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Original Article

Pigment-specific relationships between feather corticosterone concentrations and sexual coloration

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The adrenocortical stress response may divert energy away from sexual ornamentation, such that ornaments signal exposure or resistance to physiological stress. Alternatively, steroid glucocorticoids released via the stress response may support ornament development by stimulating foraging and metabolism. The relationship between glucocorticoids and ornamentation may vary with ornament type and across age and sex classes that experience different resource allocation tradeoffs. In yellow warblers (*Setophaga petechia*), we conducted the first study to simultaneously assess whether relationships between corticosterone (the primary avian glucocorticoid) and ornamentation depend on sexual pigment type, age, and sex. We quantified carotenoid- and phaeomelanin-based pigmentation using spectrometry, and assayed corticosterone in feathers ($CORT_f$) to derive an integrative metric of corticosterone levels during molt. Yellow warblers with lower carotenoid hue (lambda R50) had higher $CORT_f$, suggesting that carotenoid hue may signal stress during molt across age and sex classes. Carotenoid chroma also negatively correlated with $CORT_f$. However, this correlation was absent in older males, seemingly because these males display more saturated carotenoid pigmentation, and thus less variance in carotenoid chroma. Young males with higher $CORT_f$ also tended to have poorer quality tertial feathers, indicating poor condition at molt. Phaeomelanin-based pigmentation was largely unrelated to $CORT_f$, suggesting that pleiotropic effects do not link phaeomelanogenesis and CORT release. Finally, $CORT_f$ was repeatable across years within individuals. Thus, carotenoid- and phaeomelanin-based pigmentation communicate nonequivalent information about physiological stress, with carotenoid pigmentation having the potential to signal stable differences in stress levels that could affect fitness.

Key words: **carotenoids, feather corticosterone, melanins, sexual coloration, sexual signaling, stress physiology.**

INTRODUCTION

Sexual ornamentation may convey information about physiological condition and genetic quality, and reflect exposure and vulnerability to environmental stress. Steroid glucocorticoids (corticosterone [CORT], in birds) are released via the hypothalamus–pituitary–adrenal axis in response to environmental and physiological stressors, and may be elevated in individuals in poor condition (Romero and Wikelski 2001; Wingfield and Sapolsky 2003). Thus, the adrenocortical stress response may represent a physiological mechanism whereby energy is diverted toward immediate survival at the expense of reproductive effort, including sexual displays (Husak and Moore 2008). On the other hand, elevation of CORT could also facilitate investment toward costly ornaments (Cote et al. 2010; Grunst and Grunst 2014), because CORT can support energetically expensive activities by increasing foraging rate, metabolic rate (Astheimer et al. 1992), and gluconeogenesis (Sapolsky et al. 2000).

Indeed, recent research provides support for both negative and positive relationships between CORT levels and sexual ornamentation, particularly in the case of carotenoid-based pigmentation. Carotenoids may be in limited dietary supply and act as antioxidants and immunostimulants, as well as underlying sexual coloration in many species (von Schantz et al. 1999; McGraw and Ardia 2003; McGraw 2006a). Thus, carotenoid pigmentation often displays condition-dependent expression, and serves as a sexual ornament (McGraw 2006a; Grunst et al. 2014). Negative relationships

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between CORT concentrations and carotenoid pigmentation suggest that CORT may suppress the deposition of carotenoids into pigmentation in favor of other functions, or is correlated with poor condition and the inability to produce intense pigmentation (Mougeot et al. 2010; Martínez-Padilla et al. 2013). For example, red grouse (*Lagopus lagopus scoticus*) infected with parasites had more CORT deposited in feathers and paler red carotenoid-based sexual pigmentation than disinfected males (Mougeot et al. 2010). On the other hand, positive relationships between CORT and carotenoid pigmentation suggest that CORT may enhance uptake, processing, or mobilization of carotenoids (Cote et al. 2010; Lendvai et al. 2013; Fairhurst et al. 2014).

A potential resolution of conflicting empirical relationships between CORT concentrations and sexual pigmentation is that CORT plays a context-dependent role in mediating levels of ornamentation. For instance, in common lizards (*Lacerta vivipara*), experimental elevation of CORT led to an increase in carotenoid-based pigmentation when resources were abundant, but not when resources were limited (Cote et al. 2010). Further, correlations between CORT and sexual ornamentation might also vary with age or sex, as access to resources and optimal levels of investment into ornamentation shift. For example, in house finches (*Haemorhous mexicanus*) carotenoid-based pigmentation is more strongly related to condition at molt among first year breeders, perhaps because foraging efficiency and access to carotenoids increases with age, reducing allocation tradeoffs in older males (Badyaev and Duckworth 2003). Analogously, CORT levels might relate to coloration more strongly in younger birds, if CORT plays a role in mediating the allocation of carotenoids (or other resources) to ornamentation, and younger birds have fewer resources.

Further, in addition to carotenoid-based pigmentation, many species display melanin-based pigmentation, which may serve a nonequivalent signaling function (Møller and Pomiankowski 1993), and thus relate differently to CORT levels. Melanin-based pigmentation may be less strongly related to energy balance than carotenoid-based pigmentation (McGraw and Hill 2000; but see McGraw 2008). However, pleiotropic effects may lead to a relationship between the adrenocortical stress response and melanin-based pigmentation. In the case of phaeomelanin (a reddish, cysteine-based form of melanin), pigmentation levels may be enhanced in individuals with higher stress responses and higher CORT levels because agouti-related protein (AGRP) may stimulate the stress response in the brain, and phaeomelanogenesis in the skin (Takeuchi et al. 2000; Ducrest et al. 2008; Jenkins et al. 2013). However, to the contrary, elevating CORT reduced phaeomelanin-based pigmentation in nestling barn owls (*Tyto alba*; Roulin et al. 2008), perhaps because CORT can downregulate MC1R and tyrosinase, which are required for melanogenesis (Slominski et al. 2004; Roulin et al. 2008). Indeed, CORT could act to suppress phaeomelanogenesis in poor quality individuals to avoid potential oxidative and energetic costs (McGraw 2006b; Galván and Solano 2009).

