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METHODS

How many color metrics do we need? Evaluating how different color-scoring procedures explain carotenoid pigment content in avian bare-part and plumage ornaments

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Abstract For a variety of technical and conceptual reasons, biologists have come to use several different methods to quantify the colors of animals. However, the relative abilities of these different color-scoring procedures to capture variation in the actual color-generating mechanisms—pigment or structural composition of the integument—have never been tested systematically. Here, we examined which commonly employed color metrics predict carotenoid content of ornaments in three avian species (house finch *Carpodacus mexicanus*, mallard duck *Anas platyrhynchos*, and zebra finch *Taeniopygia guttata*). We used spectrophotometry to measure reflectance spectra from beak and feather tissue, calculated numerous color metrics (e.g., hue, chroma, brightness, principal components, and tetrahedral color space position) from these spectra, and determined carotenoid content at the site of color measurement with high-performance liquid chromatography. We found that several principal component, tristimulus, and avian visual model metrics significantly correlated with carotenoid content of house finch feathers and duck beaks. Carotenoid content of mallard beaks was most closely correlated with brightness and saturation metrics, whereas in house finch feathers, carotenoid concentration was best captured by hue and saturation metrics. According to tristimulus scores and visual models, we found that the ultraviolet portion of the spectrum was not an essential predictor of variation in carotenoid content. Also, visual model chromatic contrasts generally were not significant predictors of carotenoid content, although some achromatic contrasts and tetrahedral color space vector parameters were. Our results indicate that numerous methods, especially tristimulus scores, are suitable for capturing pigment-based color variation in two carotenoid-containing ornaments, and we discuss the merits and shortcomings of these different approaches. In contrast, there were no significant relationships between any color metrics and the carotenoid content of zebra finch beaks, suggesting that other color-generating mechanisms besides carotenoids may contribute to color variability in this species.

Keywords *Anas platyrhynchos* · *Carpodacus mexicanus* · Ornamentation · Principal components analysis · *Taeniopygia guttata* · Tristimulus · Ultraviolet · Visual model

List of symbols and abbreviations

B	Brightness
H	Hue
HPLC	High-performance liquid chromatography
LWS	Long wavelength sensitive
MWS	Medium wavelength sensitive
PC	Principal component
PCA	Principal components analysis
S	Saturation
SWS	Short wavelength sensitive
UV	Ultraviolet
UV-Vis	Ultraviolet and human-visible
UVS	Ultraviolet sensitive
VS	Violet sensitive
θ	Theta in visual model; relative stimulation of SWS, MWS, and LWS photoreceptors

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φ	Psi in visual model: stimulation of the UV/V sensitive photoreceptors
r	*R* in visual model: chromaticity or spectral purity

Introduction

Animals use a variety of signaling modalities (e.g., visual, auditory, tactile, and olfactory) to communicate information to conspecifics and heterospecifics. A fundamental challenge in the field of animal communication is determining the appropriate method to quantify signal expression. For some signals, such as tail length (e.g., Saino and Møller 1996), determining a suitable method of measurement is relatively intuitive. However, for many other types of signals (e.g., acoustic, visual), quantification is more difficult, as there are multiple axes of trait variation (e.g., amplitude and frequency of calls) and signal detection is affected by the signaling environment (e.g., ambient lighting or sound transmission properties) as well as sensory-system sensitivities of signal receivers. To accommodate this complexity, and due to several technological advances (e.g., spectrophotometry and song analysis software), signal measurement techniques have proliferated in recent years.

Among forms of visual communication in animals, colorful ornaments have often been studied in sexual and social signaling contexts (e.g., Hill 2002; Peters et al. 2004a). However, after decades of evolving methodologies, there is no consensus or best way to quantify the spectral properties of a surface (Armenta et al. 2008; Hill 1998; Zuk and Decruyenaere 1994). The earliest systems of color measurement were based on subjective human perception (e.g., Munsell color chips; Zuk et al. 1990), but this ignored aspects of coloration (e.g., ultraviolet) to which many non-human animals are sensitive (Bowmaker and Hunt 1999). The advent of portable reflectance spectrophotometers, which have since become the gold standard of color measurement hardware and the method of choice for obtaining raw reflectance data, has allowed for objective quantification of reflected light across the animal-visible wavelengths (Zuk and Decruyenaere 1994; Quesada and Senar 2006). Since that time, the variety of analytical procedures to calculate metrics based on these spectral data has proliferated widely. These include hue, saturation, and brightness values that correspond to the three major axes of color variation perceived by human observers (Wyszecki and Stiles 1982), as well as principal components that are derived by transforming reflectance values from the ultraviolet and human-visible range into orthogonal factors (PCA; Cuthill et al. 1999). There are also a variety of equations employed to calculate hue (which provides information regarding the color's wavelength), saturation (spectral purity, similar to chroma), and brightness (total reflectance of surface) values from a given ultraviolet-visible (UV-Vis) spectral output (tristimulus variables; reviewed in Montgomerie 2006). Each of these approaches has its merits (Montgomerie 2006), but also its limitations, from ignoring UV reflectance when using some colorimeters (e.g., the Colortron, which does not collect data at wavelengths below 390 nm) to the problems of cross-study and even cross-species comparisons of principal components analysis (Montgomerie 2006).

To further refine color measurement procedures, physiologically based models of color vision were developed within the past few decades to quantify colors as animals perceive them (Endler and Mielke 2005; Vorobyev and Osorio 1998; Vorobyev et al. 1998). The implementation of these visual models has furthered several areas of research, including the identification of "hidden" sexual dimorphism among bird species (Eaton 2005), the potential for "private" channels of visual communication (Håstad et al. 2005), and new ways of capturing color variation (Delhey and Peters 2008). However, these analyses have been limited to determining the contrast among colorful patches within an individual or conspicuousness of colorful patches within the environment, and have rarely been compared with other metrics of coloration to date (Loyau et al. 2007). The method itself has several limitations. The receptor noise levels by which all contrasts are scaled have been estimated in only one species of bird, and changes in receptor noise are predicted to have significant effects on color discrimination (Lind and Kelber 2009). Also, these receptor noise-based models are valid only in bright light conditions. At this early stage of research, it is unclear if and how the incorporation of visual model metrics can be applied to our understanding of the control, production, or function of animal colors.

