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Color variation in museum specimens of birds: effects of stress, pigmentation, and duration of storage

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COLOR VARIATION IN MUSEUM SPECIMENS OF BIRDS: EFFECTS OF STRESS, PIGMENTATION, AND DURATION OF STORAGE

by

Eileen A. Kennedy

A Thesis

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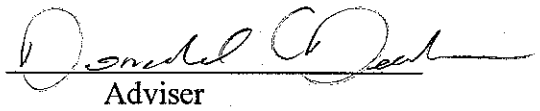

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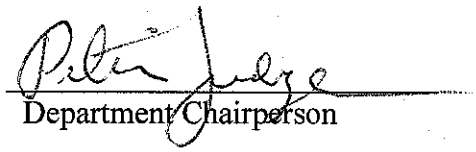

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ABSTRACT

Animal coloration often serves as a signal to others that may communicate traits about the individual such as toxicity, status, or quality. Colorful ornaments in many animals are often honest signals of quality assessed by mates, and different colors may be produced by different biochemical pigments. Investigations of the mechanisms responsible for variation in color expression among birds are best when including a geographically and temporally broad sample. In order to obtain such a sample, studies such as this often use museum specimens; however, in order for museum specimens to serve as an accurate replacement, they must accurately represent living birds, or we must understand the ways in which they differ. In this thesis, I investigated the link between feather corticosterone, a hormone secreted in response to stress, and carotenoid-based coloration in the Red-winged Blackbird (*Agelaius phoeniceus)* in order to explore a mechanistic link between physiological state and color expression. Male Red-winged Blackbirds with lower feather corticosterone had significantly brighter red epaulets than birds with higher feather corticosterone, while I found no significant changes in red chroma. I also performed a methodological comparison of color change in museum specimens among different pigment types (carotenoid and psittacofulvin) and pigments in different locations in the body (feather and bill carotenoids) in order to quantify color change over time. Carotenoids and psittacofulvins showed significant reductions in red brightness and chroma over time in the collection, and carotenoid color changed significantly faster than psittacofulvin color. Both bill and feather carotenoids showed

significant reductions in red brightness and red chroma over time, but change of both red chroma and red brightness occurred at a similar rate in feathers and bills. In order to use museum specimens of ecological research on bird coloration specimen age must be accounted for before the data can be used; however, once this is accomplished, museum-based color data may be used to draw conclusions about wild populations.

CHAPTER 1

Feather corticosterone predicts color of carotenoid-based feather ornamentation in male Red-winged Blackbirds (*Agelaius phoeniceus*)

ABSTRACT

Colorful ornaments are often honest signals of quality assessed by mates, and variation in ornament expression may impact reproductive success. Carotenoid-based plumage ornaments in birds can be honest signals that may reflect the foraging capabilities or health status of the bearer, and the degree of color expression may be mediated by physiological stress. In Red-winged Blackbirds, *Agelaius phoeniceus*, carotenoid-based epaulets play a role in male-male social interactions, territory maintenance, and female choice. Here I assess whether physiological stress measured via feather corticosterone predicts color expression of male epaulets using museum specimens. Using reflectance spectrophotometry, I obtained reflectance spectra of adult male epaulets and plucked adjacent feathers for corticosterone analysis via radioimmunoassay. I controlled for differences in social influences, specimen age, and geography by selecting only males with one mate (as indicated by the specimen tag) and only birds collected in Florida during a three-year window of time. Feather corticosterone measured as a function of feather length was a predictor of red brightness but not red chroma of the epaulet. Males

with epaulets receiving a high red brightness score had significantly lower corticosterone than males with epaulets receiving a low red brightness score; however, a high brightness score represents a color that is lighter and contains more white, so males with low feather corticosterone had lighter epaulets. This is the first study to show a link between corticosterone and carotenoid-based plumage coloration. These results are inconsistent with experimental studies on the role of corticosterone in skin-based color expression; however, other mechanisms, which are discussed here, may be affecting color expression and corticosterone levels in this species.

INTRODUCTION

Colorful ornaments have the potential to reveal information about the physiological status of individuals. Many animals exhibit red, orange, or yellow coloration that is created by carotenoids (Endler 1980, Kodric-Brown 1989, McGraw 2006). The expression of carotenoids is often condition-dependent (Olsen & Owens 1998) and carotenoid-based ornaments may be honest signals of quality (Zahavi 1975) because of the trade-offs between the use of carotenoids as pigments, as antioxidants (Bendich & Olsen 1989, Lozano 1994, Krinsky 2001, McGraw 2005), and as an important component in the immune response (Bendich 1989, McGraw & Ardia 2003, Alonso-Alvarez et al. 2004).

Carotenoids cannot be synthesized *de novo* by any animal (Goodwin 1984) and thus may be a limiting resource for overall health and fitness. Indeed, immune-challenged animals show reduced coloration (Faivre et al. 2003, Mougeot et al. 2009, Toomey et al. 2010b). Furthermore, carotenoid-restricted birds show reductions in coloration (Blount et al. 2003, McGraw et al. 2005, McGraw 2006) and circulating carotenoids (McGraw et al. 2005), while carotenoid supplementation is associated with increased coloration (Jouventin et al. 2007). Because of carotenoids' involvement in the immune system and their antioxidant properties, carotenoid-based coloration should be selected for because it might honestly signal an individual's health (von Schantz et al. 1999). While carotenoid pigments in soft tissues (such as in skin, bills, or eyes) may be available for re-uptake during an immune or oxidative challenge, it is important to note that carotenoids deposited in subsequently inert tissue such as scales or feathers cannot be

mobilized for future use; thus only healthy individuals, or those with very high amounts of available carotenoids, can afford to deposit them in feathers as an investment in showiness.

Conditions involving the activation of the immune response, and thus the use of antioxidants such as carotenoids, are often also tied to the 'stress' response. Under environmental or social challenges the hypothalamic-pituitary-adrenal (HPA) axis is stimulated, resulting in the production and release of glucocorticoid hormones from the adrenal cortex. Glucocorticoids are metabolic hormones and act to adjust the way energy is used within the body, in order to cope with and recover from stressors. In conjunction with focusing energy on tasks essential for survival, glucocorticoid hormones suppress the immune system, growth, reproduction, and digestion (for a complete review of the actions of glucocorticoids, see Sapolsky et al. 2000). When secreted over short time frames, the actions of glucocorticoids are adaptive—focusing energy on tasks essential for immediate survival and restoring homeostasis (Romero 2002). However, when the stress response is repeated or extended over long periods, the effects are costly and may be detrimental to survival.

In the context of mate choice, females might benefit from choosing mates that are either good at coping with stressors or good at avoiding stressful situations. Given two males under identical environmental conditions, it remains unclear as to whether a female should choose the male with high corticosterone because this individual is responding properly to stressors and thus likely to overcome them, or whether to choose the male with low corticosterone because this individual is avoiding the reallocation of energy

away from normal activities (Husak & Moore 2008). It has generally been assumed that high baseline corticosterone levels indicate individuals in poorer condition with reduced fitness, and this theory is referred to as the Cort-Fitness Hypothesis; however, this hypothesis has not been well supported in the literature (reviewed by Bonier et al. 2009). For example, reproduction is an energetically demanding time for an individual and may increase corticosterone levels to reallocate energy use, which may result in an increase in fitness. Under the Cort-Fitness Hypothesis females are predicted to prefer males with lower corticosterone in order to obtain the best mate, and there is some support for this in the literature. For example, Roberts et al. (2007) found that female zebra finches (*Taeniopygia guttata)* preferred males bred from low corticosterone (the primary glucocorticoid hormone in birds) lines over those bred from high corticosterone lines. However, because of the benefits to corticosterone production, this result may not be applicable to other systems.

If females actually do benefit from choosing males based on corticosterone levels there must be a phenotypic male trait that honestly reflects allostatic load. In birds, this signal could be conveyed by colorful plumage ornaments. There is evidence that corticosterone secretion has negative effects on immune function (Buchanan 2000) and that immune activation negatively alters carotenoid availability and coloration (Toomey et al. 2010b), yet, to date, few studies have looked for a link between corticosterone and coloration. A study in the common lizard, *Lacerta vivipara,* found that corticosterone mediated carotenoid-based skin coloration, but only when food was limited (Cote et al. 2010), suggesting that the relationship between corticosterone and condition-based

coloration may be more complex than previously surmised (further supported by Fitze et al. 2009). A recent study by Mougeot et al. (2009) showed that internal parasite infection among free-living red grouse (*Lagopus lagopus scoticus*) increased corticosterone levels, and reduced carotenoid-based skin pigmentation in the comb, but did not directly test the effects of corticosterone alone on carotenoid pigmentation. This is an important first step in understanding how condition-dependent carotenoid-based signals may be regulated by corticosterone. However, many more birds use plumage-based signals that might communicate quality. Regarding melanin-based plumage signals, experimental work by Roulin et al. (2008) demonstrated that owls implanted with corticosterone grew feathers with less melanic coloration, a condition-dependent trait that is assessed by both males and females when making mating decisions (Roulin & Altwegg 2007). While a link has been established in melanin coloration, to date, no studies have shown a correlation between corticosterone levels and plumage-based carotenoid color expression.