We aimed to elucidate whether the relationship between CORT concentrations and sexual pigmentation is age-, sex-, or pigment-type-dependent by measuring feather CORT levels and carotenoid- and phaeomelanin-based sexual pigmentation in the yellow warbler (*Setophaga petechia*). By measuring CORT in feathers ($CORT_f$) bearing sexual pigmentation, we derived a measure of CORT integrated across the period of pigment deposition (Bortolotti et al. 2008 2009; Fairhurst et al. 2013). In addition, we examined the relationship between $CORT_f$ and condition at molt as reflected by feather growth bars and quality, which we have previously associated with sexual pigmentation (Grunst et al. 2014). Finally, using a small sample of recaptured birds, we assessed the between-year repeatability of $CORT_f$ levels. We predicted that the relationship between both types of pigmentation and $CORT_f$ would be more negative in first year breeders than older birds, since first year breeders might be more resource limited (Badyaev and Duckworth 2003). Further, age-dependent relationships between condition at molt and carotenoid-based pigmentation are sex-specific in our study population (Grunst et al. 2014). Among males, carotenoid-based pigmentation increases with condition at molt among first-year breeders, but not among older birds. However, in females, carotenoid-based pigmentation does increase with condition at molt among older birds. Thus, we also predicted that age might affect the relationship between $CORT_f$ and carotenoid-based coloration in a sex-specific fashion.

METHODS

Capture of birds and quantification of feather quality

We collected data on yellow warblers from 2010 to 2012 at the University of California's Sierra Nevada Aquatic Research Laboratory and in the adjacent Inyo National Forest. The University of California, Riverside's Animal Care and Use Committee approved all field protocols (Protocol A-20100003E). Sample collection and fieldwork were authorized by a United States Geological Survey (USGS) bird-banding subpermit (23035-G), a California state collecting permit (SC11060), a federal migratory bird collecting permit (MB22669A-0), and the Inyo National Forest (MLD100007P).

We lured males and associated females into mist nets using a conspecific song playback and decoy. We captured the majority of birds in the preincubation period (early May to early June, $N = 107$), but captured a smaller number of individuals ($N = 28$) by placing nets near nestling-stage nests. Our sample included 135 observations (24 second year males [birds in their first breeding season], 67 after-second year males, 12 second year females, 32 after-second year females) and 119 unique individuals. Fourteen individuals were captured multiple times over the course of the study.

At capture we randomly collected ~20–25 contour feathers from the upper breast for use in CORT assays and measurement of sexual coloration (see below), and measured tarsus length (±0.01 mm) as an indicator of structural body size. Yellow warblers replace contour feathers during a prebreeding molt that occurs from January to February. Thus, we collected newly grown contour feathers bearing sexual coloration that was adopted for the current breeding season. In addition, from warblers captured in 2010 and 2011, we collected the first tertial feather (the most distal of the inner most flight feathers). We stored all feathers in closed envelopes in a dry, dark location until analysis. In yellow warblers, tertial feathers are replaced at the prebreeding molt along with contour feathers bearing sexual pigmentation (Pyle 1997; Lowther et al. 1999).

We used tertial feather quality to assess condition at prebreeding molt. To quantify tertial feather quality we measured tertial feather length (±0.01 mm) using digital calipers and tertial feather weight (±0.1 mg) using a high precision digital scale. In addition, we used digital calipers to measure the width of 4 central pairs of alternating dark and light growth bars (±0.01 mm), as a metric of feather growth rate (Grubb 2006). Since these measurements were highly correlated, we used principal component analysis to extract a single

factor (PC1), which we term "tertial feather quality." Tertial weight (0.61), length (0.61), and growth bar width (0.51) were all positively loaded on PC1, which explained 72% of total variation and had an eigenvalue of 2.17. For females, we corrected tertial PC1 scores for individual size, by extracting residuals from a regression of PC1 score on tarsus length (male tarsus length was unrelated to tertial PC1 scores, $P = 0.64$, so this correction had no effect) (Grunst et al. 2014 for detail).

Measurement of coloration

To quantify coverage of melanin-based pigmentation, we used a Stylus 800 Olympus camera to take multiple (2–3) digital photographs of both the front and side of birds. We held a 1-cm grid level to the bird for scale, and held the camera perpendicular to the bird at a distance of ~3 cm. We took all pictures outdoors in full shadow to provide a uniform lighting environment and to maximize contrast between yellow and red-brown pigmentation.

For males, we used the threshold color function in the image analysis program *ImageJ* to extract the percent coverage of red-brown (phaeomelanin) from photographs (Schneider et al. 2012). We determined percent coverage of melanin in a 2 cm by 1.5-cm rectangle centered at the top of the breast, and in a 2 cm by 1-cm rectangle centered on the side of the bird, at the top of the wing. We averaged percentages derived from 2 photographs from both the front and side of each bird to obtain a final measure of melanin coverage. For females, melanin coverage was generally too low to quantify in *ImageJ*. Thus, for females we scored melanin coverage on a scale of 0–4, where 0 corresponded to no melanin-based streaking, 1 to a trace of streaking, 2 to moderate streaking (~1–2% coverage), and 4 to heavy streaking (~5% coverage). This scoring criterion encompassed the range in melanin coverage observed in females, and was 99% repeatable.

We used 5 feathers bearing carotenoid-based pigmentation and 5 feathers bearing melanin-based pigmentation for spectrometric analyses of coloration. To obtain reflectance spectra, we arranged feathers on a black felt background (with zero reflectance) to mimic natural feather alignment. We then used an USB4000 spectrometer with a xenon light source (range: 200–1100 nm; Ocean Optics, Inc., Dunedin, FL) to obtain reflectance spectra between 300 and 725 nm, across the avian visual range. We measured reflectance relative to a white standard, and averaged 5 spectra from each feather patch to obtain a final spectrum for each bird. The probe of the spectrometer was enclosed in a black rubber sheath to exclude ambient light, held perpendicular to the sample ("coincident normal"), and slightly repositioned between each reading (Andersson and Prager 2006; Montgomerie 2006; Hegyi et al. 2007).

