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Do trace metals select for darker birds in urban areas?

An experimental exposure to lead and zinc

Running title: trace metals and plumage melanin selection

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1 **ABSTRACT**

2

3 Trace metals from anthropogenic activities are involved in numerous health impairments and
4 may therefore select for detoxification mechanisms or a higher tolerance. Melanin,
5 responsible for the black and red colourations of teguments, plays a role in metal ions
6 chelation and its synthesis is positively linked to immunity, to antioxidant capacity and to
7 stress resistance due to pleiotropic effects. Therefore, we expected darker birds to 1) store
8 higher amounts of metals into their feathers, 2) maintain lower metal concentrations in blood
9 and 3) suffer less from metal exposure. We exposed feral pigeons (*Columba livia*) exhibiting
10 various plumage darkness levels to low but chronic concentrations of zinc and/or lead, two of
11 the most abundant metals in urban areas. First, we found negative and positive effects of lead
12 and zinc respectively on birds' condition and reproductive parameters. Then, we observed
13 positive relationships between plumage darkness and both zinc and lead concentrations in
14 feathers. Interestingly, though darker adults did not maintain lower metal concentrations in
15 blood and did not have higher fitness parameters, darker juveniles exhibited a higher survival
16 rate than paler ones when exposed to lead. Our results show that melanin-based plumage
17 colouration does modulate lead effects on birds' fitness parameters but that the relationship
18 between metals, melanin, and fitness is more complex than expected and thus stress the need
19 for more studies.

20

21

22 INTRODUCTION

23

24 Current human activities generate considerable environmental disturbances, such as light,
25 noise and chemical pollutions. Like other chemical pollutions, trace metals emissions are of
26 particularly timely concern, given their implication in several human diseases (reviewed in
27 Jarup, 2003) and their noxious effects on wildlife (Hsu *et al.*, 2006). In birds, trace metals
28 negatively affect immunity in great tits and zebra finches (Snoeijs *et al.*, 2004, 2005) and
29 learning abilities in young herring gulls (Burger & Gochfeld, 2004). In addition, high levels
30 of trace metals in the environment correlate with reproduction impairments (e.g. higher nest
31 desertion, hatching failure and mortality; Eeva & Lehikoinen, 1996; Janssens *et al.*, 2003;
32 Eeva *et al.*, 2009), and oxidative damages (Berglund *et al.*, 2007) in passerine birds.

33 Because concentrations of trace metals are higher in the cities than in rural areas (Azimi *et al.*,
34 2003; Roux & Marra, 2007), trace metals likely have ecological and evolutionary
35 consequences on urban wildlife, though the exact levels organisms experience remain
36 unknown and most probably depend on the taxonomic group considered. Because of their
37 toxicity, trace metals may select for detoxification mechanisms (such as higher elimination
38 rate of ingested metals) or for higher resistance to their toxic effects (such as higher oxidative
39 stress resistance). Environments polluted with trace metals thus represent exciting
40 opportunities to study ongoing evolutionary mechanisms in the wild.

41 Interestingly, highly melanic plumage may be advantageous in environments polluted with
42 trace metals (i.e. darker individuals may suffer less in these habitats), both through direct and
43 indirect effects of melanogenesis. First, melanin is composed of negatively-charged free
44 carboxyl, hydroxyl and amine functions known to bind metal ions in vitro (Larsson & Tjälve,
45 1978; Liu *et al.*, 2004; Bridelli & Crippa, 2007). For this reason, metal chelation is suggested
46 as one of the main biological functions of melanin (McGraw, 2003; Hong & Simon, 2007;

47 Chatelain *et al.*, 2014). In birds, metal transfer from the bloodstream into melanic feathers
48 during their growth could represent an efficient detoxification mechanism through metal
49 sequestration and elimination during moulting, a hypothesis already proposed for keratin in
50 feathers (Burger, 1993). Consequently, more melanic feathers would be able to store higher
51 amounts of metals, a hypothesis supported by a positive correlation between concentrations of
52 some metals in feathers and melanin-based plumage colouration in feral pigeons, white-tailed
53 eagles and barn owls (Niecke *et al.*, 1999, 2003; Chatelain *et al.*, 2014). In habitats where
54 metals indeed have noxious effects, highly melanic birds would thus have a better
55 detoxification ability than paler birds by lowering their circulating metal burden. Although
56 this detoxification mechanism could represent a significant driver of melanin-based plumage
57 colouration polymorphism maintenance, it has been poorly investigated. Positive correlations
58 have been shown between melanin-based plumage colouration and concentrations of some
59 metals (zinc, calcium and manganese; Niecke *et al.*, 1999, 2003; Zduniak *et al.*, 2014), while
60 no such link has been demonstrated for highly toxic metals such as lead and cadmium, maybe
61 because of the correlative nature of the studies (Gochfeld *et al.*, 1991; Chatelain *et al.*, 2014).
62 Then, to the best of our knowledge, no study compared metal concentrations in feathers
63 between differently melanin-coloured birds in controlled environmental conditions (ie. under
64 the same metal exposure).

