Urbanization alters the relationship between coloration and territorial aggression, but not hormones, in song sparrows

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Melanin-based coloration is associated with aspects of phenotype, including conspecific aggression and hormone concentrations. These relationships could arise as a result of genetic and/or biochemical links between melanin production and other traits. Additionally, anthropogenic change, including urbanization, exposes animals to novel conditions that can alter pigmentation, behaviour or hormones, potentially disrupting relationships between coloration and other traits. We examined relationships among the extent and darkness of melanin spotting on the breast of male song sparrows, *Melospiza melodia*, territorial behaviour, plasma testosterone and corticosterone concentrations to determine whether coloration was reliably associated with phenotype in this species. We conducted this study in urban and rural populations to determine whether relationships between coloration, behaviour and hormones varied between habitats. Males in urban habitat had more extensive brown spotting than rural males. The relationship between melanin coloration and territoriality differed in urban and rural habitats. In rural sparrows, territoriality was negatively correlated with spotting area, while in urban sparrows territoriality was positively associated with spotting darkness. Regardless of habitat, males with more extensive spotting increased testosterone secretion more in response to gonadotropin-releasing hormone administration and males with darker spotting had greater handling restraint-induced release of corticosterone. This suggests that plumage coloration is associated with underlying physiology, but the relationship between coloration and behaviour may shift between habitats.

Melanin-based coloration is widespread in vertebrates and can signal aspects of phenotype to conspecifics (Jawor & Breitwisch, 2003; Rohwer, 1975). The size and reflectance of melanin areas are associated with a number of phenotypically plastic traits including behaviour and physiology (Roulin, 2016). Although signal honesty, a reliable association between phenotype and coloration, is essential for the maintenance of stable communication systems, the mechanisms that maintain this association in melanin-based ornaments are unclear (Dawkins & Krebs, 1978; Diep & Westneat, 2013; Wiley, 1994). Recently, it has been proposed that genetic and biochemical links between melanin production and other systems that regulate behaviour and physiology maintain associations between melanin pigmentation and other aspects of phenotype (hypotheses presented in Ducrest, Keller, & Roulin, 2008; Jenkins, Vitousek, & Safran, 2013). There are two predominant

types of melanin, eumelanin and pheomelanin, and their production is largely antagonistic such that the genetic and biochemical conditions that produce one inhibit the production of the other (Ducrest et al., 2008; Galvan, Wakamatsu, Camarero, Mateo, & Alonso-Alvarez, 2015), although most melanin ornaments are a mixture of the two pigments. Thus, the type of melanin or relative contribution of each type of melanin to an ornament could be central to generating correct predictions regarding the relationship between coloration and behaviour or physiology (Roulin, Almasi, Meichtry-Stier, & Jenni, 2011).

Much of the work that has addressed relationships between melanin-based coloration, aggression and hormone concentrations has focused on eumelanin-based traits. Individuals with darker and/or larger eumelanin ornaments are predicted to be (1) more aggressive, (2) have greater androgen concentrations and (3) be less responsive to stress. In the case of primarily pheomelanin-based ornaments, the predicted relationships are similar with the exception of reactivity of the hypothalamic—pituitary—adrenal (HPA) axis; more pheomelanic individuals are predicted to have greater baseline and stress-induced release of glucocorticoids than those that are less pheomelanic. In many vertebrates, including

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birds, fish and reptiles, more eumelanic individuals are more aggressive than less eumelanic individuals (Mafli, Wakamatsu, & Roulin, 2011; Schweitzer, Motreuil, & Dechaume-Moncharmont, 2015; Seddon & Hews, 2016; Senar, 2006). Initial testosterone is positively associated with the size of eumelanin-based traits (Buchanan, Evans, Goldsmith, Bryant, & Rowe, 2001; Evans, Goldsmith, & Norris, 2000; Gonzalez, Sorci, Smith, & de Lope, 2001), although some studies found no such relationship (Beziers, Ducrest, Simon, & Roulin, 2017; Laucht, Kempenaers, & Dale, 2010; Seddon & Hews, 2016). In the case of predominantly pheomelanin-based traits, males with darker or larger pheomelanin coloration can have higher androgen levels (Hasegawa, Arai, Sato, & Sakai, 2017; Safran, Adelman, McGraw, & Hau, 2008) while in other cases no relationship was found between coloration and androgens (Grindstaff, Lovern, Burtka, & Hallmark-Sharber, 2012). Animals may transiently increase testosterone during social interactions, and this ability can be assessed by measuring the response to exogenous gonadotropin-releasing hormone (GnRH) (Goymann, 2009; Goymann, Landys, & Wingfield, 2007). Thus, GnRH-induced testosterone concentrations may be more strongly associated with social behaviour and coloration than initial testosterone concentrations (McGlothlin et al., 2008). Relatively few studies have assessed relationships between any type of coloration and GnRH-induced testosterone secretion (Cain & Pryke, 2017; McGlothlin et al., 2008; Spinney, Bentley, & Hau, 2006).

Production of melanins may be associated with glucocorticoid concentrations as well. Biochemical links between the production of melanins and the HPA axis lead to the predictions that pheomelanin-based coloration is positively associated with glucocorticoids while eumelanin coloration is indicative of lower initial and stress-induced corticosterone release (Ducrest et al., 2008; Jenkins et al., 2013). A few studies have found that more eumelanic individuals have lower baseline corticosterone profiles (Kittilsen et al., 2009; Saino et al., 2013), although in other cases, no relationship has been found between initial corticosterone and either melanin pigment (Almasi, Jenni, Jenni-Eiermann, & Roulin, 2010; Corbel et al., 2016; Grindstaff et al., 2012; Jenkins et al., 2013). Similarly, stress-induced corticosterone concentrations can be negatively (Almasi et al., 2010; Kittilsen, Johansen, Braastad, & Overli, 2012; Saino et al., 2013) or positively (Corbel et al., 2016) associated or unassociated with both melanins.

Melanin coloration, behaviour and physiology could be influenced by environmental conditions, including human alteration of habitats. Urbanization produces novel ecological conditions that influence melanin coloration (Giraudeau et al., 2015; Senar, Conroy, Quesada, & Mateos-Gonzalez, 2014), behaviour (Bokony, Kulcsar, Toth, & Liker, 2012; Davies & Sewall, 2016; Lowry, Lill, & Wong, 2013; Shochat, 2004; Yeh, Hauber, & Price, 2007) and physiology (Atwell, Cardoso, Whittaker, Price, & Ketterson, 2014; Bonier et al., 2007; Partecke, Schwabl, & Gwinner, 2006). Examining populations in urban and rural habitats may provide novel insights into the mechanisms, including physiological linkages, ecological factors and selective pressures, that maintain associations between plumage coloration and other phenotypic traits. Given the widespread nature of urbanization, such research is increasingly important.

Song sparrows, *Melospiza melodia*, provide an ideal system for exploring relationships between melanin coloration, phenotype and urbanization. Both sexes display brown, melanin-based spotting on the breast, which is likely a mixture of eumelanin with some pheomelanin based on the reflectance spectra (Galvan & Wakamatsu, 2016), but to date no studies have determined whether the breast spotting is a signal used in conspecific

interactions. The first step towards determining whether melanin plumage pigmentation is a signal in song sparrows is to determine whether it is reliably associated with behaviour or physiology. We focused on the breast spotting because the pattern is conspicuous on the chest (dark brown spots on a white background), particularly when males sing, and there is obvious variation among individuals in the size and colour of the spotting (Fig. 1). This spotting is also similar to plumage traits that are related to physiology and behaviour in other avian species, including sparrows (Grunst & Grunst, 2015; Rohwer, 1975, 1977). One goal of our study was to determine whether the darkness and/or area of spotting predicted a bird's level of territorial aggression in response to a simulated territorial challenge, and we focused on males because females are not territorial. Previous research found that urban song sparrows were more aggressive than rural males (Davies & Sewall, 2016; Evans, Boudreau, & Hyman, 2010; Foltz et al., 2015). Based on these results, we predicted that urban birds would possess more extensive and/or darker spotting (i.e. be more melanic) than rural birds. We also predicted that males with darker plumage and/or greater spotting would respond more aggressively during a simulated territorial intrusion, independent of habitat type. Additionally, we predicted that male song sparrows with larger and/or darker spotting would have greater initial and GnRH-induced release of testosterone as this is the general pattern for both eumelanin- and pheomelanin-based traits. Because greater eumelanin production is associated with greater resistance to stressors, we predicted that males with darker spotting would have lower initial and stress-induced corticosterone than males with lighter spotting. Finally, we examined all of these relationships in urban and rural habitats, thereby testing the hypothesis that urbanization influences pigment–phenotype relationships.
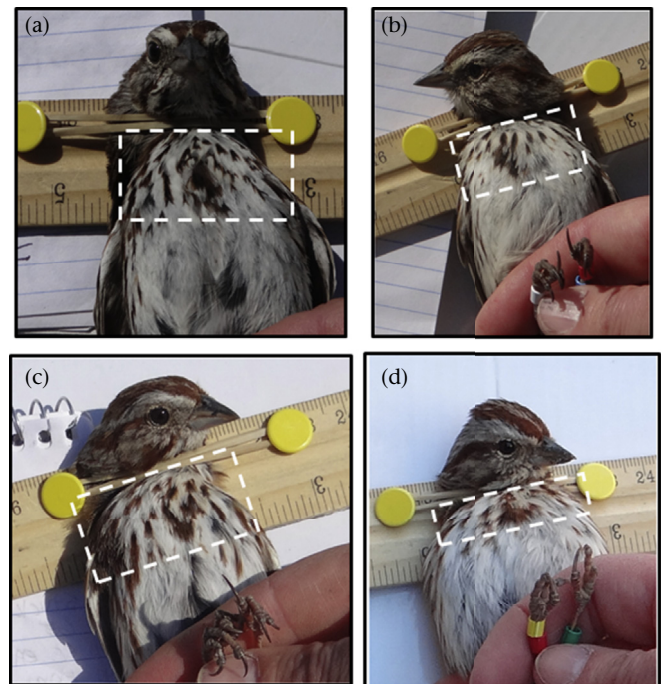

Figure 1. Variation in song sparrow chest pigmentation. The two male song sparrows depicted in (a) and (b) have rather darker brown, more eumelanic chest pigmentation compared to the two redder, more pheomelanic males depicted in (c) and (d). The amount of chest pigmentation also varies, with some males having a greater area of their chest covered (a, c) or a relatively smaller area covered (b, d).