Investigation of a link between corticosterone and ornamental coloration should use physiological measures that are matched in the time span that they sample, but this is a difficult task. Corticosterone is most often measured in blood samples, but these only provide a narrow snapshot of the endocrine status of the animal, which can change on a minute-by-minute basis (Romero & Reed 2005). Because feathers are grown over several days or weeks, comparing such a temporally narrow measure of corticosterone levels 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 found measurable amounts of corticosterone deposited in feathers, providing a longer-term, integrated measure of

corticosterone that represents the amount of corticosterone produced in response to all the stressors experienced over the time the feather grew. Corticosterone, as a steroid hormone, may be stable in the feather for decades (Bortolotti et al. 2009).

Red-winged Blackbirds (*Agelaius phoeniceus*) are an abundant, sexually dichromatic passerine in North America whose mating system has been very well studied (Nero 1955, Searcy & Yasukawa 1983, Beletsky 1996). The function of the carotenoid- and melanin-based (McGraw et al. 2004) red and yellow epaulets of the males is under some debate in the literature. Epaulets have been shown to function both as an intersexual signal of male quality displayed during territorial disputes with other males (Hansen & Rohwer 1986, Roskaft & Rohwer 1987, Metz & Weatherhead 1992), and as an intrasexual signal assessed by females during precopulatory displays (Yasukawa et al. 2009), although a recent meta-analysis revealed no significant effects of color on male-male interactions, female preference, and various measures of reproductive success (Yasukawa et al. 2010). Individuals of this species undergo delayed plumage maturation whereby second year (SY) males retain the first basic plumage through their first breeding season, and do not attain basic definitive plumage until late summer after breeding. They are easily distinguished from after second year (ASY) males by their speckled appearance and by the epaulet, which is usually orange, rather than red, and mottled with black (Meanley & Bond 1970). Molt occurs yearly thereafter in late-July through August 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.

In this study, I compared feather corticosterone to carotenoid-based epaulet coloration of male ASY Red-winged Blackbirds to make a temporally relevant comparison of stress and ornamentation under the hypothesis that stress impacts the degree of carotenoid coloration. I conducted this test with museum specimens which allowed me to explore whether these techniques can be used to ask behavioral endocrinology questions about animals that are no longer living. Because glucocorticoid hormones, such as corticosterone, affect energy use throughout the body and carotenoid-based signals are costly to produce, I predicted that individuals with higher levels of feather corticosterone would express reduced coloration compared to those individuals with lower corticosterone levels.

METHODS

Museum Specimens

I collected feather samples, reflectance spectra measurements (described below), and other data (body mass (g), testis length and width (mm), number of mates) from adult male Red-winged Blackbird specimens at the Smithsonian Institution's National Museum of Natural History (USNM), Washington, DC, USA. To control for possible geographic variation in corticosterone levels and differences in color due to specimen age (Doucet & Hill 2009), only specimens collected in Florida during May and June 1964-1966 were used. Because the USNM would not allow removal of the red epaulet feathers for

sampling, I 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 & Searcy 1995). Feathers were stored in individual paper envelopes at room temperature until extraction.

The feathers from whole, frozen Red-winged Blackbird specimens donated to Bucknell by North Dakota State University were used in the validation of the assay in order to determine if black and red feather samples contain the same amount of corticosterone, and to gauge repeatability across samples from the same specimen.

Reflectance Spectrometry

I collected reflectance spectra from 300-700nm 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. I took four measurements of one point on the leading edge of the epaulet region where the red color was most intense. The probe was held at a 45-degree angle, 1cm from the plumage surface, and I lifted and replaced the probe between measurements.

Under the Munsell color system, I calculated red brightness (sum of reflectance 600-700nm divided by number of data points in this range), and red chroma (sum of the reflectance 600-700nm divided by the sum of the total reflectance 300-700nm) for each spectrum obtained (Montgomerie 2006). Both measures of coloration were repeatable

within measures of the same individual (red brightness: $r=0.869$, $F_{65, 198}=27.540$, $p<0.001$; red chroma $r=0.784$, $F_{65, 198}=15.488$, $p<0.001$) (Lessells & Boag 1987), thus average red brightness and red chroma for each individual were used in statistical analyses.

Corticosterone Extraction and Analysis

Feathers were brought to Bucknell University, Lewisburg, PA, USA for extraction. Extraction followed the methods of Lattin et al. (in review), which follows 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 <5mm long and weighed. Three to five feathers from a single bird were combined in order to reach a sample mass of approximately 11.6mg. Because corticosterone can be affected by sample mass when the sample is very small (Lattin et al. in review), the mass of all samples was standardized to within 0.2mg (1-2% of sample mass).

Following mincing, 7ml of methanol was added to each extraction tube and tubes were placed in a sonicating water bath for 30min 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 2.5ml 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 a PBS 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, Saint Louis, MO, USA, lot 57K4791).

Assay Validation

In order to determine variability in corticosterone measurements due to extraction and due to the assay itself, I created a homogenous mixture of pooled feathers ground to dust using a Ball mill (Kleco model 4200, Visalia, CA, USA) following Lattin et al. (in review). These pooled feather samples were used as standards in the RIA.

To assess the variation in corticosterone across different feather samples from the same bird, I extracted and measured corticosterone from two different black contour feather samples (each 3-5 feathers) from the same bird (n=12 birds). These black feather sample extracts were then compared to a sample of red epaulet feathers (8-12 feathers) from the same specimen to test whether corticosterone levels are consistent across feather types and colors. Red-winged Blackbirds undergo a complete yearly molt so the red epaulet feathers and the black epaulet feathers were likely grown within the same 6-8 week period from late July to late September (Nero 1984).

Statistical Analyses

To control for effects of social hierarchy, only birds with one mate (n=38) were used in the analyses, after outliers with discontinuously high corticosterone (n=4) were removed. I calculated corticosterone (CORT) measurements as both a function of feather length (pg CORT/mm feather) and sample mass (pg CORT/mg feather), and found that the two measures were highly correlated (Pearson $r=0.984$, $p<0.001$). Bortolotti (2008) suggests that measuring corticosterone as a function of feather length is more relevant due to the way a feather grows, thus only corticosterone as a function of feather length is reported here. Corticosterone measurements were natural log transformed to obtain a normal distribution.

Red brightness and red chroma were fitted in separate stepwise regression models with corticosterone (ln(pg CORT/mm feather)), individual body mass (g), specimen age (years), and testis volume (estimated using the formula for a regular ellipsoid, vol= $4/3\pi ab^2$, where *a* and *b* are the testis length and width) (Bercovitch 1996), using Minitab statistical software, v. 16.1.0 (State College, Pennsylvania, USA). Means ±SE are reported here.

RESULTS

Assay Validation

Based on repeated analysis of the pooled feather standard, intra-assay variation was 4.1% and the inter-assay variation was 6.5%. No significant differences in corticosterone were found between samples of black contour feathers taken from the same birds (paired t-test, p=0.717), yet samples were only moderately 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 corticosterone content (paired t-test, p=0.717), yet measures from the same individual were only moderately correlated (r=0.297, $F_{11, 12}$=1.845, p=0.154). Note that the variation between these feather samples is driven partly by the intrinsic variation within and between assays (reported above) and perhaps partly by real differences in corticosterone between different feather samples from the same bird.

Corticosterone and Color

Red-winged Blackbird feathers contained a mean of 0.1509±0.0106pg CORT/mm feather (CV=43.39). Epaulets had a mean red brightness score of 70.01± 1.75 (CV=15.38), and a mean red chroma score of 0.77505± 0.00975 (CV=7.75). Red brightness and red chroma were negatively correlated among individuals (Pearson r=-0.219, p=0.187), and testis volume and body mass were highly correlated (Pearson r=0.484, p=0.002).

Corticosterone level (ln(pg CORT/mm)) was a significantly negative predictor of epaulet red brightness (r^2=0.2102, Regression coefficient= -11.4597, SE= 3.70271, p=0.0045) (Figure 1). Testis volume (mm^3), body mass (g), and specimen age did not predict red brightness in male Red-winged Blackbird epaulets (p= 0.972, 0.559, and 0.815, respectively). Red chroma was not predicted by corticosterone, testis volume, body mass, or specimen age (all p-values > 0.2).

DISCUSSION

The red epaulet coloration of male Red-winged Blackbirds varied with feather corticosterone levels. Specifically, males with lower corticosterone levels had feathers that were brighter red than individuals with higher corticosterone levels, whereas there was no relationship between corticosterone and red chroma.

