, 2003, 2008; Hõrak et al., 2000; Dauwe and Eens, 2008; Geens et al.,

389 2009; Biard et al., 2017), and our study is the first to document that birds residing close to the road have

390 reduced carotenoid-based pigmentation. Melanin-based pigmentation was highly repeatable, but

391 unrelated to metal exposure or distance from the road. These results support the idea that carotenoid-

392 based pigmentation is more condition-dependent than melanin-based pigmentation, and might serve as a

393 more reliable indicator of environmental stress exposure (Hill, 1995; McGraw and Hill, 2000; Grunst et

394 al., 2014a; but see McGraw, 2008). We proceed to discuss our findings in more detail.

395   First, metal exposure differed between the study sites, such that given an effect of metal pollution on

396 plumage coloration, we might expect site differences in coloration. Specifically, feather Pb, Cd, Cu, and

397 As, and blood Pb concentrations were higher at UM than at other sites. Blood Pb concentrations were

398 also elevated at F6 in comparison to other sites, with the exception of UM. However, this likely reflects

399 the effect of a pollution source other than the UM facility, because emissions from the smelter decrease

400 exponentially with distance. Despite differences in metal exposure levels, we found limited evidence for

401 strong site differences in plumage coloration, with only UV chroma being significantly lower at UM than

402 at the other sites. Moreover, UV chroma did not differ between birds at UM and F6, suggesting that

403 variables other than metal exposure from the smelter may also be mediating site-level differences in UV

404 chroma. In contrast to in the current study, we previously found that carotenoid chroma and hue were

405 lower at UM than at F7 and F4 (Dauwe and Eens, 2008). One explanation for this discrepancy is that

406 habitat quality and carotenoid availability was previously lower at UM, but has rebounded over time,

407 resulting in less pronounced site-level differences in coloration. UV chroma could still be affected by

408 metal exposure via an effect on feather microstructure, which can influence the UV reflectance properties

409 of feathers (Bleiweiss, 2004, 2005; Mougeot et al., 2007). Importantly, metal exposure levels also vary

410 substantially between individuals within sites (Grunst et. al., 2019b), which is why individual-level rather

411 than site-level analyses are expected to more clearly elucidate relationships between metal exposure and

412 coloration.

17

413   Indeed, our analysis using individual feather metal concentrations was consistent with metal exposure

414   affecting multiple dimensions of carotenoid coloration, including carotenoid chroma, UV chroma, and

415   hue. This individual-level analysis may be more meaningful with respect to the relationship between

416   metal exposure and coloration than the site-level analysis, since the sites also differ in other

417   characteristics besides metal levels in the environment. Interestingly, relationships with carotenoid-based

418   coloration were not observed when using blood metal concentrations. A possible explanation for this

419   result is that, despite the potential for external contamination (Jaspers et al., 2004), metal concentrations

420   measured in the feathers are indicative of metal exposure during molt, when feathers are grown and

421   pigmentation levels adopted. In contrast, in our study, blood samples were obtained later in the year. In

422   addition, circulating blood metal concentrations may poorly reflect accumulation of metals in organs,

423   which may limit the utility of blood metal levels in predicting biotic effects of metal exposure (Powolny

424   et al., 2019).

425   We found age-dependent relationships between feather metal concentrations and carotenoid-based

426   coloration, suggesting that sensitivity to metal exposure may be contingent on age-specific life-history

427   differences. UV chroma was lower in birds with high feather metal PC1 scores across age classes.

428   However, higher PC1 scores were correlated with reduced carotenoid chroma and hue only among older

429   birds. Moreover, higher PC2 scores were associated with lower carotenoid chroma, UV chroma and hue

430   only among yearlings. Variation in feather metal PC1 was related to the metal pollution gradient,

431   whereas variation in PC2 was not. Thus, coloration in older birds appears more sensitive to metal

432   pollution. In contrast, yearling coloration appears to be associated with Zn levels (highly loaded on PC2),

433   which vary independent of the metal pollution gradient, and might be correlated to other factors (e.g. food

434   quality). Average reflectance was unrelated to metal exposure, which is consistent with results from our

435   past research (Dauwe and Eens, 2008; Geens et al., 2009).

436   Differences in carotenoid-based pigmentation between study sites, and with respect to feather metal

437   concentrations, were not mediated by body condition (similar to Dauwe and Eens, 2008). Body condition

438   was lowest at the site farthest from the smelter, and was unrelated to feather and blood metal

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439 concentrations. The causation underlying lower body condition at F4 remains unclear, but could reflect

440 differences in food availability, inter- or intraspecific competition, or predation pressure. Moreover,

441 average reflectance (the coloration variable most strongly associated with condition) was not associated

442 with metal exposure. As a caveat, our metric of condition was mass-size residuals, which have been

443 frequently related to fitness metrics in great tits (Tinbergen and Boerlijst, 1990; Both et al., 1999; Naef-

444 Daenzer et al., 2001; Perrins and McCleery, 2001; Tilgar et al., 2010; Rodríguez et al., 2016; Vermeulen

445 et al., 2016; Grunst et al., 2019c), but which are incomplete indicators of physiological state.

