

Feather coloration in museum specimens is related to feather corticosterone

**Eileen A. Kennedy, Christine R. Lattin,
L. Michael Romero & Donald
C. Dearborn**

Behavioral Ecology and Sociobiology

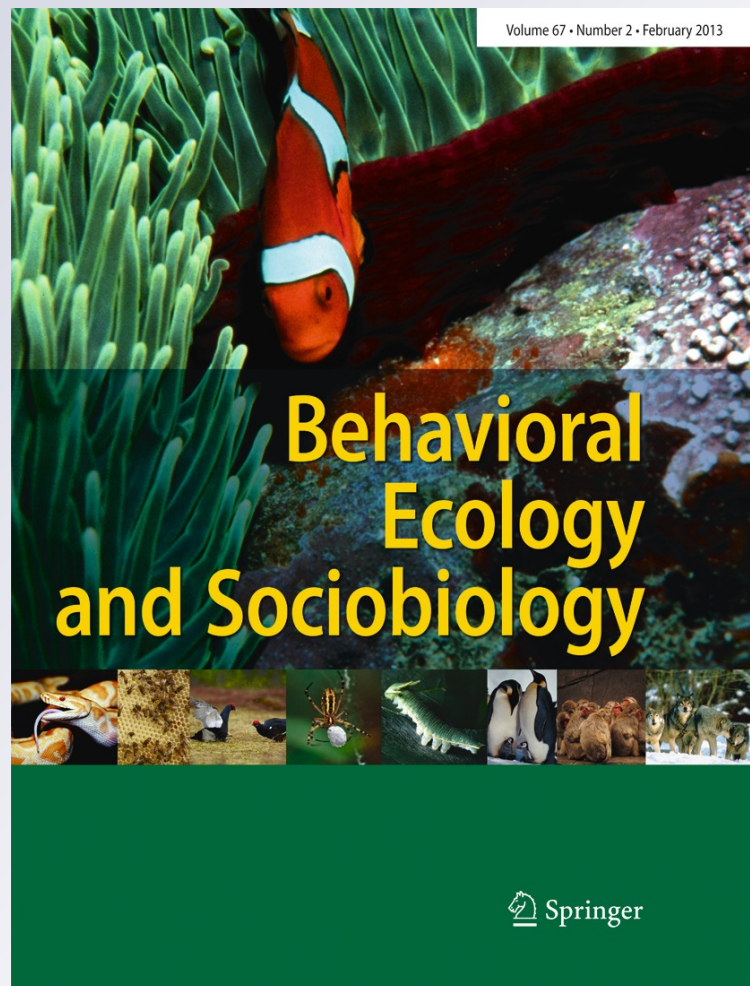
ISSN 0340-5443

Volume 67

Number 2

Behav Ecol Sociobiol (2013) 67:341-348

DOI 10.1007/s00265-012-1454-9



Your article is protected by copyright and all rights are held exclusively by Springer-Verlag Berlin Heidelberg. This e-offprint is for personal use only and shall not be self-archived in electronic repositories. If you wish to self-archive your work, please use the accepted author's version for posting to your own website or your institution's repository. You may further deposit the accepted author's version on a funder's repository at a funder's request, provided it is not made publicly available until 12 months after publication.

Feather coloration in museum specimens is related to feather corticosterone

Eileen A. Kennedy · Christine R. Lattin ·
L. Michael Romero · Donald C. Dearborn

Received: 24 July 2012 / Revised: 8 November 2012 / Accepted: 9 November 2012 / Published online: 29 November 2012
© Springer-Verlag Berlin Heidelberg 2012

Abstract Colorful ornaments in birds are often sexually selected signals of quality, and variation in ornament expression may be mediated by physiological stress through the secretion of corticosterone. However, testing for links between ornamentation and corticosterone often requires sampling live animals, and such physiological measures may not be matched in the time span in which they were sampled (e.g., very dynamic plasma corticosterone vs. plumage coloration, which is relatively static). Here, we use museum specimens to test for a link between the color of a sexual ornament and feather corticosterone at the time of ornament formation. In red-winged blackbirds, *Agelaius phoeniceus*, carotenoid-based epaulets appear to be important in male–male social interactions, territory maintenance, and female choice. We measured reflectance spectra of adult male epaulets and plucked adjacent feathers for corticosterone analysis via radioimmunoassay. We controlled for differences in the number of mates, specimen age, and geography by selecting only males with one mate and only birds collected in Florida during a 3-year period. Epaulet hue and red chroma did not vary with feather corticosterone, but males whose epaulets scored high for mean brightness and red brightness had significantly lower corticosterone than males with low brightness scores. This correlation with brightness but not hue or chroma is consistent

with an effect of corticosterone (CORT) on feather microstructure, with elevated CORT leading to lower reflectance of white light from the keratin matrix surrounding the carotenoid pigments.

Keywords Feather corticosterone · Museum specimens · Carotenoids · Coloration · Sexual selection · Structural color

Signals of many different modalities have the potential to reveal information about the physiological status of individuals. For example, elephants' odors can signal reproductive state (Goodwin et al. 2012), bird song can communicate an individual's ability to cope with stressors (Schmidt et al. 2012), and symmetry of visual ornaments can advertise developmental stability (Andersson 1994). This connection between signals and physiology can be important in intraspecific interactions, including territory defense and mate choice.

Many endocrine examples relate sexual ornaments to physiological state, most notably in regard to the vertebrate stress response. The stress response involves the activation of the hypothalamic–pituitary–adrenal axis and the release of glucocorticoid hormones from the adrenal cortex. In birds, the primary glucocorticoid hormone is corticosterone (CORT). When secreted over short time frames, CORT's actions are adaptive, reallocating energy to tasks essential for immediate survival and restoring homeostasis (Romero 2002). However, when elevated levels of CORT are maintained for long periods, the effects may be detrimental to survival (Sapolsky et al. 2000). Because CORT is so influential to energy balance, it may play an important role in sexual signaling and mate selection.

