Carotenoid-Based Plumage Coloration Predicts Leukocyte Parameters during the Breeding Season in Northern Cardinals (Cardinalis cardinalis)

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Abstract

Dichromatism in songbirds is often associated with polygyny and dimorphic parental investment, and is thought to arise via sexual selection. Northern cardinals (Cardinalis cardinalis) are not only dichromatic but also monogamous and biparental, suggesting that plumage coloration in this species may serve different functions than in more typical dichromatic species. In order to explore the role of sexual selection in the evolution of plumage coloration in cardinals, we used reflectance spectrophotometry to investigate whether two carotenoid-based ornaments, the male’s red breast and the female’s underwing coverts, contain information that potential mates or competitors could use to assess condition. We found that whereas coloration was not related to body condition (measured as the residual body mass from a regression of body mass on wing chord), more saturated carotenoid coloration was associated with higher heterophil to lymphocyte ratios in males, and with higher white blood cell counts in females. Thus, in both sexes, carotenoid coloration was positively linked to immune measures normally associated with higher levels of stress and infection. These results do not indicate that carotenoid-based coloration functions as a signal of low levels of stress or disease in this species. We propose instead that because plumage coloration may be related to competitiveness, the more saturated individuals increase their risk of injury, stress, and infection by engaging in more competitive behavior or by secreting more testosterone, or both. Our finding that carotenoid pigmentation is positively associated in males with the size of the cloacal protuberance, an androgen-sensitive sex character, supports this hypothesis.
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Carotenoid Coloration and Leukocyte Parameters in Northern Cardinals

The northern cardinal (Cardinalis cardinalis), a year-round resident passerine common to eastern and central North America, represents an unusual exception to the general trend toward monomorphism in monogamous, biparental, non-migratory birds. The males’ plumage is primarily red, with brilliant red breast and cheeks and duller red feathers on the back, wings, and tail. The female, in contrast, is primarily a drab tan or olive brown, with red plumage visible only on the wings (particularly the underwing coverts) and tail. Despite this striking dichromatism, males of this species are rarely polygynous and engage in extra-pair fertilizations relatively infrequently (Breitwisch et al. 1999; Halkin & Linville 1999). Behavioral dimorphism is also low in this species compared with other dichromatic songbirds; both males and females engage in parental care, territorial defense, and singing (Halkin & Linville 1999). Because they lack the strongly dimorphic breeding strategies and short mate-sampling periods typical of many other dichromatic species, dichromatism in northern cardinals may be subject to atypical evolutionary pressures.

Has sexual selection played a role in the evolution of the males’ red plumage? During courtship displays such as the song–dance display and the song–flight display (Shaver & Roberts 1933; Lemon 1968), the male displays his breast to the female, suggesting that the coloration could have evolved as a courtship signal. Redder males pair with earlier breeding females and have greater reproductive success than duller males (Wolfenbarger 1999a). Coloration may signal direct fitness benefits to females; the redness of the breast plumage is positively correlated with territory quality (Wolfenbarger 1999a). Whether coloration signals indirect benefits to females is less clear; Wolfenbarger (1999a) reported that redness is not associated with size or body condition, whereas Jawor & Breitwisch (2004) found positive correlations between redness of breast plumage and body size (but not condition). Whether the coloration of the male’s breast provides information that a female could use to assess the health or condition of the male requires further study.

Red plumage in this and other species is attributable to dietary carotenoids that are either deposited directly into feathers or modified before deposition (reviewed by McGraw 2006). In cardinals, red coloration is primarily due to the carotenoid pigments canthaxanthin, astaxanthin, adonirubin, and a-doradexanthin (Hudon 1991), which the birds synthesize from yellow pigments obtained in the diet (McGraw et al. 2001). Variation in carotenoid coloration among individuals may be related to variation in dietary acquisition of the proper precursors, as well as variation in carotenoid metabolism and deposition (Linville & Breitwisch 1997; McGraw & Hill 2001; McGraw et al. 2001). Dietary carotenoids used in or converted for use in plumage pigmentation also function as antioxidants and contribute to immune function (reviewed by von Schantz et al. 1999). Over the past decade, many investigators have hypothesized that carotenoid-based coloration may signal immunocompetence and therefore provide information on resistance to disease, parasites or stress. For example, dietary supplementation of carotenoids enhances immune function (e.g. Blount et al. 2003; McGraw & Ardia 2003; Fitze et al. 2007; but see Navara & Hill 2003), and many investigators have reported that brighter or more highly saturated carotenoid coloration is positively correlated with immunocompetence (e.g. Birkhead et al. 1998; McGraw & Ardia 2003; Saks et al. 2003; Peters et al. 2004; Hill & Farmer 2005; but see Camplani et al. 1999; Navara & Hill 2003).

