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

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Running title: colouration and stress response in feral pigeons

Abstract

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

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

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

Introduction

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

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

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

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

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

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

Materials and methods

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

Capture

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

Melanin-based colouration

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

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

Housing

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

level of stress captive birds are exposed to (Pryke *et al.* 2007; Morgan *et al.* 2004). In this study, colouration scores were similarly distributed among the different aviaries (Median test: $\chi^2_2 = 4.909$, $P = 0.086$; Kruskal Wallis test: $H = 3.671$, $df = 2$, $P = 0.160$) and median score was close to 50% (48.7%), thus any frequency-dependent effect of differently coloured individuals within aviaries on individual corticosterone release was very unlikely. Aviaries were strictly identical (3.10 m $\times$ 2 m $\times$ 2.40 m), adjacent and visually isolated from one another to limit perturbations. Water and maintenance diet (a mixture of peas, corn and maize) were provided *ad libitum*. This design allowed keeping pigeons in similar captive conditions in terms of housing conditions, bird density and exposure to external stressors. Pigeons were acclimatized to captivity for at least three weeks before measuring their stress responses until daily food intake and body weight were stabilized (Pascual *et al.* 1999). At the end of the acclimation period, body mass varied by only 2.2 ± 0.5 % ($n = 71$) from the values at capture, this variation being four-fold lower than that associated with an alteration of the HPA function in captive birds ($> 8\%$, Romero *et al.* 1997; Romero & Wingfield 1999; Dickens *et al.* 2009). Mass changes during acclimation were not affected by pigeon origin ($F_{2,68} = 1.319$, $P = 0.274$), colour type ($F_{1,69} = 0.005$, $P = 0.942$) or the interaction between both ($F_{2,65} = 1.306$, $P = 0.278$).

Stress response measurement

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

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

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

Corticosterone levels measurement

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

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

Statistical analyses

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

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: $P = 0.03$, $\text{AIC}_C = 884.31$; 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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

To conclude, we show that in a bird species with plumage colour variation, the degree of melanin-based colouration is associated with the ability to modulate stress response 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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All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

This article does not contain any studies with human participants performed by any of the authors.

Informed consent was obtained from all individual participants included in the study.

Conflicts of interest

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 (district number)	GPS coordinates	Urbanization	
					rate (% construced area)	Habitat type
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 $\times$ Colour type	3,105	2.40	0.07
Sampling time $\times$ Habitat	6,105	0.76	0.61
Colour type $\times$ Habitat	2,34	3.01	0.06
Sampling time $\times$ Colour type $\times$ Habitat	6,105	2.53	0.03

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

Figure captions

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

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