65 Second, both the pleiotropic effect of the gene coding for melanin synthesis (POMC) and its
66 linkage disequilibrium with various loci result in correlations between variation in melanin-
67 based plumage colouration and several biological traits, including immunity, antioxidant
68 capacity and stress resistance (Ducrest *et al.*, 2008; Mckinnon & Pierotti, 2010). Indeed,
69 darker pigeons exhibit both a lower endoparasite intensity and a greater cellular immune
70 response than paler pigeons (Jacquin *et al.*, 2011). Moreover, eumelanin level in the barn owl
71 positively correlates with resistance to oxidative stress (Ducrest *et al.*, 2008; Roulin *et al.*,

72 2011) and to physiological stress (corticosterone synthesis; Almasi *et al.*, 2010, 2012).
73 Therefore, melanin-based plumage colouration may shape birds' tolerance to trace metals.
74 Both direct (metal binding) and indirect (resistance to parasites, oxidative stress and
75 physiological stress) associations between biological traits and plumage melanism may favour
76 darker birds in environments polluted with trace metals. Accordingly, previous studies
77 observed a higher frequency of darker feral pigeons in European cities (Obukhova, 2007;
78 Jacquin *et al.*, 2013a), where environmental concentrations of metals are the highest (Azimi *et al.*
79 *et al.*, 2005; Scheifler *et al.*, 2006; Roux & Marra, 2007; Kekkonen *et al.*, 2012). However, there
80 is no experimental evidence for fitness advantages of being more melanistic in habitats
81 polluted with trace metals. Still, the existence of such melanin-based plumage polymorphism
82 in cities raises questions about its evolutionary maintenance, which could result from either a
83 transitory polymorphism (i.e. the hypothesized selective pressure induced by trace metals is
84 ongoing), some imperfect linkage disequilibrium between loci involved in melanogenesis and
85 other biological traits under selection or the co-occurrence of antagonistic selective pressures.
86
87 Partly due to large emissions by anthropogenic activities (beginning during the Roman
88 Empire and increasing with the Industrial Revolution of the 19th century Nriagu, 1996), lead is
89 the most abundant toxic metal in the environment (Azimi *et al.*, 2005; Roux & Marra, 2007).
90 Although lead used in gasoline drastically diminished since the 70s (Jarup, 2003), it remains
91 of high ecological importance due to its accumulation into the soil (Roux & Marra, 2007) and
92 to the negative biological effects of a chronic exposure, even at low levels (Patrick, 2006). In
93 addition, zinc is the most abundant metal in the environment (Azimi *et al.*, 2005). While it
94 may induce harmful effects at high concentrations (Greenberg & Briemberg, 2004; Bozym *et al.*
95 *et al.*, 2010), it is overall an essential nutrient (Mertz, 1981; Prasad, 1998, 2009), also able to
96 compensate the negative effects induced by other traces metals (Chichovska & Anguelov,

97 2006; Prasanthi *et al.*, 2006, 2010). Therefore, lead and zinc likely induce the strongest
98 (negative or positive) effects on urban wildlife. While lead and zinc effects on condition and
99 reproductive success may greatly modulate birds' fitness and population dynamics, to the best
100 of our knowledge, no experimental study demonstrated such effects and the previous
101 correlative studies cannot exclude confounding urban factors also known to impair birds'
102 reproduction (Halfwerk *et al.*, 2011; Dominoni *et al.*, 2013).

103 In this work, we chronically exposed feral pigeons (*Columba livia*) to lead and/or zinc in
104 experimentally controlled concentrations inferred from previous measures in urban areas. The
105 feral pigeon is a highly polymorphic bird species with respect to its melanin-based plumage
106 colouration and experiences an extended moulting period (Johnston & Janiga, 1995). First, we
107 evaluated the effects of such exposures on lead and zinc concentrations in feathers and in
108 blood, and on bird condition and reproductive parameters. Then, we investigated whether the
109 ability of feathers to store zinc and lead depends on their melanin-based colouration and, as a
110 consequence, whether melanistic birds maintain lower lead concentrations in blood; because
111 zinc concentration in blood is under strict homeostatic regulation in eukaryotes (Gaither &
112 Eide, 2001), no relationship was expected between plumage colouration and blood levels of
113 zinc. Finally, we tested whether melanin-based plumage colouration could be advantageous in
114 environments polluted with metals by investigating the interaction between plumage
115 colouration and metal exposure on birds' condition and reproductive parameters.

116

117 **MATERIALS AND METHODS**

118

119 *Biological model*

120 Free-living feral pigeons are considered to originate from the continuous reproduction
121 between wild, synanthropic and domesticated pigeons (Johnston & Janiga, 1995). Contrasting

122 artificial selective pressures on various phenotypic traits linked to past domestication has
123 generated polymorphism in the degree of melanin-based plumage colouration. Free-living
124 feral pigeons have then evolved in natural environments in close proximity with human
125 populations for centuries and maintained one of the highest melanin-based plumage
126 colouration polymorphism amongst birds. This polymorphism involves melanin type
127 (eumelanin and pheomelanin, respectively responsible for the black and reddish colour of
128 teguments) and melanin degree (i.e. the area of pigmentation; see below). Both parameters of
129 melanin-based plumage colouration in feral pigeons are highly heritable (heritability of
130 melanin degree: 0.82 ± 0.12 ; Jacquin *et al.*, 2013b).