We are presently left with a menu of sophisticated data-processing methods from which to choose, but without much guidance as to the appropriateness of any given method for studying particular taxa, research questions (but see Armenta et al. 2008), or types of coloration (e.g., pigmentary versus structural). However, the need for guidance is evident; many areas in biology, including visual ecology, honest signaling theory, and the evolution of aposematism, among others, would benefit greatly with the identification of the most appropriate color metrics. Certainly no one would expect a single metric or approach to be universally appropriate, but what is needed is a rigorous comparative investigation of these modern methods within a study system (e.g., Zuk and Decruyenaere 1994), to shed light on the redundancies or unique insights that these different approaches might offer.

Therefore, the goal of this study was to assess the ability of these various color metrics to capture variation in an important mechanism of color production—carotenoid pigment accumulation in colorful integuments of birds. Operationally, we consider color as the wavelength-specific surface reflectance of an object (Andersson and Prager 2006). Prior tests of pigment-color relationships have focused on single species and few coloration metrics (Inouye et al. 2001; Saks et al. 2003; Shawkey et al. 2006) and have never compared the relationships of integumentary carotenoid content to spectra-derived PCs, multiple hue and brightness metrics, or any visual model parameters. Here, we used three frequently studied species that display carotenoid-pigmented ornaments (mallard ducks (*Anas platyrhynchos*), house finches (*Carpodacus mexicanus*), and zebra finches (*Taeniopygia guttata*)). We measured their carotenoid-dependent traits using two different color-scoring instruments (Colortron and UV-Vis spectrophotometers), all major, published techniques for color metric calculation from spectral data (PCA, three brightness, 15 saturation, and five hue calculations), as well as contrast and tetrachromatic color space calculations from ultraviolet-sensitive (UVS; blue tit, *Cyanistes caeruleus*), violet-sensitive (VS; peafowl, *Pavo cristatus*), and species-specific visual models.

We chose to study the carotenoid coloration of birds for several reasons. Carotenoids are the pigments responsible for many of the yellow, orange, and red colors found in vertebrate integument, and carotenoid-based colors in birds are inherently more variable than other color-generating mechanisms (Delhey and Peters 2008). Due to the beneficial physiological roles that carotenoids play in many animals, carotenoid-based coloration, in which only high-quality individuals can deposit relatively high levels of carotenoids in their integument, has become a model system for studying honest signals and life-history trade-offs (Blount and McGraw 2006; McGraw 2006; Saks et al. 2003). Thus, by comparing coloration metrics to the carotenoid content of the tissue at the site of color measurement, we can (a) further the work of earlier studies that examined a few subsets of hue, saturation, and brightness metrics, and (b) evaluate the ability of visual models to capture carotenoid-dependent color variation and the relative importance of variables that utilize the UV portion of the spectrum (e.g., Bleiweiss 2005).

Materials and methods

Species and husbandry

We acquired 15 mallards as 1-day-old ducklings (ssp *platyrhynchos*) from McMurray Hatchery (Webster City, IA, USA) and reared them under different housing, lighting, and temperature conditions as part of a developmental study (Butler and McGraw 2009). We also captured 15 male house finches at feeder traps on Arizona State University's campus from 24 to 28 November 2006 (for details, see Toomey and McGraw 2009). Fifteen male zebra finches came from a captive population at ASU that is genetically similar to wild-caught individuals (Forstmeier et al. 2007). Male zebra finches were housed in pairs in small wire cages (McGraw 2005) in an indoor room on a 14:10 h light/dark cycle. We fed them an *ad libitum* diet of tap water, cuttlebone, and a commercial birdseed mix (Kaytee® Forti-Diet™ finch blend, Kaytee Products Inc., Chilton, Wisconsin; McGraw et al. 2002). These sample sizes are similar to those of many behavioral ecology studies that examine color (e.g., Maney et al. 2008; Reudink et al. 2009; Solís et al. 2008), including studies that utilize PCA (see below; Mahler and Kempenaers 2002; Mays et al. 2004; Parker et al. 2003).

Color measurement

We used an Ocean Optics (Dunedin, FL, USA) USB2000 spectrophotometer with a PX-2 pulsed xenon light source to collect reflectance values for wavelengths between 300 and 700 nm for all colorful tissues (integration time, 120 ms; 15 readings averaged per recording; boxcar 5; OOIBase 32, version 2.0.1.4). All measurements were taken at coincident-normal (a single probe emits the source light and collects reflected light, held perpendicular to the surface of interest), and the spectrophotometer was standardized to a Spectralon white standard (Labsphere Inc., North Sutton, NH, USA) between individuals and to a dark standard to correct for electrical noise before each testing period. We measured the reflectance of a single point of each species' colorful patch (the dorso-lateral surface of the mallards' beaks halfway between the nares and the beak tip, the pigmented distal portions of house finch breast feathers mounted on black cardstock (Quesada and Senar 2006), and the lateral surface of the zebra finch beak) five times so that we could calculate repeatability (see below). Similarly, we used a 36-band Colortron II visible-light reflectance spectrophotometer (Light Source Inc., San Rafael, CA; see Hill 1998 for details) to collect hue (H), saturation (S), and brightness (B) reflectance values from the same spot, again with five iterations. We then carefully trimmed the pigmented feather tips (house finches; 1–3 mg), removed soft beak tissue from underlying bony structure in recently euthanized mallards (10–40 mg), or excised with a razor a portion of the outer, dead beak tissue (zebra finches, 0.7–1.4 mg; *sensu* Bright et al. 2004) from which we had just

collected color data for use in subsequent integumentary carotenoid analyses.

Tissue carotenoid analyses

We weighed tissue samples to the nearest 0.001 mg, and transferred the sample to a 1.5 ml screw-top microcentrifuge tube for pigment extraction (see McGraw and Toomey 2010). Using methods optimized for each species (Inouye et al. 2001; McGraw and Toomey 2010; MWB unpublished data), we added 1 ml of 1:1 hexane/methyl tert-butyl ether (MTBE; duck and zebra finch bill) or methanol (house finch feathers) and ground them for 3 min at 30 Hz in a ball mill (MM200, Retsch GmbH and Co. KG, Haan, Germany). After centrifugation at 3,000 RPM for 5 min, the supernatant was collected, the process was repeated two more times, and then the total 3 ml of supernatant was evaporated to dryness under nitrogen and stored at −80°C until saponification (see more below). Saponification was not needed for the feather samples (McGraw et al. 2006), so they were immediately prepared for high-performance liquid chromatography (HPLC) analysis following extraction.