In the context of mate choice, females might choose mates based on ornaments that are honest indicators of their physiological or hormonal state. Depending on the context, females could benefit from choosing males with high CORT because these individuals are responding properly to stressors

Communicated by J. A. Graves

E. A. Kennedy · D. C. Dearborn
Department of Biology, Bucknell University, Lewisburg, PA, USA

C. R. Lattin · L. M. Romero
Department of Biology, Tufts University, Medford, MA, USA

D. C. Dearborn (✉)
Department of Biology, Bates College, 44 Campus Ave.,
Lewiston, ME 04240, USA
e-mail: ddearbor@bates.edu

and thus likely to overcome them, or alternatively females might benefit from choosing males with lower CORT because these individuals are avoiding the reallocation of energy away from normal activities (Husak and Moore 2008). Of these two possibilities, it has generally been thought that high baseline CORT concentrations indicate individuals in poorer condition with reduced fitness, and this theory is referred to as the CORT-fitness hypothesis (Bonier et al. 2009a); however, this hypothesis has not been well supported (reviewed by Bonier et al. 2009b). If females actually do benefit from choosing males based on CORT concentrations, there may be an observable male trait that honestly reflects these concentrations (Bortolotti et al. 2009b).

In birds, CORT exposure could be signaled by colorful plumage ornaments, such as the reds, oranges, and yellows created by carotenoid pigments. Carotenoid-based ornaments play an important role in mate choice in many species (Kodric-Brown 1989; McGraw 2006) and may be honest signals of health (von Schantz et al. 1999) or quality (sensu Zahavi 1975) because of trade-offs in the use of circulating carotenoids as pigments, as antioxidants (Bendich and Olson 1989; Krinsky 2001; McGraw 2005), and as modulators of the immune response (reviewed by Chew and Park 2004). The immune and antioxidant trade-offs of carotenoids are particularly relevant, as elevated CORT can cause both immunosuppression (Berger et al. 2005) and oxidative stress (Costantini et al. 2011). Elevated CORT also interferes with protein synthesis and can cause changes in feather structure (Strochlic and Romero 2008; DesRochers et al. 2009); this is important because carotenoid-based colors depend not only on the carotenoid pigments themselves but also on the surrounding keratin microstructure that reflects light through the carotenoid pigments (Jacot et al. 2010). As a consequence of the multiple connections between CORT, carotenoids, and feather structure, we hypothesized that elevated CORT would have a negative effect on carotenoid-based coloration, either through a reduction in availability of carotenoids as pigments or through a disruption of the microstructure of the feather.

Two additional scenarios might be involved in linking CORT and color, though our study was not intended to assess whether these scenarios might be at work in red-winged blackbirds. First, diet could independently affect both color (e.g., via carotenoid intake; McGraw et al. 2005) and CORT (via nutritional quality; Kitaysky et al. 2001). Second, epaulet color expression may affect CORT secretion by influencing social challenges (i.e., how many mates obtained or how many intruding males to defend the territory against); chronologically, the epaulet color expressed by a male during one breeding season might affect CORT concentration and the color of new epaulets formed at the postbreeding molt. Our study controlled for two aspects of social challenges by sampling only birds with one mate and by excluding males that were in their first breeding season.

Our goal with this study was to take the first step in testing for a connection between a sexual signal and physiological state in museum specimens. Such a test generally requires sampling live animals. In contrast, using museum specimens to approach similar questions about physiology opens a window to the past and allows for the inclusion of questions regarding different populations, different points on an evolutionary trajectory, or even extinct species or populations. As a step in assessing the practicality of such approaches, we used museum specimens to test for a link between the color of a sexual ornament and integrated CORT secretion at the time of ornament formation. Such a test should use physiological measures that match the time span in which they were sampled, but CORT is most often measured in plasma samples, which can change on a minute-by-minute basis (Romero and Reed 2005) and thus provide a very narrow snapshot of an animal's endocrine status. Because feathers are grown over several days or weeks, comparing a temporally narrow measure of plasma CORT concentrations to plumage traits that may reflect condition over a period of weeks is not an ideal approach.

Recent work by Bortolotti et al. (2008) has introduced a new tool for measuring CORT—through the analysis of CORT deposited in feathers. Measurement of the CORT deposited during feather growth provides an integrated assay of both the sustained baseline concentrations of CORT and the amount of CORT released in response to all the stressors experienced over the time the feather grew (though feather CORT may be more heavily influenced by acute stressors than by baseline CORT; Bortolotti et al. 2008). Because CORT appears to be stable in the feather for decades (Bortolotti et al. 2009a), analyzing CORT in the feathers of museum specimens opens up avenues to the past and allows for the pursuit of questions involving the endocrine state of animals that are no longer living.

Here, we compared feather CORT to carotenoid-based epaulet coloration of male red-winged blackbird (*Agelaius phoeniceus*) museum specimens to make a temporally relevant comparison of CORT and ornamentation. This allowed us to explore whether these techniques can be used to ask behavioral endocrinology questions about animals that are no longer living. Our hypothesis was that elevated CORT would have a negative effect on carotenoid-based coloration. If this occurs through a reduction in availability of carotenoids as pigments, we predict an effect on chromatic aspects of feather color such as hue or chroma. If this occurs by disrupting the microstructure and broad-spectrum reflective effectiveness of the keratin matrix, we predict an effect on the achromatic brightness of feather color.