131

132 *Subjects and Housing*

133 Ninety six (48 males and 48 females genetically sexed) free-living adult feral pigeons
134 (*Columba livia*) exhibiting various melanin-based plumage colourations were caught during
135 winter 2013 (February/March) in several pigeon flocks within Paris (Supplementary
136 material). Birds were all adults as indicated by reliable morphological criteria such as well-
137 formed caruncle, absence of juvenile plumage and presence of iridescent neck feathers
138 (Johnston & Janiga, 1995). The birds were immediately transferred in 8 outdoor aviaries (3.10
139 m x 2.00 m x 2.40 m) at the CEREEP field station (Centre d'Ecologie Expérimentale et
140 Prédictive-Ecotron Ile-de-France, UMS 3194, Ecole Normale Supérieure, Saint-Pierre-lès-
141 Nemours, France). Birds were fed *ad libitum* with a mix of maize, wheat and peas. The
142 aviaries were enriched with a bowl of water used for bathing and with branches as perches.
143 Birds were individually identified with a numbered plastic ring. At the end of the experiment,
144 all birds were released back to the wild at their site of capture. All experiments were carried
145 out in strict accordance with the recommendations of the "European Convention for the
146 Protection of vertebrate Animals used for Experimental and Other Scientific Purposes" and

147 were conducted under the authorizations of the “Ministère de l’éducation nationale, de
148 l’enseignement supérieur et de la recherche” (authorization N_00093.02) and the “Direction
149 Départementale des Services Vétérinaires de Seine-et-Marne” (authorization N_77-05).

150

151 *Plumage colouration measurement*

152 At their capture, birds were first categorised as eumelanic (grey to black pigmented; 37 males
153 and 45 females) or pheomelanic (red pigmented; 11 males and 3 females), which defines what
154 we called their melanin type. Pheomelanic birds are usually in low frequencies in cities
155 worldwide (Obukhova, 2007), and are particularly scarce in Paris (about 3%; based on
156 personal data on 2074 pigeons captured over 5 years); despite the particularly strong capture
157 effort provided to capture pheomelanic pigeons, their amount remained small (14 out of 96).
158 Then, birds were individually photographed to precisely quantify their eumelanin or
159 pheomelanin level. Eumelanin or pheomelanin level was calculated as the percentage of black
160 or red on the wing surface of birds respectively (number of black pixels/number of white
161 pixels x 100) using the Gimp image retouching and editing software, which is a reliable and
162 repeatable estimation of melanin concentration (Jacquin *et al.*, 2011). At the end of the
163 experiment, fledglings born during the experiment were also photographed to assess their
164 eumelanin or pheomelanin level. The percentage of pigmented surface did not significantly
165 differ between eumelanic and pheomelanic birds ($F_{1,94}=0.27$, $P=0.606$).

166

167 *Treatments*

168 Two weeks before the start of the experiment, the birds were distributed in the aviaries in
169 order to equilibrate both sex (6 females and 6 males per aviaries) and plumage colouration
170 ($F_{1,94}<0.01$, $P=0.974$). However, because of their lower number (n=14), pheomelanic
171 individuals were split in 6 aviaries only. Importantly, because birds’ precise location origin in

172 Paris is likely to affect their previous exposure to trace metals and consequently their initial
173 (prior to the experiment) trace metal body concentrations (Frantz *et al.*, 2012), birds were
174 randomly distributed in the aviaries according to their flock ($\text{Chi}^2=71.09$, $\text{df}=70$, $P=0.441$).
175 This randomization should also avoid any other correlation between birds' history (e.g. age)
176 and aviary. The aviaries were then randomly assigned to one of the 4 following metal
177 exposure treatments: exposed to lead only (*lead* group; 1ppm lead acetate in tap water,
178 Sigma-Aldrich), exposed to zinc only (*zinc* group; 10ppm zinc sulphate in tap water, Prolabo),
179 exposed to both lead and zinc (*lead+zinc* group; 1ppm lead acetate and 10ppm zinc sulphate
180 in tap water) or control (*control* group; tap water without any metal addition). This resulted in
181 2 aviaries with 12 pigeons each (24 pigeons in total) per treatment. We chose these
182 concentrations based on both lead concentrations in blood measured in feral pigeons (ranging
183 from 110 to 154ppb; personal data on feral pigeons captured in 2009 in Paris) and the
184 gastrointestinal absorption rate of lead in zebra finches (<10%) calculated from Dauwe *et al.*
185 (2002). Zinc concentrations were approximated using zinc/lead concentrations ratio in the
186 environment and bird feathers in Paris (Azimi *et al.*, 2005; Frantz *et al.*, 2012). Drinking
187 troughs and baths were filled with the corresponding treated water every other day, miming
188 part of birds' exposure to trace metals in the wild (i.e. through ingestion and deposition onto
189 the plumage).

190

191 *Scaled mass index*

192 From the start to the 20th week of the experiment, all adults were captured once a week to be
193 weighed to the nearest gram with a Pesola Newton scale. Scaled mass index was calculated
194 according to the method described by Peig & Green (2009, 2010). Briefly, scaled mass index
195 was calculated using $\widehat{M}_i = M_i \left[\frac{L_0}{L_i} \right]^{b_{SMA}}$ where M_i and L_i are the body mass and tarsus length
196 of individual i respectively; b_{SMA} is the scaling exponent estimated by the Standardized Major

197 Axis regression of M on L ; L_0 is the arithmetic mean of tarsus length in the study population;
198 and \widehat{M}_i is the predicted body mass for individual i when tarsus length is standardized to L_0 .
199 Scaled mass index is a better indicator of the relative size of energy reserves and of other
200 body components than the ordinary least squares (OLS) regression of body mass against size
201 (Peig & Green, 2009), broadly used amongst ecologists.

