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SYMPOSIUM

Melanin-Based Color of Plumage: Role of Condition and of Feathers' Microstructure

Liliana D'Alba,[1,*] Caroline Van Hemert,[†] Karen A. Spencer,[‡] Britt J. Heidinger,[§] Lisa Gill,[¶] Neil P. Evans,[||] Pat Monaghan,[||] Colleen M. Handel[†] and Matthew D. Shawkey*

*Department of Biology, University of Akron, Akron, OH, USA; [†]USGS Alaska Science Center, Anchorage, AK, USA; [‡]School of Psychology and Neuroscience, University of St. Andrews, Scotland, UK; [§]Department of Biological Sciences, North Dakota State University, Fargo, ND, USA; [¶]Max Planck Institute for Ornithology, Seewiesen, Germany; [||]Institute of Biodiversity Animal Health and Comparative Medicine, University of Glasgow, Glasgow, UK

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[1]E-mail: liliana@uakron.edu

Synopsis Whether melanin-based colors honestly signal a bird's condition during the growth of feathers is controversial, and it is unclear if, or how, the physiological processes underlying melanogenesis or the role of the microstructure of feathers in imparting structural color to feathers may be adversely affected by condition. Here, we report results from two experiments designed to measure the effect of condition on expression of eumelanic and pheomelanic coloration in black-capped chickadees (*Poecile atricapillus*) and zebra finches (*Taeniopygia guttata*), respectively. In chickadees, we compared feathers of birds affected and unaffected by avian keratin disorder, whereas in zebra finches we compared feathers of controls with feathers of those subjected to an unpredictable food supply during development. In both cases, we found that control birds had brighter feathers (higher total reflectance) and more barbules, but similar densities of melanosomes. In addition, the microstructure of the feathers explained variation in color more strongly than did melanosome density. Together, these results suggest that melanin-based coloration may in part be condition-dependent, but that this may be driven by changes in keratin and feather development, rather than melanogenesis itself. Researchers should be cautious when assigning variation in melanin-based color to melanin alone and microstructure of the feather should be taken into account.

Introduction

The bright feathers and striking plumage patterns of birds have served as classic model systems for investigation of the evolution of conspicuously colored integument of animals. The observed colors are produced either through selective absorption of certain wavelengths of light by pigments or through the scattering of light by tissues arranged at the nanometer scale (called structural colors) (Hill and McGraw 2006). Melanin pigments produce a broad range of black, brown, and gray colors through broadband light absorption across the visible spectrum, and are ubiquitously present in avian plumage (Stoddard and Prum 2011). Two chemical variants of melanin exist: black eumelanin and rusty-red (rufous) pheomelanin (Prota 1992). Melanin-based feather colors are produced by mixtures of the two in varying concentrations (McGraw 2006). These melanin pigments are housed in organelles called melanosomes that are deposited directly from melanocytes into the developing feather (Bagnara and Hadley 1973).

Although carotenoid-based coloration has been convincingly shown to reflect numerous aspects of the qualities of males (reviewed by Hill and McGraw [2006]), data for coloration based on melanin are more equivocal (reviewed by Meunier et al. [2011] and Guindre-Parker and Love [2014]).

Melanin-based coloration appears to be under tight genetic control, showing heritability estimates between 0.53 and 1.0 (Roulin and Ducrest 2013). This, along with the fact that melanin is endogenously produced, has led to the suggestion that it is unlikely to be dependent on condition. Studies examining the dependency of melanin-based traits on condition have produced mixed results (reviewed by Hill and McGraw [2006], Meunier et al. [2011], and Guindre-Parker and Love [2014]). Manipulation of levels of endoparasitism strongly reduced yellow carotenoid color in American goldfinches (*Spinus tristis*) but had no effect on black eumelanin-based color of the head (McGraw and Hill 2000). By contrast, experimental reduction of condition through increased brood size negatively affected the size of eumelanin-based dark tail bands of nestling Eurasian kestrels (*Falco tinnunculus*) (Piault et al. 2012). In turn, feeding rates of male barn owls (*Tyto alba*) with more eumelanic spots were less affected by experimental elevations of stress hormone (corticosterone) than were those of owls with fewer spots (Almasi et al. 2008). Fewer studies have examined pheomelanin, but in one case corticosterone administered to nestling barn owls led to less red (pheomelanic) plumage coloration (Roulin et al. 2008).