METHODS

Study Site and Testing

We conducted this research in Montgomery County, Virginia, U.S.A. at six locations. Three of these locations were more natural, rural habitats while the other three locations were urban sites located on the campuses of Radford University and Virginia Tech (Supplementary Table S1). Additional details of the sites and the methodology for classifying sites as urban or rural can be found elsewhere (Davies, Beck, & Sewall, 2018).

We assessed aggressive behaviour of 67 male song sparrows from 18 April to 25 May 2016 by using conspecific playback for 6 min. To do this, we identified the centre of a male's territory by briefly using conspecific playback to identify the area that a male occupied. We then placed a speaker in the centre of a territory and broadcast one of 16 playback tracks recorded from a population of song sparrows in Durham, North Carolina, U.S.A. at a volume (75−85 dB at 1 m) and rate (1 song every 10 s) that approximated natural song. We recorded the distance between the male and the speaker in standardized intervals, the number of broadcast songs, the number of soft songs and the number of wing waves into a digital voice recorder (following Nowicki, Searcy, Krueger, & Hughes, 2002; Searcy, Anderson, & Nowicki, 2006). Broadcast songs indicate an individual's identity and are produced at high amplitudes that allow the signal to cover long distances (Anderson, Searcy, Peters, & Nowicki, 2008; Dabelsteen & Pedersen, 1992). Soft songs are produced predominantly in aggressive contexts and are predictive of attack or aggressive escalation in song sparrows (Akçay, Tom, Campbell, & Beecher, 2013; Searcy et al., 2006) and other songbirds (Ballentine, Searcy, & Nowicki, 2008; Hof & Hazlett, 2010). Wing waves are also associated with aggressive escalation and are predictive of attack in song sparrows (Akçay et al., 2013). Additionally, males that remain closer to the speaker are also more likely to attack (Searcy et al., 2006).

Following the territorial aggression trial, males were captured in a mist net using conspecific song playback (average total playback duration 13.38 ± 0.9 min). Within 3 min of capture, we obtained a 100 μl blood sample from the brachial vein to quantify initial hormone concentrations, took morphological measurements and banded males. Because males heard the playback for a variable amount of time prior to capture, we considered these to be initial, rather than baseline, hormone concentrations. For 30 males, we assessed handling restraint-induced corticosterone release and GnRH-induced testosterone release. After the initial blood sample, we injected the pectoral muscle with 500 ng of GnRH-1 (H-3106, Bachem, Torrance, CA, U.S.A.) dissolved in 50 μl of PBS (following Soma & Wingfield, 2001). This method has been used repeatedly in song sparrows and other songbirds and reliably produces an increase in testosterone above initial levels and in comparison to a saline injection during the breeding season (Jawor et al., 2006; Schmidt, MacDougall-Shackleton, Soma, & MacDougall-Shackleton, 2014). For this reason, and to have a larger sample of GnRH-injected males, we did not perform a control, saline injection. Males were placed in a cloth bag, and a second 100 μl blood sample was taken from the opposite brachial vein 30 min after the injection. Blood samples were kept on ice until return to the laboratory ($\leq$6 h) where they were spun at 6708 g for 5 min and the plasma fraction frozen at $-80\,^{\circ}$C in separate aliquots for the hormone assays.

Quantification of Testosterone and Corticosterone

Details regarding the quantification of testosterone and corticosterone can be found elsewhere (Davies & Sewall, 2016; Davies et al., 2018). Briefly, we quantified plasma testosterone and corticosterone using validated enzyme-linked immunoassays (Enzo Life Sciences, Farmingdale, NY, U.S.A.) following the manufacturer's instructions. For both hormones, we assayed samples in duplicate and assigned all of the samples from a given bird to the same assay plate. The average intra- and interassay coefficients of variation were 6.6% and 9.5%, respectively, for the testosterone assay and 6.8% and 7.8%, respectively, for the corticosterone assay. The average assay sensitivities for the testosterone and corticosterone were calculated as 2 standard deviations above the blank value and were 2.7 pg/ml and 19.8 pg/ml, respectively.

Quantification of Spotting Size and Colour

We measured plumage reflectance for 70 males and spotting area for 46 males. Sample sizes were higher for plumage reflectance than for behaviour ($N = 67$) because we captured a few males inadvertently when targeting another male. Sample sizes are lower for spotting size because we did not always have time to photograph birds in the field.

To quantify spotting area, we took a photograph of each bird on a white background with a metric ruler as a size standard. The camera was held perpendicular to the bird and we took three to five images of the chest of each individual. Between pictures, we smoothed the feathers, which produced a slightly different view of the spotting. We used Image J (Rueden et al., 2017) to quantify the area covered in brown on each bird in the three pictures (Appendix, Fig. A1). We calculated the brown area from the neck to a line drawn horizontally across the chest, just below the bottom of the central portion of the badge (Fig. 1). We used the threshold tool in Image J to produce a black-and-white image that closely approximated the original and then calculated the area covered by brown pigmentation. We calculated the repeatability of these three measurements following McGraw and Wong (1996) and found that they were highly repeatable ($r = 0.87$). We used the average area from the three pictures as our estimate of spotting area.

After photographing a bird, we collected approximately 12 brown feathers from the central portion of the badge. We taped eight feathers on a piece of black construction paper with minimal reflectance in a manner similar to their appearance on the bird. We quantified plumage coloration using a JAZ reflectance spectrophotometer (Ocean Optics, Dunedin, FL, U.S.A.). A 200 μm fibreoptic probe was held in a sheath that excluded ambient light and was maintained at a constant distance of 5.5 mm from the sample. All reflectance measurements were made relative to a white standard (Labsphere, North Sutton, NH, U.S.A.). We made four measurements of the badge and used the program CLR v.1.05 (Montgomerie, 2008) to extract colour variables. We focused on average brightness (average percentage reflectance from 320 to 700 nm) and refer to this variable as darkness throughout the rest of the manuscript. This is because lower brightness should be indicative of greater melanin content and thus a darker bird (Appendix, Fig. A2). Furthermore, we estimated the relative contribution of eumelanin and pheomelanin to the spotting using relationships between reflectance spectra and concentration of the two melanin pigments from Galvan and Wakamatsu (2016). The relative contribution of eumelanin and pheomelanin to feather coloration, as measured by HPLC, was associated with the slope of the reflectance spectra from 300 to 700 nm (Galvan & Wakamatsu, 2016). Thus, we calculated the slope for the song sparrow melanin coloration and compared it to the values reported in Galvan and Wakamatsu (2016) to estimate the relative concentration of eumelanin and pheomelanin in song sparrow feathers at the population level.

Analyses

We assessed normality using Kolmogorov–Smirnov tests and normal probability plots. We used log transformations on all hormone concentrations because they deviated from assumptions of parametric tests. Spotting area and darkness were not correlated (Pearson correlation: $r_{42} = -0.013$, $P = 0.934$), so we included both variables in all of the models. We calculated the scaled mass index for all males (Peig & Green, 2009) and included this as a measure of body condition in the models. Preliminary analysis indicated that none of the variables differed among the three rural habitats or among the three urban habitats (all $P \geq 0.114$) and so we pooled the sites as urban and rural habitats for the analysis. We ran ANCOVAs for each hormone measure and included habitat as a fixed factor, spotting area, spotting darkness, sampling date, playback duration (the trial time plus the time playback was used to capture the male) and body condition as covariates. Because we wanted to determine whether relationships between coloration and phenotype are influenced by habitat, we included the interaction between habitat type and spotting area and darkness in these models. We then used backward elimination beginning with interaction terms and removed variables sequentially until only those with $P \leq 0.05$ remained in the model.