202

203 *Reproductive success measurements*

204 *Breeding success.* A week after the start of the treatments six nest boxes per aviary were
205 opened to allow birds to mate and breed (a box per couple). A bird was considered as
206 reproducing when it had laid (female) or incubated (female and male) at least one egg during
207 the breeding season. Overall, 52 pigeons (25 eumelanic and 3 pheomelanic females and 17
208 eumelanic and 7 pheomelanic males) successfully reproduced.

209 *Eggs' quality measurement.* Feral pigeons commonly produce two-egg clutches, one to 6
210 times a year. The day it had been laid, the egg was removed from the nest, weighed and
211 measured (3 measures of eggs' maximum length and maximum width were taken, and then
212 averaged). Egg volume was calculated as $V = 0.4866 \times Length \times (Width^2)$ (Paillisson *et*
213 *al.*, 2007). Eggs of the first, third and fifth clutches were put back in the nest to allow
214 incubation (n=83) whereas the others were frozen (n=65). Shell, albumen and yolk of frozen
215 eggs were separated, then weighed to the nearest μg (eggshells were previously oven-dried).
216 Dried shell thickness was measured to the nearest μm with a specimeter (Mitutoya 0-1mm).

217 *Hatching success.* The hatch was considered as successful when the chick was completely
218 cleared from its shell and alive. 52 eggs successfully hatched out of 83.

219 *Nestlings' growth measurement.* Each one-day-old hatchling was weighed and measured (3
220 measures of tarsus and wing length were taken, and then averaged; n=52). Weight, tarsus and
221 wing measures were reiterated every day until 25 days old (note that nestlings' body mass

222 slow down at 16 days old on average; Johnston & Janiga 1995). Growth (for body mass,
223 tarsus and wing length) was calculated as $W = \frac{A}{1+\exp(-k(t-t_i))}$, where W =morphological
224 measure (body mass, tarsus length or wing length), A =asymptote (final body mass, tarsus
225 length or wing length at the end of growth), k =growth rate constant t =age and t_i =the
226 inflexion point of the curve (Newbrey & Reed, 2009; Jacquin *et al.*, 2012). Therefore we
227 characterized nestlings' growth by its growth rate (k) and its age of slowing growth (t_i). Only
228 the growth of nestlings which successfully fledged was calculated (n=41). Three months after
229 the birds stopped growing, their weight, tarsus length and wing length were measured to
230 assess their scaled mass index (n=40).

231 *Fledging success.* Chick was considered as successfully fledged when it was found outside
232 the parental nest and was able to fly and to feed by itself.

233 *Juveniles' condition.* At the end of the experiment, while the younger bird was 3 months old
234 and the older was 6 months old, we measured juvenile haematocrit, corresponding to the
235 erythrocyte volume fraction of a blood sample (n=40). It is expected to be an indicator of
236 general health state (Cooper, 1975; Averbek, 1992). In addition, the number of leukocytes
237 per 10000 erythrocytes was counted from blood smear. Slides were fixed with methanol
238 during 5 minutes and coloured with GIEMSA (diluted 1:20) during 45 minutes. We identified
239 heterophils, eosinophils, lymphocytes and monocytes. Because glucocorticoid decreases the
240 number of circulating lymphocytes while it stimulates the influx of heterophils from the bone
241 marrow, leukocyte profiles are suitable for identifying some physiological stress (Davis *et al.*,
242 2008). Therefore, we calculated the heterophils/lymphocytes ratio. We also considered the
243 total number of white blood cells (total number of leukocytes per 10000 erythrocytes) that is
244 suggested to be an indication of birds' immunity (Davis *et al.*, 2008).

245 Laying date was measured to ensure that trace metals effects on reproductive parameters
246 would not be due to cumulative effects but was not considered as a reproductive success
247 measurement.

248

249 *Metal quantitative analyses*

250 *In blood.* 10 weeks after the start of the experiment, 50µl of blood were collected from the
251 brachial vein of each 96 adult pigeons and were immediately frozen until analyzed. Prior to
252 metal measurement, blood was defrosted and vortexed. Then, 200mg (± 0.1 mg) were digested
253 with 1ml HNO₃ solution (68%) during 24h at 80°C.

254 *In feathers.* 13 weeks after the start of the experiment, a secondary remige (the 5th) was
255 removed a first time. Once the regrown feather finished its development and was
256 devascularized, it was plucked off and conserved in an individual plastic bag. Feathers were
257 washed vigorously with 0.25M NaOH solution, rinsed energetically 3 times in ultrapure water
258 (Milli-Q purified) to remove external contamination (Scheifler *et al.*, 2006; Frantz *et al.*,
259 2012), left 1h in ultrapure water and dried 12h at 80°C to dry mass. Barbs were removed from
260 the rachis, weighed to the nearest 0.1 mg and digested following the method described above.
261 The product of digestion was transferred into plastic tubes and water was added to reach a
262 final volume of 8ml; then, each sample was diluted by 2.5. Total lead and zinc concentrations
263 were determined in all of the 96 feather samples and 48 blood samples (6 females and 6 males
264 amongst each of the four treatments) by mass spectrometry (quadrupole ICP-MS, XSeries II)
265 and optical emission spectrometry (ICP-OES, JY 2000) respectively.