Methods

We chose one of the most abundant species in museum collections: the red-winged blackbird, a sexually dichromatic

North American passerine whose mating system has been well studied (e.g., Nero 1955; Searcy and Yasukawa 1983; Beletsky 1996). Males display red and yellow epaulet regions that are carotenoid- and melanin-based (McGraw et al. 2004). These epaulets have been shown to function in both modes of sexual selection—as an intrasexual signal displayed during territorial disputes with other males (Hansen and Rohwer 1986; Røskaft and Rohwer 1987; Metz and Weatherhead 1992) and as an intersexual signal assessed by females during precopulatory displays (Yasukawa et al. 2009; but see Yasukawa et al. 2010). Red-winged blackbirds undergo delayed plumage maturation, thus second-year males are easily distinguished from after-second-year (ASY) males by the former's speckled appearance and by the epaulet, which is usually orange, rather than red, and mottled with black (Meanley and Bond 1970). Molt occurs yearly in late-July through September (Linz et al. 1983) and is also clearly evident in that newly molted black contour feathers have brown tips and the glossy black nuptial plumage is acquired by wear.

Museum specimens

We collected feather samples, measured reflectance spectra (described below), and recorded museum-tag data on body mass (in gram), testis length and width (in millimeter), and number of mates from adult (ASY) male red-winged blackbird specimens ($n=70$) at the Smithsonian Institution's National Museum of Natural History (USNM), Washington, DC, USA. To control for possible variation in CORT concentrations or color due to specimen age or geographic location (Doucet and Hill 2009), only specimens collected in Florida during May and June 1964–1966 were used. Because the USNM would not allow removal of red epaulet feathers for destructive sampling, we plucked black contour feathers from the upper dorsal side of the specimen. This species undergoes a complete annual molt, and thus feathers from these two areas grew during the same approximately 8-week period from late July to late September (Yasukawa and Searcy 1995). Feathers were stored in individual paper envelopes at room temperature until extraction.

Reflectance spectrometry

We collected reflectance spectra, from 300 to 700 nm, of the epaulet region of the wing (upper marginal coverts) using an Ocean Optics USB4000 spectrophotometer and a PX-2 pulsed xenon light source (Ocean Optics, Dunedin, FL, USA), relative to a white standard (WS-2), using SpectraSuite software. We took four measurements of one point on the leading edge of the epaulet region where the red color was most intense. The probe (Ocean Optics R600-7-SR/125F) was mounted in a light-excluding aluminum block (Ocean Optics RPH-1) at a 45° angle, 1 cm from the plumage surface; the block was draped

in black fabric and was lifted and replaced between measurements.

Color variables

We calculated brightness, chroma, and hue between 300 and 700 nm. Brightness is a measure of the total amount of light, or radiance, reflected from a surface across all wavelengths. We calculated mean brightness as the sum of the reflectance from 300 to 700 nm divided by the number of wavelength intervals used from 300 to 700 nm. We also calculated brightness in the red region of wavelengths as the sum of reflectance 600–700 nm divided by the number of wavelength intervals in this range. Chroma is a measure of the pureness of a color, or measure of the radiance of one part of the spectrum as compared to the whole. We calculated the chroma of the red spectrum as the sum of the reflectance from 600 to 700 nm divided by the sum of the total reflectance from 300 to 700 nm (Montgomerie 2006). Hue represents the spectral location of the color; because the red reflectance peaks in our samples were broad, we quantified hue with a metric that was not dependent on identification of the exact wavelength of maximum reflectance (see Saks et al. 2003; Jouventin et al. 2007; and Barbosa et al. 2012 for a similar approach): $\text{Hue} = \text{Arc tan}\left\{\frac{(R_y - R_b)/R_t}{[(R_r - R_g)/R_t]}\right\}$ where R_y represents the sum of the reflectance from 550 to 625 nm, R_b is the sum of the reflectance from 400 to 475 nm, R_r is equal to the sum of the reflectance from 625 to 700 nm, R_g is the sum of the reflectance from 475 to 550 nm, and R_t is the sum of the reflectance from 300 to 700 nm. All measures of coloration were repeatable within measures of the same individual (mean brightness: $r=0.864$, $F_{65, 198}=26.48$, $p<0.0001$; red brightness: $r=0.869$, $F_{65, 198}=27.540$, $p<0.0001$; red chroma: $r=0.784$, $F_{65, 198}=15.488$, $p<0.0001$; hue: $r=0.915$, $F_{65, 198}=43.86$, $p<0.0001$); average mean brightness, red brightness, red chroma, and hue for each individual were used in statistical analyses.

CORT extraction and analysis

Feathers were brought to Bucknell University, Lewisburg, PA, USA for extraction. Extraction followed the methods of Lattin et al. (2011), which follow those of Bortolotti et al. (2008) with some modifications, described here. The calamus was removed from the feather prior to measuring vane length. The remaining feather was then minced into pieces $<5 \text{ mm}^2$ and weighed. Three to five feathers from a single bird were combined in order to reach a sample mass of approximately 11.6 mg. Because feather CORT concentrations can be affected by sample mass, especially when samples are very small (Lattin et al. 2011), the mass of all samples was standardized to within 0.2 mg (1–2 % of sample mass).

Following mincing, 7 ml of methanol was added to each extraction tube and tubes were placed in a sonicating water bath for 30 min at room temperature. After sonication, samples were placed in a shaking 50 °C water bath overnight. Following shaking, feather pieces were removed from the methanol solution using vacuum filtration and a glass filter funnel. The sample tube, feather pieces, and filter apparatus were washed twice with 2.5 ml methanol, with washes added to the methanol extract. The methanol extract was dried under air in a 50 °C water bath and stored dry at –20 °C until shipment to Tufts University, Medford, MA, USA for analysis.