Under the Munsell color system, color characteristics are described in a three dimensional space, using three parameters. The first, hue, represents the principle color such as 'red' or 'blue. The five hues (Red, Yellow, Green, Blue, and Purple) are subdivided into steps and given a numerical value. The second parameter, brightness, defines a color based on the amount of black and white present. Very light colors that contain high amounts of white receive high brightness scores while dark colors containing high amounts of black receive low brightness scores. The final parameter, chroma, defines colors based on the amount of gray they contain, assigning high chroma

scores to pure colors, and low scores to colors with a closer resemblance to grey. Based on this system, keeping hue and chroma constant, a bird receiving a high brightness score may not appear more colorful that a bird receiving a low brightness score because the brighter bird will appear more white (Montgomerie 2006). Given this idea, Red-winged Blackbirds with higher corticosterone levels, that received low brightness scores, have, by definition, darker epaulets.

Interpretation of a link between epaulet color and feather corticosterone depends on an understanding of what exactly is being measured by the assay. Previous work with feather corticosterone has shown that feathers contain large amounts of metabolized corticosterone, in addition to unmodified corticosterone (Bortolotti et al. 2008). Despite the presence of corticosterone metabolites in the feather, Bortolotti et al. (2008) found identical amounts of corticosterone in the total fraction and the secreted fraction of their assay, suggesting that the antibody used (C8784 Sigma-Aldrich, Saint Louis, Missouri, USA) is only reacting with unmodified corticosterone. However, further investigation by Lattin et al. (in review) using an antibody purchased Endocrine Sciences/Esoterix (B3-163, Calabasas Hills, California, USA) did not detect any corticosterone in feather extracts, suggesting differences in antibody sensitivity, or perhaps the Sigma antibody is binding not just to unmodified corticosterone, but potentially to metabolites as well. Until analysis of feather extracts via high performance liquid chromatography and/or gas chromatography has been performed to determine which forms of corticosterone are present in feathers, caution should be exercised when interpreting results from feather corticosterone assays. Nonetheless, all work to date indicates that corticosterone is in

fact being measured in these assays and may be used as an indicator of physiological stress during feather development.

Judging the importance of a correlation between feather corticosterone and epaulet color requires an understanding of sexual selection in this system. Across a range of studies, there is mixed support for the role that epaulets play in determining male reproductive success in male Red-winged Blackbirds. Experiments altering male epaulet color demonstrate that males with experimentally blackened epaulets (those with a very low brightness score) may be less successful at obtaining and maintaining a territory than normal males (Peek 1972, Smith 1972). However further experiments by Morris (1975) showed that blackened males reproduced successfully after initial delays in the courtship sequence; it is unknown whether this delay meant that they attracted mates of lower quality than did blackened males. Female choice experiments by Yasukawa et al. (2009) found that females are more likely to associate with and perform precopulatory displays to redder-than-normal males whose epaulets were altered with red ink such that they were redder (a change in hue), more saturated (larger chroma score), and darker (lower brightness score) than unaltered birds. They also found that epaulet dulling (resulting in epaulets that were more orange (hue), less saturated (lower chroma score), and slightly darker (lower brightness score) resulted in no differences in male aggression as compared to males with normal epaulets. Color alterations by Westneat (2006) resulting in a visibly darker and duller epaulet revealed no disadvantages in territorial behavior, paternal care, pairing success, and reproductive success. Indeed, a meta-analysis by Yasukawa et al. (2010) revealed that color had no effect on male-male competition,

female choice, or male reproductive success, despite the fact that some individual studies had shown such effects. There is some evidence that epaulet size rather than color may be a more reliable predictor of dominance and reproductive success (Hansen & Rohwer 1986, Eckert & Weatherhead 1987, Yasukawa et al. 2010); however, measuring size in the present study was not possible due to the folded nature of the specimens' wings. While the exact signaling function of the epaulet remains unclear, this prominent male feature is still likely to be used in communication between and within sexes, and thus alterations of its color due to changes in corticosterone levels may still be of importance in male reproductive success.

Corticosterone and plumage color expression could be connected by perhaps four different causal pathways. The first possibility is the hypothesis that motivated the present study—that corticosterone, through alterations in energy use, affects color expression. A second possibility, however, is that color expression may influence corticosterone secretion through the social challenges of being more or less colorful (i.e. how many mates obtained, or how many intruding males to defend the territory against). Under this alternative scenario, the color expressed by a male during one breeding season may affect corticosterone levels at the time of molt (after chick-rearing has concluded), and thus affect color expression of the subsequent breeding season's plumage. There is experimental evidence in male House Finches, *Carpodacus mexicanus*, that subordinate individuals have significantly higher circulating corticosterone levels than dominant males (Belthoff et al. 1994). In the House Finch system, where color expression and aggression are significantly related (Hill 2002), the effects of social environment on color

and corticosterone are likely considerable. The present study on Red-winged Blackbirds controlled for one aspect of social challenges by sampling only birds with one mate, but perhaps there are other social mechanisms by which color affects corticosterone. Directly testing whether social environment influences corticosterone and color in Red-winged Blackbirds would require a multi-year approach with live animals.

A third explanation for a link between corticosterone and feather color is that other factors related to energy intake and use within the body, such as food intake, could affect both color and corticosterone simultaneously. Because carotenoid expression is ultimately limited by dietary intake of carotenoids, diet restrictions are expected to reduce coloration (McGraw et al. 2005). At the same time, diet restriction in itself may be a stressful event triggering the activation of the HPA axis (Kitaysky et al. 2001). Thus, diet may have direct or indirect effects on both corticosterone levels and ornament coloration. Further studies should control for this link by ensuring equal diets among treatment groups, perhaps in a captive study.

A fourth possible explanation for a link between corticosterone and color of these Red-winged Blackbird specimens is driven by the effect that corticosterone may have on feather integrity. Among birds, corticosterone levels are generally elevated during the breeding season, and are usually at their lowest during the post-breeding molt when feathers are replaced (Romero & Wingfield 1999, Romero 2002). Because one of the effects of glucocorticoids is to stimulate gluconeogenesis through protein catabolism (Sapolsky et al. 2000), down-regulation of corticosterone release during molt may act to protect the structural integrity of the growing feather. Indeed, experimental

manipulations of corticosterone indicate that corticosterone inhibits feather growth (Romero et al. 2005), and that some flight feathers grown while corticosterone levels are high show reduced mass, reduced barbicel hooking strength, and decreased inter-barb distance, which can potentially weaken the feather (DesRochers et al. 2009). It is unknown how these changes in feather microstructure affect coloration; however, if changes such as these were related to feather degradation over time in a museum collection, this feather degradation could cause changes in color reflectance as museum specimens age. In this way corticosterone may impact coloration in museum specimens in ways that would not occur in live birds. Specifically, variation among birds in corticosterone levels during molt could affect the durability of the feather, which might in turn affect color. If this type of degradation occurred during decades of museum storage, a corticosterone-color connection may be found in museum specimens that did not exist when the birds were alive.

A final area of consideration is the validation data from the feather corticosterone assay. I found that samples from the same bird (each sample consisting of 3-5 pooled feathers) were not statistically different from each other, and yet were not significantly correlated. The conflicting lack of statistical significance is likely an artifact of using a small number of birds, but the weak correlation is still troubling. Previous work with the feather corticosterone assay has tested whether corticosterone varies along the length of a feather, but not whether corticosterone content varies between feathers. Because the likelihood of sampling two feathers that were grown at the exact same time is very small, it is expected that there are differences in the corticosterone signature between feathers.

Previous work with this assay has been performed using a single feather per sample, which cannot account for variation in corticosterone between feathers (Lattin et al. in review, Harms et al. 2010, Mougeot et al. 2009, Bortolotti et al. 2008, 2009). The use of 3 -12 feathers in each of my samples could have served to reduce variation in corticosterone levels due to differences in the timing of feather growth, but still samples were not highly correlated, indicating that even samples containing several feathers may not be a consistent reflection of corticosterone levels. Given this finding, studies using only one feather per sample should be regarded with caution. Nonetheless, the detection of significant relationships between feather corticosterone and other variables both in the present study and other, suggests that such relationships might be very strong—i.e., large enough statistical effects to be detected despite between-feather variation in corticosterone levels. Further research is required to understand how much corticosterone varies between feathers, and how to control for this variability when sampling feathers.

While previous research supports the link between physiological stress and melanin plumage coloration, as well as carotenoid-based skin coloration, this is the first to find a link between corticosterone and carotenoid-based plumage coloration. Further studies should seek experimental evidence for the effects of corticosterone on carotenoid-based plumage coloration.