In this study, we tested whether in northern cardinals, carotenoid coloration of the male’s breast contains information related to immune status. We also evaluated another possible signal, the female’s orange–red underwing coverts, which some hypothesize may signal her quality to the male (Linville et al. 1998; Jawor & Breitwisch 2003; Jawor et al. 2004). The coloration of the underwing coverts, which are visible during flight, correlates positively with parental behavior (Linville et al. 1998) as well as body size and condition (Jawor et al. 2004). We analyzed the coloration of the males’ upper breast and the females’ underwing coverts using reflectance spectrophotometry and principle component analysis (PCA) (Cuthill et al. 1999; Grill & Rush 2000; Andersson & Prager 2006; Montgomery...
2006). To assess immune status, we used two fundamental indices: total white blood cell (WBC) count and a differential count of two types of WBCs, heterophils and lymphocytes (Campbell & Dein 1984; Campbell 1995; Smits 2007). These measures give accurate representations of immune activity and stress (Gross & Siegel 1983; Apanius 1998; Vleck et al. 2000; Davis et al. 2004), while at the same time are relatively non-invasive and can be accomplished rapidly in the field without retaking the birds in captivity. Because higher values of both variables indicate higher levels of infection or stress (Gross & Siegel 1983; Vleck et al. 2000), we predicted that brighter, more saturated carotenoid-based coloration would be associated with lower WBC counts and heterophil to lymphocyte (H:L) ratios. As an independent measure of condition, we also calculated body condition based on mass and size, and predicted that this variable would correlate positively with carotenoid-based coloration (Jawor & Breitwisch 2004; Jawor et al. 2004).

**Methods**

**Animals**

This study was conducted in Fernbank Forest in Atlanta, GA (33°46’N, 84°19’W), and at Stone Mountain Park in Stone Mountain, GA (33°48’N, 84°09’W). Thirty-eight northern cardinals (20 males and 18 females) were captured using mist nets between 7:30 AM and 3:30 PM EST from late February through early May 2006, which coincided with the early to mid-breeding season (C. S. pers. obs.; see also Halkin & Linville 1999). All birds were banded with numbered metal leg bands issued by the US Fish and Wildlife Service and released at the site of capture.

**Morphological Measurements**

For each bird, we recorded the age as determined by plumage and bill characteristics (DeSante et al. 2007), mass, and wing chord length. Any body molt, injuries, illness or disease were noted. All birds were adults (after hatch year), and none were undergoing body molt. Because breeding effort may be considered a possible stressor, we also looked for evidence of sexual or parental activity, as follows: for males, we rated the development of the cloacal protuberance (CP), an androgen-dependent structure that houses sperm inside the seminal vesicle (Bailey 1953). CP development was scored according to the following scale (Pyle 1997; DeSante et al. 2007): 0 = not enlarged; 1 = somewhat enlarged and noticeably swollen, conical; 2 = large, with a diameter as large near the tip as at the base (not conical); and 3 = very large, with a diameter considerably larger in the middle than at the base (bulbous). Breeding effort was noted in females by scoring the brood patch as follows (DeSante et al. 2007): 0 = absent, 1 = smooth, 2 = vascular, 3 = heavy, 4 = wrinkled, and 5 = molting.

**Spectrophotometry**

Color (reflectance) measurements were taken with a USB4000 miniature fiber optic reflectance spectrophotometer from Ocean Optics (Dunedin, FL, USA), using an LS-1 Tungsten halogen light source, an R400-7-VIS/NIR reflection probe, and an RPH-1 probe holder from Ocean Optics. Data were collected onto a PC laptop running OOIrrad2 software version 2.05.00 PR 12 from Ocean Optics. Measurements were taken while holding the probe at a 45° angle (see Cuthill et al. 1999; Andersson & Prager 2006). The angle as well as the distance from the bird to the probe were fixed and determined by the probe holder, which was held against the bird during each reading. A dark reference taken prior to each reading was subtracted by the computer software from the reflectance measurements. For males, six measurements were taken from the upper breast area, which is the plumage with the most intense carotenoid coloration. Two readings were taken from each female—one from each underwing area, which represents the most intense carotenoid coloration of the plumage in females. Each reading produced one spectrum spanning 400–700 nm and consisting of 845 reflectance measurements. For each of these wavelength intervals, the readings from each bird were averaged and expressed as a percentage of the value obtained for that interval from a WS-1 diffuse reflection standard (Ocean Optics), which is uniformly reflective across 250–2000 nm.

