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1 **Stress response varies with plumage colour and local habitat in feral pigeons**

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21 **Running title:** colouration and stress response in feral pigeons

22

23

24 **Abstract**

25 Bird populations exposed to different extrinsic conditions often differ in the responsiveness of
26 the hypothalamo-pituitary-adrenal (HPA) axis and thus in corticosterone response that
27 individuals mount when facing stressful events. However, the contribution of genetic
28 variation to among-individual variability in HPA axis responsiveness across different
29 environmental conditions is poorly understood. Melanin-based coloured types provide reliable
30 phenotypic markers of alternative genotypes underlying stress coping styles. Large variations
31 in melanin-based colouration are heritable in feral pigeons. We tested whether melanin-based
32 colouration is associated with variation in corticosterone stress response in feral pigeons. To
33 this aim, we examined how corticosterone response varies both within and between
34 differently coloured individuals across different environmental conditions. Differently
35 coloured individuals mounted different stress-induced corticosterone levels in interaction with
36 environmental conditions: dark pigeons exhibited a higher corticosterone when originating
37 from rural habitats, while such relation was not observed in pale pigeons. This suggests that
38 among-population variation in stress response is higher in dark pigeons, this variation
39 possibly reflecting adjustment and/or (epi)genetic adaptation to environmental conditions. In
40 addition, corticosterone response increased with the degree of melanin-based colouration in
41 pigeons originating from rural habitats but not in pigeons originating from more urbanized
42 populations, resulting in the coexistence of alternative stress responses in some populations,
43 but not in others. Our results suggest that species with melanin-based variation in differently

44 urbanized populations along rural-urban gradient are potentially good candidate systems for
45 studying stress coping styles under alternative selective regimes.

46

47 Key-words: glucocorticoids, inter-individual variations, local adaptation, phenotypic
48 flexibility, phenotypic plasticity, pigmentation, restraint stress, urbanization

49

50 **Introduction**

51 When facing unpredictable and threatening events due to biotic or abiotic factors, vertebrates
52 mount a stress response which helps individuals to meet energetic demands through resource
53 allocation trade-offs. The hypothalamo-pituitary-adrenal (HPA) axis which culminates in the
54 release of glucocorticoids (GC) is one of the most important physiological mediators of stress
55 response. Thus, stress-induced increases in GC concentrations redirect behaviour and
56 physiology towards immediate survival functions through neoglucogenesis, an increase in
57 locomotor activity and the inhibition of non-vital functions such as reproduction (Dhabhar &
58 McEwen 1997; Breuner *et al.* 1998; Sapolsky *et al.* 2000). Due to its broad implication in the
59 trade-off between survival and reproduction, the HPA-axis reactivity is closely related to
60 fitness (Breuner *et al.* 2008 ; Crespi *et al.* 2013). Hence the adaptive function of the HPA-axis
61 is pervasively invoked to explain differences in stress-induced GC concentrations between
62 populations or subspecies exposed to different extrinsic conditions, with a heavy bias towards
63 bird studies (e.g. altitude: Addis *et al.* 2011; habitat quality: Busch & Hayward 2009;
64 predation risk: Thiel *et al.* 2008; urbanization level: Parteke 2006). Indeed, rising GC stress
65 response could facilitate a fast recovery of positive energy balance in contexts of great
66 energetic demands and low energy stores (McEwen & Wingfield 2003; Addis *et al.* 2011)
67 whereas lowering the response reduces the negative impacts of chronically elevated GC on
68 non-vital functions (e.g. immunity and resistance to oxidative stress; Stier *et al.* 2009; Silverin

69 1998). For instance, up- or down-modulations of stress response would be of primary
70 importance for all animals coping with frequent anthropogenic disturbances (Partecke,
71 2006), possibly facilitating the expansion of species ranges in novel urban environments as
72 suggested by persistent differences detected in GC responses between recently urbanized
73 populations of dark-eyed juncos *Junco hyemalis* and wildland populations in the ancestral
74 range (Atwell *et al.* 2012).

75 Comparing bird populations or subspecies, what drives differences in the HPA stress response
76 remains unclear. On one hand, such differences may arise because populations or subspecies
77 adjust stress response to the prevailing conditions that birds experience at the time of
78 sampling. Indeed, the HPA-axis activity is highly flexible to e.g. food availability (Schoech
79 2006; Schoech *et al.* 2007) or human disturbances (Cockrem *et al.* 2006). The HPA-axis
80 activity also varies as a function of internal state, e.g. body condition (Corbel *et al.* 2010) or
81 stage of life cycle (Romero 2002). On the other hand, stable differences between populations
82 may result from differential selective pressures favouring individuals with respective specific
83 corticosterone stress response through fitness consequences (Satterlee & Johnson 1988; Evans
84 *et al.* 2006; Wada *et al.* 2009; Almasi *et al.* 2010). Indeed, inter-individual variation in the
85 magnitude of elevated GC concentrations is repeatable and partly under genetic control
86 (Evans *et al.* 2006; Partecke *et al.* 2006; Rensel & Schoech 2011; Jenkins *et al.* 2014). The
87 heritability of the GC response to stress is supported by cross-fostering experiments in the
88 field (Jenkins *et al.* 2014) and by selection studies in captive settings (Evans *et al.* 2006 ;
89 Satterlee & Johnson 1988), which confirm that individual variation in GC stress response is
90 underlain by an appreciable amount of standing additive genetic variation. Stable differences
91 may also arise because of environmental factors that have affected the development of the
92 HPA axis during early life and, thus, the GC stress response that adults can mount (Love &
93 Williams 2008a; Love & Williams 2008b; Wada *et al.* 2009). Thus, the degree to which

94 phenotypic adjustments to environmental stimuli and genetic differences resulting from local
95 adaptation underlie between-population differences in stress response is often unclear while
96 the interplay of both processes is likely to be significant. The maintenance of different
97 populations in common conditions is the first step for disentangling acute adjustments from
98 more stable, possibly genetically-determined, divergences in the HPA-axis reactivity
99 (Angelier *et al.* 2011; Atwell *et al.* 2012). To have a full view about the regulation of active
100 blood circulating corticosterone, we should also consider the regulation of CBG and hence of
101 free corticosterone and corticosterone bound to CBG (Almasi *et al.* 2009).