At Tufts, extracts were reconstituted in PBSG buffer (pH 5.7) and run in duplicate through a standard radioimmunoassay (RIA) as described by Wingfield et al. (1992), using an anti-CORT antibody (Sigma-Aldrich C8784, St. Louis, MO, USA, lot 57K4791). Previous work with feather CORT has shown that feathers contain large amounts of metabolized CORT, in addition to unmodified CORT (Bortolotti et al. 2008; Koren et al. 2012). Despite the presence of CORT metabolites in the feather, Bortolotti et al. (2008) found identical amounts of CORT in the total fraction and the secreted fraction of their assay, suggesting that the antibody used in that study and in the present study (C8784 Sigma-Aldrich, St. Louis, Missouri, USA) is reacting only with unmodified CORT. Reactivity and results may be different with different antibodies (Lattin et al. 2011).

Assay validation

Because we were appropriately limited in the extent to which we could destructively sample the Smithsonian Institution's specimens, assay validation was conducted with feathers from whole, frozen red-winged blackbird specimens ($n=12$ birds) donated to Bucknell University by the U.S. Department of Agriculture's National Wildlife Research Center. We used these feathers for two purposes: to determine if black and red feather samples contain the same amount of CORT and to gauge repeatability across samples from the same specimen.

In order to determine variability in CORT measurements due to extraction and the assay itself, we created a homogeneous mixture of pooled red-winged blackbird feathers ground to dust using a ball mill (Kleco model 4200, Visalia, CA, USA) following Lattin et al. (2011). These pooled feather samples were used as standards in the RIA.

To assess the variation in CORT across different feather samples from the same bird, we extracted and measured CORT from two different black contour feather samples (each consisting of three to five feathers) from the same bird ($n=12$ birds). These black feather sample extracts were then compared to a sample of red epaulet feathers (consisting of eight to 12 feathers) from the same specimen to test whether CORT concentrations are consistent across feather types and colors.

Statistical analyses

To control for effects of social hierarchy, only ASY birds with one mate ($n=42$) were used in the analyses. Outlying individuals with discontinuously high CORT ($n=4$) were subsequently removed, yielding a final sample size of 38; all four outliers exceeded the third quartile by more than 150 % of the interquartile range. They also were >2 SD away from the mean and >1 SD away from the closest non-outlier value. We calculated CORT as both a function of total length of the group of feathers in the sample (in picograms CORT per millimeter feather) and total sample mass (in picograms CORT per milligram feather) and found that the two measures were highly correlated (Pearson $r=0.984$, $p<0.001$). Bortolotti et al. (2008) suggest that measuring CORT as a function of feather length is more relevant due to the way a feather grows; thus, only CORT as a function of feather length is reported here. Because our alternate mechanisms for a CORT–color relationship differ in their direction of causality, we performed Spearman's rank correlations to test for relationships between CORT concentration and mean brightness, red brightness, red chroma, and hue.

We also tested for correlations between CORT and body mass and testis size, as ascertained from museum tag data, in case a relationship with these variables (e.g., Dunlap and Schall 1995; Harding et al. 2011) could increase our power to test for a connection between CORT and epaulet color. Specifically, we performed Spearman's rank correlations to test for relationships between CORT concentration and testis volume (estimated using the formula for a regular ellipsoid, $\text{vol} = \pi \times \text{width}^2 \times \text{length}$; Bercovitch 1996), body mass (in gram), and relative testis size (using residuals from a regression of testis volume vs. body mass). Testis volume and body mass were positively correlated ($r=0.492$, $n=42$, $p=0.0013$), but testis volume, body mass, and relative testis size were not rank-correlated with CORT concentration (all $p>0.28$) and thus were excluded from subsequent analyses.

Analyses were performed using Minitab statistical software, v. 16.1.0 (State College, PA, USA), and JMP v. 8. 0. 2 (SAS Institute, Inc.; Cary, NC). Means \pm SE are reported here.

Results

Assay validation

Based on repeated analysis of the pooled feather standard, intra-assay variation was 4.1 % and inter-assay variation was 6.5 %. No significant differences in CORT were found between samples of black contour feathers taken from the same birds (paired t test, $p=0.717$), yet sample CORT concentrations were not significantly correlated ($r=0.330$, $F_{11, 12}=1.984$, $p=0.127$). Likewise, black feathers and red feathers

from the same birds did not differ in their CORT concentration (paired t test, $p=0.717$), yet, again, measures from the same individual were not significantly correlated ($r=0.297$, $F_{11, 12}=1.845$, $p=0.154$). In a linear random effects model using REML to estimate variance components for log-transformed CORT concentration, differences between birds accounted for 14.4 % of variance in CORT measurements, while differences between red and black feathers accounted for 0 % of the variance in CORT measurements.