**Analysis of Spectral Shape**

Data collected using the spectrophotometer were analyzed using PCA (Cuthill et al. 1999; Grill & Rush 2000; Montgomerie 2006), which allows the researcher to collapse the large number of reflectance data into a small number of principle component (PC) scores for each bird. This method offers several advantages over more traditional calculations of color variables such as hue, saturation, and brightness (HSB); unlike HSB variables, PC scores
are statistically independent from each other, do not rely on assumptions about color vision, and can describe and account for complex variation across the spectrum (Montgomerie 2006). Reflectance data from male and female birds were analyzed separately. Normalized reflectance spectra were divided into relatively large bins spanning 20 nm (e.g. 400–419, 420–439 nm, etc.), and PCA was performed on the median values from each bin using SPSS version 11 for Macintosh (Chicago, IL, USA).

In order to characterize the variation that each PCA captured and thus interpret the PCs, we generated correlation coefficients between each PC and reflectance for each wavelength bin, and examined how these coefficients varied according to the wavelength. Based on the published literature (Cuthill et al. 1999; Grill & Rush 2000; Montgomerie 2006), we expected that the coefficients for PC1 would be similar in magnitude across all wavelengths, indicating a relationship between PC1 and overall brightness, and that the coefficients for any other PCs would depend on the wavelength, indicating a possible relationship between those coefficients and chroma and/or hue. Because we were primarily interested in testing the hypothesis that condition is related to the carotenoid content of plumage, we paid particular attention to coefficients in the ranges of wavelengths in which light is reflected and absorbed by carotenoid pigments (approx. 700 and 450 nm, respectively; see Andersson & Prager 2006).

**Calculation of Condition Variables**

*Body condition.* Condition was estimated using the residual body mass from a regression of body mass on wing chord (Brown 1996). These regressions were positive and statistically significant (males: \( F_{1,16} = 22.20, \ r^2 = 0.527, \) slope = 0.553, \( p < 0.001; \) females: \( F_{1,16} = 4.48, \ r^2 = 0.170, \) slope = 0.428, \( p = 0.05)\). Note that several other authors (e.g. Wolfenbarger 1999a,c; Jawor et al. 2003, 2004; Jawor & Breitwisch 2004) have used tarsus rather than wing chord to determine size or calculate body condition, so any comparisons of our results with theirs should be made with caution. We measured tarsus length in some but not all of the birds we captured, so tarsus could not be used in our calculations.

*H:L ratio.* Slides with blood smears were fixed with methanol and stained with Giemsa (Davis et al. 2004). We examined each smear with a microscope (Carl Zeiss AG; Oberkochen, Germany) at 1000× magnification (using the 100× objective) and counted all heterophils, lymphocytes, eosinophils, monocytes, and basophils following Campbell (1995). Slides were scanned until 100 WBCs or 150 fields of view were counted, whichever came first. All smears were examined by the same observer. The total number of heterophils was divided by the total number of lymphocytes to arrive at an H:L ratio for each bird. These ratios were arcsine square-root transformed to normalize them prior to analysis.

*WBC count.* The total number of WBCs per 10 000 red blood cells was estimated using the method of Campbell & Dein (1984), which has been used in wild passerines (e.g. Ots & Horak 1998; Figuerola et al. 1999; Horak et al. 2002; Ilmonen et al. 2003; Davis et al. 2004; Lobato et al. 2005; Owen & Moore 2006). These ratios were square-root transformed to normalize them prior to analysis.