A criticism of all of these studies, however, is that they have used either plumage patch size or spectrophotometric measurements to evaluate melanin content. Melanic color patches in birds rarely contain either purely pheomelanin or eumelanin (McGraw 2006). In addition, since the biochemical pathways for production of the two forms of melanin differ, it is important to know the chemical composition of a plumage patch (and how this composition correlates with color) (McGraw et al. 2005) in order to assess any effects of stress or condition. Furthermore, if melanin is costly to produce then its concentration in feathers should be positively related to condition during molt. However, concentration of melanin has rarely been directly measured (but see McGraw et al. 2005; Roulin et al. 2013).

Although chemical composition and concentration of melanin is clearly critical to variation in the production of color, other components of feathers may also contribute. For example, the duller non-iridescent, structural blue feather coloration of female relative to male bluebirds (*Sialia sialis*) is caused in part by a greater number of melanized barbules (Shawkey et al. 2005). Similarly, Galván (2011) found that greater numbers of barbules and increased thickness of the barb's cortex were associated with darker melanin-based color of feathers in great tits (*Parus major*). Development of feathers is a sensitive and costly process that is subject to perturbation (Bortolotti et al. 2002). For example, food restriction during the growth of feathers is known to negatively affect feather microstructure by decreasing both the number of barbules and the tensile strength of entire feathers (DesRochers et al. 2009). Thus, feather microstructure may affect coloration based on melanin and may itself be condition-dependent.

Here, we report results of two tests of the effects of disease and of unpredictability of food on eumelanin-based and pheomelanin-based feather coloration, focusing on melanosome density and feather microstructure as they relate to variation in color. In the first experimental model, we assessed the effects of an outbreak of disease in a wild population of black-capped chickadees (*Poecile atricapillus*) on their black coloration, which is likely eumelanic based on similarities to the black plumage of red-winged blackbirds (*Agelaius phoeniceus*) (McGraw 2006). Avian keratin disorder is a recently described disease affecting populations of birds in Alaska and the Pacific Northwest (see Handel et al. [2010] and Van Hemert and Handel [2010] for details). The disease affects the production of keratin throughout birds' bodies, including feathers, beak, and claws (Van Hemert et al. 2013) and affects plumage color due to the increased soiling of feathers resulting from the inability to preen (D'Alba et al. 2011). In the second experiment, the effects of a psychological stress, unpredictability of food, on pheomelanic (McGraw and Wakamatsu 2004) cheek color were assessed in a captive population of zebra finches (*Taeniopygia guttata*). In both cases, the patches being characterized are used in mate choice: female chickadees prefer males with more UV-reflective patches (Doucet et al. 2005), whereas female zebra finches prefer males with more chromatic and UV-reflective cheek patches (Roberts et al. 2007). Thus, under honest advertisement theory (e.g., Zahavi 1975), both chickadees and finches in better condition should grow plumage with higher UV reflectance than would birds in worse condition. The mechanistic basis of such color differences is unknown but must be caused by differences in melanin content and/or microstructure of the feathers. To test condition-dependence of these melanin-based colors, we therefore compared color, melanosome density, and feather structure between the two experimental groups (unpredictable food/predictable food, affected/unaffected by disease) of each species.

Methods

Collection of samples

For black-capped chickadees, between March and April 2009, three feathers were collected from the black bib of 10 healthy chickadees and 10 chickadees affected by avian keratin disorder that were held captive at the University of Alaska Fairbanks for approximately 5 months as part of a different study of avian keratin disorder (D'Alba et al. 2011; Van Hemert et al. 2012). These birds were captured as adults from south-central and interior Alaska after fall molt; therefore, feathers used in this study were grown before the birds were in captivity. All feathers were washed with a solution of 50% ethanol before we took all color measurements because heavy soiling consequent to the keratin disorder strongly affects total reflectance and UV-chroma (D'Alba et al. 2011).

Zebra finches used in this study were part of a separate, long-term study on the effects of developmental stress on adult phenotypes (see Spencer et al. 2009, 2010; Monaghan et al. 2012). Three cheek feathers from 31 unrelated males that were between 70 and 80 days of age were collected. The study birds were chosen at random among the offspring produced during the second breeding attempt by focal birds in the aforementioned studies. On the day of hatching and then every 3 days until 30 days of age, we used a digital balance to measure body mass (to the nearest 0.1 g) and used calipers to measure tarsus length (to the nearest 0.01 mm).