102 Large variations in the magnitude of GC response to stress also occur within populations with
103 some individuals having constantly low or constantly high GC responses across time, together
104 with a suite of correlated behavioural and physiological traits, leading to distinct, repeatable
105 stress coping styles (Cockrem 2007; Cockrem *et al.* 2009; Koolhaas *et al.* 1999). Genetic
106 studies using offspring-parent regression found significant narrow-sense heritability estimates
107 of coping styles (25%, Drent *et al.* 2003 ; 22% : Dingemanse *et al.* 2002 ; reviewed in van
108 Oers *et al.* 2005). Stress-coping styles define coherent sets of behavioural and physiological
109 responses, including corticosterone release, to challenges faced by animals (Koolhaas *et al.*
110 1999). Based on the empirical findings that different coping styles influence fitness
111 parameters (Dingemanse *et al.* 2003; Dingemanse *et al.* 2004), individual variations in stress
112 responses may have major eco-evolutionary implications and consequences (Wolf &
113 Weissing 2012). First, the coexistence of coping styles occurs only under certain selective
114 regimes, presumably involving fluctuating and/or frequency-dependent selective pressures
115 (Carere *et al.* 2010; Wolf & McNamara 2012). Second, the potential for evolvability in
116 response to new selective regimes should be particularly high for populations with genetic
117 differences in coping styles (Wolf & Weissing 2012). Thus, the consideration of among-

118 individual variation while comparing stress response between populations is crucial, although
119 largely overlooked (Williams *et al.* 2008; but see Atwell *et al.* 2012).

120 Interestingly, melanin-based coloured morphs provide reliable phenotypic markers of
121 alternative genotypes (Theron *et al.* 2001; Mundy *et al.* 2004) that are associated with stress
122 coping styles in several taxa (Ducrest *et al.* 2008; Almasi *et al.* 2010; Kittilsen *et al.* 2009).

123 This is thought to be due to the pleiotropic effects that the genes coding for the melanocortin
124 system exert on stress response (Daynes *et al.* 1987; Racca *et al.* 2005) and suites of
125 correlated phenotypic traits (e.g. aggressiveness, Ducrest *et al.* 2008, Morgan *et al.* 2004).

126 Accordingly, differently coloured individuals differently regulate the HPA axis as reflected by
127 different stress-induced GC levels, the association between colouration and stress response
128 being heritable (Almasi *et al.* 2010). In addition, facing varying levels of stress, differently
129 coloured individuals display alternative reaction norms on energy balance and trade-offs
130 between competing physiological functions (Dreiss *et al.* 2010; Jacquin *et al.* 2012) as
131 expected if stress response is colour-specific. However, whether direction and/or magnitude
132 of the covariation between stress-induced GC levels and plumage colouration differs between
133 different populations living in different environmental conditions has not been tested yet.

134 In this study, we investigated stress response in the feral pigeon *Columba livia*. Feral pigeons
135 originate from domestic pigeons and artificially-selected variation in melanin-based
136 colouration persists in feral populations (Johnston & Janiga 1995; Sol 2008). Such variation
137 in colouration is heritable and associated with alternative physiological norms of reaction as
138 also found in other species with plumage colour variation (Jacquin *et al.* 2012; Jacquin *et al.*
139 2013). After escaping from captivity in the early XIXth century, pigeons have established in a
140 variety of habitats and undergone natural selection ever since (Johnston & Janiga 1995; Sol
141 2008). Habitats of feral pigeons markedly differ notably in their level of urbanization, ranging
142 from rural areas to city centers. Because urbanization dramatically affects both abiotic and

143 biotic environmental conditions (e.g. climate, human disturbance, population density,
144 resource availability, predation risk; Shochat *et al.* 2006; McDonnell & Hahs 2009; Niemela
145 *et al.* 2011), it can alter selection on morphological, behavioral, and physiological traits (Liker
146 *et al.* 2008; Møller 2008; Jacquin *et al.* 2013; Atwell *et al.* 2012), including stress response
147 (e.g. Partecke *et al.* 2006). Accordingly, differences in stress response between differently
148 urbanized populations have been shown in several bird species (reviewed in Bonier 2012),
149 however we crucially lack information on how this trait varies across rural-urban gradients
150 (but see Atwell *et al.* 2012). A noticeable effect of human activities is the buffering of
151 temporal changes in environmental conditions notably for those species relying on humans for
152 food resources as is the case in feral pigeons (Johnston & Janiga 1995; Shochat 2006).
153 Because instability of environment is a potent selective mechanism explaining the
154 maintenance of distinct stress copying styles within populations (Carere *et al.* 2010), the
155 magnitude of among-individual variation in stress response can be hypothesized to vary along
156 a rural-urban gradient, being lower in more stable, urban habitat.