To characterize reflectance spectra, we used colorimetric measurements of brightness (reflectance), chroma (saturation or spectral purity), and hue (spectral location). Yellow carotenoid pigmentation that is not fully saturated displays a bimodal reflectance spectrum, with reflectance peaks for both ultraviolet and yellow light, and high absorbance of blue-green light. To characterize carotenoid reflectance spectra, we calculated carotenoid chroma (saturation), average reflectance, and lambda R50 (Parker et al. 2003; Andersson and Prager 2006; Hegyi et al. 2007). Lambda R50 is the wavelength at which reflectance is halfway between its minimum and maximum value, and is a measurement of hue. Previous studies indicate that as the concentration of yellow carotenoids in feathers increases, carotenoid chroma and lambda R50 increase (hue shifts toward orange), and average reflectance may decrease (Andersson and Prager 2006). Melanin pigmentation displays a simpler reflectance spectrum, with reflectance steadily increasing across the visible wavelengths (McGraw et al. 2004; Safran and McGraw 2004). We only measured melanin reflectance in males due to limited coverage of melanin in females. To characterize melanin spectra, we calculated red chroma, average reflectance, and lambda R50. More concentrated melanin-based pigmentation is less reflective and displays greater red chroma and lambda R50 (McGraw et al. 2004 2005; Safran and McGraw 2004; Andersson and Prager 2006; Parejo et al. 2011). We list the formulas used to calculate colorimetric variables in Table 1. We performed analyses on each colorimetric variable separately, because past studies on the relationship between $CORT_f$ and coloration have obtained contrasting results regarding which aspect of coloration is related to CORT levels. For instance, Kennedy et al. (2013) found a relationship with brightness, whereas Lendvai et al. (2013) found a relationship with hue.

Feather preparation

We weighed feathers using a 4-place digital scale (±0.1 mg) to obtain a final feather mass as close as possible to 6.1 mg (range 5.8–6.2 mg). We also measured all feathers using digital calipers (±0.01 mm). A nonlinear relationship between feather mass and the amount of CORT/mg detected in feathers may occur at low feather masses (<20 mg) (Lattin et al. 2011). Using equivalent feather masses avoids problems associated with this nonlinearity. Feather mass was not correlated with the pg CORT/mg in our dataset (Linear model (LM): $F_{1,133} = 0.59$, $\beta = -17.28 \pm 22.32$, $P = 0.44$, $R^2 = 0.004$). Feathers used in the CORT assay were primarily contour feathers, which were also used to measure sexual coloration. However, for yellow warblers captured in 2010 and 2011, we also used the tertial feather from individuals for which we did not have a sufficient number of contour

Table 1

Formulas used to calculate colorimetric variables from carotenoid- and phaeomelanin-based reflectance spectra

Variable	Formula	Interpretation
Carotenoid chroma (saturation)	$\frac{\text{Median R700} - \text{Medium R450}}{\text{Median R700}}$	Saturation of coloration in the yellow wavelengths
Red chroma (saturation)	$\frac{\sum(\text{R605} - \text{R700})}{\sum(\text{R300} - \text{R700})}$	Saturation of coloration in the red wavelengths
Average reflectance	Average (R300–R700)	Reflectance averaged across all wavelengths
Lambda R50	λ_{R50}, where, R50 = (R725 ± R450)/2	A measurement of spectral location or hue

R = Percent reflectance at the following wavelength in nanometer. For carotenoid chroma, we calculated median R700 nm across R505–R710 nm, because reflectance tended to rise toward maximum by 505 nm. We calculated median R450 nm between 400 and 500 nm, where reflectance was at a minimum for the lutein reflectance curve. λ = Wavelength in nanometer.

feathers to reach our target weight. In yellow warblers, tertial feathers and contour feathers bearing sexual pigmentation are replaced at the prebreeding molt (Pyle 1997; Grunst et al. 2014), and previous studies have reported that feathers molted during the same time period do not differ in CORT concentrations (Lendvai et al. 2013). Whether or not a tertial feather was included in a sample had no effect on $CORT_f$ (LM: $F_{1,133} = 3.67$, $\beta = 5.46 \pm 2.85$, $P = 0.06$, $R^2 = 0.01$). We removed the calamus from tertial feathers, but not from contour feathers, due to their small size. We used a surgical scissors to mince both contour and tertial feathers into pieces $<5\,mm^2$ in surface area. We transferred samples to 12 mL conical tubes for transport to Tufts University, where CORT assays were performed.

CORT assay

We extracted CORT from feather samples by adding 7 mL of methanol (HPLC grade, Fischer Scientific, Pittsburgh, PA), placing tubes in a sonicating water bath at room temperature for 30 min, and then incubating samples overnight in a shaking water bath at 50°C. After incubation, we used vacuum filtration to separate feather particles from methanol. We used #4 Whatman filters and filtration funnels. We rinsed filter paper 2 times with an additional 2.5 mL of methanol, which was added to the methanol extract (Lattin et al. 2011). We dried methanol extracts using nitrogen gas under a fume hood, with samples placed in a 50°C water bath. We reconstituted extracts in 550 μL of PBS phosphate buffer (0.05 M, 7.6 pH). We then employed standard radioimmunoassay techniques (Wingfield et al. 1992) to determine $CORT_f$ in a single assay, using an anti-CORT antibody purchased from Sigma–Aldrich (C8784, St. Louis, MO). We separated bound and unbound CORT using dextran-coated charcoal. Due to the large number of samples involved, we processed samples in batches corresponding to the 4 separate centrifuge spins needed to accomplish separation of bound and unbound hormone fractions. Intra-assay coefficients of variation, calculated from $CORT_f$ concentrations in sample duplicates, averaged 10.6%. The detection limit of the assay was 33.24 pg/tube, and the lowest sample measurement was 58.53 pg/tube.