CORT and color

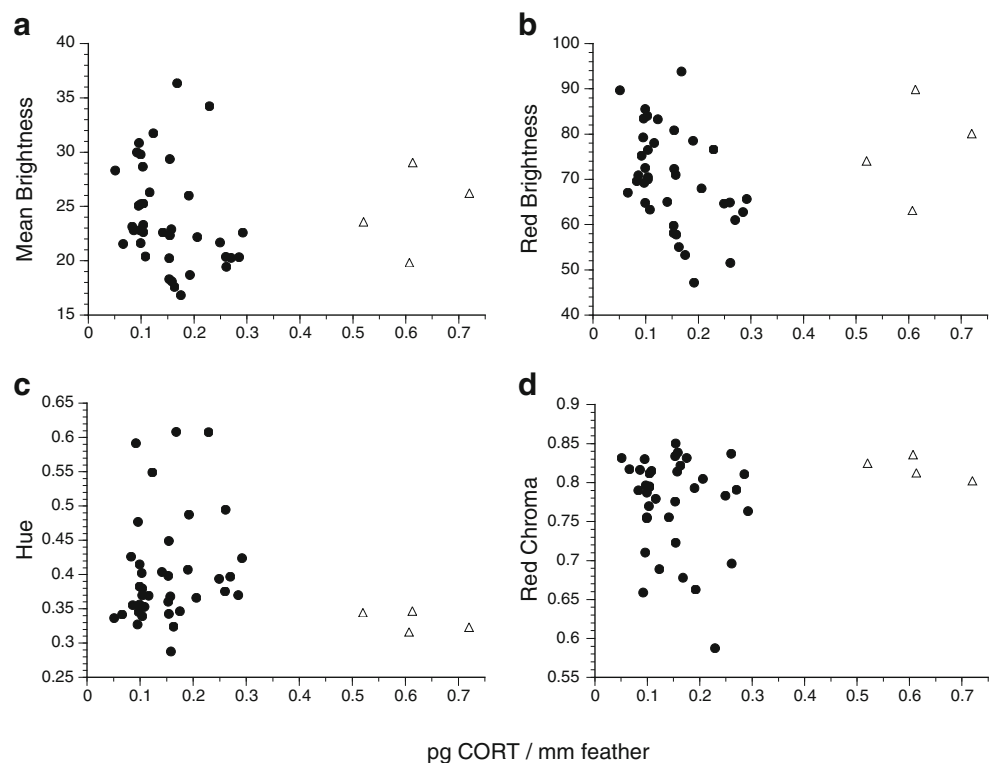
Red-winged blackbird feathers contained a mean of 0.151 ± 0.01 pg CORT/mm feather ($SD=0.07$) (including outlier feathers increased the mean to 0.195 ± 0.02 pg CORT/mm feather ($SD=0.15$)). Epaulets had an overall mean brightness score of 24.01 ± 0.71 ($SD=4.62$), a mean red brightness score of 70.65 ± 1.67 ($SD=10.85$), a mean red chroma score of 0.779 ± 0.01 ($SD=0.06$), and a mean hue of 0.40 ± 0.01 ($SD=0.08$). Considering just the relationships among the measures of color, there were significant correlations between total brightness and red brightness ($r=0.809$, $n=42$, $p<0.0001$), total brightness and red chroma ($r=-0.588$, $n=42$, $p<0.0001$), total brightness and hue ($r=0.616$, $n=42$, $p<0.0001$), and hue and red chroma ($r=-0.932$, $n=42$, $p<0.0001$), but not between red chroma and red brightness ($r=-0.174$, $n=42$, $p=0.270$) or between hue and red brightness ($r=0.233$, $n=42$, $p=0.138$).

CORT concentration (in picograms CORT per millimeter) showed significant negative relationships with mean brightness (Spearman's $\rho=-0.411$, $n=38$, $p=0.010$) and red brightness (Spearman's $\rho=-0.463$, $n=38$, $p=0.003$) (Fig. 1), though these relationships were not significant when the four CORT outliers were included (CORT and total brightness: $n=42$, $\rho=-0.284$, $p=0.068$; CORT and red brightness: $n=42$, $\rho=-0.277$, $p=0.075$). CORT concentration was not correlated with red chroma (Spearman's $\rho=-0.069$, $n=38$, $p=0.139$) or hue (Spearman's $\rho=0.245$, $n=38$, $p=0.139$).

Discussion

Aspects of the red epaulet coloration of male red-winged blackbirds correlated with feather CORT concentration. Specifically, males with lower CORT had epaulets that reflected more light—both averaged over the whole spectrum and only in the red part of the spectrum—whereas the hue and saturation of the red pigment did not show systematic variation with feather CORT. This pattern is most consistent with elevated CORT having a negative effect on feather microstructure: because the keratin-based structure reflects light through the carotenoid pigments, a less effective microstructure would result in lower achromatic reflectance (Shawkey and Hill 2005), which is captured in our measures of epaulet brightness. The role of this structural matrix in the production of carotenoid-based coloration is a

Fig. 1 Scatter plots of the relationship between feather CORT (in picograms CORT per millimeter feather), and **a** mean brightness, **b** red brightness, **c** hue, and **d** red chroma of male epaulet regions. Circles represent the average of four measurements of a single individual ($n=38$), while points indicated by a triangle ($n=4$) represent outliers with discontinuously high CORT that were not included in statistical analyses. CORT concentrations correlated with mean brightness and red brightness of male red-winged blackbird epaulets but not with red chroma or hue



little-studied topic, and research thus far has happened to focus only on yellow plumage (Matrková and Remes 2012); it is possible that the role of the structural matrix is different for yellow carotenoids vs. red carotenoids. Despite this caveat, it is certainly intriguing that a recent experiment with great tits showed that nests manipulated to have fewer chicks exhibited both higher nestling growth rates (i.e., better condition) and greater total brightness in carotenoid-based coloration (Jacot et al. 2010).

In contrast, chromatic aspects of reflectance spectra (i.e., variation in hue or chroma) are more likely to be driven by changes in the amount of carotenoids in the feathers (Jacot et al. 2010). As carotenoid concentrations increase, this should cause the absorbance of more of the blue and violet portions of the broad-spectrum light being reflected by the keratin matrix, with a resulting shift of the viewable light towards a redder hue and greater red chroma (Jacot et al. 2010). Because we did not observe a relationship between CORT and either hue or chroma, it seems likely that CORT was impacting only feather structure and not feather carotenoid content (but see Jouventin et al. 2007). This mechanism is consistent with any of the three scenarios outlined previously: that CORT during molt affects color in newly formed feathers, that color during the breeding season drives social challenges in a way that affects both the CORT and color of the epaulets formed at the subsequent molt, or that diet or energy balance at the time of molt affects both CORT and color.