157 In this study, we tested whether the variation in melanin-based colouration was related to
158 alternative stress responses in feral pigeons. To this aim, we examined how stress responses
159 varies both within and between differently coloured individuals in differently urbanized
160 populations. We maintained wild feral pigeons captured in three differently urbanized habitats
161 in similar environmental conditions to disentangle immediate phenotypic adjustments from
162 more stable divergences in the HPA-axis activity (Angelier *et al.* 2011). Captive feral pigeons
163 were submitted to a standard acute restraint stress to compare their corticosterone (main avian
164 GC) response. If melanin-based colouration is a phenotypic marker of stress responses in feral
165 pigeons, we expected differently coloured feral pigeons to display alternative stress responses.
166 Furthermore, if differently coloured feral pigeons display alternative stress responses
167 depending on their originating population, we expected differently coloured individuals to

168 mount different stress-induced corticosterone levels in interaction with habitat. In addition,
169 we expected to detect lower differences in corticosterone stress response between differently
170 coloured pigeons in those populations thriving with more stable environmental conditions as
171 found in urban centers.

172

173 **Materials and methods**

174 Capture and experiments were all approved by the Direction Départementale des Services
175 Vétérinaires de Seine-et-Marne (activity permit # 77-06; aviary licence # A77-431-1).

176 **Capture**

177 Feral adult pigeons were caught with trap cages at the beginning of February 2010 in three
178 different habitats (Urban center, n = 25 individuals; Urban park, n = 22 individuals; Rural
179 farm, n = 24 individuals) of the Parisian region (Table 1). Urban park and center were located
180 in the city of Paris. The farm was situated in a rural area in the Parisian suburbs. Because the
181 distance between capture sites was higher than the recently estimated distance covered by the
182 Parisian feral pigeons within their local environment (less than 800 m; Frantz *et al.* 2012), we
183 assume that these 3 habitats reflect distinct populations with restricted movements between
184 them, thus experiencing different local environmental conditions which can be described as
185 rural farm, urban park and urban center. Urbanization rate of each site was estimated using
186 land use inventory for Paris area (MOS 2003, Institut d'Aménagement et d'Urbanisme Ile-de-
187 France) and MapInfo Professional 8.5 SCP (Pitney Bowes Software Inc.). It was calculated as
188 the proportion of area covered by buildings and roads on the total circular area within a radius
189 of 1000 m around the capture site (Jacquin *et al.* 2013b; Table 1).

190 **Melanin-based colouration**

191 Feral pigeons display a continuous variation in melanin-based plumage colouration from
192 entirely white to entirely black (Johnston & Janiga 1995). The continuous variation of the

193 surface of dark area on the wings across colouration patterns corresponds to different levels of
194 melanin deposited in feathers (Haase *et al.*, 1992; Jacquin *et al.*, 2011). Melanin-based
195 colouration was assessed following Jacquin *et al.* (2011) using pictures of the upper wing
196 taken in standardized light conditions. Light conditions were standardized by the use of a 1 m²
197 reflecting white sheet placed 50 cm in the background of the wing, and of two studio lamps
198 placed 1 m both at the left and the right sides from the wing. The wing was stretched by an
199 experimenter and pictures were taken at a resolution of 96 DPI using a camera (Sony DSC-
200 HX1) placed on tripod 1 m from the wing, being saved under JPG files. Pictures were binary
201 transformed using Gimp (GNU image manipulation program) and colouration score was
202 determined as the proportion of black pixels (%) on the upper wing using ImageJ (U.S.
203 National Institutes of Health, Bethesda, USA). For analyses purposes, pale and dark colour
204 groups were defined as pigeons with colouration scores < 50 % and > 50 % respectively
205 (Jacquin *et al.* 2013a). This colouration score has been shown to be highly heritable (82 %;
206 Jacquin *et al.* 2013a).

207 **Housing**

208 Immediately after capture, body mass was measured using a Pesola spring balance (\pm 5g) and
209 pigeons were brought to the Centre de Recherche en Ecologie Expérimentale et Prédictive
210 (CEREEP, Saint-Pierre-lès-Nemours, France). Pigeons from each habitat were kept in
211 separate outdoor aviaries to limit social and physiological perturbations. Indeed, behavioural
212 and immune traits such as aggressiveness levels and parasite loads often differ between
213 differently urbanized bird populations (e.g. Evans 2010; Jacquin *et al.* 2013b). Thus mixing
214 pigeons from different origins would potentially have confounded the results as both social
215 and parasite environments impact individual corticosterone release in birds (Raouf *et al.*
216 2006; Pryke *et al.* 2007). In addition, social interactions among different colour morphs can
217 generate frequency-dependent effects in the local environment, which may directly affect the

218 level of stress captive birds are exposed to (Pryke *et al.* 2007; Morgan *et al.* 2004). In this
219 study, colouration scores were similarly distributed among the different aviaries (Median test:
220 $\chi^2 = 4.909$, $P = 0.086$; Kruskal Wallis test: $H = 3.671$, $df = 2$, $P = 0.160$) and median score
221 was close to 50% (48.7%), thus any frequency-dependent effect of differently coloured
222 individuals within aviaries on individual corticosterone release was very unlikely. Aviaries
223 were strictly identical (3.10 m \times 2 m \times 2.40 m), adjacent and visually isolated from one
224 another to limit perturbations. Water and maintenance diet (a mixture of peas, corn and
225 maize) were provided *ad libitum*. This design allowed keeping pigeons in similar captive
226 conditions in terms of housing conditions, bird density and exposure to external stressors.
227 Pigeons were acclimatized to captivity for at least three weeks before measuring their stress
228 responses until daily food intake and body weight were stabilized (Pascual *et al.* 1999). At
229 the end of the acclimation period, body mass varied by only 2.2 ± 0.5 % ($n = 71$) from the
230 values at capture, this variation being four-fold lower than that associated with an alteration of
231 the HPA function in captive birds ($> 8\%$, Romero *et al.* 1997; Romero & Wingfield 1999;
232 Dickens *et al.* 2009). Mass changes during acclimation were not affected by pigeon origin
233 ($F_{2,68} = 1.319$, $P = 0.274$), colour type ($F_{1,69} = 0.005$, $P = 0.942$) or the interaction between
234 both ($F_{2,65} = 1.306$, $P = 0.278$).