**Data Analysis**

Color measurements were analyzed separately for males and females as described under ‘Analysis of Spectral Shape’ above. We looked for correlations between each of the PC’s, which by definition are statistically independent, and the other variables (CP score, brood patch, and the condition variables, see above). We controlled for the effect of date on CP and brood patch scores by including date as a variable in partial correlation tests, which, because CP and brood patch were scored on ordinal scales, were conducted on rank-transformed data (Conover & Iman 1981). Thus, we calculated a partial Spearman’s \( \rho \) for each test, which was then followed by a two-tailed significance test \( (\text{SPSS}) \). To look for correlations between each PC and the three condition variables, we used Pearson’s \( r \) with two-tailed significance tests \( (\text{SPSS}) \). Because leukocyte parameters...
may vary according to the time of day (Horak et al. 1998), tests on H:L ratio and WBC count were conducted using partial correlation tests to control for time as well as date of capture. The sequential Bonferroni correction was applied for multiple tests (Rice 1989). Significant correlations were then tested for robustness using jackknifing, or repeating the PCA and the correlation tests on a number of pseudo-replicate datasets, each lacking a different data point (Montgomerie 2006). We looked for effects of sex on body condition, H:L ratio, and WBC count, as well as effects of site of capture on those variables plus the PCs using MANOVAs with time of day and date of capture as covariates. We looked for effects of site of capture on CP and brood patch scores using Mann–Whitney U-tests.

Results

We noted no obvious injuries, disease or other possible stressors. Eleven males had discernable CPs (x̄ = 1, IQR 2.00) and six females had brood patches (x̄ = 0, IQR 1.00). The average H:L ratio was 0.150 ± 0.028 SEM for males and 0.134 ± 0.023 SEM for females, which was within the range reported for wild passerines (see Davis et al. 2004). The average WBC count was 33.7 ± 4.14 SEM for males and 40.4 ± 3.35 SEM for females, which is also within the range of values obtained using this method of estimation (compared with 17.1 in pied flycatchers, Ficedula hypoleuca, Lobato et al. 2005; 22.8 in cirl buntings, Emberiza cirlus, Figuerola et al. 1999; and 74.9 in house finches, Carpodacus mexicanus, Davis et al. 2004). There were no sex differences in body condition, H:L ratio or WBC count (MANOVA: F₃,₃₃ = 0.871, p = 0.466), and no effects of site of capture on those variables or the PCs in males (MANOVA: F₅,₁₁ = 0.786, p = 0.581) or in females (MANOVA: F₅,₁₀ = 1.172, p = 0.387). There were no effects of site of capture on CP score (U = 16.5, p = 0.944) or brood patch score (U = 31.5, p = 0.910).

Males

Reflectance spectra from the males’ upper breast area are plotted in Fig. 1a. The peak reflectance occurred at approx. 675 nm and the minimum reflectance at approx. 490 nm. The PCA yielded two PCs; the first explained 77% of the total variance, and the two PCs together explained 94%. Figure 1b shows how each PC was related to reflectance across the spectrum. The coefficients for PC1 were all positive and of similar magnitude across all wavelengths, indicating that as expected, PC1 correlated with reflectance broadly. PC2 was related to reflectance positively at longer wavelengths and negatively at shorter wavelengths, indicating that it describes the variation in the relative ratio of longer- to shorter-wavelength reflectance. Carotenoid chroma or saturation is defined as the percentage of total reflectance that falls in the upper range of the spectrum as opposed to the lower range (i.e. it is proportional to the difference between reflectance at 700 nm and that at 450 nm; see Andersson & Prager 2006), suggesting that PC2 was positively related to carotenoid coloration of the upper breast area in males.

We found that PC2 was significantly correlated with CP score (partial Spearman’s ρ = 0.575, p = 0.010; Fig. 2) and H:L ratio (r = 0.653, p = 0.004; Fig. 3). Jackknifing analysis showed that
these relationships were robust and their statistical significance and large effect size did not rely on any single data point. PC1 was not correlated with CP score (partial Spearman's \( r = -0.032, \ p = 0.900 \)), and neither PC was significantly related to body condition or WBC (Table 1). Because carotenoid saturation increased with increasing PC2 scores (Fig. 1b), this relationship suggests that carotenoid-based coloration is positively related to CP score.

In males, PC2 was significantly correlated with H:L ratio (see Fig. 3). In females, WBC count was significantly correlated with PC2 (see Fig. 5). H:L ratio, heterophil to lymphocyte ratio; WBC, white blood cell.