Understanding the evolutionary consequences of a correlation between feather CORT and particular aspects of epaulet color would be aided by a more nuanced understanding of sexual selection on epaulets. Unfortunately, there are no experimental studies of sexual selection that have manipulated epaulet coloration in a way that exactly matches the brightness variation that we found to correlate with feather CORT. For example, a recent experiment by Yasukawa and colleagues (2009) manipulated epaulet color to be duller, using a blackening dye; the manipulation not only reduced the total brightness and red brightness of the epaulets but also affected hue and chroma. Females showed a trend towards associating less with males whose epaulet color was manipulated in a way that, in part, made them darker red (i.e., a lower brightness score)—which in our study was correlated with high CORT. Although the specific effects of color on territory acquisition and mate attraction are quite varied across studies of this species (Westneat 2006; Yasukawa et al. 2010), multiple experiments have shown that epaulets play some role in sexual selection in red-winged blackbirds (Peek 1972; Smith 1972; Morris 1975; Røskaft and Rohwer 1987; Metz and Weatherhead 1992), and thus alterations of epaulet color due to changes in CORT concentrations at least have the potential to be of importance in male reproductive success.

In considering the value of future studies of color and CORT—or of other aspects of physiology using museum

specimens—we now discuss two methodological issues. One is the validation data from the CORT assay. We found no evidence for significant differences in CORT between red feathers and black feathers, but two different analyses suggested that variation in CORT samples within birds was high relative to variation between birds. This may be due to a combination of methodological issues (extraction efficiency, intra-assay variation, inter-assay variation) and real differences in CORT between different feather samples from the same bird. Because the likelihood of sampling two feathers that were grown at the exact same time is very small (unless measuring feathers regrown after removal), we would expect to see some differences in the CORT signature between feathers. It is also possible that CORT is deposited differentially into feathers that are or are not used for sexual signaling or that feathers of different colors are also different in their tendency to take up hormones (as has been shown with human hair; Rivier 2000); this is not consistent with our failure to find a difference in CORT between red and black feathers, but that analysis was based on a small number of birds. Because the measurement of feather CORT is still a new approach, there is simply a great deal that is not yet known.

Most previous work with this assay used a single, large feather per sample, which cannot account for variation in CORT between feathers (Bortolotti et al. 2008, 2009a; Harms et al. 2010; Mougeot et al. 2010; but see Latin et al. 2011). The use of three to 12 small feathers in each of our samples could have served to reduce error variation in CORT concentrations due to differences in the timing of feather growth, but samples still were not highly correlated, indicating that even samples containing several feathers may not be a consistent reflection of CORT concentrations. Given this finding, studies using only one or a few feathers per sample should be regarded with caution. Nonetheless, the detection of significant relationships between feather CORT and other variables, both in the present study and others, suggests that such relationships might be very strong—i.e., large enough statistical effects to be detected despite between-feather variation in CORT. Further research is required to understand how much CORT varies between feathers and how to control for this variability when sampling feathers.

The second methodological question concerns a possible negative effect of CORT on feather integrity. Among birds, circulating CORT concentrations are generally elevated during the breeding season and are usually at their lowest during the postbreeding molt when feathers are replaced (Romero 2002; Romero and Wingfield 1999). This is important because one of the effects of glucocorticoids is to stimulate gluconeogenesis through protein catabolism (Sapolsky et al. 2000). Thus, downregulation of CORT release during molt may act to protect the growth or structural integrity of the new feather (Romero et al. 2005); consistent with this idea, flight

feathers grown while CORT concentrations are experimentally elevated can show reduced mass, reduced barbicular hooking strength, and decreased inter-barb distance, which can potentially weaken the feather (DesRochers et al. 2009). If elevated CORT during molt was to reduce the durability of the feather, an important question is whether this in turn could affect the persistence of color over time in a live bird or perhaps even more so in a museum specimen.

Although there are unresolved questions about CORT in feathers, it clearly has the potential to be an important tool and it may broaden the types of questions that can be addressed with museum specimens. Our study's primary finding that feather CORT correlates with brightness but not hue or chroma is consistent with an effect of CORT on feather microstructure, with elevated CORT leading to lower reflectance of white light from the keratin matrix surrounding the carotenoid pigments. Further studies should build on recent experimental evidence for the effects of CORT on structural vs. pigment components of carotenoid-based coloration (Jacot et al. 2010; Matrková and Remes 2012) and should explore the way that color and feather structure change over long time periods.

Acknowledgments We thank KB Boomer for consultations regarding the statistical analyses and George Linz at the U.S. Department of Agriculture Wildlife Service, National Wildlife Research Center, Bismarck, ND for providing the whole red-winged blackbird specimens, as well as James Dean, Eileen Spade, Morgan Gilmour, and Jameson Clarke for the logistical support. We thank DeeAnn Reeder, Mark Haussmann, and three anonymous reviewers for providing helpful feedback on the drafts of the manuscript. Thank you to the National Museum of Natural History for access to the specimens. Funding was provided by the Smithsonian Institution to EA Kennedy, by the Bucknell University Biology Department and Herbert L. Spencer endowed funds to DC Dearborn and the National Science Foundation (USA) IOS-1048529 to LM Romero.

Declaration of compliance The authors declare that the experiments herein comply with the current laws of the USA.