For male behaviour, we used principal components analysis (PCA) to combine correlated behaviours into a single principal components score (Appendix, Table A1). We initially included the number of broadcast songs, soft songs, wing waves and the average distance from the speaker in a single principal components analysis. However, this analysis produced two principal component scores that each explained a modest amount of the variance in male behaviour (38 and 36%, respectively). This seemed largely due to the number of broadcast songs loading onto the second PC score with little contribution from the other variables. Because of this, we reran the PCA without the number of broadcast songs. Results regarding broadcast song can be found elsewhere, and there was no difference in the number of broadcast songs between habitats (Davies et al., 2018). The final PCA included the number of soft songs and wing waves and the average distance from the speaker, which together produced a single territorial behaviour PC1 score that explained 58.4% of the variance in male behaviour. We used the territorial behaviour PC1 score as the dependent variable in a third ANCOVA and included spotting area, spotting darkness, habitat, body condition, sample date and the interactions between habitat and spotting area/darkness in the model as described previously. All analyses were performed in SPSS 23 (IBM, Armonk, NY, U.S.A.) and we set $\alpha = 0.05$.

Ethical Note

This research adheres to the ABAS/ABS Guidelines for the use of animals in research. All of our methods were approved by the Institutional Animal Care and Use Committee of Virginia Tech (BIOL 15-185), the Virginia Department of Game and Inland Fisheries (VA-DGIF permit 48639), the U.S. Geological Survey (USGS) Bird Banding Lab (permit 23818) and the U.S. Fish and Wildlife Service (MB08005B-0). We sampled 67, after-hatch year, wild, male song sparrows. All of these animals were wild-caught and released at their point of capture in Montgomery County, Virginia, U.S.A. During observations, we remained 10 m away from the focal male, which should have limited our effect on his behaviour, and each trial lasted only 6 min. Males were captured in mist nets. Nets were monitored continuously and males were removed immediately upon capture (generally <3 min). All males were banded with one USGS metal band (size 1B) and three coloured leg bands (diameter 2.8 mm). The metal band was attached using banding pliers and the coloured leg bands were attached using a small band spreader that temporarily widened the band. These bands were not removed so

that birds could be identified in future years. Leg bands are small, lightweight, commonly used and should have minimal effect on a bird. From each male, we obtained a total of 200 µl blood sample from the brachial vein, which is well below 1% of a male song sparrow's body mass.

In this study, we briefly altered male hormone concentrations by giving 30 males an injection of 500 ng GnRH dissolved in 50 µl of PBS and holding them for 30 min. This caused an increase in concentrations of testosterone (GnRH) and corticosterone (in response to being held for 30 min). These manipulations produce short-term changes in hormone concentrations similar to those seen during natural social interactions in the wild. Male hormone concentrations would have returned to normal baseline levels in 1–2 h following the manipulation. We detected no negative effects of this manipulation, capture or blood sampling on these males. All of the males were resighted on their territory for at least 2 weeks post manipulation.

RESULTS

Plumage Colour and Habitat

The average reflectance spectra of the song sparrows in this study produced a slope of 0.017 from 300 to 700 nm (Appendix, Fig. A2), similar to the value found for dark brown coloration in other species (Galvan & Wakamatsu, 2016). Thus, we can infer that the plumage of males from our population contains somewhat more eumelanin than pheomelanin.

We predicted that males in urban habitats would have more extensive or darker spotting and we found that the spotting area differed between urban and rural habitats but darkness did not. Males in urban habitat had significantly greater spotting than males in rural habitat ($F_{1, 43} = 4.848$, $P = 0.033$; Fig. 2a). Body condition was unrelated to spotting area ($P = 0.51$). Spotting darkness did not differ significantly between urban and rural birds ($P = 0.096$) and was not related to body condition or capture date (both $P \geq 0.399$; Fig. 2b).

Plumage Colour and Male Behaviour

We predicted that spotting area and/or darkness would be positively correlated with territorial aggression. Territorial behaviour PC1 was related to body condition and interactive effects between habitat and spotting area and darkness but was not related to playback duration or the individual effects of spotting area or darkness (Table 1). In rural habitat, there was a negative association between spotting area and territorial behaviour PC1 scores, indicating that males with more extensive spotting were less aggressive in rural habitat (Fig. 3a). In contrast, there was no relationship between spotting area and territorial behaviour PC1 scores in urban habitat (Appendix, Table A2). Rather, in urban habitat, males with darker spotting were less territorially aggressive than males with brighter spotting (Table 1, Fig. 3b). In rural habitat, there was no association between male spotting darkness and male behaviour (Appendix, Table A2).

Plumage Colour and Hormone Concentrations

We predicted that males with more extensive and darker spotting would have higher testosterone but lower corticosterone than males with less extensive and brighter spotting. Initial testosterone was unrelated to the independent variables (Appendix, Table A3). The final model for GnRH-induced testosterone included sampling date, habitat and spotting area (Table 2), but it excluded playback duration, spotting darkness, body condition and the interaction between habitat and spotting area or darkness (Appendix, Table A3). There was a positive association
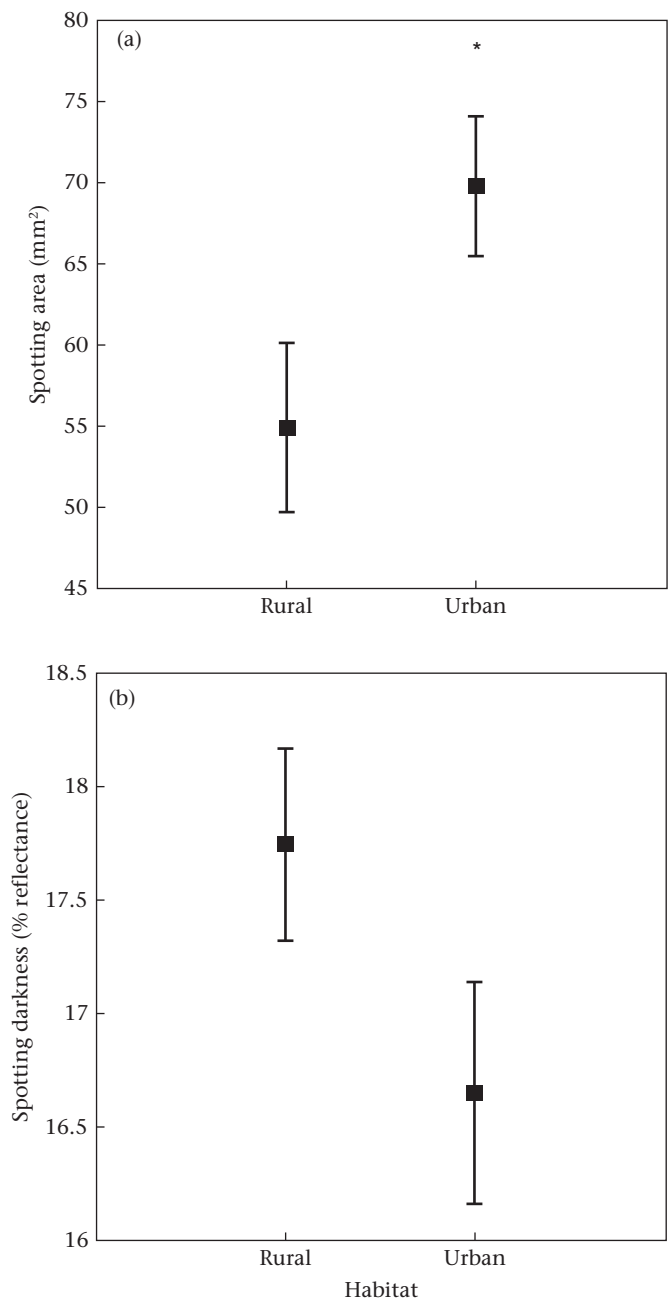
Figure 2. Comparisons between (a) spotting area and (b) spotting darkness in urban and rural birds. Means ± SE are presented. *$P < 0.05$.
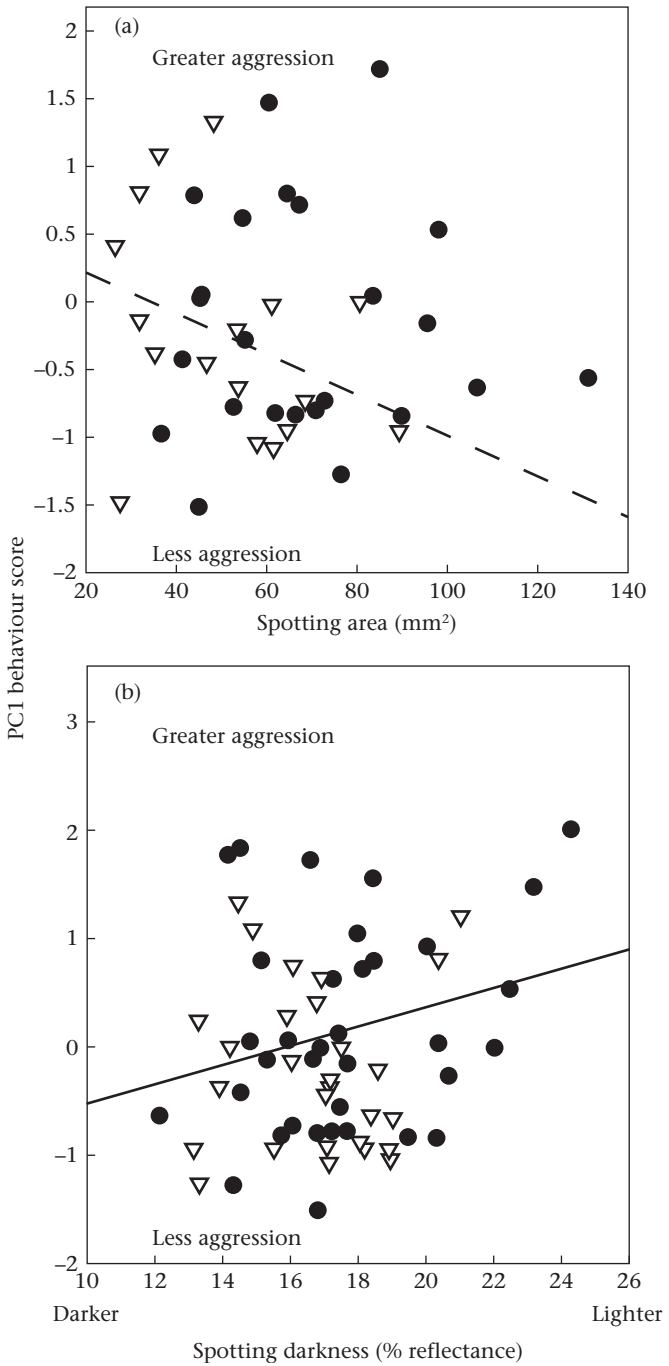