<table>
<thead>
<tr>
<th>Condition measurement</th>
<th>Males</th>
<th>Females</th>
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<tbody>
<tr>
<td></td>
<td>PC1</td>
<td>PC2</td>
</tr>
<tr>
<td>Body condition</td>
<td>-0.214</td>
<td>0.277</td>
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<tr>
<td>H:L ratio</td>
<td>0.257</td>
<td>0.653*</td>
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<tr>
<td>WBC count</td>
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<td>0.060</td>
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In males, PC2 was significantly correlated with H:L ratio (see Fig. 3). In females, WBC count was significantly correlated with PC2 (see Fig. 5). H:L ratio, heterophil to lymphocyte ratio; WBC, white blood cell. *\( p = 0.004 \).

CP, and between PC2 and H:L ratio indicate that males with higher carotenoid saturation in the upper breast area had higher CP scores (Fig. 2) and higher H:L ratios (Fig. 3) than males with lower carotenoid saturation in that area.

**Females**

Reflectance spectra from the female underwing area are plotted in Fig. 4a. The peak reflectance occurred at approx. 670 nm and the minimum reflectance at approx. 460 nm. The PCA yielded two PCs: the first explained 86% of the total variance, and the two PCs together explained 95%. Figure 4b shows how each PC was related to reflectance across the spectrum. The coefficients for PC1 were, as for the males, positive and similar across all wavelengths, indicating that PC1 described achromatic variation in brightness. The relationship between PC2 and reflectance was, interestingly, opposite from what was found for male breast coloration. PC2 in females was *inversely* related to reflectance at longer wavelengths, and *positively* related to reflectance at shorter wavelengths. Therefore, birds with high values for PC2 had lower than average reflectance in the wavelength range reflected by carotenoid pigments, and higher than average values in the range where the pigments absorb light, suggesting that unlike in males, PC2 was *inversely* related to the carotenoid content of underwing coverts. Therefore, any correlations between PC2 and the condition variables in females should, according to our hypotheses, be in a direction opposite from those observed in males.

All \( r \) values for correlations between the PCs and the condition variables are given in Table 1. PC2 was significantly correlated with WBC count (\( r = -0.680, \ p = 0.004; \) Fig. 5). Jacknifing analysis showed that the large effect size and statistical significance of this correlation did not rely on any single data point.
Because carotenoid saturation of underwing coverts decreased with increasing PC2 scores (Fig. 4b), the negative relationship between PC2 and WBC count indicates that females with higher carotenoid saturation in the underwing coverts had higher WBC counts than females who were less saturated in that area (Fig. 5). Neither PC was significantly related to body condition, H:L ratio (Table 1) or brood patch score (PC1: partial Spearman’s \( \rho = -0.247, p = 0.339 \); PC2: \( \rho = -0.310, p = 0.225 \)).

**Discussion**

In this study, we analyzed plumage coloration using PCA of reflectance spectra (Cuthill et al. 1999; Andersson & Prager 2006). Each of our analyses produced two PCs, the first of which was related to brightness uniformly across the spectrum and the second related to carotenoid saturation (Figs 1b and 4b). In both sexes, PC2 was related to an immune parameter. Because of the type of variation captured by PC2, our results suggest that males with higher carotenoid content in the upper breast feathers had higher H:L ratios than males with relatively low carotenoid saturation in that area (Fig. 3). Furthermore, our analysis suggests that females with higher carotenoid content in the underwing coverts had higher WBC counts than females with lower carotenoid saturation in that area (Fig. 5). In both sexes, therefore, carotenoid-based coloration was positively linked to immune parameters that increase during an active immune response or stress (e.g. Gross & Siegel 1983; Vleck et al. 2000; Ruiz et al. 2002; Davis et al. 2004).

If carotenoid coloration evolved in northern cardinals as an ornament that signals condition, one would predict carotenoid ornamentation to correlate negatively, not positively, with variables associated with stress or infection. The correlations we report between PC2 and H:L ratio in males and WBC count in females were therefore unexpected. Other investigators have reported positive relationships between red coloration in this species and measures of mate quality such as body condition, territory quality, and parental behavior (Linville et al. 1998; Wolfenbarger 1999a; Jawor & Breitwisch 2004; Jawor et al. 2004). Many studies in other avian species have suggested that deposition of carotenoids in plumage and other ornaments is associated with better body condition,
lower leukocyte counts, lower parasite loads, higher immunocompetence, and sexual attractiveness (e.g. Thompson et al. 1997; Birkhead et al. 1998; Blount et al. 2003; Figuerola et al. 2003; Saks et al. 2003; Alvarez et al. 2005; Hill & Farmer 2005; Mougeot et al. 2007). We are not the first, however, to report evidence of a positive association between carotenoid ornamentation and leukocyte parameters; the intensity of yellow plumage in cirl buntings and great tits (Parus major) is positively related to the relative proportions of heterophils (Duva & Allander 1995; Figuerola et al. 1999). In both cases, the authors concluded that the increased heterophils in the brightly colored individuals indicated enhanced resistance to parasites and superior immune function rather than an active infection or stress. Studies such as these and others have thus created some confusion in the literature regarding the interpretation of leukocyte profiles. The vast majority of published work, however, supports the idea that increased numbers of heterophils relative to other leukocytes, particularly lymphocytes (H:L ratio), represent a response to an active infection, disease, or stressor. This parameter is widely used as a condition index, particularly within the poultry industry, with high values invariably indicating a poorer state of health; birds with high H:L ratios have been deemed ‘sensitive’ to stressors whereas those with low ratios are deemed ‘resistant’ (e.g. Al-Murrani et al. 2006).