We verified the parallelism of the assay for our study species by creating a pool of yellow warbler feathers. We pulverized feathers using a ball mill (Kleco model 4200, Visalia, CA) to create a fine dust. We then extracted CORT from 50 mg of pool, resuspended the extraction in 1000 μL of PBS, and generated a dilution series ranging in concentration from 1:1 through 1:8. Serial dilutions produced a curve parallel to the standard curve.

We calculated CORT concentrations as a function of feather mass (pg CORT/mg), although Bortolotti et al. (2008 2009) suggest that standardizing by length is preferable. We chose to standardize by weight because of the high potential for measurement error inherent to measuring many, small contour feathers. Lendvai et al. (2013) also adopted this approach. Further, past studies report that CORT values calculated as a function of mass and length are highly correlated (Kennedy et al. 2013), and this was also true in our dataset (Spearman correlation: $r_s = 0.94$, $P < 0.001$). Procedures for measuring CORT in feathers are relatively new. However, experimental work employing the same assay techniques as we used indicates that feather CORT concentrations are elevated in birds that have higher plasma CORT concentrations during molt (Lattin et al. 2011).

Statistical analyses

We performed statistical analyses using R 2.15.2 (R Core Team 2012). Since 14 birds were captured in multiple years, we employed linear mixed effects models (LMMs, R package lme4, Bates et al. 2012), with individual entered as a random effect. We specified Helmert contrasts so that beta estimates for main effects were estimated across sex and age categories, and employed Sattherwaite approximations to calculate degrees of freedom for final *F*-tests (R package lmerTest; Kuznetsova et al. 2013). We used coloration variables (saturation, brightness, and hue for melanin and carotenoid pigmentation, melanin coverage/score) as the dependent variable in 8 independent models. For carotenoid pigmentation variables (saturation, hue, and brightness) we initially entered the 3-way interaction between sex, age, and $CORT_f$ as the predictor term, with year entered as a covariate. Melanin pigmentation variables differed for males and females (percent coverage and reflectance variables for males and melanin score for females), so we constructed separate models for the 2 sexes. In these models we entered the interaction between age and $CORT_f$ as the predictor term, with year again entered as a covariate. For the model involving female melanin scores, we used a generalized linear mixed model (GLMM) with a Poisson distribution, to account for the distribution of scores. We sequentially removed nonsignificant predictors from models, by always removing the predictor with the highest *P*-value first.

In addition, we assessed the relationship between $CORT_f$ and our metric of condition at prebreeding molt (tertial feather PC1), and whether $CORT_f$ levels varied with age or sex. We used LMMs to predict tertial feather PC1 from $CORT_f$, sex, age, and year, as for coloration variables. We assessed if $CORT_f$ levels were related to age and sex by using LMMs to predict $CORT_f$ from the interaction between age and sex.

Finally, to assess the between-year repeatability of $CORT_f$ and whether $CORT_f$ changed longitudinally with age we used the 14 birds for which we obtained $CORT_f$ measurements in 2 years. We first constructed an LMM to predict $CORT_f$ from whether an observation represented the first or second capture of a bird (to test for a longitudinal change in $CORT_f$), year (2010, 2011, 2012), and the random effect of individual identity. Year was significant in this model, so we retained this covariate when estimating repeatability. We then used R package rptR (function rpt.remlLMM.adj, for conditional repeatability) to calculate repeatability with standard error (SE) and 95% confidence intervals (CI) based on variance components extracted from the LMM. *P*-values for repeatability estimates derive from log-likelihood ratio tests (Nakagawa and Schielzeth 2010).

Feather CORT concentrations correlated with position in the assay. Specifically, samples run in the 4th spin of the centrifuge had lower $CORT_f$ values. Thus, before performing analyses, we corrected $CORT_f$ for this "spin effect" by taking the residuals from an ANOVA model predicting $CORT_f$ from spin number. We obtained qualitatively equivalent results when dropping samples from the 4th spin from the dataset.

RESULTS

Variation in $CORT_f$ across age and sex classes

Yellow warblers had mean ± SE $CORT_f$ concentrations of 40.55 ± 13.90 pg/mg, with levels comparable between males (mean: 41.81 ± 14.19) and females (mean: 37.94 ± 13.07) (LMM: $F_{1,125} = 0.13$, $\beta = -3.01 \pm 2.59$, $P = 0.72$, $N = 135$ observations, 119 individuals). After-second year (mean: 39.98 ± 14.35) and second year (mean: 42.13 ± 12.66) birds also had similar $CORT_f$ concentrations ($F_{1,130} = 0.53$, $\beta = -5.65 \pm 3.93$, $P = 0.47$), and

the sex × age interaction term was nonsignificant ($F_{1,130}$ = 2.65, β = 7.81 ± 4.79, P = 0.11).

Carotenoid pigmentation and $CORT_f$

We found negative correlations between metrics of carotenoid-based pigmentation and $CORT_f$. Yellow warblers with higher $CORT_f$ had lower carotenoid chroma (Table 2; Figure 1). However, there was a positive $CORT_f$ × sex interaction, which reflected a weaker negative relationship between $CORT_f$ and carotenoid chroma in males than females (Table 2). This interaction was driven by after-second year males, in which $CORT_f$ was not associated with carotenoid chroma (Figure 1). Further, there was also a negative $CORT_f$ × age interaction (Table 2), which reflected a stronger negative relationship between $CORT_f$ and carotenoid chroma in second year birds (LM: $F_{1,32}$ = 4.52, β = −0.002 ± 0.001, P = 0.04, N = 36) than after-second year birds (LMM: $F_{1,89}$ = 2.99, β = −0.001 ± 0.001, P = 0.09, N = 99 observations, 87 individuals). The $CORT_f$ × age interaction was driven by males, and was significant within males alone ($F_{1,74}$ = 7.03, β = −0.001 ± 0.0003, P = 0.009, N = 91 observations, 78 individuals). The model predicting carotenoid chroma also retained a positive effect of male sex, a negative effect of being a second year bird, and a positive sex × age interaction (Table 2). The sex × age interaction reflects the fact that carotenoid chroma was much higher in after-second year females than in second year females, whereas after-second year and second year males were more similar in coloration (Grunst et al. 2014). Finally, the model predicting carotenoid chroma included a year effect to account for lower carotenoid chroma among birds captured during 2011 (Table 2).