235 **Stress response measurement**

236 At the end of the acclimation period, all pigeons were subjected to a standard acute restraint
237 stress following a modified version of a protocol by Romero & Wingfield (2001).
238 Experiments were done from the end of February to mid-March 2010 between 9:00 h and
239 13:00 h to minimize the effects of seasonal and circadian changes in corticosterone plasma
240 levels, respectively (Breuner *et al.*, 1999; Romero 2002). The time of day was not related to
241 baseline levels of corticosterone ($R^2 = 0.001$, $P = 0.845$, $N = 41$) however it was weakly but
242 significantly related to the levels of stress-induced corticosterone ($R^2 = 0.096$, $P = 0.049$, $N =$

243 41). Because neither did the time of day differ between the aviaries ($F_{2,38}=1.860$, $P=0.170$) nor
244 was it related to colouration scores ($R^2 = 0.0003$, $P = 0.918$, $N=41$), we feel confident that the
245 time of day did not interact with the outcomes of this study. Each pigeon was stressed only
246 once, and a single intervention was done per day and per aviary in order to avoid
247 physiological habituation to stress created by human intervention as demonstrated in
248 chronically stressed birds (Cyr & Romero 2009). Within each aviary, pigeons were randomly
249 sampled with respect to colour type. A rapid screening of feathers was done to ensure that no
250 bird was engaged in moult process as feather renewal may affect the HPA-axis activity
251 (Romero 2002). The onset of the stress was considered as the time at which pigeons detected
252 our presence nearby the aviary, usually 15 s before capture. Pigeons were captured with a net
253 and thereafter kept motionless in an opaque cloth bag in order to create a restraint, stressful
254 condition (Romero & Wingfield 2001). At different times along the restraint stress, blood
255 samples of 0.5 ml were taken from the brachial vein, using a 26 g needle and a 1 ml
256 heparinized syringe. A total of 13% of blood samples, corresponding to 30 pigeons could not
257 be used because of hemolysis or insufficient volume. Thus, analyses hereafter in this study
258 were done on 41 pigeons. Blood was first collected at 3 min after the onset of stress (mean
259 value: 3.30 ± 0.10 min, $n = 41$). These samples were expected to provide baseline
260 corticosterone levels, since increase in corticosterone levels generally does not occur within 3
261 min following initiation of a stressful stimulus (Wingfield *et al.* 1982). Subsequent samples
262 were collected at 10 min (mean value: 10.71 ± 0.41 min, $n = 41$), 20 min (mean value: 20.46
263 ± 0.20 min, $n = 41$) and 30 min (mean value: 29.89 ± 0.10 min, $n = 41$) after the onset of
264 stress, to obtain a time course of the stress response (Romero & Wingfield 2001). Total blood
265 volume collected from any individual did not exceed 2 ml *i.e.* less than 1 % of body mass as
266 recommended by McGuill & Rowan (1989). Immediately after restraint, pigeons were
267 weighed to the nearest 5g. Body mass at restraint was similar between individuals displaying

268 alternative colour types ($F_{1,39} = 1.304, P = 0.260$) or from different habitats ($F_{2,38} = 0.670, P =$
269 0.517) as this was also the case for body mass at capture (colour type effect: $F_{1,39} = 0.951, P =$
270 0.336 ; habitat effect: $F_{2,38} = 0.958, P = 0.393$). Finally, a blood smear was taken to determine
271 parasite intensity (*Haemoproteus spp.*) as the number of infected red blood cells among 10
272 000 cells from different microscopic fields forming a monolayer (Sol *et al.* 2000; Jacquin *et*
273 *al.* 2011). Parasite intensity did not vary between colour types ($F_{1,39} = 2.002, P = 0.165$) or
274 habitats ($F_{2,38} = 0.625, P = 0.541$). Blood samples were immediately centrifuged for 10
275 minutes at 14000 rpm to separate plasma from red blood cells. Plasma were then kept at -20°
276 C until corticosterone analyses (see below). Red blood cells were kept in a PBS/EDTA
277 anticoagulant solution and were used for molecular sex determination. Briefly, DNA was
278 extracted using a Qiagen kit (DNeasy Blood and Tissue kit, cat. 69506, Qiagen Sciences) and
279 amplified by polymerase chain reaction using the P8 and P2 primers following Griffiths *et al.*
280 (1998). Sex ratio did not differ significantly for pale and dark pigeons ($\chi^2_1 = 0.028, P = 0.867$)
281 and for different habitat types ($\chi^2_2 = 0.196, P = 0.907$). Because the HPA axis activity is likely
282 to be influenced by body mass, parasite intensity and sex (e.g. O'Reilly & Wingfield 2001;
283 Cockrem *et al.* 2006; Raouf *et al.* 2006), all these factors were included in the model
284 explaining the variation in corticosterone levels along restraint stress (see below).