266

267 *Statistical analyses*

268 To distinguish the respective effects of lead and/or zinc exposure treatments on the variables
269 measured, we binary coded (absence/presence) the exposure to lead on one hand and the

270 exposure to zinc on the other (table 1). First, we tested for the existence of correlations
271 between the explanatory variables considered (lead exposure, zinc exposure, plumage melanin
272 type and plumage eumelanin level among adults and among juveniles separately). Colinearity
273 between eumelanin level and metal exposure among juveniles did not allow to include metal
274 exposure and juvenile eumelanin level in the same model (see results). There was no
275 significant relation between adult eumelanin level and metal exposure whatever the adult
276 group (all adults or adults that bred, produced hatchlings or fledglings successfully).

277 For each dependent variable listed in table 1, we performed three successive models:

278 - in models 1, we tested ecotoxicological effects of lead exposure, zinc exposure and their
279 interaction in all birds whatever their plumage melanin type (pheomelanic and eumelanic,
280 thus not included in the model);

281 - in models 2, we tested the effects of melanin type (pheomelanic vs. eumelanic), lead and
282 zinc exposures and their interactions in all birds; note that the interaction between zinc
283 exposure and lead exposure was not tested because no pheomelanic birds have been exposed
284 to both zinc and lead;

285 - in models 3, we tested the effects of plumage eumelanin level in interaction with lead and
286 zinc exposure in eumelanic birds only (note that the number of pheomelanic pigeons was too
287 low to test the effect of pheomelanin level).

288 First, we investigated metal concentrations in feathers using linear mixed models. Age (adult
289 vs. juvenile) was added into the models when testing the effects of metals alone (model 1) and
290 of melanin type (model 2). However, there were too few pheomelanic juveniles (N=4) to test
291 the interaction between melanin type and age. When testing the effects of eumelanin level
292 (model 3), colinearity issues (see above) did not allow us to test the effects of metal exposure
293 and eumelanin level in the same model, so that we performed separate models for adults and
294 juveniles. In juveniles, we first tested the effect of eumelanin level alone; when significant,

295 we then tested the effect of lead exposure, zinc exposure and their interaction, and compared
296 the two models using their AIC. Lead is undetectable in the feathers after one year of
297 captivity in our study site (Chatelain *et al.*, 2014). Therefore, lead detected in the feathers of
298 adult not experimentally exposed to this metal arose mostly from birds' original environment
299 prior to our experiment, which could conceal the potential link between plumage colouration
300 and lead concentrations in feathers. Thus, we performed additional models investigating lead
301 concentrations in feathers according to plumage colouration in birds exposed to lead (*lead* and
302 *lead+zinc* groups), both in adults and juveniles, which exhibited significantly higher lead
303 feather concentrations (see results).

304 Second, we investigated metal concentrations in blood using linear mixed models.

305 Third, we performed linear mixed models with time (expressed as the number of weeks after
306 the beginning of the experiment) and its interactions with the other variables listed above as
307 fixed effects to explain adult scaled mass index variation along time; individual was added as
308 a random effect.

309 Finally, we investigated the effects of metal exposure on reproductive parameters using linear
310 mixed models (egg quality, nestling growth and scaled mass index at 3 days of age) or general
311 linear mixed models for binomial distribution (breeding success, hatchling success, fledging
312 success, juvenile total white blood cell and heterophils/lymphocytes ratio); mother identity
313 was added as random effect. When testing the effect of plumage colouration (parental or
314 juveniles), plumage colouration was included as melanin type (model 2) or eumelanin level
315 (model 3) of both parents for egg quality and reproductive success (breeding, hatchling,
316 fledging), or eumelanin level of juvenile (model 3) for juvenile growth, scaled mass index and
317 physiological state (the effect of melanin type was not tested for these variables because
318 pheomelanic juveniles were rare (4 birds out of 40)). When testing the effects of juvenile
319 eumelanin level (model 3) and to take colinearity issues into account (see above), we first

320 tested the effect of eumelanin level alone; if significant, we then tested the effect of lead
321 exposure, zinc exposure and their interaction, and compared the two models using the AIC
322 (i.e. when performing linear mixed models). In all performed models, the aviary was added as
323 random effect. There was no significant relationship between laying date and trace metal
324 exposure, suggesting that even though cumulative effect might exist, it would not be
325 significantly different between the treatments. Therefore, laying date was not added as
326 random effect in the models.

327

328 Statistical analyses were performed using R software (version 3.0.2) and a type 3 approach
329 was used to take the unbalance of the design into account. We did not correct p-values for
330 multiple testing as suggested by Moran (2003), García (2004) and Nakagawa (2004).

331

332 **RESULTS**

333

334 **Trace metal concentrations in feathers**

335 Among all pigeons (pheomelanic and eumelanic), zinc concentrations in feathers were higher
336 in birds exposed to zinc (*zinc* and *lead+zinc* groups; $F_{1,104}=4.25$, $P=0.042$; table 1) and in
337 juveniles ($F_{1,104}=6.64$, $P=0.011$; $91.36\text{ppm}\pm 1.68$ and $99.27\text{ppm}\pm 2.28$ in adults and in
338 juveniles respectively). Moreover, lead concentrations in feathers were higher in birds
339 exposed to lead (*lead* and *lead+zinc* groups) than in the others (*zinc* and *control* groups;
340 $F_{1,105}=15.09$, $P<0.001$; table 1).