In passerines, H:L ratios increase in response to a wide variety of stressors, including long-distance migration (Owen & Moore 2006), parasitic infection (Davis et al. 2004; Lobato et al. 2005), and radioactive contamination (Camplani et al. 1999). In general, this measure appears to be reliable, even more reliable than plasma corticosterone, as an indicator of mild-to-moderate stress (McFarlane & Curtis 1989; Maxwell 1993; Vleck et al. 2000). Similarly, although decreases in total WBC under stressful conditions have been reported (Owen & Moore 2006), increases in total WBC count most likely indicate a response to a pathogenic agent (Apanius 1998) and are inversely related to recruitment (Lobato et al. 2005). Our results are therefore not consistent with the hypothesis that highly saturated carotenoid pigmentation signals a vigorous state of good health.

Why would more intense carotenoid coloration be associated with higher levels of infection or stress? We consider two hypotheses here, both of which have been discussed extensively in the literature. First, because carotenoids are important for immune function and cannot be used by the immune system once they are deposited in feathers, there may be a trade-off between investing carotenoids in plumage and investing them in immune defense (see von Schantz et al. 1999). If such a trade-off exists, then brightly colored individuals may pay the price for their plumage by suffering from more infections. For example, brightly colored common redpolls (Carduelis flammea) were less likely than duller individuals to survive a salmonellosis epidemic (van Oort & Dawson 2005; but see Nolan et al. 1998). More direct tests of the trade-off hypothesis show that carotenoid supplementation enhances both ornament pigmentation and immune function (Blount et al. 2003; McGraw & Ardia 2003) and that mounting an immune response can result in duller carotenoid ornamentation (Faiivre et al. 2003; Fitze et al. 2007). The biochemical mechanisms underlying a potential trade-off, however, namely how and whether plumage coloration and immune function are limited by carotenoid intake, metabolism or deposition, are complex issues still under intense scrutiny (Hill 1999; McGraw et al. 2005; Fitze et al. 2007). In any case, if the immune system incurs a cost by the deposition of carotenoids in ornaments, it seems unlikely that this cost would manifest itself as an increase in H:L ratio or total WBC as we saw here, because these measures represent active immune responses. Our results are not necessarily consistent with the hypothesis that more vividly colored individuals are immunocompromised compared with duller ones, but rather that they are more likely to be exposed to stressors, disease or infections that trigger immune responses. In other words, the link we report between plumage coloration and immune parameters may have a behavioral explanation.