Figure 3. Relationship between the first principal component behaviour score (PC1) and (a) spotting area and (b) spotting darkness in urban (filled circles, solid line) and rural (open triangles, dashed line) male song sparrows. Higher territorial aggression PC1 scores were associated with males that spent more time near the speaker and performed more wing waves and soft songs.

Table 1

Final model for the relationship between habitat, coloration and behaviour during a simulated territorial intrusion

	F	df	P
PC1 behaviour final model			
Habitat	8.824	1, 31	**0.006**
Residual body mass	4.075	1, 31	**0.052**
Spotting darkness	0.247	1, 31	0.622
Spotting area	0.488	1, 31	0.490
Habitat × spotting darkness	4.877	1, 31	**0.035**
Habitat × spotting area	7.916	1, 31	**0.008**

Significant P values are shown in bold.

between spotting area and GnRH-induced testosterone release ($F_{1, 25} = 4.236$, $P = 0.050$; Fig. 4a).

Initial corticosterone was unrelated to all of the independent variables (all $P \geq 0.277$; Appendix, Table A3). In contrast, handling restraint-induced corticosterone concentrations were negatively associated with spotting darkness ($F_{1, 24} = 5.013$, $P = 0.034$; Table 2, Fig. 4b) but not with spotting area, body condition, sample date or the interaction between habitat and spotting area or darkness (all $P \geq 0.381$; Appendix, Table A3).

Table 2
Final models for the relationship between male coloration and plasma testosterone and corticosterone concentrations

Final models	F	df	P
Initial testosterone			
Spotting darkness	2.752	1, 62	0.102
Induced testosterone			
Habitat	9.074	1, 25	**0.006**
Day of year	8.158	1, 25	**0.009**
Spotting area	4.236	1, 25	**0.050**
Initial corticosterone			
Playback duration	3.504	1, 38	0.069
Induced corticosterone			
Habitat	5.655	1, 24	**0.026**
Spotting darkness	5.013	1, 24	**0.035**

In cases where no variables remained in the final model, we present results from the penultimate model. Significant P values are shown in bold. Full models are available in the Appendix.

DISCUSSION

Melanin coloration is associated with physiology and behaviour in a variety of taxa, but the mechanisms that maintain this association are not always clear. Melanin coloration could be associated with phenotype by genetic or biochemical links between eumelanin or pheomelanin synthesis that also influence other aspects of physiology and behaviour (Ducrest et al., 2008; Jenkins et al., 2013). In the case of primarily eumelanin-based ornaments, degree of melanization is predicted to be positively associated with territorial aggression and testosterone concentration but negatively associated with corticosterone concentration (Ducrest et al., 2008). In contrast, predominantly pheomelanin-based ornaments have been positively associated with testosterone in previous studies (Hasegawa et al., 2017; Safran et al., 2008) and are predicted to be positively associated with corticosterone concentration (Jenkins et al., 2013; Xiao, Xia-Zhang, Vulliémoz, Ferin, & Wardlaw, 2003). From our reflectance data, we inferred that song sparrow spotting was somewhat more eumelanic than pheomelanic. Somewhat counterintuitively, we found that males with lighter and less extensive spotting displayed greater territorial aggression than those with darker and more extensive spotting, similar to a recent study of melanin coloration and boldness (van den Brink, Dolivo, Falourd, Dreiss, & Roulin, 2012). As predicted, we found that males with greater spotting had greater GnRH-induced testosterone release. However, males with darker spotting had greater handling restraint-induced corticosterone release, which could be related to pheomelanin production. These endocrine results indicate that song sparrow chest spotting is reliably associated with endocrine profiles, could serve as signals to conspecifics of aspects of phenotype associated with hormone levels, and thus be considered an ornament. These findings also support the hypothesis that biochemical processes associate melanin spotting with hormone profiles in song sparrows, although the relationship between melanin and behaviour may be more plastic.

We found that males with more extensive spotting had greater GnRH-induced testosterone release than males with less extensive spotting. However, we found no relationship between spotting darkness or area and initial testosterone concentration, nor did we find a relationship between initial or GnRH-induced testosterone and male behaviour (Davies et al., 2018). While some studies have found the predicted positive relationship between initial testosterone and melanin traits (Peters, Delhey, Goymann, & Kempenaers, 2006), others have not (Grindstaff et al., 2012; Laucht et al., 2010). Testosterone concentrations change rapidly in response to social interactions, such as mate attraction and conspecific aggression (Goymann et al., 2007; Pinxten, de Ridder, & Eens, 2003; Wingfield, Hegner, Dufty, & Ball, 1990; Wingfield, Lynn, & Soma, 2001), and these socially induced testosterone concentrations are correlated
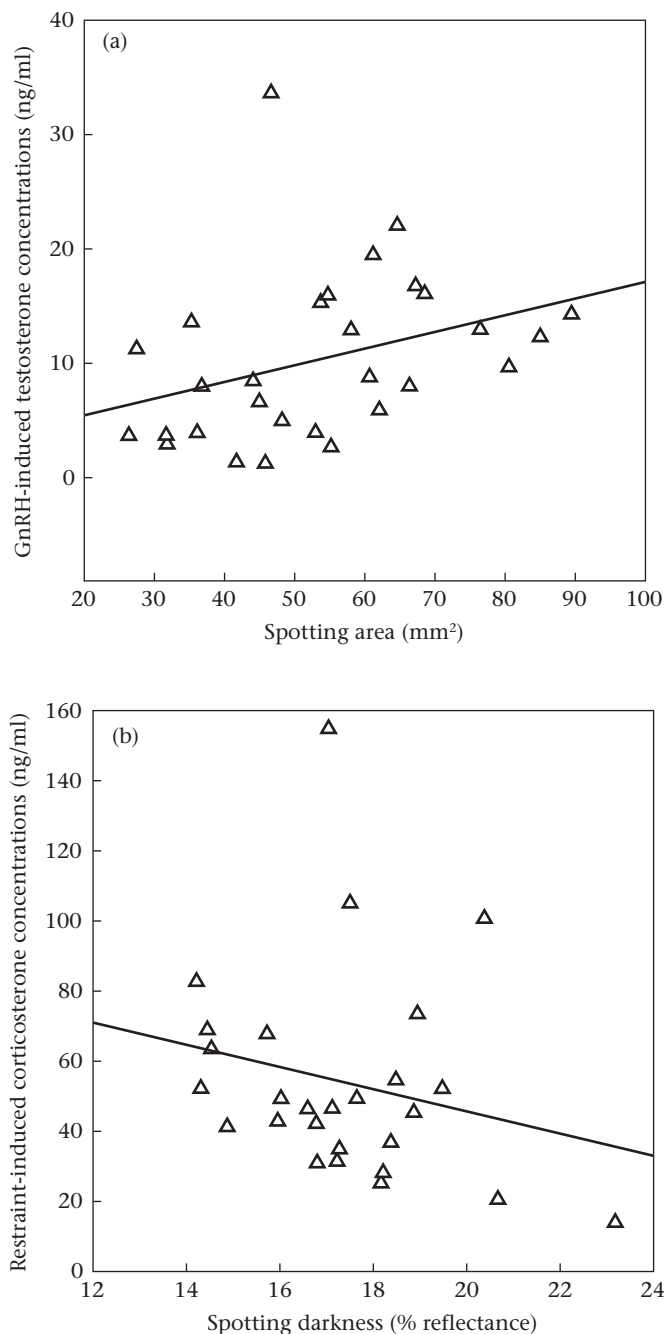
Figure 4. (a) Relationship between spotting area and GnRH-induced testosterone concentration in male songs sparrows. (b) Relationship between spotting darkness and handling restraint-induced corticosterone concentration in male songs sparrows.

with GnRH-induced testosterone release (Goymann et al., 2007). Because more ornamented males are often involved in more social interactions, ornamentation should be related to GnRH-induced testosterone, perhaps to a greater extent than initial testosterone profiles (McGlothlin et al., 2008). Indeed, a few studies have found positive relationships between male ornamentation and GnRH-induced testosterone concentration (McGlothlin et al., 2008; Spinney et al., 2006) or the daily maximum in testosterone concentrations (Laucht, Dale, Mutzel, & Kempenaers, 2011). Our findings support continued use of GnRH-induced testosterone concentrations in studies of behaviour and ornamentation.