Beak extracts were saponified to remove fatty acid esters from carotenoids, which interfere with HPLC elution, by adding 1 ml of a basic solution (0.5 M methanolic NaOH for mallards, 0.02 M methanolic KOH for zebra finches), capping under nitrogen, and holding in the dark at room temperature for 6 h (*sensu* McGraw and Toomey 2010). We then added 2 ml of saturated salt solution, vortexed the mixture, and added 3 ml of 1:1 hexane/MTBE and again shook the solution. The mixture was then centrifuged at 3,000 RPM at room temperature for 5 min; the supernatant was transferred, dried down, and reconstituted for HPLC analysis as described in McGraw et al. (2008). Major carotenoid types were identified for each species by comparison to authentic reference pigments, and concentrations determined based on external standard curves and tissue sample mass. Mallard beaks contained predominantly lutein and zeaxanthin (mean: 30% and 37%, respectively). House finch feathers were made up of "yellow" xanthophylls (canary xanthophylls A and B, dehydrolutein, lutein, and zeaxanthin) and "red" keto-carotenoids (astaxanthin, canthaxanthin, echinenone, 3-hydroxy-echinenone, adonirubin, and 4-oxo-rubixanthin), making up, on average, 41% and 59% of total carotenoids, respectively (see McGraw et al. 2006). Zebra finch beaks contained predominately keto-carotenoids (α-doradexanthin, adonirubin, astaxanthin, and canthaxanthin, mean: 90% of total) along with small amounts of xanthophylls (lutein and anhydrolutein; McGraw and Toomey 2010). The concentrations of all individual carotenoid types were correlated with total carotenoid titer within all species (all $r>0.8$, all $P<0.003$), so we used total carotenoid concentration in subsequent statistical analyses.

Color quantification—Colortron

Hue, saturation, and brightness values were provided directly to us by the Colortron software (ColorShop 2.6.1, San Rafael, CA, USA) using a human-based cone capture model.

Color quantification—UV-Vis reflectance spectrophotometry

To calculate coloration from UV-Vis spectral data, we binned all reflectance values using the JAVA-based program CLR (version 1.05, Montgomerie 2008) by 1 nm for all color metric calculations except for principal components analysis (PCA; see below). We further used CLR to calculate brightness (overall reflectivity, B), saturation (proportional reflectance, S), and hue (specific wavelength, H) values via the most commonly used functions in the signaling literature (B1, B2, B3, S1R, S1G, S1B, S1U, S1v, S1Y, S2, S3, S5a, S5b, S5c, S6, S7, S8, S9, H1, H3, H4a, H4b, and H4c; mathematically defined in Montgomerie 2006; CLR version 1.05 README file; Appendix 1) for each carotenoid-pigmented trait. The five replicate measurements for each individual were used to calculate repeatability (Lessells and Boag 1987) and averaged for subsequent analyses. Because it is advisable to have five times the number of subjects as variables in PCA (Grimm and Arnold 1995), researchers should use larger bin sizes (Montgomerie 2006), although larger bins can result in a less precise reflectance curve. Therefore, to test the effect of bin size on the ability of PCA to capture variation in carotenoid content, we used a principal component analysis (SAS 9.2, Cary, NC, USA; Proc PRINCOMP) to acquire orthogonal variables from data binned at 1, 2, 5, 10, and 20 nm, using all eigenvectors with eigenvalues that were greater than one (1 nm loadings; Fig. 1).

Color quantification—avian visual modeling

The spectral sensitivities of avian visual systems are relatively conserved among species (Hart and Hunt 2007), with the major recognized difference occurring in the tuning of the UV/VS cone. Thus, visual systems have been broadly categorized as UVS or VS, depending on the tuning of this photoreceptor (Ödeen and Håstad 2003). Among our study species, the mallard duck has been identified as VS (Jane and Bowmaker 1988; Ödeen and Håstad 2003), the zebra finch as UVS (Bowmaker et al. 1997; Ödeen and Håstad 2003), and although the spectral sensitivities of the house finch have not been directly