341 Among all pigeons (pheomelanic or eumelanic), zinc concentrations in feathers were higher in
342 eumelanic birds than in pheomelanic ones ($F_{1,104}=25.64$, $P<0.001$; $96.26\text{ ppm}\pm 1.15$ in
343 eumelanic birds and $72.54\text{ ppm}\pm 5.13$ in pheomelanic birds). Melanin type was not retained in

344 the final model fitted for lead concentrations in feathers (among all birds and among birds
345 exposed to lead).

346 Among eumelanic adults, zinc concentrations in feathers increased with eumelanin level
347 ($F_{1,63}=11.21$, $P<0.001$; Fig. 1). Among eumelanic adults exposed to lead (*lead* and *lead+zinc*
348 groups), lead concentrations in feathers increased with plumage eumelanin level ($F_{1,33}=5.12$,
349 $P=0.030$; Fig. 2). Among eumelanic juveniles, eumelanin level was not retained for the
350 models fitted for zinc and for lead concentrations in feathers among all juveniles and juveniles
351 exposed to lead.

352

353 **Trace metal concentrations in blood**

354 Among all adults (pheomelanic and eumelanic), lead concentrations in blood tended to
355 depend on the interaction between zinc exposure and lead exposure ($F_{3,43}=3.64$, $P=0.063$;
356 table 1): birds exposed to zinc only (*zinc* group) exhibited lower lead concentrations in blood
357 than birds exposed to both lead and zinc (*lead+zinc* group; $F_{1,21}=10.79$, $P=0.004$), birds
358 exposed to lead only (*lead* group; $F_{1,21}=6.04$, $P=0.023$) and controls (*control* group;
359 $F_{1,21}=4.18$, $P=0.054$). None of the considered variables were retained in the models fitted for
360 zinc concentrations in blood.

361

362 **Scaled mass index variation**

363 Among all adults (pheomelanic and eumelanic), scaled mass index depended on the
364 interaction between time, zinc exposure and lead exposure ($F_{1,96}=8.93$, $P=0.003$): scaled mass
365 index decreased along time in *lead* ($F_{1,24}=52.39$, $P<0.001$), *control* ($F_{1,24}=28.11$, $P<0.001$) and
366 *lead+zinc* groups ($F_{1,24}=57.92$, $P<0.001$) while time was not retained in the final model fitted
367 for scaled mass index in *zinc* group.

368 Among eumelanic adults, scaled mass index depended on the interaction between time, zinc
369 exposure, lead exposure and eumelanin level ($F_{1,82}=19.29$, $P<0.001$; Fig. 3): scaled mass
370 index decreased along time in *control* ($F_{1,20}=35.64$, $P<0.001$) and *lead+zinc* group
371 ($F_{1,24}=57.92$, $P<0.001$). In *lead* group, scaled mass index depended on the interaction between
372 time and eumelanin level ($F_{1,19}=40.02$, $P<0.001$), with scaled mass index decreasing along
373 time among the darkest birds only ($F_{1,9}=87.10$, $P<0.001$). Neither time nor eumelanic level
374 was retained in the final model fitted for scaled mass index among *zinc* group.

375 Melanin type was not retained in the model fitted for scaled mass index.

376

377 **Reproductive success**

378 None of the variables considered was retained in the final model fitted for birds' breeding
379 success.

380 Among all parents (pheomelanic and eumelanic), metal exposure was not retained in the
381 models fitted for egg weight and volume, albumen or eggshell weight. However, yolk was
382 heavier in eggs from parents exposed to zinc (*zinc* and *lead+zinc* groups) than from the others
383 (*control* and *lead* groups; $F_{1,53}=7.36$, $P=0.007$; mean \pm se 4.22g \pm 0.08 and 3.89g \pm 0.09
384 respectively; table 1). Moreover, eggshell was thicker in eggs from parents exposed to zinc
385 (*zinc* and *lead+zinc* groups; $F_{1,62}=5.18$, $P=0.023$; mean \pm se 0.49mm \pm 0.01 and 0.47mm \pm 0.00
386 respectively; table 1) while it was thinner in eggs from parents exposed to lead (*lead* and
387 *lead+zinc* groups; $F_{1,62}=8.24$, $P=0.004$; mean \pm se 0.47mm \pm 0.00 and 0.49mm \pm 0.01
388 respectively; table 1).

389 None of the variables considered was retained in the model fitted for hatching success.

390 Among all parents, nestlings of parents exposed to lead (*lead* and *lead+zinc* groups) were
391 significantly lighter than the other ones (*control* and *zinc* groups; $F_{1,52}=4.17$, $P=0.041$;
392 mean \pm se 14.94g \pm 0.72 and 17.20g \pm 0.67 respectively; table 1). None of the variables

393 considered was retained in the models fitted for tarsus and wing length of one-day-old chick.
394 With regard to nestling growth, none of the variables considered was retained in the models
395 fitted for weight, tarsus and wing growth rate. However, the age at which weight and tarsus
396 growth slowed down depended on the interaction between lead and zinc exposure ($F_{1,24}=5.53$,
397 $P=0.019$; and $F_{1,41}=9.66$, $P=0.002$; Fig. 4). Indeed, growth slowed down earlier in juveniles of
398 parents exposed to lead only (*lead* group) than in juveniles of parents exposed to both lead
399 and zinc (*lead+zinc* group; $F_{1,24}=5.53$, $P=0.019$ and $F_{1,24}=6.01$, $P=0.014$ respectively) and of
400 controls parents (*control* group; $F_{1,25}=11.46$, $P=0.002$ and $F_{1,25}=19.52$, $P<0.001$ respectively).
401 Zinc and lead exposure was not retained in the models fitted for the age at which wing growth
402 slowed down, and melanin type and eumelanin level were not retained in any of the models
403 fitted for nestlings' growth.