285 **Corticosterone levels measurement**

286 Plasma corticosterone concentrations were determined with an enzyme-immunoassay
287 procedure (corticosterone EIA kit, cat. AC-14F1, IDS Ltd) using polyclonal antiserum against
288 corticosterone coated onto the inner surface of polystyrene microtitre wells. Calibrators,
289 controls and diluted (1:2) plasma samples were incubated overnight at 28°C with peroxidase-
290 labeled corticosterone in the antibody-coated wells. Wells were then washed and a colour
291 reaction was developed using the tetramethylbenzidine chromogen. The absorbance of the
292 reaction mixtures was read in a microplate reader. Colour intensity developed is inversely

293 proportional to the concentration of corticosterone in the diluted samples. The sensitivity of
294 assays was 0.55 ng.ml^{-1} and intra- and inter-assay repeatability were 87.4 % and 79.4 %, respectively.
295

296 **Statistical analyses**

297 The aim of this study was to investigate the interacting effect of melanin-based colouration
298 with habitat on corticosterone levels. To do this, we used general linear mixed models with
299 corticosterone levels as the dependent variable, blood sampling time along with restraint (*i.e.*
300 the time since the onset of stress), habitat, colour type (pale and dark pigeons) and their
301 interactions as fixed factors, and individual nested within habitat as a random factor. Sex,
302 body mass and parasite intensity were also added as covariates in the model. The best model
303 was chosen according to the AIC_C criterion (Burnham & Anderson 2002) using a descending
304 model selection procedure starting with a model containing the following predictor variables:
305 time, habitat, colour type¹ and all second- and three-term associated interactions plus sex, body
306 mass and parasite intensity. When an interaction was significant, we conducted post-hoc
307 pairwise t-tests using a Bonferroni correction applied on α level of significance for the t-
308 statistic computed by Fishers's LSD method, to control for multiple comparisons of
309 corticosterone levels. *P*-values were adjusted so that a significant level of 0.05 was
310 consistently used (see below). The interacting effect of colour type with habitat on
311 corticosterone levels (see results) was further examined by separate analyses of regression of
312 stress-induced corticosterone levels on colouration scores (continuous variable) in pigeons
313 from each origin. All analyses were run under SPSS 20.0.0. Significance levels were set to
314 0.05 and tests were two-tailed. Means are computed with their standard errors.

315

316

317 **Results**

318 Corticosterone levels increased from $2.07 \pm 0.16 \text{ ng.ml}^{-1}$ (at 3 min after capture) to $14.27 \pm$
319 1.38 ng.ml^{-1} (at 30 min after capture; $n = 41$, $F_{3,120} = 63.422$, $P < 0.001$) across successive
320 blood sampling times, showing that restraint induced a significant stress in individuals.
321 Corticosterone levels measured at 30 minutes after capture were significantly higher than
322 baselines in all groups of pigeons as defined by each combination of colouration \times habitat
323 levels (Bonferroni correction, all $P \leq 0.012$).

324 Changes in corticosterone across blood sampling times was significantly associated with the
325 interacting effect of colour type with habitat, as resulting from the best model (effect of
326 Sampling time \times Colour type \times Habitat: $P = 0.03$, $AIC_C = 884.31$; Table 2). Corticosterone
327 levels measured in restrained individuals at 3 min and at 10 min after capture were neither
328 associated with colour type (post hoc Bonferroni correction, all $P > 0.473$) nor with habitat
329 (post hoc Bonferroni correction, all $P > 0.999$, Figure 1). By contrast, at 20 min and at 30 min
330 after capture, darker individuals reached significantly higher corticosterone levels than paler
331 individuals, this difference being detected in pigeons from rural habitat (post-hoc Bonferroni
332 correction, at 20 min: $P = 0.01$; at 30 min : $P < 0.001$; Figure 1), but not in pigeons from
333 other habitats (post hoc Bonferroni correction, at 20 min: all $P > 0.37$; at 30 min : all $P >$
334 0.08 ; Figure 1). In addition, corticosterone levels measured at 20 min and at 30 min after
335 capture significantly differed among habitats, being higher in pigeons from the rural habitat
336 than in pigeons from the urban center habitat, these differences being detected in darker
337 individuals (post hoc Bonferroni correction for urban center *versus* rural farm at 20 min: $P =$
338 0.046 ; at 30 min: $P < 0.001$; Figure 1), but not in paler individuals (post-hoc Bonferroni at 20
339 min: all $P > 0.93$; at 30 min: all $P > 0.67$; Figure 1). A positive, linear relation between stress-
340 induced corticosterone levels at 30 min and colouration scores (continuous variable) was
341 detected in pigeons from rural farm ($F_{1,11} = 7.190$, $P = 0.021$, $R^2 = 0.395$, $n = 13$; Figure 2c)
342 suggesting than darker pigeons mounted a stronger corticosterone response, but not in pigeons

343 from urban center ($F_{1,13} = 1.773$, $P = 0.206$, $R^2 = 0.120$, $n = 15$; Figure 2a) or urban park ($F_{1,11}$
344 $= 1.094$, $P = 0.318$, $R^2 = 0.090$, $n = 13$; Figure 2b).

345

346

347 **Discussion**

348 The aim of this study was to compare stress response both within and between differently
349 coloured pigeons across differently urbanized habitats.

350 Our results show that dark pigeons from the rural habitat reached higher levels of
351 corticosterone levels in response to stress as compared to dark pigeons from the urban center
352 habitat and to pale pigeons from either habitat. Differences between mean corticosterone
353 levels at 30 min of restraint that are necessary for statistical tests to have an acceptable power
354 (80%) have been defined by a recent meta-analysis using the coefficients of variation
355 obtained in several bird species (Cockrem *et al.* 2009). A rule of thumb is that the difference
356 between means must be at least 80 % and 50 % of the lowest mean for sample sizes of 5 and
357 10, respectively. In this study, mean corticosterone levels at 30 min differed by 143 % and
358 137 % of the lowest mean between differently coloured pigeons in rural habitat (9.80 ng.ml⁻¹
359 *versus* 23.21 ng.ml⁻¹, $N > 6$) and between dark pigeons from differently urbanized habitats
360 (9.55 ng.ml⁻¹ *versus* 23.21 ng.ml⁻¹, $N \geq 5$), respectively. Thus, we were confident that the
361 significant interacting effect of colour type with habitat on stress-induced corticosterone
362 levels did not arise by sampling error.