Warblers with higher $CORT_f$ also had lower carotenoid hue (lambda R50), when controlling for a positive effect of male sex, and a negative effect of being captured in 2011 (Table 2; Figure 2). Carotenoid hue was not related to age ($F_{1,130}$ = 2.61, β = −0.71 ± 0.44, P = 0.11). Further, sex (LMM: $F_{1,124}$ = 0.12, β = 0.008 ± 0.02, P = 0.73) and age ($F_{1,124}$ = 0.73, β = −0.01 ± 0.01, P = 0.40) did not interact with $CORT_f$ in the model predicting hue, and there was no sex × age interaction ($F_{1,127}$ = 0.31, β = 0.13 ± 0.24, P = 0.58). The negative relationship between $CORT_f$ and carotenoid hue was significant in males alone ($F_{1,87}$ = 6.12, β = −0.05 ± 0.01, P = 0.01, N = 91 observations, 78 individuals). This relationship was not significant in females alone, but the coefficient estimate was in the same direction ($F_{1,41}$ = 1.75, β = −0.05 ± 0.04, P = 0.19, N = 44 observations, 41 individuals; Figure 2).

The brightness of carotenoid-based pigmentation was not related to $CORT_f$, or to age, sex, year of capture, or interaction terms (P > 0.20 in all cases; Supplementary Table S1).

In models predicting carotenoid coloration variables, none of the 3-way interactions between and $CORT_f$, age, and sex were statistically significant (LMM: P > 0.10 in all cases).

Melanin pigmentation and $CORT_f$

In contrast to carotenoid-based pigmentation, we did not find any significant relationships between melanin-based pigmentation in males and $CORT_f$ (LMM: P > 0.40 in all cases, Supplementary Table S2). Age and the $CORT_f$ × age interaction term were also unrelated to melanin pigmentation in males (P > 0.10 in all cases, Supplementary Table S2). However, year of capture was related to melanin chroma ($F_{1,65}$ = 4.88, β = −0.02 ± 0.01, P = 0.03) and tended to be related to melanin hue ($F_{1,58}$ = 3.79, β = −5.90 ± 3.03, P = 0.06). In parallel to results for carotenoid pigmentation, birds captured in 2011 had lower melanin chroma and hue than birds captured in the other 2 years.

In females, second year birds with higher melanin scores had lower $CORT_f$, whereas this relationship was not present among

Table 2
Final LMMs predicting carotenoid chroma and hue (lambda R50) from $CORT_f$ and covariates

	N	Estimate (β ± SE)	F	Denom (df)	P (>F)
Carotenoid chroma	135, 119[a]				
Intercept		0.85 ± 0.006			
$CORT_f$		−0.002 ± 0.0004	12.15	124.08	<0.001
Sex		0.10 ± 0.004[b]	426.37	116.40	<0.001
Age		−0.02 ± 0.004[c]	22.45	122.88	<0.001
Year		−0.03 ± 0.006[d]	19.64	48.12	<0.001
$CORT_f$ × sex		0.001 ± 0.0004	4.77	125.80	0.03
$CORT_f$ × age		−0.001 ± 0.004	5.01	100.28	0.02
Sex × age		0.02 ± 0.004	16.75	126.38	<0.001
Carotenoid hue	135, 119				
Intercept		507.13 ± 0.39			
$CORT_f$		−0.05 ± 0.02	138.94	119.03	0.004
Sex		4.99 ± 0.42	8.37	127. 98	<0.001
Year		−0.93 ± 0.39	5.83	108.5	0.02
Carotenoid brightness	135, 119				
Intercept		16.85 ± 0.23			
$CORT_f$		0.002 ± 0.01	0.02	124.72	0.88
Sex		−0.009 ± 0.19	0.002	121.42	0.96
Age		0.05 ± 0.19	0.09	126.97	0.77
Year		−0.21 ± 0.30	0.50	96.68	0.48
$CORT_f$ × sex		−0.01 ± 0.01	0.79	125.18	0.38
$CORT_f$ × age		−0.01 ± 0.01	0.68	121.53	0.41
Sex × age		−0.22 ± 0.19	1.39	126.61	0.24

[a]N = Number of observations, number of individuals.
[b]Males relative to females.
[c]Second year relative to after-second year.
[d]2011 relative to other years.