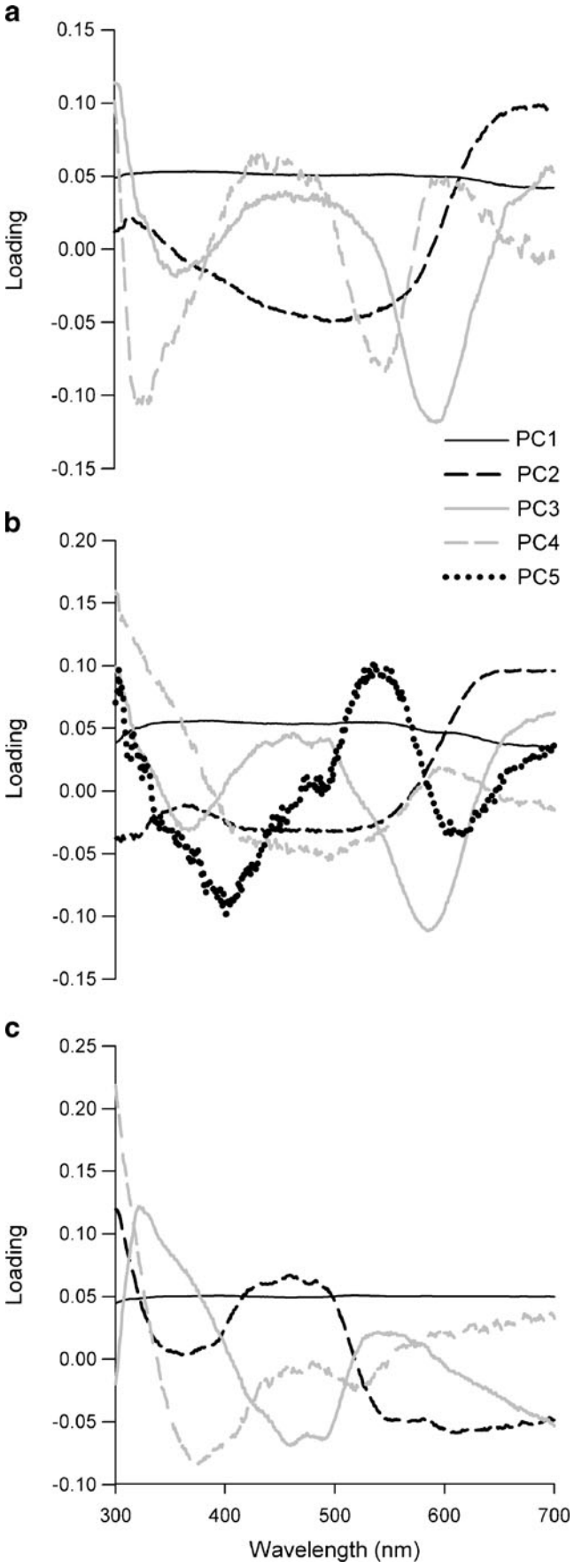

Fig. 1 Loading of principal components by wavelength for mallard duck beaks (**a**), house finch feathers (**b**), and zebra finch beaks (**c**)

measured, a member of the same subfamily, the canary *Serinus canaria,* is known to have a UVS visual system (Das et al. 1999). To capture this variation in visual systems, we calculated (see below, Appendix 2) the spectral sensitivity data of two species that have been used as model visual systems (blue tit, UVS; peafowl, VS; Avilés et al. 2008; Gomez and Théry 2007; Håstad et al. 2005), as well as species-specific visual systems (mallard, Jane and Bowmaker 1988; house finch using canary sensitivities, Das et al. 1999; zebra finch, Bowmaker et al. 1997), using models that account for oil droplet visual tuning (Appendix 2).

To model carotenoid-based coloration in the avian visual system, we took two approaches. Firstly, we calculated chromatic contrasts using the noise-limited receptor model of Vorobyev and Osorio (1998; see also Vorobyev et al. 1998) by contrasting ornament coloration against adjacent plumage regions, rather than against environmental structures (e.g., leaf litter and pond water), which allowed us to avoid making assumptions about background spectra in the natural environment that vary over space and time. For house finches, we calculated the contrast between red breast plumage and the light brown/gray plumage of the vent region. For mallard ducks, we compared yellow beak coloration to the dark brown breast plumage. For zebra finches, we calculated the contrast of the beak against the dark gray striped breast of the males. We used an ambient light spectrum collected outside at 1200 h during late summer in Tempe, AZ, and contrasts were calculated according to Avilés et al. (2008; Appendix 2). Secondly, we mapped the location of each ornament in tetrahedral color space, defined by the relative stimulation of the four single-cone photoreceptors involved in color vision (Stoddard and Prum 2008). We plotted the locations of each carotenoid ornament in tetrahedral color space following Stoddard and Prum (2008) using the UVS, VS, and species-specific visual parameters. We then plotted each ornament as a vector in spherical coordinates extending from the achromatic central point of the tetrahedron. This vector was defined by two angles θ and φ and a magnitude *r*. The angle θ encoded the relative stimulation of the short- (s), medium- (m), and long- (l) wavelength sensitive photoreceptors. The angle φ encoded the stimulation of the UV/VS cone. The magnitude *r* was a measure of the chromaticity or spectral purity of the ornament.

Statistical analyses

Firstly, we tested the repeatability of all coloration metrics acquired from the Colortron, the UV-Vis spectrophotometer, and visual models. Then, to test for relationships

between color metrics and carotenoid content of tissues, we used several approaches. We tested all variables for normality, and found that some variables (and the residuals from parametric correlation analyses) departed from normality, a subset of which could not be transformed to achieve normality. Therefore, to compare all statistical relationships within the study in a consistent manner, we ran non-parametric tests on single variables, and even though this may have reduced our power (Zar 1999), this seemed to be the most conservative and informative approach. We ran Spearman rank correlations within each species to test which color metrics were associated with total carotenoid content of the tissue.

Due to the number of analyses performed (nearly 140 rank correlations; Tables 1, 2, 3, and 4), we do not address every relationship that trended toward significance ($0.05< P<0.1$), or even every traditionally significant relationship ($P<0.05$) in the text, largely due to our reluctance to advocate significance at the $\alpha=0.05$ level when we have such a large number of comparisons (although all analyses are available; Tables 1, 2, 3, and 4). Nor did we strictly correct for multiple comparisons here (as has been done previously with questions of similar scope; Cohen et al. 2008), because our goal was to generally evaluate whether color metrics reliably captured the carotenoid content of the colorful tissue, rather than testing for explicit mathematical relationships for any given color metric.

Results

Repeatability

Repeatability estimates varied widely among the coloration metrics and ranged from 0.08 to 0.88 (mean=0.53) in mallards, 0.00 to 0.83 (mean=0.47) in house finches, and 0.02 to 0.82 (mean=0.43) in zebra finches (Table 5). However, the intraclass correlations (Zar 1999) demonstrated that variation within individuals was smaller than variation among individuals ($P<0.05$) for all but seven variables (one mallard, four house finch, and two zebra finch), meaning that most variables were significantly repeatable (Table 5).

Principal component analysis

Bin size did not qualitatively affect the correlation between carotenoid content and principal components (e.g., for house finches, only the third PC was significantly positively related to carotenoid content for all bin sizes; Table 1). Using the data binned at 1 nm, only the first principal component of the spectral data, indicative of overall brightness (Fig. 1a), was negatively related to total carotenoid concentration in the yellow beak of mallards (Table 2). In house finches, only the third principal component, indicative of the slope of the reflectance spectrum (Fig. 2b), was positively related to total carotenoid content of breast feathers (Table 3). No principal component was significantly related to total carotenoid content in the red beaks of zebra finches (all $P>0.3$).