CHAPTER 2

Color fading in museum specimens: effects of pigment type and pigment location

ABSTRACT

Museum specimens are becoming commonly used for ecological research, particularly concerning avian morphology and plumage characteristics. However, plumage and skin-based colors have been observed to change over time in museum collections. Because of differences in their chemical properties, different pigments may change at different rates. To date, fading of structural, melanin- and carotenoid-based plumage colors have been assessed. This study aims to expand on these studies by evaluating and comparing color change of carotenoid- and psittacofulvin-based red plumage and by comparing change of carotenoid-based plumage and bill coloration. Using reflectance spectrophotometry, I collected spectral data on five species expressing carotenoid-based red color, seven species expressing psittacofulvin-based red color, and three species expressing both feather- and bill-based carotenoid color. Red brightness and red chroma of both carotenoid- and psittacofulvin-based plumage colors decreased over time, and carotenoid-based red brightness and red chroma decreased at a significantly faster rate than that of psittacofulvins. Both plumage- and bill-based red brightness and red chroma underwent significant changes over time; however, there were no significant differences in the rate

of change of plumage- and bill-based carotenoid color. While bill-based color has been observed to change very rapidly in both living birds and museum specimens, I found no such rapid change here, likely due to the fact that the newest specimen included in the bill-fading analysis was collected nine years ago, and significant fading may have already occurred in that time. While I found significant changes in the coloration of museum specimens, some of the slopes associated with these changes were small, such that in some cases very little change in red brightness or red chroma was actually occurring. Possible mechanisms underlying the differences in color change based on pigment type and location are discussed.

INTRODUCTION

The use of museum specimens for research on ecological questions is becoming increasingly common (Pyke & Erlich 2009). Museum collections are particularly useful resources for studies of comparative avian anatomy or morphology (Desrochers 2010,Francis & Guralnick 2010) , and plumage characteristics (Dyck 1992, Schmitz-Ornes 2006, Toomey et al. 2010a). While the temporal range of specimens in a collection is beneficial to studies of historical population changes, feather color has been observed to change over time (Gabrielson & Lincoln 1951), so research on coloration using museum specimens should proceed with caution (McNett & Marchetti 2005).

Changes in plumage coloration across the duration of storage have received recent attention. An analysis of living and museum specimens of wood warblers (Parulidae) revealed significant differences in brightness, chroma, and hue of carotenoid-based plumage patches (McNett & Marchetti 2005), suggesting that museum specimen coloration may not accurately represent that of wild birds. Expanding on these findings using a variety of passerine species, Armenta et al. (2008) found no effects of age on rust, black, or blue colors (derived from either melanin pigments or nanostructures), yet significant decreases in brightness of blue-grey, white, and red colors with age. Armenta et al. (2008) also report that changes in the brightness of a color do not necessarily result in changes in the other axes of Munsell color space, chroma, and hue. Analysis by Doucet and Hill (2009) found an increase in brightness in the carotenoid-based red mantle of Long-tailed Manakins, *Chiroxiphia linearis*; however, they found no effect of

age on blue, black and grey colors (which are typically made by either melanin pigments or structural mechanisms), suggesting that color change may be related to the mechanism that produced the color.

Because colors produced by different mechanisms or expressed in different tissue types may change differently over time (Doucet & Hill 2009), it is important to analyze and report these differences on a pigment-by-pigment or tissue-by-tissue basis. To date, there is some fading information regarding carotenoid- and melanin-based plumage coloration from individual species as described above (McNett & Marchetti 2005, Doucet & Hill 2009). Less is known, however, about post-mortem changes in pigments that have a narrower taxonomic distribution among birds (such as porphyrins, pterins, and psittacofulvins), and in pigments that are not localized in feathers. Dramatic changes in bill coloration over short periods of time have long been observed to take place post-mortem in museum specimens (McGraw, 2006, *pers. observation*), but quantitative analysis of this change is currently lacking.

With these issues in mind, the first aim of this study is to assess fading of carotenoid- and psittacofulvin-derived plumage coloration in order to expand our knowledge of pigment fading in museum specimens. The second goal of this study is to quantify and compare fading of carotenoid pigments in feathers and in the soft tissue of bills.

Carotenoid and Psittacofulvin Color Change

The red, orange, and yellow coloration due to carotenoid pigmentation is the focus of much research because expression of this coloration is widespread across taxa, is often condition dependent, and is often assessed by mates during reproduction (reviewed by McGraw 2006). These pigments cannot be synthesized *de novo* by animals and must be obtained through diet (Goodwin 1984). An alternative source of red coloration, the psittacofulvin family of pigments, is found only in parrots (Psittaciformes). In contrast to the diet-derived carotenoids, psittacofulvins are synthesized by the animal at the site of feather growth (McGraw & Nogare 2004, 2005). Because of the taxonomic limitations of the occurrence of psittacofulvin pigmentation, this pigment group has received far less attention from researchers than carotenoid pigments. Predictions about post-mortem color changes are difficult to make because little is known about the synthesis, deposition, and degradation of psittacofulvins in feathers. Nonetheless, studies of the effect of light on psittacovulvin-based plumage suggest that colors derived from these pigments may be some of the most vulnerable to fading (Horie 1990, Solajic et al. 2002, Hudon 2005), and characteristics of their chemical structure (described by Stradi et al. 2001) may provide support for this idea. Because of the potential vulnerability of psittacofulvins to the effects of light, I predicted that specimen age should have a greater effect on color change in psittacofulvins than in carotenoids.

Bill and Feather Color Change

Carotenoid-pigmented bare parts (i.e. skin, bills, feet and areas of the face) often serve as signals of quality (Burley & Coopersmith 1987, Perez-Rodriguez & Vinuela 2008, Baird et al. 2010). Unlike feather-based signals, which only change to update the status of the individual upon annual or semi-annual molt, skin-based ornamental color may change much more rapidly. For example, bill coloration of male goldfinches (*Carduelis tristis*) faded from orange to yellow within 24h of being brought into captivity (Rosen & Tarvin 2006), and carotenoid-based foot color in male blue-footed boobies (*Sula nebouxii*) became duller within 48h of food deprivation, while brightness returned upon feeding (Velando et al. 2006). Thus individual signals of status may be updated quickly to accurately reflect current conditions.

While the mechanisms behind the dynamic changes in bare-part coloration remain in question, rapid color changes post mortem have still been observed and I predicted that color change will occur more rapidly in bare parts than in feathers.

METHODS

I collected reflectance spectral measurements (described below) from adult specimens of 14 species at the Smithsonian Institution's National Museum of Natural History, Washington, DC, USA.

I collected reflectance spectra from 300-700nm 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. I took four measurements of one point on the plumage (see Table 1) or bill (Table 2), with the probe at a 45-degree angle and at a distance of 1 cm from the plumage or bill surface. Plumage areas were chosen based on the presence of red coloration, and bill measurements were taken from an area of the bill that was known to have been red while the bird was alive (based on photographs of live birds). Both areas measured were kept the same for all members of a given species.

Using the Munsell color system, I calculated red brightness (sum of reflectance 600-700nm divided by number of data points in this range), and red chroma (sum of the reflectance 600-700nm divided by the sum of the total reflectance 300-700nm) for each spectrum obtained (Montgomerie 2006).

Psittacofulvins and Carotenoids

To compare fading of carotenoids and psittacofulvins I collected samples from a taxonomically broad group of carotenoid-pigmented birds, and a necessarily narrower group of psittacofulvin-pigmented birds (given that the taxonomic distribution of this pigment is limited to Psittaciformes only (McGraw & Nogare 2005)), focusing on red plumage only (Table 1). Carotenoid-pigmented birds were collected mainly from the eastern United States from Maine to Florida and as far west as Texas, and Wisconsin, and ranged in collection date from 2009 to 1843. Carotenoid-pigmented birds were all male

(or were unlabeled but suspected to be male), as the carotenoid-based color in these species is much more strongly expressed in males. Psittacofulvin-pigmented birds were collected from areas of Australia, Central and South America and Africa, as well as some obtained from the National Zoo or other captive setting, and ranged in collection date from 1997 to 1859. Both male and female psittacofulvin-pigmented specimens were sampled, as red coloration in Psittaciformes is often expressed by both sexes (Table 1).

Both measures of carotenoid and psittacofulvin feather coloration were repeatable within samples (carotenoid red brightness: $r=0.997$, $F_{198, 597}=1290.366$, $p<0.001$; carotenoid red chroma: $r=0.991$, $F_{198, 597}=418.603$, $p<0.001$; psittacofulvin red brightness: $r=0.982$, $F_{89, 270}=219.260$, $p<0.001$; psittacofulvin red chroma: $r=0.953$, $F_{89, 270}=82.602$, $p<0.001$) (Lessells and Boag 1987).