404 In all parents, fledging success was lower in juveniles from pheomelanic fathers ($F_{1,59}=8.13$,
405 $P=0.004$) and tended to be also lower among birds exposed to lead (*lead* and *lead+zinc*
406 groups; $F_{1,59}=3.62$, $P=0.057$). Eumelanin level was not retained in the model fitted for
407 fledging success.

408 In all juveniles, scaled mass index was smaller in lead groups (*lead* and *lead+zinc* groups)
409 than in the other groups (*control* and *zinc* groups; $F_{1,40}=6.43$, $P=0.011$; $\text{mean}\pm\text{se}$
410 293.91 ± 19.11 and 349.73 ± 20.02 respectively; table 1). Moreover, the number of white blood
411 cells was higher in zinc groups (*zinc* and *lead+zinc* groups) than in the other groups
412 ($F_{1,40}=5.14$, $P=0.023$; $6.0\%\pm 0.5$ and $4.8\%\pm 0.3$ respectively; table 1). The number of
413 heterophils among the number of lymphocytes depended on the interaction between lead and
414 zinc exposure ($F_{1,37}=5.79$, $P=0.016$, $P=0.016$; table 1): it was higher in *lead* group than in
415 *control* group ($F_{1,20}=4.65$, $P=0.031$) and *lead+zinc* group ($F_{1,19}=4.01$, $P=0.046$). Among
416 eumelanic juveniles, eumelanin level was not retained in the model fitted for scaled mass
417 index, the number of white blood cells and for the heterophils/lymphocytes ratio.

418 Finally, the plumage eumelanin level of surviving juveniles significantly depended on lead-
419 exposure ($F_{3,30}=6.69$, $P=0.015$; Fig. 5), with eumelanin level being higher among juveniles
420 exposed to lead only (*lead* group) than among juveniles from other groups (*zinc*, *control*, and
421 *lead+zinc* groups).

422

423 **DISCUSSION**

424

425 To investigate whether and how trace metals may affect pigeons and may select for darker
426 pigeons in urban areas, we tested whether 1) trace metals have ecotoxicological effects, 2)
427 darker individuals store higher amounts of metals into their feathers, 3) darker individuals
428 maintain lower metal levels in their blood and 4) darker individuals are more tolerant to the
429 exposure to toxic metals. To this aim, we used an experimental approach; zinc and lead
430 supplementations successfully increased zinc and lead concentrations in feathers respectively,
431 while exposure to a given metal did not raise its concentration in the blood (table 1). Overall,
432 metal concentrations in blood may not always reliably estimate recent exposure as they can
433 result from numerous mechanisms (lead clearance from bones, lead and zinc transfer into
434 organs, bones and feathers (Cosson, 1989; Gulson *et al.*, 1996; Kim *et al.*, 1998; Agusa *et al.*,
435 2005)).

436

437 First, our study consistently demonstrated detrimental effects of lead and beneficial effects of
438 zinc on some of bird condition and reproductive success parameters (table 2). The scaled
439 mass index decreased over the 20-week-long experiment in birds exposed to lead (*lead* and
440 *lead+zinc* groups) and in *control* group, while remaining constant in *zinc* group. Eggshell was
441 thinner in eggs from lead-exposed parents. This negative effect, previously observed in pied
442 flycatchers (Eeva & Lehikoinen, 1995) and likely due to limitation of calcium deposition

443 (Clunies *et al.*, 1992; Simons, 1993), may impair eggshell permeability and resistance to
444 impacts (King & Robinson, 1972). On the contrary, zinc increased eggshell thickness. Zinc
445 also increased yolk mass, potentially elevating egg nutritive content (Noy & Sklan, 1998).
446 Accordingly, previous work reported a positive link between plasma zinc and vitellogenin
447 production (Mitchell & Carlisle, 1991). Since zinc exposure did not influence egg size nor
448 one-day-old chick size, this extra yolk mass may have been allocated to other physiological
449 traits, such as immunity (see Li *et al.*, 1998). Accordingly, juveniles exposed to zinc (*zinc* and
450 *lead+zinc* groups) had a higher amount of white blood cells, an index of the immune system
451 (Davis *et al.*, 2008), than the other groups (*lead* and *control* groups). In addition, lead-
452 exposure induced lighter one-day-old chicks. Because lead did not affect egg total, yolk nor
453 albumen mass, it may not alter maternal investment in eggs but may be maternally transferred
454 into the eggs and affect embryonic development (Burger, 2002). Juveniles exposed to lead
455 only (*lead* group) tended to have a lower fledging success, possibly a consequence of a poorer
456 condition at hatching (Grant, 2008). Among fledglings, lead exposure also induced a shorter
457 growth period of body mass and tarsus and a smaller scaled mass index at three months of
458 age, which could impair their future survival or reproduction (“catch-up” hypothesis, see
459 Criscuolo *et al.*, 2008). Consistently, juveniles exposed to lead only (*lead* group) had a higher
460 number of heterophils/lymphocytes ratio, suggesting a higher stress hormone level (Davis *et*
461 *al.*, 2008). Finally, zinc-exposure had protective effects against lead: when provided along
462 with zinc, lead did not induce negative effects on several of the measured traits (table 1, Fig.
463 4). This protective effect may result from zinc ability to reduce the absorption and retention of
464 ingested lead (Cerklewski & Forbes, 1976; El-Gazzar *et al.*, 1978), as suggested by lower lead
465 concentrations in blood in birds exposed to zinc only (*zinc* group) than in the other birds
466 (*control*, *lead zinc+lead* groups). The effects of trace metals observed on juveniles may be

467 due to either direct effects on juveniles or to indirect effects through parental investment that
468 further experimental studies should disentangle.