We found that spotting darkness was positively associated with restraint-induced corticosterone concentration although not with

initial concentration. There are several potential explanations for this finding. First, this result is partially consistent with the hypothesis that biochemical processes generate relationships between melanin coloration and the HPA axis. In barn swallows, *Hirundo rustica*, darker individuals have relatively more pheomelanin than eumelanin in their feathers than do lighter individuals (Saino et al., 2013). Darker males have greater corticosterone concentrations than lighter males, although this relationship is also influenced by brood size (Saino et al., 2013). Pheomelanin production is facilitated by agouti-stimulating protein, which also stimulates the HPA axis (Xiao, Xia-Zhang, Vulliémoz, Ferin, & Wardlaw, 2003) and could lead to the positive association between coloration and restraint-induced corticosterone we detected. However, this should also influence initial corticosterone levels, which we did not find. Alternatively, spotting darkness could be part of an alternative strategy. Stress-induced corticosterone concentrations direct resources and behaviours towards self-maintenance and can lead to reductions in parental care (Wingfield & Sapolsky, 2003). In some cases, the stress-induced release of corticosterone is dampened during the breeding season, which prevents a loss of fitness (Bokony et al., 2009; Breuner, Delehanty, & Boonstra, 2013; Wingfield & Sapolsky, 2003). It is possible that male song sparrows with darker spotting direct resources towards self-maintenance and maintain the ability to release greater concentrations of corticosterone after exposure to a stressor. In contrast, male song sparrows with brighter spotting may invest more in parental care and dampen their stress-induced release of corticosterone to facilitate the maintenance of high parental care. Assessing resistance to stress using approaches other than quantification of glucocorticoids, such as evaluating expression of receptors (Lattin & Romero, 2013, 2015) or concentrations of corticosterone-binding globulin (Breuner et al., 2003), may provide additional insight into the relationship between stress resistance and melanin coloration.

Many aspects of phenotype, including coloration, are influenced by environmental conditions such as the degree of urbanization (Atwell et al., 2014; Davies & Sewall, 2016; Partecke et al., 2006). We found that males in urban habitat had more extensive spotting than rural birds, but spotting darkness did not differ between habitats. This result is consistent with studies in feral pigeons, in which melanic phenotypes are more common in urban areas and lighter phenotypes are more common in rural areas (Jacquin et al., 2013; Obukhova, 2007) although the reverse is true for dark-eyed juncos, *Junco hyemalis* (Atwell et al., 2014). There are at least two explanations for the difference in spotting across habitats in song sparrows. First, song sparrows may perceive urban habitat as higher quality and, thus, more ornamented (this study) or more aggressive individuals may settle in urban areas (Davies & Sewall, 2016; Foltz et al., 2015; Hyman, Hughes, Searcy, & Nowicki, 2004). Suitable nesting habitat may be more limited in urban areas and only the most aggressive and ornamented song sparrows may be able to hold these territories. Second, males with more extensive spotting may be more likely to survive in urban areas than males with less spotting due to the unique ecological conditions caused by urbanization. Recent studies have indicated that potentially toxic metals are more abundant in urban areas than in rural ones (Imperato et al., 2003; Manta, Angelone, Bellanca, Neri, & Sprovieri, 2002) and melanin can play an important role in detoxification (Bridelli & Crippa, 2008), which could promote the survival of more melanic individuals.

Urban males tended to have darker spotting and had more extensive spotting and were more territorially aggressive than rural males. However, with both habitats combined, there was no relationship between spotting darkness or area and territorial aggression. Instead, the relationship between chest spotting and behaviour differed between habitats. In rural habitat, males with

more extensive spotting were less territorially aggressive than males with less extensive spotting, but there was no relationship between spotting area and behaviour in urban males. In contrast, in urban habitat, males with darker spotting were less aggressive than males with brighter spotting, but there was no relationship between spotting darkness and behaviour in rural habitat. This indicates that within a habitat, the least ornamented males are the most aggressive while more ornamented males are less aggressive. In dark-eyed juncos and house finches, *Carpodacus mexicanus*, the relationship between aggression and ornamentation differs between urban and rural environments, with urban birds being both less ornamented and less aggressive than rural birds (Atwell et al., 2012; Hasegawa, Ligon, Giraudeau, Watanabe, & McGraw, 2014). These two studies indicate that relationships between ornaments and behaviour can be maintained despite novel environmental conditions, while our results indicate that novel conditions can alter the relationship between melanin traits and behaviour. Indeed, a growing number of studies indicate anthropogenic change can alter the expression of ornaments and potentially the relationships between ornamentation, phenotype and fitness (Iglesias-Carrasco, Head, Jennions, & Cabido, 2016; Rodewald, Shustack, & Jones, 2011; Scordato, Bontrager, & Price, 2012).

Although aspects of spotting were associated with behaviour in the two habitats, the relationships were counterintuitive: contrary to our prediction, less colourful males were more aggressive in both habitats. An inverse relationship between dominance, aggression and ornamentation has been found in birds displaying carotenoid-based ornaments (Jones et al., 2017; McGraw & Hill, 2000a, 2000b). In house finches and golden-winged warblers, *Vermivora chrysoptera*, drab males are more aggressive or dominant to more highly ornamented males (Jones et al., 2017; McGraw & Hill, 2000a, 2000b). Male song sparrows with less or lighter spotting may have more to gain by being aggressive, or it may be necessary for them to respond more strongly to an intruder to maintain their territory compared to males with more or darker spotting. A second possibility is that less ornamented male song sparrows may be at greater risk of losing paternity at their nest, so they use higher levels of territorial aggression as a paternity guard. In other avian species, females prefer males with larger or darker patches as both social and extrapair mates, so less ornamented males may be more likely to be cuckolded than more ornamented males (Safran, Neuman, McGraw, & Lovette, 2005; Thusius, Peterson, Dunn, & Whittingham, 2001). In other song-birds, territorial defence can function as a paternity assurance behaviour in addition to its role in resource defence (Langmore, 1996; Tobias & Seddon, 2000). Rates of extrapair paternity in eastern song sparrows range from 8% to 15% (Major & Barber, 2004; Potvin, Crawford, MacDougall-Shackleton, & MacDougall-Shackleton, 2015), and in western song sparrows, song characteristics are unrelated to extrapair and within-pair mating success (Hill, Akçay, Campbell, & Beecher, 2011; Sardell, Arcese, Keller, & Reid, 2011). It is possible that visual cues contribute to reproductive success in song sparrows although additional work is needed to determine whether females show a preference for spotting darkness and area in social and extrapair mates. Also, experimentally manipulating spotting area and darkness in males or using models with manipulated spotting area could be used to determine how signaller and receiver coloration influence territorial aggression.

In this study, we assessed the relationship between melanin-based coloration, urbanization and a number of phenotypically plastic traits to determine what factors maintain the relationship between melanin coloration and other aspects of phenotype and whether urbanization influences relationships between melanin coloration and phenotype. Male song sparrows in urban habitat had more extensive spotting than males in rural habitat and the relationship between spotting area, darkness and behaviour

differed between urban and rural sites. The differences in spotting area and the trend for darkness to differ may be related to differences in social interactions that affect settlement in the two habitats (Maia, Brasileiro, Lacava, & Macedo, 2012; Vitousek, Zonana, & Safran, 2014) or to abiotic factors that promote greater melanization in urban birds than in rural birds (Chatelain, Gasparini, & Frantz, 2016). We hypothesize that territorial aggression is relatively plastic across habitats and that this plasticity alters the relationship between coloration and behaviour across sites. However, within a habitat, individuals may be very consistent in their level of territorial aggression (Davies & Sewall, 2016; Krippel, Ballentine, & Hyman, 2017). Importantly, spotting features were reliably linked with endocrine profiles across habitats, making them potentially reliable signals of physiological phenotypes. Urbanization and other novel habitats can have diverse effects on organisms (e.g. Atwell et al., 2014; Davies & Sewall, 2016; Partecke et al., 2006), but relatively few studies have assessed how these novel habitats can influence correlates between plumage coloration and physiology or behaviour (but see Atwell et al., 2014; Iglesias-Carrasco et al., 2016). Novel habitats provide a unique opportunity to assess mechanisms underlying the relationships between plumage traits and phenotype and to examine the degree of plasticity that exists between coloration and other aspects of phenotype such as behaviour. Assessing relationships across novel sites could ultimately be used to identify factors that can alter the strength or direction of sexual selection and enhance our understanding of the factors that maintain genetic variation in traits under strong directional selection. Such relationships may be difficult to detect in natural conditions or require multiple years of data in order to compare phenotypic relationships among years that differ in environmental conditions (Cockburn, Osmond, & Double, 2008; Scordato et al., 2012). Research in urban areas, as well as other landscapes modified by humans, will also be increasingly important as global change continues at an unprecedented rate. Examining spotting area and darkness relative to territorial aggression and additional aspects of reproduction, such as parental care, could resolve how life-history trade-offs associated with ornament expression are mediated by hormones. Furthermore, conducting this research in different habitats will reveal the degree of plasticity that exists between these aspects of phenotype.