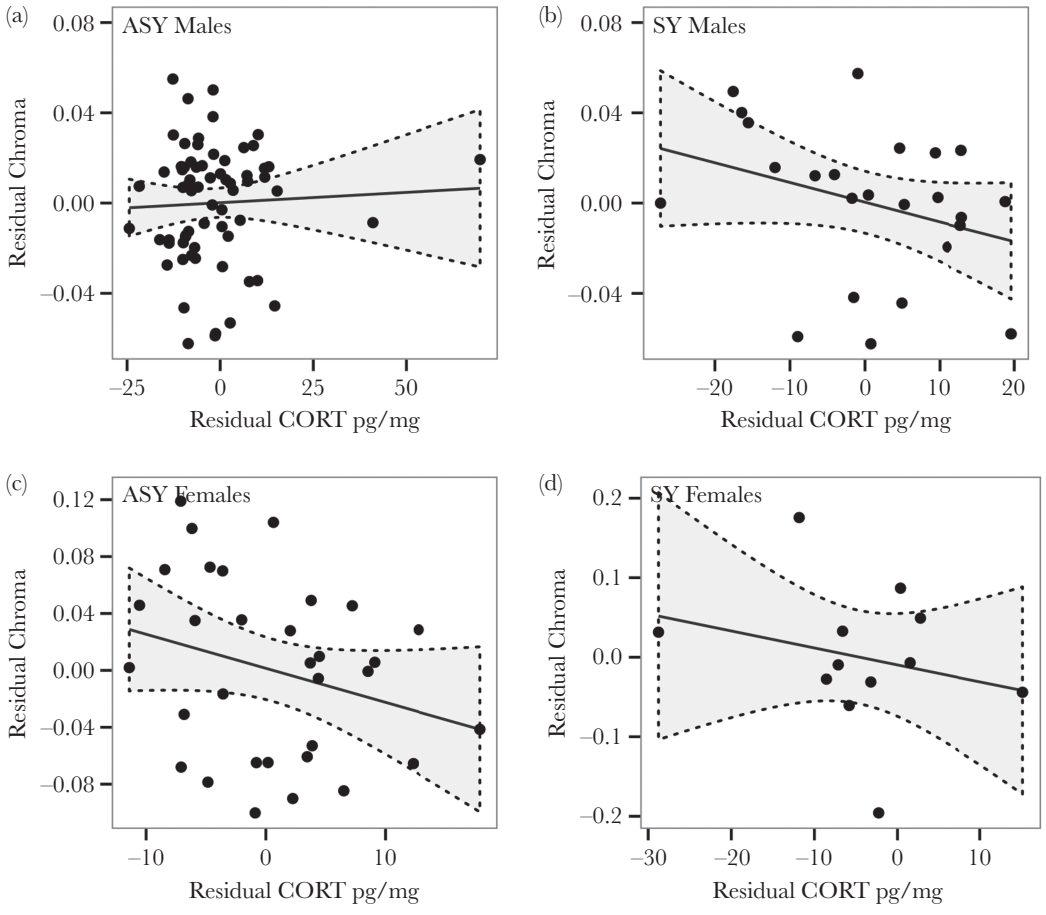

Figure 1
Relationship between residual $CORT_f$ (controlling for spin number) and residual carotenoid chroma (controlling for year) across sex and age classes. SY, second year; ASY, after-second year. Removing the 2 after-second year males with outlying $CORT_f$ values did not qualitatively alter results. Shaded regions represent the 95% confidence intervals.

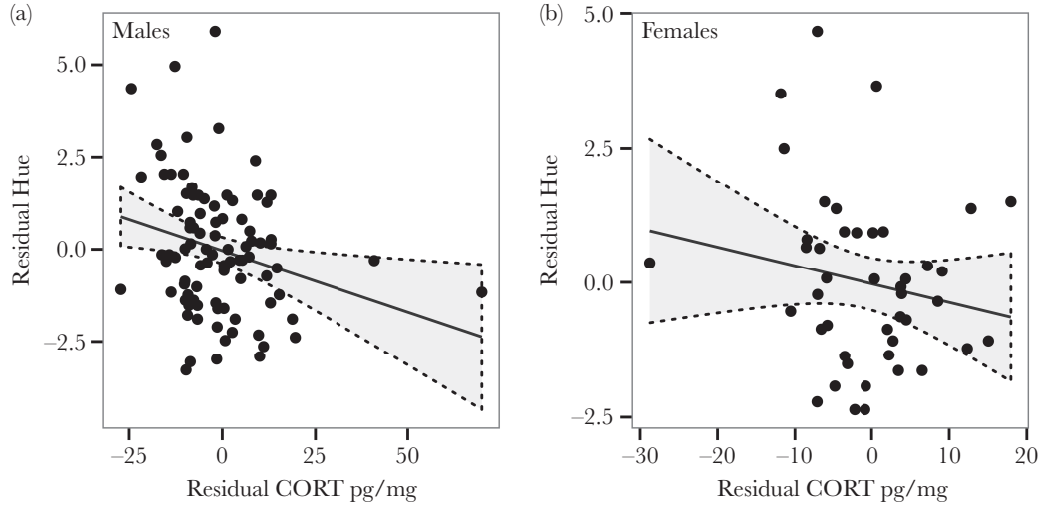

Figure 2
Relationship between residual $CORT_f$ (controlling for spin number) and residual carotenoid hue (lambda R50; controlling for year) in males and females. Removing the 2 males with outlying $CORT_f$ values did not qualitatively alter results. Shaded regions represent the 95% confidence intervals.

after-second year birds, as indicated by a negative interaction (GLMM: $\mathcal{Z} = -2.87$, $\beta = -0.16 \pm 0.05$, $P = 0.004$, $N = 44$ observations, 41 birds) between age ($\mathcal{Z} = -2.35$, $\beta = -1.16 \pm 0.49$, $P = 0.01$) and female melanin score ($\mathcal{Z} = -1.35$, $\beta = -0.04 \pm 0.03$, $P = 0.17$) in the model predicting $CORT_f$ levels. In second year females alone, there was a negative relationship between melanin score and $CORT_f$

(GLM: $\chi = -3.31$, $\beta = -0.11 \pm 0.03$, $P = 0.001$, $N = 12$), whereas this relationship was nonsignificant in after-second year females alone (GLMM: $\chi = 1.44$, $\beta = 0.04 \pm 0.03$, $P = 0.15$, $N = 32$ observations, 30 birds). However, the result in second year females was driven by 2 individuals, so these results must be viewed with caution.

Tertial feather quality and $CORT_f$

Relationships between tertial feather quality and $CORT_f$ were weak. The main effect of $CORT_f$ on tertial feather quality was nonsignificant (Table 3). However, there was a marginally significant 3-way interaction term (Table 3). This interaction term reflected the fact that there was a negative $CORT_f \times$ age interaction in males (LMM within males: $F_{1,43} = 4.70$, $\beta = -0.02 \pm 0.01$, $P = 0.03$, $N = 58$ observations, 51 individuals), but not females (LMM within females: $F_{1,23} = 1.56$, $\beta = 0.04 \pm 0.04$, $P = 0.22$). The significant $CORT_f \times$ age interaction in males arose due to a tendency toward lower tertial feather quality in second year males with higher $CORT_f$ (LM: $F_{1,16} = 3.26$, $\beta = -0.05 \pm 0.02$, $P = 0.09$), but no relationship between $CORT_f$ and tertial feather quality in after-second year males (LMM: $F_{1,37} = 0.06$, $\beta = -0.003 \pm 0.01$, $P = 0.80$, $N = 40$ observations, 34 males; Figure 3).