Bill and Feather Carotenoids

To compare fading of carotenoid pigments of the bill and feather, I selected species that expressed carotenoid-based red coloration both in the plumage and on the bill. I measured both plumage and bill coloration from the same individuals of Northern Cardinals, *Cardinalis cardinalis,* White-throated Toucans, *Ramphastos tucanus,* and Keel-billed Toucans, *Ramphastos sulfuratus* (Table 2). Sampling was limited to these three species by their abundance in the museum collection. *C. cardinalis* specimens were all male, collected from the eastern United States from Florida to New York, and west to Wisconsin and Illinois, and were collected from 1843 to 2001 to. *R. tucanus* specimens were both male and female, were collected throughout northern South America from

1891 to 1999. *R. sulfuratus* specimens were all male, collected from Central and South America and Gambia in West Africa, and were collected from 1911 to 1990.

Both measures of coloration were repeatable within both bill and feather samples (bill red brightness: r=0.999, $F_{71, 216}$=2769.386, p<0.001; bill red chroma: r=0.995, $F_{71,216}$=846.416, p<0.001; feather red brightness: r=0.995, $F_{70, 213}$=219.260, p<0.001; feather red chroma: r=0.995, $F_{70, 213}$=730.302, p<0.001).

Statistical Analyses

Because there was a large amount of variability in the mean age of the specimens across a species, I centered age within species (group mean centering; centered age=specimen age-species mean age, hereafter referred to as 'age') for use in the analyses, as described by Enders and Tofighi (2007). Statistical analyses were performed using SAS statistical software (v. 9.2, Cary, North Carolina, USA). Means ±SE are reported throughout.

Carotenoids and Psittacofulvins

To compare the effects of age on carotenoid- and psittacofulvin-based red brightness of feathers, I performed a hierarchical linear mixed model which models the effect of age on pigment types, and models the variability within a species nested within a pigment group, using 'species' within each pigment as a random effect to control for variability in the effects of age on the individual species, and using 'age' as a covariate. There was no significant variability in the linear effect of age across species (p=0.3780), so this effect was removed from the final model.

A hierarchical linear mixed model was similarly used to compare the effects of age on red chroma, again using 'species' as a random effect, and 'age' as a covariate. There was so little variability in the linear effect of age across species that it was estimated to be approximately zero, and this effect was removed from subsequent models; however, the variability in mean red chroma (e.g. intercept) for each species was controlled and accounted for.

Bill and Feather Carotenoids

To compare the effects of age on bill- and feather-based carotenoid red brightness, I performed a hierarchical linear mixed model using 'species' as the random effect to control for variability in the effects of age on color between the individuals of a single species. 'Age' was used as a covariate. This model also accounted for the paired nature of the data—that bill and feather measurements were taken from the same individual.

I used a similar hierarchical mixed model to test for the effects of specimen age on red chroma in bill- and feather- based pigmentation using 'species' as a random effect, and 'age' as a covariate. The model accounting for variation in the effect of age on different species could not converge, and the random effect of age could not be estimated, suggesting that the variance in the effects of age of the three species is very small.

RESULTS

Carotenoids and Psittacofulvins

Plumage red brightness and red chroma varied within and between species (Table 3).

Red Brightness

The first model compared the effect of age on red brightness for each of the pigment types. The slopes representing the effect of age on red brightness for both pigments were significantly different from zero ($F_{2, 10}$=18.81, p=0.0004) and negative, indicating that red brightness decreased significantly with age.

The second model tested for differences in the slopes representing the effect of age on red brightness for carotenoids and psittacofulvins. The slopes representing the effect of age on red brightness for carotenoids and psittacofulvins were significantly different from each other ($F_{1, 1142}$=10.33, p=0.0013, Figure 2), indicating that age is affecting carotenoid and psittacofulvin-based coloration differently, with the slope for carotenoids (-0.071± 0.01724) being more negative than the slope for psittacofulvins (-0.0159±0.01589). Thus there is strong evidence suggesting that carotenoid-based plumage coloration changes at a faster rate than psittacofulvin-based coloration.

Red Chroma

The first model compared the effects of age on red chroma using only the intercepts (species mean red chroma) as the random effect, fitting one slope for each pigment type.

The slopes representing the effect of age on carotenoid and psittacofulvin pigments were significantly different from zero ($F_{2, 1142}$=25.66, p<0.0001), thus red chroma of both pigments types are decreasing significantly with age.

The second model tested for differences in the slopes of the lines representing the effect of age on red chroma for the two pigments. The slopes for these lines were significantly different from each other (F_{1142}=4.31, p=0.0382), and the slope for carotenoids (-0.00036±0.000131) was more negative than that for psittacofulvins (-0.00009±0.000121) (Figure 3). This suggests that carotenoid-based pigment red chroma changes at a faster rate than that of psittacofulvin-based pigments.

Bill and Feather Carotenoids

In a simple correlation analysis that pooled all specimens (*n*=144) regardless of species, bill red brightness and red chroma were strongly correlated (Pearson r=0.694, p<0.001); however, plumage red brightness and red chroma were not (Pearson r=0.089, p=0.457). Bill red brightness and plumage red brightness were strongly correlated (Pearson r=0.416, p<0.001) in addition to bill red chroma and plumage red chroma (Pearson r=-0.245, p=0.038). In addition, red brightness and red chroma varied within and between body parts, individuals, and species (Table 4).

Red Brightness

The first model tested for the random effect of age on red brightness of the individuals within a species. The effect of age on red brightness among the individuals of a species

did not vary significantly (p=0.237), thus age affected the individuals within a species similarly. This model also tested for the variability in the effect of age on the mean red brightness for individuals within a species, and found little variability (p=0.1808).

The next model tested whether the slopes of the lines representing the effects of age on red brightness for each body part are different from zero. The slopes for bill and for plumage were both significantly different from zero ($F_{2, 146}$=7.37, p<0.0009), thus there were significant negative effects of age on red brightness of both bills (slope= -0.04539±0.03414) and plumage (slope= -0.15799±0.03126).

A final model tested for differences in the slopes of the effect of age on red brightness for bills and plumage. There were no significant differences in the two slopes ($F_{1, 146}$=2.11, p=0.1488) (Figure 4), thus there are no significant differences in the effect of age on bill and plumage brightness, and we can conclude that they are changing at the same rate.

Red Chroma

The first model fit a line for each species in the two body part groups. Because this model tested for variability in the slopes and intercepts of the lines for each species, slopes and intercepts were used as random effects. There was no evidence of significant variability in the effect of age on red chroma across the species within each pigment group. The slopes were so small and so similar that a test statistic could not be calculated.

Next, I fit a model using only the intercepts as the random effect, fitting one slope for each pigment type to test whether the slopes were different from zero. There was strong evidence that the slopes of the lines representing the change in red chroma across age are different from zero ($F_{2, 146}$=6.41, p<0.0021), thus age is having a significant effect on red chroma in both bill and feather coloration. There were no significant differences between the slope for bill (-0.00091±0.00031) and the slope for feather (-0.00058±0.00028) ($F_{1, 146}$=0.19, p=0.6627) (Figure 5), suggesting that bill and feather coloration are fading at the same rate.

DISCUSSION

Carotenoids and psittacofulvins

The goals of this study were to test for patterns in color change of carotenoids and psittacofulvins as individual pigment groups. Because previous studies suggest that the chemical structure of psittacofulvin pigments may make them more prone to degradation over time, I predicted that psittacofulvin-based plumage would undergo color change at a faster rate than carotenoid-based plumage. While I found significant changes in red brightness and red chroma of both carotenoid and psittacofulvin-based plumage areas, carotenoid-based plumage was found to undergo color change at a significantly faster rate than psittacovulvin-based plumage. Despite these statistically significant changes, the total change in brightness and chroma was fairly small. For example, red brightness of

carotenoid-based plumage as a group decreased by 1.62% over 10 years while red chroma decreased by 0.25% over ten years. In 100 years, red brightness decreased 15.19% and red chroma decreased by 2.31% for the carotenoid group. Red brightness psittacofulvin-based plumage as a group decreased 0.33% over 10 years, while red chroma decreased 0.34%. In 100 years red brightness decreased 3.27% and red chroma decreased 3.41% for the psittacofulvin group. These somewhat small changes in coloration, while statistically different from zero, may suggest that little relevant change in coloration is likely occurring in either carotenoid- or psittacofulvin-based coloration; however, the degree to which these changes affect future research will depend on the range in the age of specimens used. If the specimens to be sampled were all collected within a reasonably small number of years, the effect of storage on coloration may be minimal, however these effects are likely to me much larger if the range in specimen age is larger.A similar study by Armenta et al. (2008) found significant differences in plumage coloration between living and museum specimens of a single species. They found significant effects of age on some plumage colors (red) but not others (green, brown, yellow), indicating that different pigments may age differently. Similarly, Doucet and Hill (2009) found significant changes in brightness among carotenoid-based plumage, but only changes in hue and chroma among blue and black feather regions colored by either melanin or nanostructures. Neither of these studies reported color change rates (slopes) or percent changes over time, making a comparison to their findings somewhat difficult.