Competing Interests

We have no competing interests.

Supplementary Material

Supplementary material related to this article can be found at https://doi.org/10.1016/j.anbehav.2018.06.012.

References

Akçay, Ç.l., Tom, M. E., Campbell, S. E., & Beecher, M. D. (2013). Song type matching is an honest early threat signal in a hierarchical animal communication system. *Proceedings of the Royal Society of London, Series B: Biological Sciences, 280*(1756), 1−8.

Almasi, B., Jenni, L., Jenni-Eiermann, S., & Roulin, A. (2010). Regulation of stress response is heritable and functionally linked to melanin-based coloration. *Journal of Evolutionary Biology, 23*(5), 987−996.

Anderson, R. C., Searcy, W. A., Peters, S., & Nowicki, S. (2008). Soft song in song sparrows: Acoustic structure and implications for signal function. *Ethology, 114*(7), 662−676.

Atwell, J. W., Cardoso, G. C., Whittaker, D. J., Campbell-Nelson, S., Robertson, K. W., & Ketterson, E. D. (2012). Boldness behavior and stress physiology in a novel urban environment suggest rapid correlated evolutionary adaptation. *Behavioral Ecology, 23*(5), 960−969.

Atwell, J. W., Cardoso, G. C., Whittaker, D. J., Price, T. D., & Ketterson, E. D. (2014). Hormonal, behavioral, and life-history traits exhibit correlated shifts in relation to population establishment in a novel environment. *The American Naturalist, 184*(6), E147−E160.

Ballentine, B., Searcy, W. A., & Nowicki, S. (2008). Reliable aggressive signalling in swamp sparrows. *Animal Behaviour, 75*, 693−703.

Beziers, P., Ducrest, A. L., Simon, C., & Roulin, A. (2017). Circulating testosterone and feather−gene expression of receptors and metabolic enzymes in relation to melanin-based colouration in the barn owl. *General and Comparative Endocrinology, 250*(2017), 36−45.

Bokony, V., Kulcsar, A., Toth, Z., & Liker, A. (2012). Personality traits and behavioral syndromes in differently urbanized populations of house sparrows (*Passer domesticus*). *PLoS One, 7*(5), e36639.

Bokony, V., Lendvai, A. Z., Liker, A., Angelier, F., Wingfield, J. C., & Chastel, O. (2009). Stress response and the value of reproduction: Are birds prudent parents? *The American Naturalist, 173*(5), 589−598.

Bonier, F., Martin, P. R., Sheldon, K. S., Jensen, J. P., Foltz, S. L., & Wingfield, J. C. (2007). Sex-specific consequences of life in the city. *Behavioral Ecology, 18*(1), 121−129.

Breuner, C. W., Delehanty, B., & Boonstra, R. (2013). Evaluating stress in natural populations of vertebrates: Total CORT is not good enough. *Functional Ecology, 27*(1), 24−36.

Breuner, C. W., Orchinik, M., Hahn, T. P., Meddle, S. L., Moore, I. T., Owen-Ashley, N. T., et al. (2003). Differential mechanisms for regulation of the stress response across latitudinal gradients. *American Journal of Physiology − Regulatory, Integrative, and Comparative Physiology, 285*(3), 594−600.

Bridelli, M. G., & Crippa, P. R. (2008). Theoretical analysis of the adsorption of metal ions to the surface of melanin particles. *Adsorption, 14*(1), 101−109.

van den Brink, V., Dolivo, V., Falourd, X., Dreiss, A. N., & Roulin, A. (2012). Melanic color-dependent antipredator behavior strategies in barn owl nestlings. *Behavioral Ecology, 23*(3), 473−480.

Buchanan, K. L., Evans, M. R., Goldsmith, A. R., Bryant, D. M., & Rowe, L. V. (2001). Testosterone influences basal metabolic rate in male house sparrows: A new cost of dominance signalling? *Proceedings of the Royal Society of London, Series B: Biological Sciences, 268*(1474), 1337−1344.

Cain, K. E., & Pryke, S. R. (2017). Testosterone production in response to exogenous gonadotropin releasing hormone (GnRH challenge) depends on social environment and color polymorphism. *General and Comparative Endocrinology, 244*(2017), 77−85.

Chatelain, M., Gasparini, J., & Frantz, A. (2016). Do trace metals select for darker birds in urban areas? An experimental exposure to lead and zinc. *Global Change Biology, 22*(7), 2380−2391.

Cockburn, A., Osmond, H. L., & Double, M. C. (2008). Swingin' in the rain: Condition dependence and sexual selection in a capricious world. *Proceedings of the Royal Society of London, Series B: Biological Sciences, 275*(1635), 605−612.

Corbel, H., Legros, A., Haussy, C., Jacquin, L., Gasparini, J., Karimi, B., et al. (2016). Stress response varies with plumage colour and local habitat in feral pigeons. *Journal of Ornithology, 157*(3), 825−837.

Dabelsteen, T., & Pedersen, S. B. (1992). Song features essential for species discrimination and behaviour assessment by male blackbirds (*Turdus merula*). *Behaviour, 121*(3−4), 259−287.

Davies, S., Beck, M. L., & Sewall, K. B. (2018). Territorial aggression in urban and rural song sparrows is correlated with corticosterone but not testosterone. *Hormones and Behavior, 98*(2018), 8−15.

Davies, S., & Sewall, K. B. (2016). Agonistic urban birds: Elevated territorial aggression of urban song sparrows is individually consistent within a breeding period. *Biology Letters, 12*(6), 20160315.

Dawkins, R., & Krebs, J. R. (1978). Animal signals: Information or manipulation. In J. R. Krebs, & N. B. Davies (Eds.), *Behavioural ecology: An evolutionary approach* (pp. 282−309). Oxford, U.K.: Blackwell.

Diep, S. K., & Westneat, D. F. (2013). The integration of function and ontogeny in the evolution of status signals. *Behaviour, 150*(9−10), 1015−1044.

Ducrest, A. L., Keller, L., & Roulin, A. (2008). Pleiotropy in the melanocortin system, coloration and behavioral syndromes. *Trends in Ecology & Evolution, 23*(9), 502−510.

Evans, J., Boudreau, K., & Hyman, J. (2010). Behavioural syndromes in urban and rural populations of song sparrows. *Ethology, 116*(7), 588−595.

Evans, M. R., Goldsmith, A. R., & Norris, S. R. A. (2000). The effects of testosterone on antibody production and plumage coloration in male house sparrows (*Passer domesticus*). *Behavioral Ecology and Sociobiology, 47*(3), 156−163.

Foltz, S. L., Ross, A. E., Laing, B. T., Rock, R. P., Battle, K. E., & Moore, I. T. (2015). Get off my lawn: Increased aggression in urban song sparrows is related to resource availability. *Behavioral Ecology, 26*(6), 1548−1557.

Galvan, I., & Wakamatsu, K. (2016). Color measurement of the animal integument predicts the content of specific melanin forms. *RSC Advances, 6*(82), 79135−79142.

Galvan, I., Wakamatsu, K., Camarero, P. R., Mateo, R., & Alonso-Alvarez, C. (2015). Low-quality birds do not display high-quality signals: The cysteine−pheomelanin mechanism of honesty. *Evolution, 69*(1), 26−38.

Giraudeau, M., Mateos-Gonzalez, F., Cotin, J., Pagani-Nunez, E., Torne-Noguera, A., & Senar, J. C. (2015). Metal exposure influences the melanin and carotenoid-based

colorations in great tits. *The Science of the Total Environment, 532*(2015), 512–516.

Gonzalez, G., Sorci, G., Smith, L. C., & de Lope, F. (2001). Testosterone and sexual signalling in male house sparrows (*Passer domesticus*). *Behavioral Ecology and Sociobiology, 50*(6), 557–562.

Goymann, W. (2009). Social modulation of androgens in male birds. *General and Comparative Endocrinology, 163*(1–2), 149–157.

Goymann, W., Landys, M. M., & Wingfield, J. C. (2007). Distinguishing seasonal androgen responses from male–male androgen responsiveness: Revisiting the challenge hypothesis. *Hormones and Behavior, 51*(4), 463–476.

Grindstaff, J. L., Lovern, M. B., Burtka, J. L., & Hallmark-Sharber, A. (2012). Structural coloration signals condition, parental investment, and circulating hormone levels in eastern bluebirds (*Sialia sialis*). *Journal of Comparative Physiology A – Neuroethology Sensory Neural and Behavioral Physiology, 198*(8), 625–637.

Grunst, A. S., & Grunst, M. L. (2015). Context-dependent relationships between multiple sexual pigments and paternal effort. *Behavioral Ecology, 26*(4), 1170–1179.

Hasegawa, M., Arai, E., Sato, M., & Sakai, H. (2017). Plasma testosterone levels increase with expression of male ornaments during mating, but not incubation, in Japanese barn swallows. *Zoological Science, 34*(4), 261–266.

Hasegawa, M., Ligon, R. A., Giraudeau, M., Watanabe, M., & McGraw, K. J. (2014). Urban and colorful male house finches are less aggressive. *Behavioral Ecology, 25*(3), 641–649.

Hill, C. E., Akçay, Ç. L., Campbell, S. E., & Beecher, M. D. (2011). Extrapair paternity, song, and genetic quality in song sparrows. *Behavioral Ecology, 22*(1), 73–81.