Repeatability and longitudinal changes in $CORT_f$

We found no evidence that $CORT_f$ changes longitudinally with age, as indicated by a nonsignificant relationship between $CORT_f$ and whether or not an observation represented the first or second capture of a bird (LMM: $F_{1,17} = 1.47$, $\beta = 4.58 \pm 3.77$, $P = 0.24$). However, $CORT_f$ was repeatable within individuals between years ($r_1 = 0.40$, SE = 0.20, CI = 0–0.76, $P < 0.001$), when controlling for a year effect on $CORT_f$ levels (LMM: $F_{1,19} = 5.20$, $\beta = -9.92 \pm 4.35$, $P = 0.03$). Birds captured in 2012 had lower $CORT_f$ levels than birds captured in the other 2 years. When dropping the year effect from the model, $CORT_f$ remained significantly repeatable ($r_1 = 0.23$, SE = 0.20, CI = 0–0.67, $P = 0.01$).

DISCUSSION

Consistent with some past research in other species (Loiseau et al. 2008; Mougeot et al. 2010; Martínez-Padilla et al. 2013), our results suggest that carotenoid-based pigmentation in yellow warblers is negatively correlated with CORT concentrations. Thus, carotenoid-based pigmentation may indicate lower levels of stress exposure, or the ability to better cope with elevated stress, to prospective mates (Husak and Moore 2008). CORT levels are often expected to be lower in higher quality individuals that experience fewer energetic, oxidative, and immune challenges (Raouf et al. 2006; Angelier et al. 2010), or are better able to cope with these challenges (Saino et al. 2002). In our case, $CORT_f$ concentrations reflect stressors experienced on the wintering grounds, where warblers undergo the prebreeding molt. These stressors may include long-term (chronic) stressors such as resource limitation or

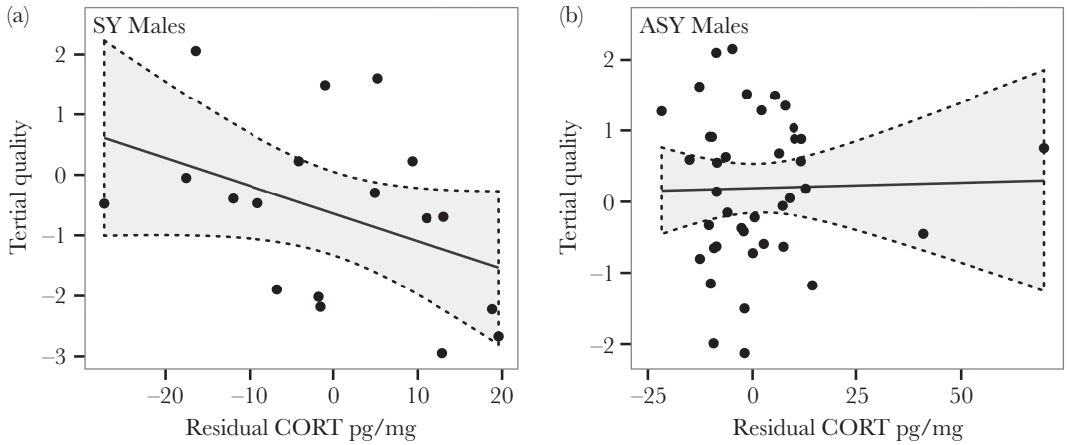

Figure 3
Relationship between residual $CORT_f$ and tertial feather quality (PC1) in second year males (SY) versus after-second year (ASY) males. Shaded regions represent the 95% CI.

Table 3
Final LMM predicting tertial feather quality (condition at molt) from $CORT_f$ and covariates

	Estimate (β ± SE)	*F*	Denom (df)	*P* (>*F*)
Intercept	0.21 ± 0.15			
$CORT_f$	0.03 ± 0.02	2.65	77.81	0.11
Age	−0.34 ± 0.14[a]	5.43	77.97	0.02
Sex	0.001 ± 0.15[b]	<0.001	71.97	0.99
$CORT_f \times$ sex	−0.05 ± 0.01	8.09	77.81	0.01
$CORT_f \times$ age	0.01 ± 0.02	0.33	75.60	0.57
Sex × age	−0.08 ± 0.14	0.34	77.97	0.56
$CORT_f \times$ age × sex	−0.03 ± 0.01	3.35	84.99	0.07

$N = 86$ observation, 76 individuals.
[a]Second year relative to after-second year.
[b]Males relative to females.

infection, which may be lower in individuals that obtain good wintering territories (Marra and Holberton 1998), or acute stressors such as extreme weather events or predation threats. In support of a negative relationship between CORT levels at molt and individual quality, $CORT_f$ is negatively correlated with survival in house sparrows (*Passer domesticus*; Koren et al. 2012) and plasma CORT degrades feather quality in European starlings (*Sturnus vulgaris*; DesRochers et al. 2009). Further, past studies have found a negative correlation between baseline plasma CORT and fitness metrics (e.g., Angelier et al. 2010), although the opposite relationship has also been observed (Bonier et al. 2009 for review). High CORT levels in low quality individuals may suppress allocation of carotenoids toward pigmentation at the expense of alternative functions including immune stimulation and antioxidant defense, promoting reliability of carotenoid-based signals.

Our results suggest that carotenoid hue more consistently indicates $CORT_f$ concentrations than carotenoid chroma, and may thus serve as a more consistent signal of stress perceived or encountered in the environment. Carotenoid hue was negatively correlated with $CORT_f$ levels across the sexes and age classes, including among after-second year males, which have the most saturated carotenoid pigmentation. In contrast, consistent with our previous finding that carotenoid pigmentation is less condition-dependent in older males (Grunst et al. 2014), carotenoid chroma was negatively correlated with $CORT_f$ in females and young males, but not in after-second year males. Variance in carotenoid chroma (saturation) decreases as pigmentation approaches complete saturation (Andersson and Prager 2006), and is low in after-second year male yellow warblers (Grunst et al. 2014). As a result, carotenoid chroma appears to have little potential to reflect individual differences in quality and $CORT_f$ among after-second year males, whereas hue continues to indicate $CORT_f$ among these birds.