446 Despite the lack of a correlation between body condition and metal levels, suppressed sexual

447 coloration in great tits exposed to metal pollution could be mediated by effects of metal exposure on

448 stress hormone levels, or other hormonal systems (the *stress hormone hypothesis*; Eeva et al., 2006). For

449 instance, if exposure to metal pollution elevated corticosterone concentrations, this may induce birds to

450 direct energy expenditure towards survival and away from costly sexual displays. Similarly, effects of

451 metal exposure on testosterone levels could induce changes in sexual signaling dynamics, and

452 consequently affect the phenotypic expression of plumage coloration (Eeva et al., 2006).

453 In addition to demonstrating associations with metal pollution, our study is the first to demonstrate a

454 negative relationship between proximity to the road and carotenoid-based pigmentation. Birds found

455 closer to the road had lower carotenoid chroma. In addition, males found farther from the road had higher

456 hue and yearlings found farther from the road had lower average reflectance. Males may invest more into

457 hue than females, deeming male hue more sensitive to elevated stress near roads. Yearlings found closer

458 to the road may molt later in the year, and their feathers may consequently have higher reflectance.

459 Indeed, yearlings had higher average reflectance than older birds, and yearling great tits generally molt

460 later in the season than older birds (Flegg and Cox, 1969). Reduced carotenoid coloration in birds found

461 close to roads could arise through different mechanisms, including reduced carotenoid availability linked

462 to edge effects and habitat degradation (Kociolek et al., 2011; Biard et al., 2017) or physiological stress

463 related to air, light and noise pollution (Alonso-Alvarez et al., 2008; Kociolek et al., 2011; Grunst et al.,

464 2014b, c; Injaian et al., 2018a, b). Furthermore, feather Cu and Zn were elevated near the road, and

465   feather metal PC1 and PC2, which were negatively associated with carotenoid-based coloration in adults

466   and yearlings, respectively, tended to be lower farther from the road. Thus, these results suggest that

467   exposure to roadway-associated metal pollution could contribute to reduced coloration. Finally, birds

468   residing near the road tended to be in poor condition, which could contribute to reduced carotenoid-based

469   pigmentation near roadways (Pulliam and Danielson, 1991; Holmes et al., 1996).

470   We found no relationship between metal exposure or proximity to the road and the area of the

471   melanin-based breast stripe. Researchers have proposed that the capacity for melanin to bind metal ions

472   could deem melanic phenotypes adaptive in polluted areas (Dauwe and Eens, 2008; Chatelain et al., 2014,

473   2016). In addition, modulatory effects of metals on melanin synthesis could arise because metal ions

474   serve as cofactors for tyrosinase, an enzyme involved in melanogenesis (McGraw, 2003; Niecke et al.,

475   2003; Roulin, 2016; Chatelain et al., 2016). Indeed, in contrast to our current results, we previously

476   found that birds at UM had more melanin coverage than birds at F7 and at F4, but this previous study had

477   a much smaller sample size. Giraudeau et al. (2015b) found a positive effect of Cu and total metal

478   concentrations on melanin coverage of great tits. Studies on pigeons (*Columba livia*; Chatelain et al.,

479   2014) and barn owls (*Tyto alba*; Niecke et al., 2003) also found that more melanic individuals had higher

480   feather Zn concentrations. Whether a relationship arises between melanin-based pigmentation and metal

481   exposure may depend on the nature of the pollution regime and characteristics of the study species, but in

482   the case of our current study, this trait was highly repeatable and unrelated to pollution levels.

483   Finally, a limitation to our study is that we did not differentiate between different types of roads. The

484   roads that pass by the study areas are generally type 3 or type 4 roads, as classified by the European Open

485   Transport Map (http://opentransportmap.info), and thus have similar traffic volumes. However, some F6

486   birds were capture in nest boxes located near a major freeway. In future work it would be informative to

487   differentiate between classes of roadways, or to measure traffic volume and noise levels. In addition,

488   comprehensively measuring pollution variables associated with roads, such as noise, light, and air

489   pollution levels could shed light on unresolved mechanistic underpinnings, as could experimental studies.

490   In conclusion, we found negative relationships between individually-based metrics of metal exposure

491   and carotenoid-based pigmentation traits, and we report for the first time that birds residing close to roads

492   have reduced carotenoid-based pigmentation. Thus, multiple disturbance factors within the urban matrix

493   may combine to influence the expression of carotenoid-based pigmentation. We also found evidence for

494   age- and sex-dependent effects on carotenoid-based pigmentation, suggesting the need to differentiate

495   between classes of individuals when assessing impacts of environmental stress. Our study provides

496   precedence for a more thorough examination of how anthropogenic disturbance factors affect sexual

497   signaling dynamics and fitness outcomes. In particular, road networks are a ubiquitous and still

498   increasing feature of landscapes worldwide. Thus, there is now an urgent need to examine whether

499   individuals residing near roadways also have reduced carotenoid-based pigmentation in other populations

500   and species, and what implications this has for individual fitness and population dynamics.

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507

508   **Data availability**: Data will be available in the Dryad Digital Repository.

509

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