Hof, D., & Hazlett, N. (2010). Low-amplitude song predicts attack in a North American wood warbler. *Animal Behaviour, 80*, 821–828.

Hyman, J., Hughes, M., Searcy, W. A., & Nowicki, S. (2004). Individual variation in the strength of territory defense in male song sparrows: Correlates of age, territory tenure, and neighbor aggressiveness. *Behaviour, 141*(1), 15–27.

Iglesias-Carrasco, M., Head, M. L., Jennions, M. D., & Cabido, C. (2016). Condition-dependent trade-offs between sexual traits, body condition and immunity: The effect of novel habitats. *BMC Evolutionary Biology, 16*(135), 1–10.

Imperato, M., Adamo, P., Naimo, D., Arienzo, M., Stanzione, D., & Violante, P. (2003). Spatial distribution of heavy metals in urban soils of Naples city (Italy). *Environmental Pollution, 124*(2), 247–256.

Jacquin, L., Récapet, C., Prévot-Julliard, A., Leboucher, G., Lenouvel, P., Erin, N., et al. (2013). A potential role for parasites in the maintenance of color polymorphism in urban birds. *Oecologia, 173*(3), 1089–1099.

Jawor, J. M., & Breitwisch, R. (2003). Melanin ornaments, honesty, and sexual selection. *The Auk: Ornithological Advances, 120*(2), 249–265.

Jawor, J. M., McGlothlin, J. W., Casto, J. M., Greives, T. J., Snajdr, E. A., Bentley, G. E., et al. (2006). Seasonal and individual variation in response to GnRH challenge in male dark-eyed juncos (*Junco hyemalis*). *General and Comparative Endocrinology, 149*(2), 182–189.

Jenkins, B. R., Vitousek, M. N., & Safran, R. J. (2013). Signaling stress? An analysis of phaeomelanin-based plumage color and individual corticosterone levels at two temporal scales in North American barn swallows, *Hirundo rustica erythrogaster*. *Hormones and Behavior, 64*(4), 665–672.

Jones, J. A., Tisdale, A. C., Bakermans, M. H., Larkin, J. L., Smalling, C. G., & Siefferman, L. (2017). Multiple plumage ornaments as signals of intrasexual communication in golden-winged warblers. *Ethology, 123*(2), 145–156.

Kittilsen, S., Johansen, I. B., Braastad, B. O., & Overli, O. (2012). Pigments, parasites and personalitiy: Towards a unifying role for steroid hormones? *PLoS One, 7*(4), e34281.

Kittilsen, S., Schjolden, J., Beitnes-Johansen, I., Shaw, J. C., Pottinger, T. G., Sorensen, C., et al. (2009). Melanin-based skin spots reflect stress responsiveness in salmonid fish. *Hormones and Behavior, 56*(3), 292–298.

Krippel, J., Ballentine, B., & Hyman, J. (2017). Reproductive consequences of aggression in a territorial songbird. *Ethology, 123*(4), 261–269.

Langmore, N. (1996). Territoriality and song as flexible paternity guards in dunnocks and alpine accentors. *Behavioral Ecology, 7*(2), 183–188.

Lattin, C. R., & Romero, L. M. (2013). The size of a melanin-based plumage ornament correlates with glucocorticoid receptor concentrations in the skin of that ornament. *Biology Letters, 9*(5), 20130440.

Lattin, C. R., & Romero, L. M. (2015). Seasonal variation in glucocorticoid and mineralocorticoid receptors in metabolic tissues of the house sparrow (*Passer domesticus*). *General and Comparative Endocrinology, 214*(2015), 95–102.

Laucht, S., Dale, J., Mutzel, A., & Kempenaers, B. (2011). Individual variation in plasma testosterone levels and its relation to badge size in house sparrows *Passer domesticus*: It's a night-and-day difference. *General and Comparative Endocrinology, 170*(3), 501–508.

Laucht, S., Kempenaers, B., & Dale, J. (2010). Bill color, not badge size, indicates testosterone-related information in house sparrows. *Behavioral Ecology and Sociobiology, 64*(9), 1461–1471.

Lowry, H., Lill, A., & Wong, B. B. M. (2013). Behavioural responses of wildlife to urban environments. *Biological Reviews, 88*(3), 537–549.

Mafli, A., Wakamatsu, K., & Roulin, A. (2011). Melanin-based coloration predicts aggressiveness and boldness in captive eastern Hermann's tortoises. *Animal Behaviour, 81*, 859–863.

Maia, R., Brasileiro, L., Lacava, R. V., & Macedo, R. H. (2012). Social environment affects acquisition and color of structural nuptial plumage in a sexually dimorphic tropical passerine. *PLoS One, 7*(10), e47501. https://doi.org/10.1371/journal.pone.0047501.

Major, D. L., & Barber, C. A. (2004). Extra-pair paternity in first and second broods of eastern song sparrows. *Journal of Field Ornithology, 75*(2), 152–156.

Manta, D. S., Angelone, M., Bellanca, A., Neri, R., & Sprovieri, M. (2002). Heavy metals in urban soils: A case study from the city of Palermo (Sicily), Italy. *The Science of the Total Environment, 300*(1–3), 229–243.

McGlothlin, J. W., Jawor, J. M., Greives, T. J., Casto, J. M., Phillips, J. L., & Ketterson, E. D. (2008). Hormones and honest signals: Males with larger ornaments elevate testosterone more when challenged. *Journal of Evolutionary Biology, 21*(1), 39–48.

McGraw, K. J., & Hill, G. E. (2000a). Carotenoid-based ornamentation and status signaling in the house finch. *Behavioral Ecology, 11*(5), 520–527.

McGraw, K. J., & Hill, G. E. (2000b). Plumage brightness and breeding-season dominance in the house finch: A negatively correlated handicap? *The Condor: Ornithological Applications, 102*(2), 456–461.

McGraw, K. O., & Wong, S. P. (1996). Forming inferences about some intraclass correlation coefficients. *Psychological Methods, 1*(1), 30–46.

Montgomerie, R. (2008). *CLR (Version 1.05)*. Kingston, Ontario, Canada: Queen's University.

Nowicki, S., Searcy, W. A., Krueger, T., & Hughes, M. (2002). Individual variation in response to simulated territorial challenge among territory-holding song sparrows. *Journal of Avian Biology, 33*(3), 253–259.

Obukhova, N. (2007). Polymorphism and phene geography of the blue rock pigeon in Europe. *Russian Journal of Genetics, 43*(5), 492–501.

Partecke, J., Schwabl, I., & Gwinner, E. (2006). Stress and the city: Urbanization and its effects on the stress physiology in European blackbirds. *Ecology, 87*(8), 1945–1952.

Peig, J., & Green, A. J. (2009). New perspectives for estimating body condition from mass/length data: The scaled mass index as an alternative method. *Oikos, 118*(12), 1883–1891.

Peters, A., Delhey, K., Goymann, W., & Kempenaers, B. (2006). Age-dependent association between testosterone and crown UV coloration in male blue tits (*Parus caeruleus*). *Behavioral Ecology and Sociobiology, 59*(5), 666–673.

Pinxten, R., de Ridder, E., & Eens, M. (2003). Female presence affects male behavior and testosterone levels in the European starling (*Sturnus vulgaris*). *Hormones and Behavior, 44*(2), 103–109.

Potvin, D. A., Crawford, P. W., MacDougall-Shackleton, S. A., & MacDougall-Shackleton, E. A. (2015). Song repertoire size, not territory location, predicts reproductive success and territory tenure in a migratory songbird. *Canadian Journal of Zoology, 93*(8), 627–633.

Rodewald, A. D., Shustack, D. P., & Jones, T. M. (2011). Dynamic selective environments and evolutionary traps in human-dominated landscapes. *Ecology, 92*(9), 1781–1788.

Rohwer, S. (1975). The social significance of avian winter plumage variability. *Evolution, 29*(4), 593–610.

Rohwer, S. (1977). Status signaling in Harris sparrows: Some experiments in deception. *Behaviour, 61*(1–2), 107–129.

Roulin, A. (2016). Condition-dependence, pleiotropy and the handicap principle of sexual selection in melanin-based colouration. *Biological Reviews, 91*(2), 328–348.

Roulin, A., Almasi, B., Meichtry-Stier, K. S., & Jenni, L. (2011). Eumelanin- and pheomelanin-based colour advertise resistance to oxidative stress in opposite ways. *Journal of Evolutionary Biology, 24*(10), 2241–2247.

Rueden, C. T., Schindelin, J., Hiner, M. C., DeZonia, B. E., Walter, A. E., Arena, E. T., et al. (2017). ImageJ2: ImageJ for the next generation of scientific image data. *BMC Bioinformatics, 18*(529).

Safran, R. J., Adelman, J. S., McGraw, K. J., & Hau, M. (2008). Sexual signal exaggeration affects physiological state in male barn swallows. *Current Biology, 18*(11), R461–R462.

Safran, R. J., Neuman, C. R., McGraw, K. J., & Lovette, I. J. (2005). Dynamic paternity allocation as a function of male plumage color in barn swallows. *Science, 309*(5744), 2210–2212.