Observational studies of parrot feathers used in ancient cultural pieces suggest that psittacofulvin pigments may be one of the most susceptible to fading (Solajic et al. 2002, Hudon 2005); however, no biochemical tests of this prediction have been cited, and no support for this prediction was generated from my study.

Bill and Feather Carotenoids

Bare tissue coloration in birds has long been observed to change rapidly post mortem (McGraw 2006, *pers. observation*); however, this is the first study to my knowledge to quantify this change. Because of these observations, I predicted that bill red brightness and red chroma would change at a much faster rate than feather red brightness and red chroma. While I found that significant color change occurs over time in both of these body parts, I found no differences in the rate of color change over time in bills and feathers. For example, carotenoid-based bill coloration as a group showed a reduction in red brightness of -0.94%% in 10 years, while bill red chroma decreased 1.24%. In 100 years bill red brightness decreased 9.39%, and bill red chroma decreased 12.44%. Likewise, carotenoid-based bill coloration as a group showed a reduction in red brightness of 2.18% and red chroma decreased 0.91% over a 10-year period. In 100 years feather red brightness decreased 21.79%, and feather red chroma decreased 9.03%. These changes in red brightness and red chroma of both bill and feather suggest a noticeable change is occurring over long periods of time. The importance of this color change to future research will depend on the variability of specimen age with the sample. If the range in specimen age is kept fairly small, the variability in coloration due to

storage is likely to be minimal, however if the range in specimen age is large, duration of storage is likely to have important effects on color.

Using these same specimens and the slopes generated from the model for carotenoid pigment fading (described above), I found that plumage brightness of *C. cardinalis* decreased by only 1.03% in a 10-year period, while chroma decreased only 0.24%. Thus, the exact rate of change of carotenoid-based plumage coloration varied between models. For *C. cardinalis* red brightness may decrease between 1.03-7.12% every 10 years, while red chroma may decrease between 0.24 and 2.92% every 10 years. This variability in color change is due to differences in the statistical model, and thus color change estimates are best given as a range.

It is important to note that the most recent specimen included in my sample was collected in 2001, meaning it has been dead for at least 9 years. Given the many observations of rapid post-mortem color change in bare tissue, it is likely that much of the color change in the bills that I sampled had already occurred during the 9+ years of storage in the collection, and my sample was not able to capture this rapid color change. The mean bill red brightness and red chroma of all the specimens included in this study was much lower than that for plumage (see Figures 4 & 5). It is not currently known whether this pattern is similar for live birds, but this large difference between bills and plumage in red brightness and red chroma may suggest that significant bill fading occurred in the first decade of storage. If much of the color change occurred in the first decade of storage, there may be little overall color change left to occur beyond a decade. Visual comparison of the bill coloration of the newest *R. tucanus* specimen in my sample

and the oldest (collected in 1891) (Figure 6) reveal striking similarities, suggesting that little noticeable color change occurs after a decade of storage.

Interpreting the color change of bills and feathers is made difficult by how little is known about the deposition of carotenoids into the integument and what qualities of the bill make it different than a feather. Both structures are keratinized (Frenkel & Gillespie 1976, Stettenheim 2000), making them strong yet lightweight; however, carotenoids are usually deposited in feathers in the *trans* form, while pigments deposited in bare parts are often esterified (Czeczuga 1979). Carotenoids bind to keratins, a group of inert and insoluble proteins, in such a way that extraction is difficult (Britton et al. 2008). Such binding may suggest that carotenoid deposition in either feather or bill should be irreversible, yet rapid bill color change has been shown to occur in live birds (Rosen & Tarvin 2006) suggesting re-uptake may be occurring. Another notable difference likely leading to the rapid color change of bills on live birds (Rosen & Tarvin 2006) is the presence of a blood supply to the bill, whereas a feather is vascularized only during growth.

Future research should study bill fading in the initial decade post-mortem. Studies of the mechanisms of pigment deposition in the bill, and the processes involved in color expression are also an important step toward better a understanding of the mechanisms responsible for rapid color change seen in live birds (Rosen & Tarvin 2006), and potential post-mortem color change.

Summary and Conclusions

Doucet and Hill (2009) describe three general factors that may influence color change among specimens. The first, soiling, can occur through dust accumulation or deposition of oils from handing by researchers. Dust and oils have the capability to reduce light reflection—including doing so differently across wavelengths—and, therefore, care should be used in handing and storing collections. The second factor involves physical damage of the feather barbules, including that by bacteria, lice, and again from handling; therefore, investment in pest management may be a crucial component of color maintenance. The third mechanism for fading is through biochemical breakdown of the pigment molecules via oxidation or isomerization, which would result in increases in feather brightness, as more of the white microstructure of the feather shows through. Isomerization is the change in stereochemistry of the pigments (Britton 1995) and can be the result of exposure to light, heat, oxygen, humidity, or acidity (Shi &Le Maguer 2000), thus care should be taken to ensure that the museum environment is not extreme in any of these cases. Because I found significant reductions in brightness, this may indicate that pigment breakdown (which would cause an increase in brightness) is not occurring, and feather soiling, and feather breakdown may be the factors contributing the most to color change over time.

While I report that color change is significant both in carotenoid- and psittacofulvin-pigment plumage as well as in carotenoid-pigmented bills, application of these results to future studies using museum specimens will depend on the types of questions under consideration. Whether or not the color change described here is

important to a particular line of future research is up to the researcher, but the quantification of color change in different pigments and tissues is a critical first step. The findings from this study support the use of museum specimens for ecological or environmental research, but interpretation of color spectra obtained from specimens should proceed with caution, as significant color change may be occurring in both plumage and bare parts. An important next step in the quantification of color change over time in museum specimens is to include living and very recently dead birds into the sample to quantify color change during the first years of storage. Because of the potential for very large changes in color due to degradation of tissue or pigments, continued investment in quality museum care should be sought.

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Table 1: The species used to compare fading of carotenoid and psittacofulvin pigments.

Pigment	Common Name	Species	Order	Family	Plumage Region	*n*	Sex
Carotenoid	Red-winged Blackbird	*Agelaius phoeniceus*	Passeriformes	Icteridae	epaulet	31	m
	Red-bellied Woodpecker	*Melanerpes carolinus*	Piciformes	Picidae	nape	36	m
	Scarlet Tanager	*Piranga olivacea*	Passeriformes	Cardinalidae	back	49	m
	Northern Cardinal	*Cardinalis cardinalis*	Passeriformes	Cardinalidae	breast	46	m
	Rose-breasted Grosbeak	*Pheucticus ludovicianus*	Passeriformes	Cardinalidae	breast	37	m
Psittacofulvin	Crimson Rosella	*Platycercus elegans*	Psittaciformes	Psittacidae	crown	9	7f, 1m, 1u
	Eastern Rosella	*Platycercus eximius*	Psittaciformes	Psittacidae	under tail coverts	9	6m, 3f
	Eclectus Parrot	*Eclectus roratus*	Psittaciformes	Psittacidae	nape	13	f
	Red-and-Green Macaw	*Ara chloroptera*	Psittaciformes	Psittacidae	nape	12	6m, 6f
	Scarlet Macaw	*Ara macao*	Psittaciformes	Psittacidae	crown	13	8m, 4f, 1u
	African Grey Parrot	*Psittacus erithacus*	Psittaciformes	Psittacidae	tail	17	13m, 2f, 2u
	Mealy Amazon	*Amazona farinosa*	Psittaciformes	Psittacidae	speculum	17	3m, 13f, 1u

Table 2: The species used to compare feather and bill carotenoid pigment fading.

Common Name	Species	Order	Family	Plumage Region	*n*	Sex
Northern Cardinal	*Cardinalis cardinalis*	Passeriformes	Cardinalidae	breast	46	m
White-throated Toucan	*Ramphastos tucanus*	Piciformes	Ramphastidae	under tail	17	11m, 6f
Keel-billed Toucan	*Ramphastos sulfuratus*	Piciformes	Ramphastidae	under tail	9	m

Table 3: Descriptive statistics of the species used to compare fading of carotenoid and psittacofulvin pigments.