Higher, less variable levels of carotenoid chroma in after-second year males may suggest fewer resource limitations and more efficient acquisition of carotenoids relative to other birds. Indeed, as for carotenoid chroma, condition at molt (tertial feather quality) and $CORT_f$ tended to be negatively correlated within second year males but not after-second year males, consistent with stronger resource limitations in second year males. However, contrary to relaxed resource constraints in older males, Fairhurst et al. (2014) found that CORT deposited in feathers was positively correlated with carotenoid pigmentation in adult male common redpolls (*Acanthis flammea*), but not in females or young males. They suggest that their results reflect a stronger tradeoff between allocating carotenoids to ornamentation and alternative functions in older, more ornamented males, and that only high quality males can tolerate the CORT levels necessary to produce intense pigmentation.

In contrast to our results, but like Fairhurst et al. (2014), Lendvai et al. (2013) also reported a positive correlation between carotenoid-based pigmentation and $CORT_f$ concentrations, in this case in the house finch. These positive correlations might reflect a role of CORT in increasing foraging rate, metabolism, and release of carotenoids from the tissues (Lendvai et al. 2013; Fairhurst et al. 2014). Varying correlations between carotenoid-based pigmentation and $CORT_f$ are difficult to interpret because it is unclear whether a mechanistic link between CORT and carotenoid-based pigmentation exists, or whether differences reflect covariation between CORT levels, carotenoid-based pigmentation, and some third variable. However, $CORT_f$ concentrations could show different relationships to distinct types of carotenoid-based pigmentation. To date, the species in which positive relationships between CORT levels and carotenoid-based pigmentation have been reported have expressed red, ketocarotenoid forms of carotenoid-based pigmentation (Fairhurst et al. 2013; Lendvai et al. 2013), although some studies have also found negative relationships between red, carotenoid-based pigmentation and CORT levels (Mougeot et al. 2010). Ketocarotenoid pigmentation generally requires more processing of carotenoids obtained from the diet than yellow, hydroxycarotenoid (usually lutein-based) forms of carotenoid-based pigmentation, as expressed in the yellow warbler (McGraw 2006a; Pérez-Rodríguez et al. 2013). Therefore, more energy may be required to produce red pigmentation, necessitating upregulation of glucocorticoids in highly pigmented individuals to support the associated energetic challenge. In contrast, intense yellow pigmentation could function as a marker of foraging efficiency (García-Navas et al. 2012), and negatively covary with CORT levels because CORT is elevated in individuals with a poorer capacity to meet energetic needs (Romero and Wikelski 2001).

In contrast to carotenoid-based pigmentation, we found little evidence that melanin-based pigmentation was correlated with $CORT_f$ concentrations, suggesting that phaeomelanin-based pigmentation communicates little information regarding stress levels in yellow warblers. Consistent with a recent study in barn swallows (*Hirundo rustica*; Jenkins et al. 2013), no metric of male phaeomelanin-based pigmentation was correlated with CORT concentrations. There was a negative relationship between phaeomelanin-based pigmentation and $CORT_f$ concentrations among second-year females. This negative relationship is consistent with lower expression of phaeomelanin-based pigmentation in second year females of poorer quality and thus under elevated stress, as previously observed in barn owls (Roulin et al. 2008), and could arise if CORT inhibits melanogenesis in the skin (Slominski et al. 2004; Roulin et al. 2008; Almasi et al. 2010). A negative relationship between phaeomelanin-based pigmentation and CORT concentrations might be more likely than the proposed positive relationship mediated by pleiotropic effects of AGRP (Takeuchi et al. 2000; Jenkins et al. 2013). Indeed, phaeomelanogenesis is primarily controlled by agouti-signaling protein (ASIP) acting on MC1R in the skin (Hida et al. 2009), whereas AGRP is expressed primarily in the brain (Xiao et al. 2003). However, the negative relationship between phaeomelanin-based pigmentation and $CORT_f$ in second year females was driven by only 2 individuals, and did not occur in the other age and sex classes, so cannot be considered robust.

Although phaeomelanin-based pigmentation is not strongly related to CORT concentrations at molt in our study population, phaoemelanin-based pigmentation does show a strong, positive correlation with feather quality and growth rate (Grunst et al. 2014). Thus, carotenoid- and phaeomelanin-based pigmentation may most strongly reflect different aspects of individual quality and physiological state. For example, phaeomelanin-based pigmentation might be related to resource holding capacity, since past studies in male yellow warblers relate phaeomelanin-based pigmentation to territorial aggressiveness and social status (Studd and Robertson 1985).

Finally, to our knowledge, our study is the first to demonstrate between-year repeatability of $CORT_f$ concentrations, although other studies have reported that feather quality is repeatable within individuals across multiple molts (De la Hera et al. 2009). The repeatability of $CORT_f$ concentrations, which emerged despite a relatively small sample size, suggests that $CORT_f$ levels may reflect stable differences in individual quality and the ability to avoid or cope with stress. Further, carotenoid-based pigmentation was negatively correlated with $CORT_f$ concentrations, and may thus signal

these stable differences in individual stress levels to prospective mating partners.

In summary, our study is one of the first to investigate correlations between multiple components of sexual coloration and $CORT_f$ concentrations, which represent an integrated metric of CORT concentrations during the period of pigment deposition. Our results suggest that carotenoid- and phaeomelanin-based pigmentation communicate nonequivalent information about CORT concentrations during molt, with intense carotenoid-based pigmentation indicating lower levels of stress during molt, and phaeomelanin-based pigmentation being largely unrelated to CORT concentrations at molt. Further, sex and age modified the relationship between carotenoid chroma and CORT concentrations, seemingly because after-second year male warblers obtain highly saturated carotenoid-based pigmentation, which shows little variation in chroma. In contrast, carotenoid hue was correlated with lower CORT concentrations across age and sex classes. Finally, we demonstrate repeatability of $CORT_f$ concentrations, such that carotenoid-based pigmentation might signal stable differences in individual stress levels that could be associated with fitness.

SUPPLEMENTARY MATERIAL

Supplementary material can be found at http://www.beheco.oxfordjournals.org/

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