Table 1 Spearman's rank correlations between total carotenoid content (μg/g) of avian integument and principal component scores at a variety of bin sizes in mallards (MADU), house finches (HOFI), and zebra finches (ZEFI)

Spp	Bin size	PC	r_S	P
MADU	2	1	−0.511	0.0303
		2	−0.455	0.0577
		3	0.164	0.5153
		4	0.203	0.4184
	5	1	−0.511	0.0303
		2	−0.455	0.0577
		3	0.164	0.5153
	10	1	−0.511	0.0303
		2	−0.455	0.0577
	20	1	−0.511	0.0303
HOFI	2	1	−0.314	0.2539
		2	0.146	0.6025
		3	0.654	0.0082
		4	−0.168	0.5499
	5	1	−0.304	0.2714
		2	0.146	0.6025
		3	0.639	0.0103
		4	−0.093	0.7420
	10	1	−0.296	0.2834
		2	0.154	0.5848
		3	0.661	0.0073
	20	1	−0.296	0.2834
		2	0.239	0.3904
		3	0.700	0.0037
ZEFI	2	1	−0.250	0.3688
		2	0.264	0.3412
		3	0.129	0.6479
		4	0.011	0.9698
	5	1	−0.250	0.3688
		2	0.264	0.3412
		3	0.129	0.6479
	10	1	−0.250	0.3688
		2	0.264	0.3412
	20	1	−0.250	0.3688
		2	0.264	0.3412

All eigenvectors with an eigenvalue greater than one were included

Table 2 Spearman's rank correlations between total carotenoid content (μg/g) of mallard duck beaks and a variety of coloration metrics

Independent variable	r_S	P
PC1	−0.69	0.0014
PC2	−0.45	0.0603
PC3	−0.07	0.7977
PC4	0.17	0.4941
B1	−0.69	0.0014
B2	−0.69	0.0014
B3	−0.71	0.0009
S1R	0.58	0.0111
S1G	0.47	0.0493
S1B	−0.78	0.0002
S1U	−0.15	0.5424
S1v	−0.21	0.3994
S1Y	0.56	0.0147
S2	0.50	0.0361
S3	0.10	0.6987
S5a	0.27	0.2722
S5b	0.23	0.3583
S5c	0.13	0.6099
S6	−0.34	0.1626
S7	−0.25	0.3155
S8	0.59	0.0101
S9	−0.79	0.0001
H1	0.19	0.4529
H3	−0.25	0.3155
H4a	0.03	0.8933
H4b	0.46	0.0552
H4c	0.36	0.1372
ColortronB	−0.10	0.6950
ColortronH	−0.37	0.1302
ColortronS	0.63	0.0050
Chromatic contrast—S	0.06	0.8040
Chromatic contrast—B	−0.37	0.1256
Chromatic contrast—P	0.03	0.8933
Achromatic contrast—S	−0.47	0.0504
Achromatic contrast—B	−0.47	0.0493
Achromatic contrast—P	−0.47	0.0504
θ—S	0.30	0.2260
φ—S	−0.46	0.0528
r—S	0.11	0.6568
θ—B	0.22	0.3762
φ—B	0.17	0.5100
r—B	−0.30	0.2293
θ—P	0.11	0.6746
φ—P	−0.65	0.0037
r—P	0.12	0.6215

Tristimulus scores are mathematically defined in Appendix 1, after Montgomerie (2008). Only PCs with eigenvalues greater than 1 were included. For visual model parameters (in the last 15 rows of the table), P signifies peafowl, B signifies blue tit, and S signifies species-specific visual parameters

Table 3 Spearman's rank correlations between total carotenoid content (μg/g) of house finch feathers and a variety of coloration metrics

Color metric	r_S	P
PC1	−0.31	0.2539
PC2	0.15	0.6025
PC3	0.65	0.0082
PC4	−0.14	0.6205
PC5	0.40	0.1435
B1	−0.20	0.4748
B2	−0.20	0.4748
B3	0.25	0.3688
S1R	0.47	0.0786
S1G	−0.64	0.0109
S1B	0.07	0.8003
S1U	−0.26	0.3549
S1v	−0.25	0.3760
S1Y	−0.70	0.0037
S2	−0.15	0.5936
S3	0.47	0.0786
S5a	0.08	0.7710
S5b	0.12	0.6757
S5c	0.12	0.6757
S6	0.24	0.3977
S7	0.54	0.0365
S8	0.54	0.0380
S9	−0.34	0.2212
H1	0.07	0.8141
H3	0.67	0.0061
H4a	−0.66	0.0078
H4b	−0.50	0.0557
H4c	−0.48	0.0711
ColortronB	−0.83	0.0001
ColortronH	−0.60	0.0180
ColortronS	−0.01	0.9798
Chromatic contrast—S	−0.10	0.7229
Chromatic contrast—B	−0.14	0.6296
Chromatic contrast—P	−0.18	0.5243
Achromatic contrast—S	−0.46	0.0839
Achromatic contrast—B	−0.45	0.0953
Achromatic contrast—P	−0.46	0.0839
θ—S	−0.70	0.0039
φ—S	0.15	0.6025
r—S	−0.11	0.6945
θ—B	−0.54	0.0380
φ—B	−0.21	0.4588
r—B	−0.16	0.5585
θ—P	−0.66	0.0073
φ—P	0.10	0.7229
r—P	−0.16	0.5585

See Table 1 caption for abbreviations

Table 4 Spearman's rank correlations between total carotenoid content (μg/g) of zebra finch beaks and a variety of coloration metrics

Color metric	r_S	P
PC1	−0.25	0.3688
PC2	0.28	0.3083
PC3	0.14	0.6296
PC4	0.04	0.8894
B1	−0.27	0.3344
B2	−0.27	0.3344
B3	−0.23	0.4126
S1R	0.32	0.2483
S1G	−0.48	0.0687
S1B	−0.30	0.2773
S1U	0.03	0.9195
S1v	−0.09	0.7517
S1Y	−0.25	0.3760
S2	0.29	0.2895
S3	0.32	0.2483
S5a	0.05	0.8695
S5b	0.18	0.5327
S5c	0.18	0.5159
S6	0.29	0.3019
S7	−0.21	0.4510
S8	0.33	0.2265
S9	−0.30	0.2714
H1	0.19	0.4895
H3	0.34	0.2130
H4a	−0.35	0.2009
H4b	−0.42	0.1212
H4c	−0.37	0.1773
ColortronB	−0.44	0.0993
ColortronH	−0.28	0.3167
ColortronS	0.17	0.5413
Chromatic contrast—S	0.28	0.3147
Chromatic contrast—B	0.28	0.3147
Chromatic contrast—P	0.26	0.3412
Achromatic contrast—S	0.26	0.3480
Achromatic contrast—B	0.28	0.3083
Achromatic contrast—P	0.27	0.3278
θ—S	−0.43	0.1110
φ—S	−0.09	0.7613
r—S	0.24	0.3977
θ—B	−0.40	0.1396
φ—B	−0.18	0.5327
r—B	0.26	0.3412
θ—P	−0.41	0.1247
φ—P	−0.37	0.1728
r—P	0.26	0.3412

See Table 1 for abbreviations

Spectrophotometer-generated tristimulus scores

All brightness values (B1, B2, and B3; note that B1 and B2 are very similar mathematically) were negatively related to total carotenoid content in the yellow beaks of male mallards (all $P<0.0014$; Table 2). However, spectrophotometer-generated brightness values were not significant predictors of carotenoid content in house finch feathers (all $P>0.37$; Table 3) or zebra finch beaks (all $P>0.33$; Table 4).