			Red Brightness				Red Chroma			
Pigment	**Species**	***n***	**mean**	**STDEV**	**min**	**max**	**mean**	**STDEV**	**min**	**max**
Carotenoid	*Agelaius phoeniceus*	31	62.20	5.23	33.90	85.21	0.8067	0.0550	0.7006	0.9393
	Melanerpes carolinus	36	20.88	6.03	9.50	36.81	0.8452	0.0591	0.6961	0.9248
	Piranga olivacea	49	56.68	7.00	38.74	69.49	0.8275	0.0552	0.6788	0.9259
	Cardinalis cardinalis	46	48.64	8.54	29.61	70.27	0.7002	0.0587	0.5667	0.8116
	Pheucticus ludovicianus	37	47.24	8.82	29.26	67.26	0.7179	0.0629	0.5751	0.8356
Psittacofulvin	*Platycercus elegans*	9	36.45	5.88	29.68	46.85	0.7995	0.0608	0.6849	0.8920
	Platycercus eximius	9	46.64	5.10	41.05	54.34	0.7717	0.0577	0.6488	0.8362
	Eclectus roratus	13	36.68	5.23	28.54	42.78	0.7836	0.0370	0.7072	0.8395
	Ara chloroptera	12	44.01	5.23	36.21	52.52	0.8176	0.0477	0.7504	0.8719
	Ara macao	13	47.89	13.82	17.45	68.68	0.7907	0.0499	0.6782	0.8685
	Psittacus erithacus	17	49.33	9.53	34.74	67.52	0.7486	0.0508	0.6373	0.8330
	Amazona farinosa	17	55.08	3.91	47.83	62.32	0.7187	0.0423	0.6355	0.7801

Table 4: Descriptive statistics of the species used to compare bill and plumage fading.

			Red Brightness			Red Chroma		
species	***n***	**body part**	**mean**	**STDEV**	**CV**	**mean**	**STDEV**	**CV**
Cardinalis cardinalis	46	Bill	25.46	7.43	29.20	0.5601	0.0564	10.08
		Plumage	48.64	8.54	17.56	0.7002	0.0587	8.38
Ramphastos tucanus	17	Bill	5.29	6.57	124.33	0.3963	0.1326	33.45
		Plumage	37.58	12.62	33.58	0.7986	0.2140	26.80
Ramphastos sulfuratus	9	Bill	19.21	7.26	37.77	0.6151	0.0627	10.20
		Plumage	33.66	11.42	33.92	0.8287	0.0446	5.38

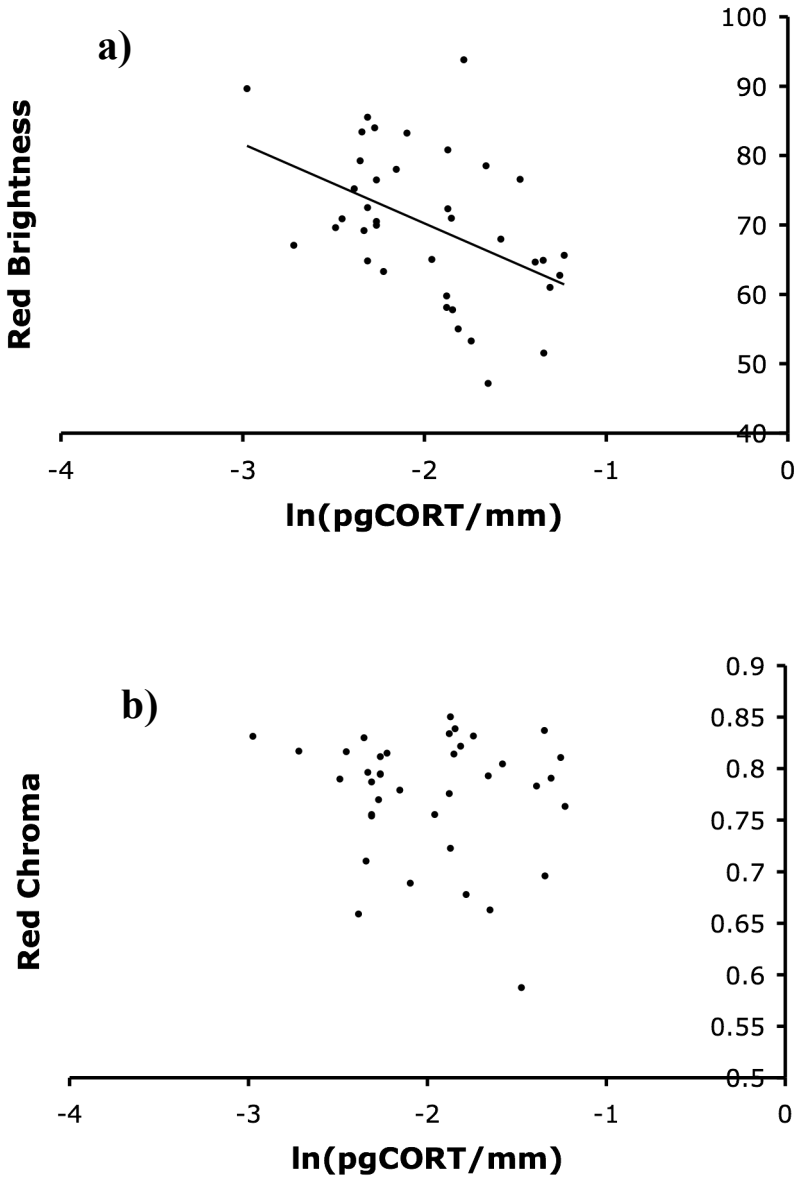

Figure 1. Scatter plots of the relationship between feather corticosterone (ln (pgCORT/mm)), and (a) red brightness and (b) red chroma of male epaulets. Circles represent the average red brightness or red chroma of four measurements of a single individual (n=38). Corticosterone levels predict red brightness but not red chroma of male Red-winged Blackbird epaulets.

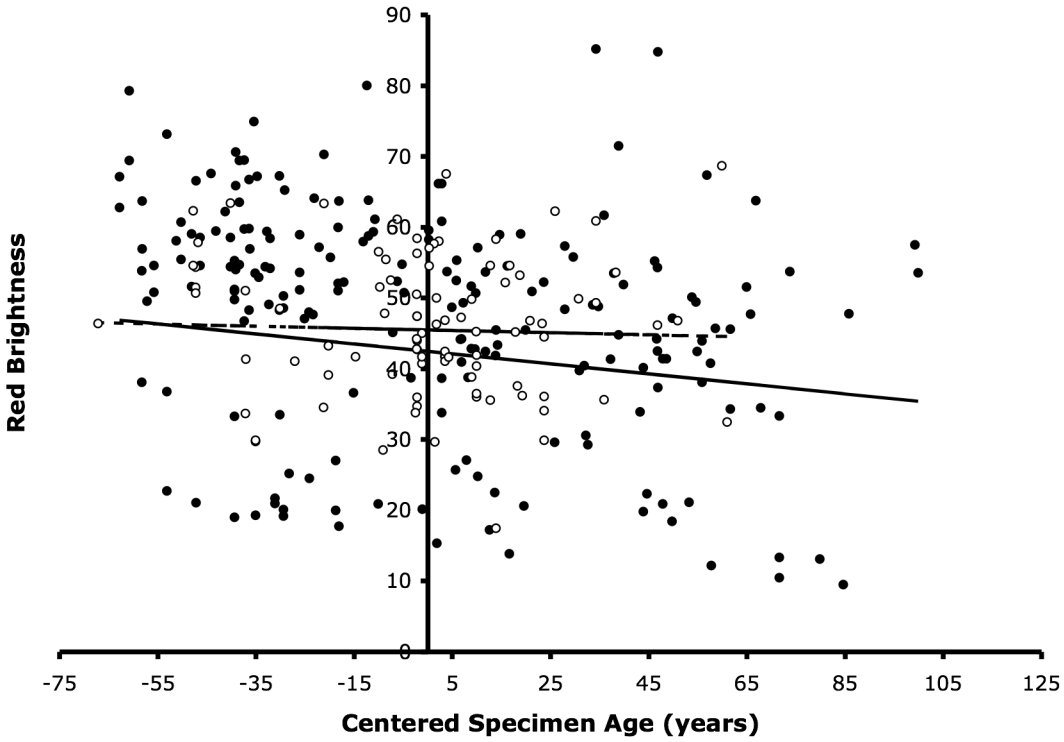

Figure 2. The relationships between red brightness and centered age of carotenoid- (closed circles, solid line) and psittacofulvin-based (open circles, dashed line) plumage coloration. Both carotenoid- and psittacofulvin-based plumage red brightness significantly decreased with age, and carotenoid-based plumage did so at a significantly faster rate than psittacofulvin-based plumage.