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

References

- Andersson M (1994) Sexual selection. Princeton University Press, Princeton
- Barbosa AM, Palacios J, Valera F, Martinez A (2012) Geographic variation in beak colouration in gentoo penguins *Pygoscelis papua*. *Polar Biol* 35:725–731
- Beletsky L (1996) The red-winged blackbird. Academic, London
- Bendich A, Olson JA (1989) Biological actions of carotenoids. *FASEB J* 3:1927–1932
- Bercovitch FB (1996) Testicular function and scrotal coloration in patas monkeys. *J Zool Lond* 239:93–100
- Berger S, Martin Ii LB, Wikelski M, Romero LM, Kalko EKV, Vitousek MN, Rödl T (2005) Corticosterone suppresses immune activity in territorial Galápagos marine iguanas during reproduction. *Horm Behav* 47:419–429
- Bonier F, Moore IT, Martin PR, Robertson RJ (2009a) The relationship between fitness and baseline glucocorticoids in a passerine bird. *Gen Comp Endocr* 163:208–213
- Bonier F, Martin PR, Moore IT, Wingfield JC (2009b) Do baseline glucocorticoids predict fitness? *Trends Ecol Evol* 24:634–642
- Bortolotti GR, Marchant TA, Blas J, German T (2008) Corticosterone in feathers is a long-term, integrated measure of avian stress physiology. *Funct Ecol* 22:494–500
- Bortolotti GR, Marchant T, Blas J, Cabezas S (2009a) Tracking stress: localization, deposition and stability of corticosterone in feathers. *J Exp Biol* 212:1477–1482
- Bortolotti GR, Mougeot F, Martinez-Padilla J, Webster LMI, Piernney SB (2009b) Physiological stress mediates the honesty of social signals. *PLoS ONE* 4:e4983
- Chew BP, Park JS (2004) Carotenoid action on the immune response. *J Nutr* 134:257S–261S
- Costantini D, Marasco V, Møller AP (2011) A meta-analysis of glucocorticoids as modulators of oxidative stress in vertebrates. *J Comp Physiol B* 181:447–456
- DesRochers DW, Reed JM, Awerman J, Kluge JA, Wilkinson J, van Griethuisen LI, Aman J, Romero LM (2009) Exogenous and endogenous corticosterone alter feather quality. *Comp Biochem Physiol A* 152:46–52
- Doucet SM, Hill GE (2009) Do museum specimens accurately represent wild birds? A case study of carotenoid, melanin, and structural colours in long-tailed manakins *Chiroxiphia linearis*. *J Avian Biol* 40:146–156
- Dunlap KD, Schall JJ (1995) Hormonal alterations and reproductive inhibition in male fence lizards (*Sceloporus occidentalis*) infected with the malarial parasite *Plasmodium mexicanum*. *Physiol Zool* 68:608–621
- Goodwin TE, Broederdorf LJ, Burkert BA, Hirwa IH, Mark DB, Waldrip ZJ, Kopper RA, Sutherland MV, Freeman EW, Hollister-Smith JA, Schulte BA (2012) Chemical signals of elephant musth: temporal aspects of microbially-mediated modifications. *J Chem Ecol* 38:81–87
- Hansen AJ, Rohwer S (1986) Coverable badges and resource defense in birds. *Anim Behav* 34:69–76
- Harding AMA, Welcker J, Steen H, Hamer KC, Kitaysky AS, Fort J, Talbot SL, Cornick LA, Karnovsky NJ, Gabrielsen GW, Grémillet D (2011) Adverse foraging conditions may impact body mass and survival of a high Arctic seabird. *Oecologia* 167:49–59
- Harms NJ, Fairhurst GD, Bortolotti GR, Smits JEG (2010) Variation in immune function, body condition, and feather corticosterone in nestling tree swallows (*Tachycineta bicolor*) on reclaimed wetlands in the Athabasca oil sands, Alberta, Canada. *Environ Pollut* 158:841–848
- Husak JF, Moore IT (2008) Stress hormones and mate choice. *Trends Ecol Evol* 23:532–534
- Jacot A, Romero-Diaz C, Tschirren B, Richner H, Fitze PS (2010) Dissecting carotenoid from structural components of carotenoid-based coloration: a field experiment with great tits (*Parus major*). *Am Nat* 176:55–62
- Jouventin P, McGraw KJ, Morel M, Celerier A (2007) Dietary carotenoid supplementation affects orange beak but not foot coloration in gentoo penguins *Pygoscelis papua*. *Waterbirds* 30:573–578
- Kitaysky AS, Kitaiskaia EV, Wingfield JC, Piatt JF (2001) Dietary restriction causes chronic elevation of corticosterone and enhances stress response in red-legged kittiwake chicks. *J Comp Physiol B* 171:701–709
- Kodric-Brown A (1989) Dietary carotenoids and male mating success in the guppy: an environmental component to female choice. *Behav Ecol Sociobiol* 25:393–401