Saino, N., Canova, L., Costanzo, A., Rubolini, D., Roulin, A., & Møller, A. (2013). Immune and stress responses covary with melanin-based coloration in the barn swallow. *Evolutionary Biology, 40*(4), 521–531.

Sardell, R. J., Arcese, P., Keller, L. F., & Reid, J. M. (2011). Sex-specific differential survival of extra-pair and within-pair offspring in song sparrows, *Melospiza melodia*. *Proceedings of the Royal Society B: Biological Sciences, 278*(1722), 3251–3259.

Schmidt, K. L., MacDougall-Shackleton, E. A., Soma, K. K., & MacDougall-Shackleton, S. A. (2014). Developmental programming of the HPA and HPG axes by early-life stress in male and female song sparrows. *General and Comparative Endocrinology, 196*(2014), 72–80.

Schweitzer, C., Motreuil, S., & Dechaume-Moncharmont, F. X. (2015). Coloration reflects behavioural types in the convict cichlid, *Amatitlania siquia*. *Animal Behaviour, 105*, 201–209.

Scordato, E. S. C., Bontrager, A. L., & Price, T. D. (2012). Cross-generational effects of climate change on expression of a sexually selected trait. *Current Biology, 22*(1), 78–82.

Searcy, W. A., Anderson, R. C., & Nowicki, S. (2006). Bird song as a signal of aggressive intent. *Behavioral Ecology and Sociobiology, 60*(2), 234–241.

Seddon, R. J., & Hews, D. K. (2016). Phenotypic correlates of melanization in two *Sceloporus occidentalis* (Phrynosomatidae) populations: Behavior, androgens, stress reactivity, and ectoparasites. *Physiology & Behavior, 163*(2016), 70–80.

Senar, J. C. (2006). Color displays and intrasexual signals of aggression and dominance. In G. E. Hill, & K. J. McGraw (Eds.), *Bird coloration function and evolution* (Vol. 2, pp. 87–136). Cambridge, MA: Harvard University Press.

Senar, J. C., Conroy, M. J., Quesada, J., & Mateos-Gonzalez, F. (2014). Selection based on the size of the black tie of the great tit may be reversed in urban habitats. *Ecology and Evolution, 4*(13), 2625–2632.

Shochat, E. (2004). Credit or debit? Resource input changes population dynamics of city-slicker birds. *Oikos, 106*(3), 622–626.

Soma, K. K., & Wingfield, J. C. (2001). Dehydroepiandrosterone in songbird plasma: Seasonal regulation and relationship to territorial aggression. *General and Comparative Endocrinology, 123*(2), 144—155.

Spinney, L. H., Bentley, G. E., & Hau, M. (2006). Endocrine correlates of alternative phenotypes in the white-throated sparrow (*Zonotrichia albicollis*). *Hormones and Behavior, 50*(5), 762—771.

Thusius, K. J., Peterson, K. A., Dunn, P. O., & Whittingham, L. A. (2001). Male mask size is correlated with mating success in the common yellowthroat. *Animal Behaviour, 62*, 435—446.

Tobias, J., & Seddon, N. (2000). Territoriality as a paternity guard in the European robin, *Erithacus rubecula. Animal Behaviour, 60*, 165—173.

Vitousek, M. N., Zonana, D. M., & Safran, R. J. (2014). An integrative view of the signaling phenotype: Dynamic links between signals, physiology, behavior and social context. *Current Zoology, 60*(6), 739—754.

Wiley, R. H. (1994). Errors, exaggeration, and deception in animal communication. In L. A. Real (Ed.), *Behavioral mechanisms in evolutionary ecology* (pp. 157—189). Chicago, IL: Chicago University Press.

Wingfield, J. C., Hegner, R. E., Dufty, A. M., & Ball, G. F. (1990). The 'challenge hypothesis': Theoretical implications for patterns of testosterone secretion, mating systems, and breeding strategies. *The American Naturalist, 136*(6), 829—846.

Wingfield, J. C., Lynn, S. E., & Soma, K. K. (2001). Avoiding the 'costs' of testosterone: Ecological bases of hormone—behavior interactions. *Brain, Behavior and Evolution, 57*(5), 239—251.

Wingfield, J. C., & Sapolsky, R. M. (2003). Reproduction and resistance to stress: When and how. *Journal of Neuroendocrinology, 15*(8), 711—724.

Xiao, E., Xia-Zhang, L., Vulliémoz, N. R., Ferin, M., & Wardlaw, S. L. (2003). Agouti-related protein stimulates the hypothalamic—pituitary—adrenal (HPA) axis and enhances the HPA response to interleukin-1 in the primate. *Endocrinology, 144*(5), 1736—1741.

Yeh, P. J., Hauber, M. E., & Price, T. D. (2007). Alternative nesting behaviours following colonisation of a novel environment by a passerine bird. *Oikos, 116*(9), 1473—1480.

Appendix

Table A1
Factor loadings for principal components analysis of male behaviour during a simulated territorial intrusion

Behaviour	Loading
Average distance	−0.762
Number of soft songs	0.820
Number of wing waves	0.706
Eigen value	1.752
% Variance	58.39%

Table A2
Full model for the relationship between habitat, male coloration and male behaviour during a simulated territorial intrusion

PC1 behaviour	F	df	P
Full model			
Habitat	8.484	1, 30	**0.007**
Day of year	0.284	1, 30	0.560
Residual body mass	3.220	1, 30	0.056
Spotting darkness	0.286	1, 30	0.597
Spotting area	0.592	1, 30	0.401
Habitat × spotting darkness	4.771	1, 30	**0.037**
Habitat × spotting area	5.609	1, 30	**0.014**

Significant P values are shown in bold.

Table A3
Full models for the relationship between male coloration and plasma testosterone and corticosterone concentrations

	F	df	P
Full model: initial testosterone			
Habitat	0.068	1, 29	0.797
Day of year	1.935	1, 29	0.175
Residual body mass	0.483	1, 29	0.493
Seconds of playback	0.227	1, 29	0.638
Spotting area	0.808	1, 29	0.376
Spotting darkness	3.142	1, 29	0.087
Habitat × spotting area	0.347	1, 29	0.560
Habitat × spotting darkness	0.398	1, 29	0.533
Full model: induced testosterone			
Habitat	0.039	1, 17	0.846

Table A3 (*continued*)

	F	df	P
Day of year	5.080	1, 17	**0.038**
Residual body mass	0.023	1, 17	0.882
Seconds of playback	1.426	1, 17	0.249
Spotting area	5.449	1, 17	**0.032**
Spotting darkness	0.240	1, 17	0.630
Habitat × spotting area	0.405	1, 17	0.533
Habitat × spotting darkness	0.656	1, 17	0.429
Full model: initial corticosterone			
Habitat	0.657	1, 31	0.424
Day of year	0.201	1, 31	0.657
Residual body mass	0.260	1, 31	0.613
Playback duration	2.936	1, 31	0.097
Spotting area	0.238	1, 31	0.629
Spotting darkness	0.001	1, 31	0.997
Habitat × spotting area	2.897	1, 31	0.099
Habitat × spotting darkness	0.010	1, 31	0.921
Full model: induced corticosterone			
Habitat	0.856	1, 17	0.368
Day of year	0.392	1, 17	0.540
Residual body mass	0.087	1, 17	0.771
Seconds of playback	0.344	1, 17	0.566
Spotting area	0.175	1, 17	0.681
Spotting darkness	2.437	1, 17	0.137
Habitat × spotting area	0.071	1, 17	0.794
Habitat × spotting darkness	1.912	1, 17	0.185

Significant P values are shown in bold.

Figure A1. Example of (a) an original song sparrow image taken in field, (b) an image imported into ImageJ and converted to black and white and (c) an image cropped to quantify badge size.
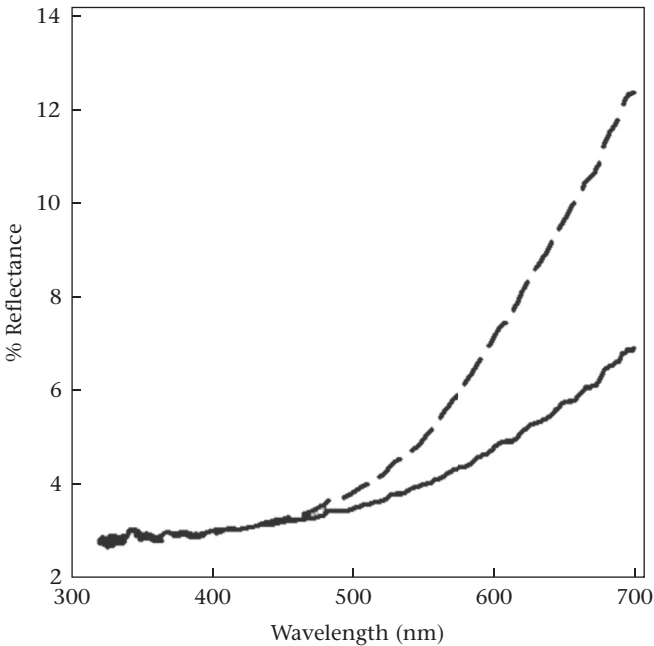

Figure A2. Male song sparrows vary in plumage reflectance and can be relatively darker (solid line) or lighter (dashed line) in plumage colour. These are representative reflectance spectra from two males.