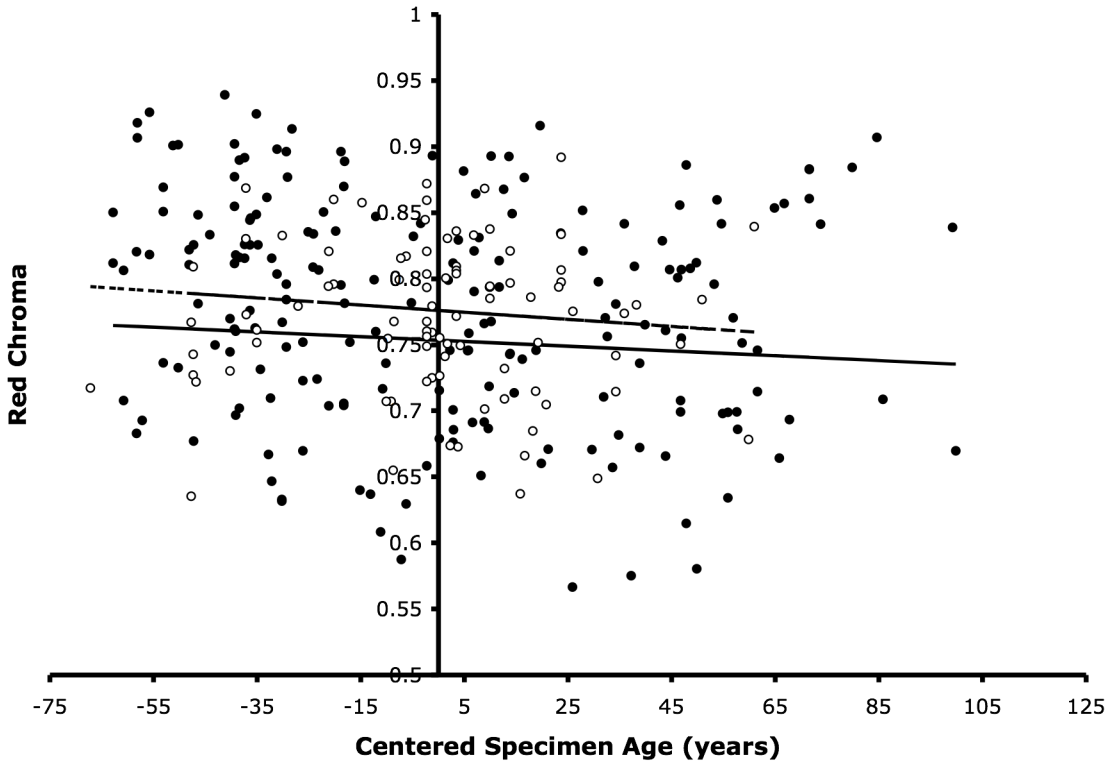

Figure 3. Closed circle, yellow line=carotenoid; open circle, blue line=psittacofulvin

Figure 3. The relationships between red chroma and centered age of carotenoid- (closed circles, solid line) and psittacofulvin-based (open circles, dashed line) plumage coloration. Both carotenoid- and psittacofulvin-based plumage red chroma significantly decreased with age, and carotenoid-based plumage did so at a significantly faster rate than psittacofulvin-based plumage.

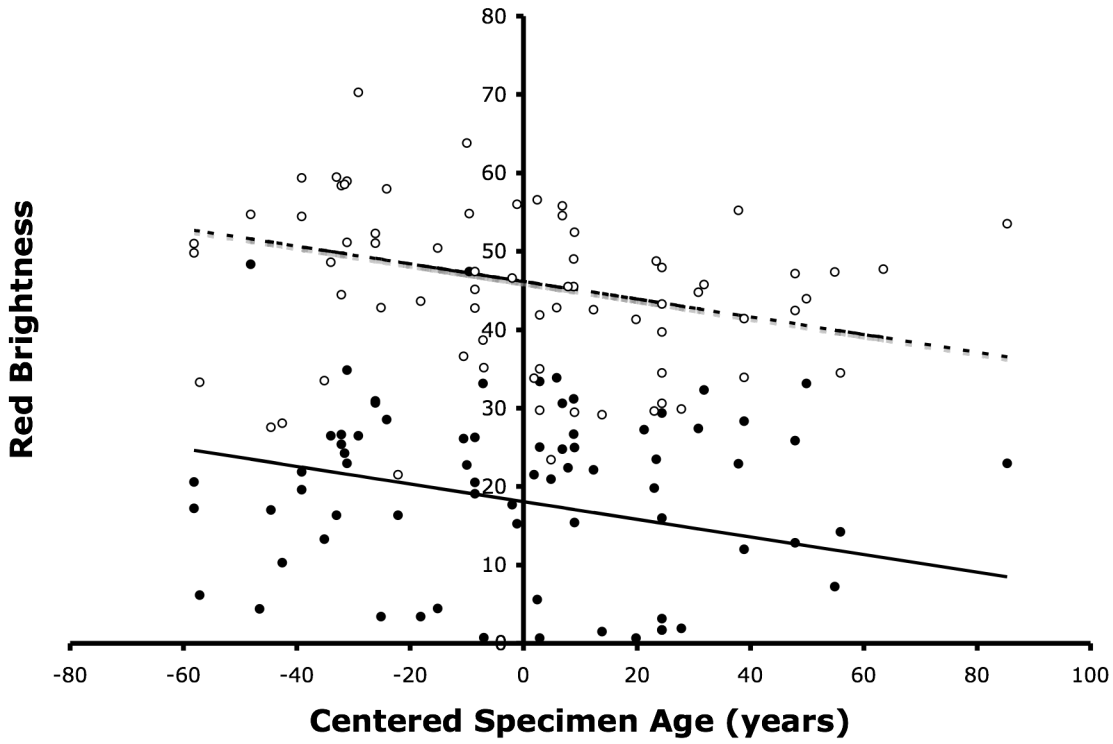

Figure 4. The relationships between red brightness and centered age of bill- (closed circles, solid line) and feather-based (open circles, dashed line) carotenoid coloration. Both bill- and feather-based red brightness significantly decreased with age, and there were no significant differences in their rates.

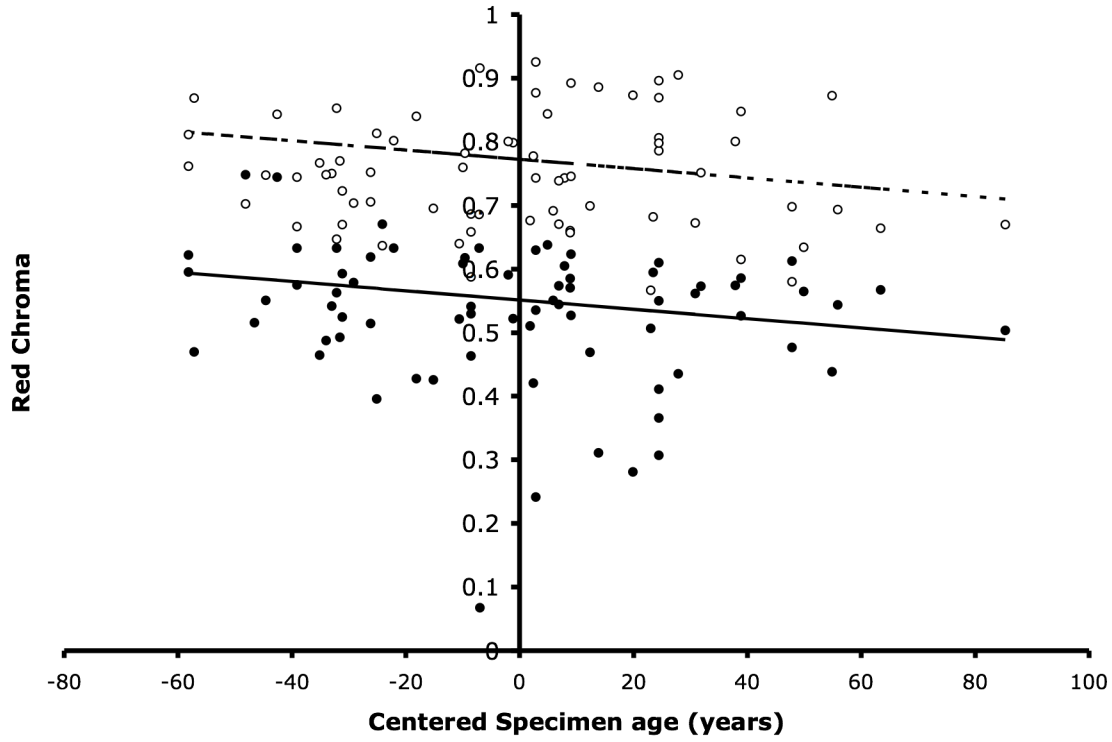

Figure 5. The relationships between red chroma and centered age of bill- (closed circles, solid line) and feather-based (open circles, dashed line) carotenoid coloration. Both bill- and feather-based red chroma significantly decreased with age, and there were no significant differences in their rates.

Figure 6. Bill color comparison of the newest *R. tucanus* specimen sampled (lower), collected in 2001, to that of the oldest specimen sampled (upper), collected in 1891. Visual inspection reveals many color similarities, supporting the idea that rapid color change occurs in bills that could not be captured with the sample obtained here. See Hilty (2003), plate 35 for an illustration of a live *R. tucanus*.