The second hypothesis we consider here, which is more consistent with our results than the trade-off hypothesis, is that coloration in northern cardinals is positively related to indices of stress because the more colorful individuals engage in more potentially stress-inducing behaviors, such as territorial defense and parental care. Previous research indicates that redder males pair with earlier breeding females and defend higher quality territories (Wollenberger 1999a), requiring them to engage in more competitive behavior. If plumage coloration is manipulated prior to the establishment of dominance hierarchies in this species, a male’s rank is predicted by how red his breast was before, not after, the manipulation, suggesting that aggressive behavior can be predicted by plumage coloration (Wollenberger 1999b). Either as a cause or a result of aggressive interactions, the more competitive males may have higher levels of testosterone (reviewed by Wingfield et al. 1990; see
also Jawor 2007), which increases glucocorticoid levels and susceptibility to parasitic infection and injury (reviewed by Wingfield et al. 2001; Roberts et al. 2004). Although we did not measure testosterone directly, we found a significant correlation between PC2, which captured variation in carotenoid saturation, and the size of an androgen-dependent secondary sex character, the CP (Fig. 2). CP size is probably not a good indicator of plasma testosterone at the time of measurement (but see van de Crommenacker et al. 2004); however, its development is under androgenic control (Witschi 1945; Bailey 1953; Schwabl & Farner 1989; Deviche 1992) and may indicate the cumulative exposure to testosterone over several days or weeks (Bailey 1953). Variation in the cumulative androgen exposure over time may explain variation in the immune parameters that we measured; exogenous testosterone treatment has been shown to increase WBC counts in house sparrows (Passer domesticus; Puerta et al. 1995) and H:L ratios in red jungle fowl (Gallus gallus; Zuk et al. 1995), which is consistent with the results we report here. Although others have shown evidence that testosterone may enhance carotenoid coloration by increasing carotenoid bioavailability and transport (Blas et al. 2006; McGraw et al. 2006), our results do not address the role of androgens in plumage coloration because CP development does not provide information on androgen levels during molt, which occurs in fall (Halkin & Linville 1999). Our results are, however, consistent with the hypothesis that plumage coloration attained in the fall may predict androgen levels during the following breeding season.

We hypothesize that variation in plumage coloration in males is a direct result of variation in competitive behavior that enhances nutritional state (Linville & Breitwisch 1997; Wolfenbarger 1999b; McGraw et al. 2005; Fitz et al. 2007), and that during the breeding season may drive increases in plasma androgens (reviewed by Wingfield et al. 1990). Competitive behavior and an increased level of androgens may each lead to more stress and infections (reviewed by Wingfield et al. 2001; Roberts et al. 2004). In this study, we provide indirect evidence suggesting that more saturated, redder males may experience higher androgen levels (Fig. 2) and that these males also have higher H:L ratios (Fig. 3). Our hypothesis would be supported further by future work showing that carotenoid-based coloration relates positively to territorial aggression during the breeding season, and that territoriality and androgen levels are linked to leukocyte parameters in this species. Similar mechanisms may be operating in females, which in this species sing and participate actively in territorial defense (Halkin & Linville 1999; Jawor et al. 2004; Vondrasek 2006) and have higher levels of testosterone than females of other passerine species (Jawor 2007). Alternatively, brightly colored females may be more susceptible to stress because they engage in more parental care (Linville et al. 1998), which in pied flycatchers increases both H:L ratio and WBC counts (Ilmonen et al. 2003). We saw no evidence, however, that carotenoid coloration was related to brood patch development.

We found no relationships between plumage coloration and body condition, which is consistent with work by Wolfenbarger (1999a) but not Jawor and colleagues, who reported positive relationships between the redness of the male breast and size (Jawor & Breitwisch 2004) and between female underwing redness and both size and condition (Jawor et al. 2004). We may have missed such correlations because our sample size was smaller, or because we used different methods for calculating body condition (see Methods) and measuring coloration. Whereas we entered binned reflectance data into the PCA (Cuthill et al. 1999; Grill & Rush 2000; Montgomerie 2006), Jawor entered measures of hue, saturation, and brightness. Other published methods, such as those employed by Wolfenbarger (1999a,b,c) have relied on human visual comparisons of plumage to color standards. As methods of color analysis become more standardized, comparing across studies will become more straightforward.

Our findings that plumage color is not associated with body condition and positively associated with indicators of stress and infection do not support the hypothesis that higher carotenoid-based coloration is a reliable signal of better health in this species. Interestingly, behavioral studies conducted in this species have failed to show evidence that coloration is used as a courtship or status signal. Preference tests conducted on captive individuals showed no evidence that females prefer brighter or redder males (Wolfenbarger 1999c). In social groups of captive males, coloration was not used as a status signal during the formation of dominance hierarchies (Wolfenbarger 1999b). The dominance studies were conducted during the non-breeding season, however, and do not rule out a role for coloration during territory defense. Jawor et al. (2003) showed that males and females mate assortatively according to plumage color; however, this finding does not address whether the birds use plumage color as a signal or
whether they are using another factor that itself correlates with color, such as territory quality (Wolfenbarger 1999a) or dominance (Wolfenbarger 1999b). Given the behavioral dissimilarities between northern cardinals and other dichromatic songbirds, it is certainly possible that dichromatism in this species has been governed by an unusual set of rules. The evolutionary function of the remarkable red coloration in this species will require further study.

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