363 Inter-individual variations in corticosterone stress response can arise from altered pituitary
364 and/or adrenal functions due to chronic stress generated by captive (Romero & Wingfield
365 1999; Dickens *et al.* 2009) or natural conditions (Walker *et al.* 2006; Cyr & Romero 2009),
366 but this possibility seems unlikely in our study. First, acclimation to captivity lasted for at
367 least three weeks (following Pascual *et al.* 1999) and resulted in low body mass variation (2

368 % only) and in corticosterone levels close to, or lower than, those of free-living pigeons
369 (baseline levels: 2 *versus* 9 ng.ml⁻¹; stress-induced levels; 14 *versus* 15 ng.ml⁻¹; Romero &
370 Wingfield 2001). This contrasts with expectations under stressful captive conditions (Romero
371 *et al.* 1997; Romero & Wingfield 1999; Dickens *et al.* 2009). Second, corticosterone baseline
372 levels did not depend on the factors of interest (colour types and habitats; Figure 1),
373 suggesting that different stress responses to restraint experiment were not due to differences
374 in physiological adjustments to captivity. Additionally, it is very unlikely that any seasonal
375 flexibility of stress response can account for differences in corticosterone levels found in our
376 work because pigeons were all synchronized for moult and were all submitted to the same
377 light, climate and feeding conditions. Finally, health parameters known to be affected by
378 chronic stress (body mass and parasite intensity; Cyr & Romero 2009) did not vary according
379 to colour types or habitats, nor did they contribute to predict variations in corticosterone
380 levels. Hence, variations in stress responses observed between pigeons in our study were
381 unlikely to result from chronic stress or associated alteration of the HPA-axis.

382 Although we found no significant difference in health marks of chronic stress between
383 differently coloured pigeons, the role for other physiological factors *e.g.* oxidative stress
384 and/or social factors affecting corticosterone levels can not be ruled out (Senar 1999;
385 Giraudau & McGraw 2014). Alternatively, the difference in stress response may rather have
386 a genetic basis. Such association between colouration and stress response may be due to the
387 pleiotropic effects of the melanocortin system (Ducrest *et al.* 2008). Melanocortins (α -, β -,
388 and γ -MSH and ACTH) of *POMC*-gene bind to the melanocortin-1 receptor (MC1-R) thereby
389 triggering melanin synthesis, but also to other receptors involved in the HPA-axis activation
390 (MC2-R and MC4-R), thus inducing some pleiotropic effects on glucocorticoid production
391 and resistance to stress (reviewed in Ducrest *et al.* 2008). Accordingly, systemic injections of
392 α -MSH in rodents induce changes in stress-induced corticosterone levels (Daynes *et al.* 1987;

393 Racca *et al.* 2005). In wild barn owls *Tyto alba*, genetic association between colouration and
394 stress response has been inferred from parent-to-offspring inheritance of covariation between
395 the degree of melanic colouration and stress-induced corticosterone levels (Almasi *et al.*
396 2010). Furthermore, in domestic rainbow trout *Oncorhynchus mykiss*, melanin pigmentation
397 patterns are associated with, and can be artificially co-selected with, the responsiveness of the
398 hypothalamus-pituitary-interrenal axis (the equivalent of HPA axis in fishes; Kittilsen *et al.*
399 2009). Here, we found that stress-induced corticosterone levels covaried with the degree of
400 melanic colouration, as reflected by black colouration scores, in one population of feral
401 pigeons. In the same species, food restriction affects the physiological trade-offs between
402 maintenance and reproduction as a function of black colouration scores in the same species
403 Indeed, under food restriction, darker eumelanic females had a higher egg production, but
404 darker adults tended to lose more body mass than paler conspecifics (Jacquin *et al.* 2012).
405 Because the HPA-axis is broadly implicated in such trade-offs (Sapolsky *et al.* 2000), it is
406 likely that a responsiveness of the HPA-axis to nutritional stress depending on plumage
407 colouration partly explains the results of Jacquin *et al.* 2012., which is in full accordance our
408 results. Overall, our results support the idea that melanin-based colouration is associated with
409 the ability to cope with stress (Fargallo *et al.* 2007; Roulin *et al.* 2008; Almasi *et al.* 2012)
410 through a differential regulation of corticosterone release (Almasi *et al.* 2010).

411 The effect of origin on the covariation between colouration and stress response could
412 reflect either an aviary effect, although the aviaries did not differ in any characteristics (size
413 or any biotic or abiotic parameter measured), and/or a habitat effect. The effect of origin on
414 the covariation between colouration and stress response could reflect either an aviary effect or
415 a habitat effect, as our design could not disentangle these factors. While the first possibility
416 cannot be ruled out, it seems unlikely because aviaries did not differ in any characteristic
417 (size, exposition, or any biotic or abiotic parameter measured). Differently coloured pigeons