Spectrophotometer-generated saturation metrics (S) of mallard beaks effectively captured variation in tissue carotenoid content, accounting for one half of the significant relationships between coloration metrics and carotenoid content (seven out of 14 uncorrected significant relationships, $P<0.05$). S9 and S1B (which are more chromatic at more negative values; Fig. 2) showed the strongest correlations with total carotenoid content (Table 2). Similarly, total carotenoid content of house finch feathers was significantly related to multiple saturation metrics (four out of 12 significant relationships, $P<0.05$), including S1G and S1Y (Table 3), whereas there were no saturation metrics that significantly predicted the carotenoid content of zebra finch beaks (all $P>0.069$), although S1G showed the strongest relationship (Table 4).

There were no significant relationships between total carotenoid content and any hue metrics in mallard beaks ($P>0.05$). For house finch feathers, total carotenoid content of feathers was associated with more red-shifted reflectance spectra (H3, $P=0.0061$; Fig. 3). There were no hue metrics that significantly predicted the carotenoid content of zebra finch beaks (all $P>0.12$).

Colortron-generated tristimulus scores

Total carotenoid concentration of duck beaks increased with saturation (ColortronS; Table 2; $P=0.005$). In house finches, a greater total carotenoid content was associated with more red-shifted (ColortronH; Table 3; $P=0.018$) and less bright (B; Table 3; $P=0.0001$) feathers. There was a trend for zebra finch beaks to be less bright with an increasing carotenoid concentration (ColortronB; Table 4; $P=0.0993$), but this relationship was not significant.

Avian visual model

Increasing carotenoid concentration of mallard beaks was marginally associated with decreasing achromatic contrast regardless of visual system (Table 2; all $0.049<P<0.051$). The φ of mallard beaks decreased (indicating a reduction in the relative stimulation of the VS cone) with increasing carotenoid content using VS visual systems ($P=0.0037$), while there was a negative trend using the species-specific visual system ($P=0.0528$) and no relationship using the

Table 5 Repeatabilities of color metrics as calculated by Lessells and Boag (1987), with associated *P* values from intraclass regressions

Variable	Mallard		House finch		Zebra finch	
	Repeatability	*P*	Repeatability	*P*	Repeatability	*P*
Achromatic contrast—B	0.64	<0.0001	0.44	<0.0001	0.44	<0.0001
Achromatic contrast—P	0.65	<0.0001	0.44	<0.0001	0.42	<0.0001
Achromatic contrast—S	0.65	<0.0001	0.44	<0.0001	0.43	<0.0001
B1	0.58	<0.0001	0.40	0.0007	0.31	<0.0001
B2	0.58	<0.0001	0.41	0.0007	0.31	<0.0001
B3	0.57	<0.0001	0.43	0.0087	0.22	<0.0001
Chromatic contrast—B	0.28	0.0009	0.59	<0.0001	0.37	<0.0001
Chromatic contrast—P	0.33	<0.0001	0.58	<0.0001	0.35	0.0002
Chromatic contrast—S	0.34	<0.0001	0.57	<0.0001	0.37	<0.0001
ColortronB	0.88	<0.0001	0.74	<0.0001	0.20	0.0937
ColortronH	0.84	<0.0001	0.66	<0.0001	0.42	0.0040
ColortronS	0.84	<0.0001	0.49	0.0009	0.02	0.4209
H1	0.42	<0.0001	0.00	0.4841	0.23	<0.0001
H3	0.08	0.1512	0.63	<0.0001	0.82	<0.0001
H4a	0.84	<0.0001	0.70	<0.0001	0.79	<0.0001
H4b	0.49	<0.0001	0.54	<0.0001	0.63	<0.0001
H4c	0.49	<0.0001	0.53	<0.0001	0.59	<0.0001
r—B	0.28	0.0008	0.61	<0.0001	0.37	<0.0001
r—P	0.58	<0.0001	0.56	<0.0001	0.62	<0.0001
r—S	0.35	<0.0001	0.60	<0.0001	0.37	<0.0001
S1B	0.71	<0.0001	0.52	0.0004	0.33	<0.0001
S1G	0.63	<0.0001	0.67	<0.0001	0.79	<0.0001
S1R	0.62	<0.0001	0.47	<0.0001	0.49	<0.0001
S1U	0.44	<0.0001	0.38	0.0003	0.33	<0.0001
S1v	0.44	<0.0001	0.38	0.0003	0.34	<0.0001
S1y	0.66	<0.0001	0.83	<0.0001	0.54	<0.0001
S2	0.32	0.0002	0.08	0.1612	0.36	<0.0001
S3	0.28	0.0007	0.47	<0.0001	0.48	<0.0001
S5a	0.76	<0.0001	0.44	<0.0001	0.46	<0.0001
S5b	0.79	<0.0001	0.44	<0.0001	0.46	<0.0001
S5c	0.78	<0.0001	0.44	<0.0001	0.46	<0.0001
S6	0.70	<0.0001	0.42	<0.0001	0.49	<0.0001
S7	0.14	0.0470	0.73	0.0006	0.32	0.0070
S8	0.39	<0.0001	0.41	<0.0001	0.48	<0.0001
S9	0.68	<0.0001	0.39	<0.0001	0.38	<0.0001
θ—B	0.54	<0.0001	0.48	<0.0001	0.60	<0.0001
θ—P	0.36	<0.0001	0.60	<0.0001	0.36	<0.0001
θ—S	0.57	<0.0001	0.10	0.1183	0.61	<0.0001
φ—B	0.15	0.0325	0.16	0.0364	0.37	<0.0001
φ—P	0.46	<0.0001	0.09	0.1438	0.52	<0.0001
φ—S	0.49	<0.0001	0.42	0.0012	0.36	<0.0001

Metrics are considered repeatable if $P<0.05$

UVS visual system ($P=0.51$). For house finch feathers, θ decreased (indicating a shift towards increase stimulation of LWS versus MWS cones, consistent with a red-shifted spectrum) with increasing carotenoid content under VS ($P=0.0073$), UVS ($P=0.0380$), and canary ($P=0.0039$; Table 3) visual systems. There were no visual system model values that significantly predicted the carotenoid content of zebra finch beaks (all $P>0.11$; Table 4).