- Koren L, Nakagawa S, Burke T, Soma KK, Wynne-Edwards KE, Geffen E (2012) Non-breeding feather concentrations of testosterone, corticosterone and cortisol are associated with subsequent survival in wild house sparrows. *Proc R Soc Lond B* 279:1560–1566
- Krinsky NI (2001) Carotenoids as antioxidants. *Nutrition* 17:815–817
- Lattin CR, Reed LM, DesRochers DW, Romero LM (2011) Elevated corticosterone in feathers correlates with corticosterone-induced decreased feather quality: a validation study. *J Avian Biol* 42:247–252
- Linz GM, Bolin SB, Cassel JF (1983) Postnuptial and postjuvinal molts of red-winged blackbirds in Cass County, North Dakota. *Auk* 100:206–209
- Matrková J, Remes V (2012) Environmental and genetic effects on pigment-based vs. structural component of yellow feather colouration. *PLoS ONE* 7:e36640
- McGraw KJ (2005) The antioxidant function of many animal pigments: are there consistent health benefits of sexually selected colourants? *Anim Behav* 69:757–764
- McGraw KJ (2006) Mechanics of carotenoid-based coloration. In: Hill GE, McGraw KJ (eds) *Bird coloration: mechanisms and measurements*, vol. 1. Harvard University Press, Cambridge, pp 177–242
- McGraw KJ, Wakamatsu K, Clark AB, Yasukawa K (2004) Red-winged blackbirds *Agelaius phoeniceus* use carotenoid and melanin pigments to color their epaulets. *J Avian Biol* 35:543–550
- McGraw KJ, Hill GE, Parker RS (2005) The physiological costs of being colourful: nutritional control of carotenoid utilization in the American goldfinch, *Carduelis tristis*. *Anim Behav* 69:653–660
- Meanley B, Bond GM (1970) Molts and plumages of the red-winged blackbird with particular reference to fall migration. *Bird Band* 41:22–27
- Metz KJ, Weatherhead PJ (1992) Seeing red: uncovering coverable badges in red-winged blackbirds. *Anim Behav* 43:223–229
- Montgomerie R (2006) Analyzing color. In: Hill GE, McGraw KJ (eds) *Bird coloration: mechanisms and measurements*, vol 1. Harvard University Press, Cambridge, pp 90–147
- Morris L (1975) Effect of blackened epaulets on the territorial behavior and breeding success of male red-winged blackbirds, *Agelaius phoeniceus*. *Ohio J Sci* 75:168–176
- Mougeot F, Martinez-Padilla J, Bortolotti GR, Webster MI (2010) Physiological stress links parasites to carotenoid-based colour signals. *J Evol Biol* 23:643–650
- Nero RW (1955) A behavior study of the red-winged blackbird. *Wilson Bull* 68:5–35
- Peek FW (1972) An experimental study of the territorial function of vocal and visual display in the male red-winged blackbird (*Agelaius phoeniceus*). *Anim Behav* 20:112–118
- Rivier L (2000) Is there a place for hair analysis in doping controls? *Forensic Sci Int* 107:309–323
- Romero LM (2002) Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates. *Gen Comp Endocrinol* 128:1–24
- Romero LM, Reed JM (2005) Collecting baseline corticosterone samples in the field: is under 3 min good enough? *Comp Biochem Physiol A* 140:73–79
- Romero LM, Wingfield JC (1999) Alterations in hypothalamic–pituitary–adrenal function associated with captivity in Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*). *Comp Biochem Physiol B* 122:13–20
- Romero LM, Strohlic D, Wingfield JC (2005) Corticosterone inhibits feather growth: potential mechanism explaining seasonal down regulation of corticosterone during molt. *Comp Biochem Physiol A* 142:65–73
- Røskaft E, Rohwer S (1987) An experimental study of the function of the red epaulettes and the black body colour of male red-winged blackbirds. *Anim Behav* 35:1070–1077
- Saks L, Ots I, Hõrak P (2003) Carotenoid-based plumage coloration of male greenfinches reflects health and immunocompetence. *Oecologia* 134:301–307
- Sapolsky RM, Romero LM, Munck AU (2000) How do glucocorticoids influence the stress response? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocr Rev* 21:55–89
- Schmidt KL, Furlonger AA, Lapierre JM, MacDougall-Shackleton EA, MacDougall-Shackleton SA (2012) Regulation of the HPA axis is related to song complexity and measures of phenotypic quality in song sparrows. *Horm Behav* 61:652–659
- Searcy WA, Yasukawa K (1983) Sexual selection and red-winged blackbirds. *Am Sci* 71:166–174
- Shawkey MD, Hill GE (2005) Carotenoids need structural colours to shine. *Biol Lett* 1:121–124
- Smith DG (1972) The role of the epaulets in the red-winged blackbird, (*Agelaius phoeniceus*) social system. *Behaviour* 41:251–258
- Strohlic DE, Romero LM (2008) The effects of chronic psychological and physical stress on feather replacement in European starlings (*Sturnus vulgaris*). *Comp Biochem Physiol A* 149:68–79
- von Schantz T, Bensch S, Grahn M, Hasselquist D, Wittzell H (1999) Good genes, oxidative stress and condition-dependent sexual signals. *Proc R Soc Lond B* 266:1–12
- Westneat DF (2006) No evidence of current sexual selection on sexually dimorphic traits in a bird with high variance in mating success. *Am Nat* 167:E171–E189
- Wingfield JC, Vleck CM, Moore MC (1992) Seasonal changes of the adrenocortical response to stress in birds of the Sonoran Desert. *J Exp Biol* 264:419–428
- Yasukawa K, Searcy WA (1995) Red-winged blackbird (*Agelaius phoeniceus*). In: Poole A, Gill F (eds) *The birds of North America*, no. 184. The Academy of Natural Sciences, Philadelphia
- Yasukawa K, Butler LK, Enstrom DA (2009) Intersexual and intra-sexual consequences of epaulet colour in male red-winged blackbirds: an experimental approach. *Anim Behav* 77:531–540
- Yasukawa K, Enstrom DA, Parker PG, Jones TC (2010) Male red-winged blackbirds with experimentally dulled epaulets experience no disadvantage in sexual selection. *J Field Ornithol* 81:31–41
- Zahavi A (1975) Mate selection—a selection for a handicap. *J Theor Biol* 53:205–214