418 may display different levels of expression of genes involved in the HPA axis activity in
419 relation to environmental factors (either under captive or natural conditions). These factors
420 may involve peri-natal exposure to corticosterone which has profound organizational effects
421 on the HPA axis that shape stress response later in life (Love & Williams 2008a; Love &
422 Williams 2008b; Wada *et al.* 2009). Corticosterone levels in developing birds primarily
423 depend on maternal condition, nest-site characteristics and parental provisioning, all of these
424 factors being likely affected by the level of urbanization of habitats (Crino *et al.* 2011, Bonier
425 2012). An interacting effect of melanic colouration with environment on the level of
426 melanocortins has been well identified in tawny owls *Strix aluco*. In this species, the level of
427 *POMC*-gene expression, as reflected by the circulating levels of POMC prohormone, as well
428 as the level of gene expression of convertase enzyme *PC1/3* which cleaves POMC
429 prohormone to obtain ACTH, covary with the degree of melanic colouration under favorable
430 conditions (Roulin *et al.* 2011; Emaresi *et al.* 2013). By contrast, in birds experiencing natural
431 or experimentally-induced higher levels of stress, no colour-specific difference is detected
432 potentially leading to up- and down-regulation of melanocortins acting on the HPA axis
433 activity depending on conditions (Noguchi *et al.* 2006; Roulin *et al.* 2011). Whether variation
434 in stress-induced corticosterone levels is linked to those of *POMC*-gene expression in feral
435 pigeons remains to be tested. Finally among-population variation in stress response could be
436 due to some genetic variation between the rural and the center-urbanized populations,
437 independently of the colour phenotype and genetic underpinning. Indeed, important genetic
438 variation exists between *C. livia* populations, for instance in the allele frequencies of
439 numerous polymorphic proteins (Johnston & Janiga, 1995). Such variations are notably
440 measured between domestic and feral populations (Johnston & Janiga, 1995). In our study, it
441 is likely that rural pigeons are genetically closer to domestic lines than are the center-

442 urbanized pigeons. Such a possibility requires future investigations examining the relationship
443 between the genetic transmission of stress response and that of plumage coloration.

444 Alternatively, our results may reflect genetically-based, colour-specific divergences in
445 stress response between differently urbanized populations due to selective forces associated
446 with the urban environment. Such divergence despite the relatively recent history of urban
447 colonization in feral pigeons would imply that evolutionary processes act quickly to shape the
448 corticosterone response to stress, as also suggested in other bird species (Partecke *et al.* 2006;
449 Angelier *et al.* 2011; Atwell *et al.* 2012). Rationales for strong selection on stress response is
450 that it is genetically determined and heritable (Evans *et al.* 2006; Jenkins *et al.* 2014) and it
451 has important fitness consequences (Breuner *et al.* 2008). Among the possible mechanisms of
452 inheritance of stress response in polymorphic species, a role for imprinting on the genes
453 coding for the receptors to regulate the HPA axis has been proposed (Almasi *et al.* 2010;
454 Chong *et al.* 2007). Such genomic imprinting would occur in darker individuals and impair
455 under some circumstances the pleiotropically-mediated genetic association between stress
456 response and melanin-based colouration (Almasi *et al.* 2010). A similar mechanism may
457 operate in dark feral pigeons established in highly urbanized areas, potentially conferring
458 offspring with selective advantages as imprinted genes are targeted by selection and allow for
459 rapid adaptive evolution (Danchin *et al.* 2011). So far, two studies have examined the genetic
460 underpinning of differences in stress responses between differently urbanized populations of
461 birds. Both studies found that urban birds raised in a common garden showed an attenuated
462 corticosterone response to a 30-min restraint stress when compared with their wildland
463 counterparts, the difference between corticosterone levels being highly comparable to that
464 observed in dark feral pigeons (mean stress-induced corticosterone levels in less urbanized
465 versus more urbanized populations: European blackbirds *Turdus merula* : 30 versus 15 ng.ml⁻¹
466 ¹, Partecke *et al.* 2006; dark-eyed junco *Junco hyemalis* : 20 versus 12 ng.ml⁻¹, Atwell *et al.*

467 2012; feral pigeons: 23 versus 10 ng.ml⁻¹, this study). Thus our findings further extend the
468 idea of urban selective pressures driving genetically-determined attenuated HPA
469 responsiveness. This hypothesis should be further examined using replicates of habitats for
470 each level of urbanization.

471 Independently of their exact underpinnings, a fundamental aspect of our results is
472 that variation in stress response across different environmental conditions was observed in
473 dark, but not in pale pigeons. This suggests that the genetic basis of stress response in darker
474 pigeons allows for a greater plasticity or flexibility to environmental conditions (genotype-by-
475 environment effect) and/or is more easily targeted by selection. Jacquin *et al.* (2013a) recently
476 showed that feral pigeons are darker in more urbanized areas of the Parisian agglomeration
477 despite important gene flux between populations (Jacob, Prévot-Julliard and Baudry,
478 unpublished), suggesting strong effect of selection on plumage colouration (Kawecki and
479 Ebert 2004, Antoniazza *et al.* 2010). Whether any modulation of the HPA responsiveness
480 accounts for some processes of local adaptation in dark pigeons requires extended field
481 studies measuring the relation between stress physiology and fitness (reproductive success
482 and survival) in differently coloured and differently urbanized individuals. The precise urban
483 selective pressures involved in such local adaptation remain unknown, but may involve any
484 factor associated with urban environments including trace metals (Chatelain *et al.* 2014) and
485 human presence (Møller 2008).