469

470 Second, we predicted a positive relationship between plumage darkness and metal
471 concentrations in feathers. Our prediction was validated for both zinc, confirming previous
472 work (Chatelain *et al.*, 2014) and lead (table 2). Our results suggest that more melanic
473 feathers would be able to store higher amounts of both zinc and lead. Although washed
474 feathers may still have some amounts of trace metals on their surface, such residual
475 concentrations likely do not correlate with plumage eumelanin level. Therefore, melanin
476 contained in the plumage could play a significant role in metal detoxification. Interestingly,
477 zinc concentrations in feathers were higher in eumelanic pigeons than in pheomelanic ones;
478 such differences could be due to differences in chemical composition between melanin types
479 (e.g. more carboxylic acid groups in eumelanin; Hong & Simon, 2007), to different
480 physiological requirements of zinc (e.g. oxidative stress; Prasad, 2009) or to differences in
481 immune responses (Chatelain *et al.* unpublished data).

482

483 Third, we did not find the negative correlation between plumage eumelanin level and metal
484 concentrations in blood expected from our detoxification hypothesis (table 2). Metal
485 concentration in blood does not reliably reflect recent exposure as it results from numerous
486 mechanisms (Cosson, 1989; Gulson *et al.*, 1996; Kim *et al.*, 1998; Agusa *et al.*, 2005) that
487 may hide detoxification process. Future work should investigate whether such potential
488 detoxification through melanin lowers metal burden in organs. Although feral pigeons moult
489 all over the year (Johnston & Janiga, 1995), metal detoxification may be more efficient when
490 moulting is most intense (i.e. fall) and during juvenile growth (i.e. synchronized growth of all
491 feathers).

492

493 Fourth, we did find a statistical interaction between plumage eumelanin level and metal
494 exposure on scaled mass index (table 2). In birds exposed to lead only (*lead* group), paler
495 birds maintained their initial condition over the course of the experiment, while darker birds
496 lost weight. Note however that darker birds had an initial lower scaled mass index than the
497 paler ones, so that we cannot distinguish whether this result was due to the effect of initial
498 scaled mass index or to eumelanin level. In the latter case, this result may suggest a
499 disadvantage of a more melanic plumage in environments polluted with lead; alternatively, it
500 may also be the result of a trade-off between condition maintenance and other biological
501 traits, such as parental investment, which would be in line with the higher survival rate of
502 darker juveniles among birds exposed to lead only (*lead* group). Indeed, plumage eumelanin
503 level of three-month-old juveniles was higher in *lead* group than in the other groups (table 2).
504 This result was not due to different reproductive success (breeding, hatching and fledging
505 success) between differently coloured adults. In addition, because melanin-based plumage
506 colouration is highly heritable in feral pigeons (0.82 ± 0.12 ; Jacquin *et al.*, 2013b), it is
507 unlikely that a direct effect of lead on melanogenesis explains the higher plumage darkness of
508 juveniles under lead-exposure. More likely, this result may reflect higher survival rate of
509 darker juveniles when exposed to lead only as compared to paler ones. Indeed, fledging
510 success tended to be lower under lead-exposure, which could be due to higher mortality in
511 paler offspring. This hypothesis is in accordance with the higher survival rate of darker
512 pigeon juveniles in a Parisian suburban environment (Récapet *et al.*, 2013) and with the
513 higher frequency of darker pigeons observed in European cities (Obukhova, 2007).

514

515 In conclusion, we found several lines of evidence supporting the possibility that trace metals
516 exert selective pressures on melanin-based plumage colouration (table 2). Indeed, darker birds

517 stored higher amounts of zinc and lead in their feathers and likely had a higher juvenile
518 survival under lead-exposure. However, scaled mass index of darker birds decreased over
519 time when exposed to lead only. Moreover, bird sensitivity to trace metals did not depend on
520 their plumage colouration for several traits. The lack of results may originate from the low
521 beneficial effect of plumage melanin under metal exposure, from the experimental exposure
522 to metal concentrations underestimating the natural range (zinc and lead in the feathers were
523 respectively 80 and 1.5 times less concentrated than measured in wild feral pigeons (Nam *et*
524 *al.*, 2004; Adout *et al.*, 2007; Brait & Filho, 2011; Frantz *et al.*, 2012; Chatelain *et al.*, 2014)),
525 from high inter-individual variation (i.e. in trace metal exposure prior to the experiment)
526 reducing the probability to detect some effects, or from ongoing selection ultimately
527 favouring dark plumage along with a series of other traits. More experimental studies
528 involving exposure to a cocktail of metals in their urban range of concentrations are needed to
529 better understand how this new selective pressure may favour particular phenotypes,
530 especially melanin-based colouration.

531

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533

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539

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