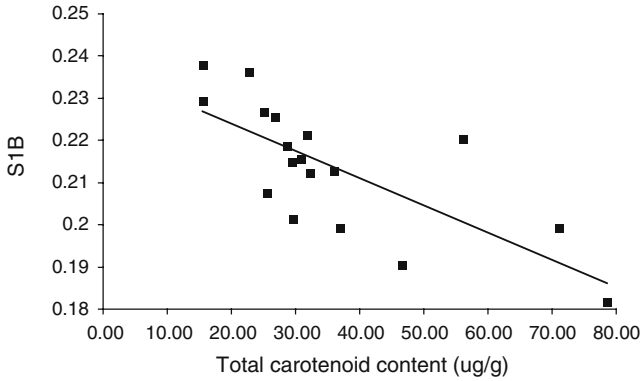

Fig. 2 Relationship between carotenoid content (μg/g) of mallard beaks and saturation (S1B), with an increasing carotenoid concentration associated with lower saturation values. Simple linear regression $F_{1,\ 16}$=18.76, P=0.005, residuals normally distributed

Discussion

We found that many of the frequently employed color variables (e.g., tristimulus scores, PCs) were significantly correlated with carotenoid content in bare parts and feathers of two avian species. Carotenoid content in mallard beaks corresponded with variables that capture brightness (including tristimulus brightness scores, achromatic contrasts, and PC1) and saturation, while carotenoid content in house finch feathers was modeled by hue (including PC3 and θ values in the visual model) and saturation metrics. These color parameters are the same as those previously recognized to be useful in mate selection, as female mallards prefer males with more yellow and presumably saturated beaks (Omland 1996) and female house finches prefer males with more red-shifted plumage (reviewed in Hill 2002).

Previous studies in mallards have shown correlations between individual quality and PC3 (described as UV chroma; Peters et al. 2004a, b), but not PC1. However, in our study it was PC1, and not PC3, that was associated with beak carotenoid content. This discrepancy is difficult to address (factor loadings are qualitatively similar between studies, based on graphical representations of the loadings), as inter-study principal components do not have intrinsic meaning to the biology of the system. This makes the results difficult to interpret because we cannot evaluate how the variation among our measurements compares to that of Peters et al. (2004a, b). Furthermore, while carotenoid content was correlated with PC1 in mallard beaks in our study, it was correlated with PC3 in house finch feathers. Thus, an investigator working with our house finch data and using the first two PCs would be neglecting the portion of spectral variance that related to the carotenoid content of the tissue. Therefore, while PCA has the advantage of generating a relatively small number of orthogonal metrics from a large number of reflectance values, our findings demonstrate that PCA is not inherently superior to other color metrics (e.g., tristimulus scores) in capturing variation in the carotenoid content of the tissue, nor is there consistency in which PC (e.g., PC1, PC2) would be associated with tissue carotenoid content. Also, although bin size did not qualitatively change the rank correlations between carotenoid content and principal components for any species, we caution that larger bin sizes may be more statistically appropriate, but result in spectral curves that are biologically less precise. Thus, for questions relating to the deposition of carotenoid content in avian integument, it is advisable to utilize other color metrics (e.g., tristimulus scores) that can capture variation in carotenoid content that avoid some of the drawbacks associated with PCA (e.g., difficulty in cross-studies comparisons, illustrated above). For example, many of the brightness and saturation (mallards) and saturation and hue (house finch) tristimulus scores not only outperformed PCs in predicting carotenoid content, but they are also directly comparable among studies and have *a priori* definitions regarding the component of coloration they capture. This finding does not invalidate the use of PCA; indeed, PCA can capture much of the variation in carotenoid content (mallard, PC1) and due to their orthogonal nature, PCs may be more statistically appropriate within models that require a large number of uncorrelated independent variables.

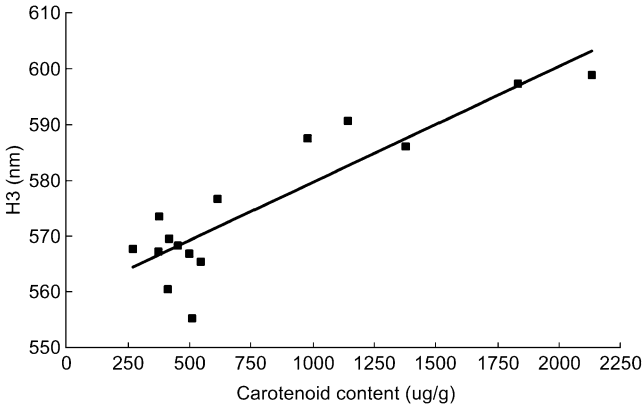

Fig. 3 Relationship between carotenoid content (μg/g) and hue (H3, nm) of house finch feathers, with an increasing carotenoid concentration associated with higher hue values. Simple linear regression $F_{1,\ 13}$= 50.83, P<0.0001, residuals normally distributed

Our study was the first to assess the relationship between variables generated from visual models of color perception and the pigmentary basis for color production. We found that visual models were able to detect differences in carotenoid content of house finch and mallard ornaments, with qualitatively similar results regardless of the visual system (UVS, VS, species-specific) utilized in the model. We also noted that chromatic contrasts did not capture as much variation in carotenoid content as color space vector parameters. Contrast measures depend strongly upon the

background spectrum that is chosen to calculate the contrast (e.g., Uy and Endler 2004), and contrast values provide little information about spectral shape (i.e., it is possible for patches of very different colors to produce similar contrast values). For studies on the signal content of coloration (as opposed to conspicuousness), measures of color space location may capture more of the relevant variation and provide results that can be compared across studies and species.