486 Another important finding is that the covariation between melanin-based colouration
487 and stress-induced corticosterone levels was observed in rural pigeons but not in more
488 urbanized ones. This result opens the possibility that the genetic, pleiotropically-mediated
489 association between colouration and stress response may be disrupted for some reasons and
490 under some circumstances, resulting in the coexistence of alternative stress responses in some
491 populations with plumage colour variation, but not in others. Theoretical models predict that

492 the instability of environment is a potent selective mechanisms explaining individual
493 variations in stress coping styles within populations (Dingemanse *et al.* 2004, Dingemanse &
494 Réale 2005; Carere *et al.* 2010) and more generally, that distinct personalities should emerge
495 only under moderately favourable ecological conditions (*i.e.* under neither unfavourable nor
496 highly favourable conditions; Luttbeg & Sih 2010). Our results support the above predictions
497 as the coexistence of colour-specific stress responses occurs in rural but not in urbanized areas
498 which are characterized by a hampering of environmental variability (e.g. the seasonality of
499 resource availability and climate) and likely constitute highly favourable ecological
500 conditions for feral pigeons (Sacchi *et al.* 2002; Shochat *et al.* 2006). Another selective factor
501 on the diversity of coping styles is the relative frequency of occurrence of similarly or
502 differently behaving individuals (Wolf & McNamara 2012), which likely varies between
503 differently urbanized populations of feral pigeons (Jacquin *et al.* 2013b). We are aware that
504 measuring stress response only once within individuals and only in captive birds limits the
505 inferences that can be made, both with respect to the repeatability and the generalization
506 under natural conditions. However, our results suggest that habitats along rural-urban gradient
507 are potentially good candidates for alternative selective regimes acting on stress coping styles
508 and more generally personalities. This hypothesis has already been partially validated by
509 recent studies in birds (Evans *et al.* 2010; Atwell *et al.* 2012; Bokony *et al.* 2012; Miranda *et*
510 *al.* 2013; Mueller *et al.* 2013) and humans (Malan *et al.* 2012). Research efforts are now
511 needed towards the measurements of suites of behavioural and physiological traits along the
512 proactive-reactive coping style axis in urban versus rural populations of pigeons and other
513 species with plumage colour variation.

514 To conclude, we show that in a bird species with plumage colour variation, the
515 degree of melanin-based colouration is associated with the ability to modulate stress response
516 as a function of environmental conditions. The extent to which phenotypic adjustments,

517 (epi)genetic divergences and the interaction between both processes explain differences in
518 colour-specific stress responses across environmental conditions requires further studies. The
519 issue could be addressed using common garden studies examining the reaction norms on
520 corticosterone release in differently coloured individuals from distinct habitats. Whatever the
521 exact cause of variation, the changes in stress response across environments being likely
522 genetically associated with colouration provides promising perspectives for the evolvability of
523 the HPA-axis in species with plumage colour variation. Thus our results suggest the
524 suitability of biological models with melanin-based variation in questioning the evolvability
525 of the HPA axis and perhaps that of copying styles under different selective regimes, at a time
526 when flourishing theoretical models explaining the evolution of personalities urgently call for
527 empirical tests (Wolf *et al.* 2007; Dingemanse & Wolf 2010; Dingemanse *et al.* 2010; Luttbeg
528 & Sih 2010; Carere *et al.* 2010; Wolf & McNamara 2012).

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540 This article does not contain any studies with human participants performed by any of
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542 Informed consent was obtained from all individual participants included in the study.

543

544 **Conflicts of interest**

545 The authors declare that they have no conflict of interest.

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842 Table 1

843 Characteristics of habitats

Site	Capture date	Site name	City		Urbanization	Habitat type
			(district number)	GPS coordinates	rate (% constructed area)	
A	02/03/2010	Porte Saint-Denis	Paris (X)	48°52'11.194"N 2°21'9.342"E	97	Urban center
B	04/02/2010	Arènes de Lutèce	Paris (V)	48°50'42.269"N 2°21'8.614"E	79	Urban park
C	11/02/2010	Mandres-Les-Roses	Mandres-Les-Roses (94)	48°42'23.536"N 2°33'15.557"E	47	Rural farm

844

845 Table 2
 846 Effects of blood sampling time, plumage colour type and habitat on corticosterone levels ($n =$
 847 164 measurements) along restraint stress in feral pigeons ($n = 41$ pigeons).

848

Effect	<i>df</i>	<i>F</i>	<i>P</i>
Sex	1,34	5.52	0.03
Sampling time	3,105	64.06	< 0.001
Colour type	1,34	3.96	0.05
Habitat	2,34	1.37	0.27
Sampling time × Colour type	3,105	2.40	0.07
Sampling time × Habitat	6,105	0.76	0.61
Colour type × Habitat	2,34	3.01	0.06
Sampling time × Colour type × Habitat	6,105	2.53	0.03

849 Significant *P*-values (< 0.05) are in bold

850 **Figure captions**

851

852 **Fig. 1** Corticosterone levels (CORT) measured at 3 min (1st blood sample, baselines), 10 min
853 (2nd blood sample), 20 min (3rd blood sample) and 30 min (4th blood sample) after capture in
854 pale (white circles) and dark (black circles) restrained feral pigeons from 3 habitats (urban
855 center: pale, $n = 10$, dark, $n = 5$; urban park: pale, $n = 5$, dark, $n = 8$; rural area: pale, $n = 7$,
856 dark, $n = 6$). Values are means \pm SE. Different superscript letters represent significant
857 differences ($P < 0.05$) between habitats within each colour type morph and each sampling
858 time following post hoc tests. The letters can not be compared within habitats. Asterisks
859 represent significant differences ($P < 0.05$) between colour type morphs within each habitat
860 following post hoc tests

861

862 **Fig. 2** Relation between stress-induced corticosterone levels (CORT) measured at 30 min
863 after captures and black colouration scores in feral pigeons from 3 sites corresponding to a)
864 urban center (N =15), b) urban park (N = 13), and c) rural area (N = 15). The line is for the
865 significant regression of CORT on colouration scores ($P = 0.021$, see results).