Before the widespread use of spectrophotomers, many studies quantified coloration of carotenoid-based ornaments solely in the human-visible spectrum (Omland 1996; Hill 1998). In support of the validity of such studies, we found that contribution from the UV portion of the spectrum was not necessary to capture variation in integumentary carotenoid content. For example, while there were a multitude of color metrics that utilize at least some portion of the UV range to accurately predict carotenoid content (e.g., mallard: φ, B1; house finch: PC3), there are also several metrics that achieve similar, or even stronger, relationships with carotenoid content that do not utilize any component of the UV range (e.g., mallard, S9; house finch, ColortronB). Furthermore, spectral data from the Colortron, which does not measure reflectance throughout the UV wavelengths, accurately modeled differences in carotenoid content in both the mallard beak and house finch feathers. It has been shown previously that variation in UV reflectance of carotenoid-containing ornaments has been linked to male quality (Peters et al. 2004a,b), that carotenoid deposition may reduce UV reflectance (Mougeot et al. 2007), and that UV reflectance is not always correlated to variation in the human-visible spectrum (Bleiweiss 2005). However, our results suggest that relevant variation in carotenoid content in house finches and mallards can be observed in just the human-visible portion of the spectrum. To be clear, this finding does not invalidate coloration metrics that utilize UV reflectance data; indeed, we identified several UV-sensitive color metrics that did account for a significant portion of the variation in carotenoid content, and some of the best predictors of carotenoid content (e.g., S1B in mallards) do utilize the UV portion of the reflectance spectrum. Rather, it suggests that measuring reflectance in the UV is not necessary for assessing carotenoid content of a tissue, as variation within the human-visible wavelengths frequently captured high levels of variation in carotenoid content of the tissue.

It should be noted that the lack of concordance between color metrics and carotenoid content of zebra finch beaks was pervasive. Therefore, the color metrics frequently used to assess their beak coloration may not relate directly to the carotenoid content of the tissue. The somewhat smaller coefficient of variation of carotenoid content in zebra finch beaks (CoV=31.4), compared with mallard beaks (CoV=48.7) or house finch feathers (CoV=72.5), may have made it more difficult to identify significant statistical correlations, although this does not satisfactorily account for the total absence of concordance between zebra finch color metrics and beak carotenoid concentration. In a previous study, a weak, non-significant relationship was uncovered between carotenoid content and bill color in zebra finch males (McGraw and Toomey 2010), and although a statistically significant relationship was also identified in females in that study, the data presented here suggest that, at least among unmanipulated zebra finch males on standard diets, beak carotenoid content does not significantly relate to beak coloration. Even if the zebra finch beaks had reached the saturation point and carotenoid concentration was no longer linearly related to chroma, there should still be relationships between carotenoid concentration and hue or brightness (Andersson and Prager 2006). Therefore, while carotenoids are demonstrably a component of the coloration of hardened zebra finch beak tissue (McGraw and Toomey 2010), other factors, including blood flow or carotenoid content of live tissue beneath the dead keratin layers, may be equally, if not more, important in determining coloration.

There have been a number of technological advances in color measurement in recent decades, resulting in several comparisons of color metrics (Grill and Rush 2000; Zuk and Decruyenaere 1994) and their applicability to comparative questions (e.g., dichromatism; Armenta et al. 2008). The data presented here further these investigations by examining the concordance between a broad array of frequently utilized color metrics and the quantity of pigment directly used in color production. While we did not control for underlying (non-carotenoid-based) structural coloration, previous work has shown that coloration of yellow feathers is driven more by variation in carotenoid content than by variation in the underlying white structural coloration (Shawkey et al. 2006). Additionally, melanin content of feathers can predict hue, saturation, and brightness of other feather colors in birds (e.g., brown and chestnut; McGraw et al. 2005), and fossilized melanin granules have even been used to reconstruct the plumage color of extinct dinosaurs (Li et al. 2010). We eagerly await analyses comparable to those presented here that systematically explore the utility of the wide range of color metrics available for predicting concentration of other pigment types (e.g., eumelanin, pheomelanin, pterins, porphyrins, and psittacofulvins; Toral et al. 2008) and in quantifying structural and iridescent coloration to comprehensively improve our methods for quantifying animal coloration.

We have identified several areas in which the research presented here may be improved in future studies. For example, while we utilized non-parametric rank correla-

tions due to the non-normality of variables and difficulty in identifying alternate distribution types, future studies with larger sample sizes may be able to use a series of linear or curvilinear models to examine the nature of the relationships between coloration and pigment content. This would allow for the identification of color metrics that are linearly dependent upon carotenoid content of the tissue, or may saturate at certain threshold pigment concentrations, which would be useful in identifying optimal pigment deposition strategies within species. Additionally, we acknowledge that, even though we used all of the most commonly invoked metrics available, it is possible, and even likely, that superior metrics have yet to be defined. We encourage future modeling exercises where spectral reflectance data, integument microstructure, and pigment concentration of ornaments are used to generate and test new metrics that may more effectively capture pigmentary bases for colorful ornaments.

In sum, we found evidence that several of the commonly used measurement and quantification techniques, notably saturation and hue tristimulus scores, were highly repeatable ($r \geq 0.7$) and able to capture the variation in carotenoid content of colorful bird ornaments. More specifically, we found support for fundamental color-generating actions of carotenoids in avian tissues (Andersson and Prager 2006), in that carotenoid content could be effectively captured by variation in tristimulus scores, including saturation (house finch, mallard), hue (house finch), and brightness (mallard) measures. Also, while color metrics that used portions of the UV spectrum could predict carotenoid content, UV was not necessary (e.g., Colortron) and sometimes even reduced the predictive power of a metric (e.g., in house finches, H4a, which does not use wavelengths below 400 nm, significantly predicted carotenoid content, whereas H4b and H4c, which use reflectance values down to 320 and 300 nm, respectively, exhibited a weak association with carotenoid content despite formula similarity; Appendix 1). Lastly, chromatic contrast scores derived from visual models, while they are demonstrably useful in quantifying conspicuousness, may be limited in their ability to capture the variation in the mechanism of color production and the information content of a signal. Within the avian visual model, measures of color space location (Stoddard and Prum 2008) may be better suited to capturing the information content of the signal.

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