We took one digital portrait picture from the right side of each male's head when he was between 70 and 80 days of age. Photos were taken with a Panasonic Lumix (FZ300) digital camera from approximately 30 cm in front of a gray background (Supplementary Fig. S1). We measured the size of the orange cheek patch from these photographs using the "magic wand" tool in Photoshop (measured in pixels with a resolution of 118.11 pixels/cm). Each patch was measured three times and the average size was used for further analyses.

Manipulation of developmental conditions in zebra finches

Birds were hatched and raised at the University of Glasgow between April and October 2008. Parents of our study birds were bred in cages that were $60 \times 50 \times 50$ cm, kept under a photoperiod of 14:10 h light:dark and temperatures between 20°C and 24°C. Zebra finches commonly experience unpredictable food in the wild (Zann 1996). To mimic this natural stress, the predictability of access to food was manipulated as follows. Pairs in the unpredictable food group received restricted access to food for periods of 3.5 h each day between 9:00 and 15:00 h on a random schedule. Access to food was denied in experimental cages by removing the food bowl and placing a transparent plastic mat over the floor of the cage to prevent access to seed within the cage litter. The same manipulation was performed for control pairs, but food was left available on top of the plastic mats. This manipulation of food started 10 days before the parents were bred and lasted until all chicks of the brood had fledged or died, or all eggs failed to show signs of development. Individuals in the study (nestlings produced by the "unpredictable food" or "control" pairs) experienced the food manipulation for an average of the first 30 days of their lives, after which time food was available *ad libitum* until feathers were collected at 70–80 days of age. Body mass of control birds ($n = 16$) at 30 days of age was higher than that of experimental fledglings ($n = 15$) that had experienced unpredictable food ($t = 2.37$, $P = 0.02$). Control birds also showed slightly faster growth during the linear phase (3–15 days of age) compared with birds in the unpredictable food group, although the differences were only marginally significant ($t = 1.89$, $P = 0.06$). Fledged chicks were kept in family groups until 60 days of age and then separated into sex-specific and treatment-specific groups ($n = 8$–10 birds per cage).

Measurements of color

For both experiments, three feathers per color patch per individual were taped to gloss-free black construction paper, and spectral data (measured as brightness and UV-chroma) from the distal portion of feathers were recorded using an AvaSpec 2048 spectrometer (range 250–880 nm; Avantes, Broomfield, CO, USA). Color data were recorded at normal (0° incident light/0° measurement) incidence using a bifurcated micron fiber optic probe held by a probe holder (RPH-1, Avantes) with matte black interior that excluded ambient light. All data were generated relative to a white standard (WS-2, Avantes). We used AvaSoft software (Avantes) to record and average 20 spectra sequentially, and recorded and averaged three measurements from randomly chosen points on each sample of feathers. Brightness was calculated as the average percent reflectance in the 300–700 nm range and UV-chroma as the proportion of total reflectance occurring within 300–400 nm.

Measurements of the microstructure of feathers

We compared feather microstructure of affected and healthy chickadees and zebra finches in the control and unpredictable food group using scanning electron microscopy (SEM). Single feathers were mounted on stubs with carbon tape, sputter-coated with silver and viewed on a scanning electron microscope (JSM7401F; JEOL, Japan).

SEM feather images were analyzed using ImageJ software (Rasband 2004). From the third and fourth barbs from the distal tip of each of these feathers, the following measurements were taken: (1) barbule density (number of barbules along each side of a 500-μm barb transect; Fig. 1A), (2) distance between barbules from base of one barbule to base of the following barbule (Fig. 1B), (3) thickness of barbs (the distance between the top and bottom surfaces of a cross-section of a barb taken at five random points per barb; Fig. 1B), (4) thickness of the barb's cortex (the distance from the edge of the barb to the edge of the central vacuole, taken at five different points per barb), (5) area of the vacuole, and (6) melanosome density (number of melanin granules observed in the barb cross-section divided by the area of the barb cross-section).

Statistical analyses

We tested the hypotheses that (1) melanin-based feather coloration and (2) feather microstructure are affected by health status or by unpredictability of food during development by comparing brightness and UV-chroma, as well as measures of feather microstructure, between healthy and affected chickadees and between control and experimental zebra finches. We used two-tailed Student *t*-tests to perform these comparisons.

To explore the relationship between microstructure and color parameters of feathers, we used an all-subsets approach to fit a set of linear models for each species with either feather brightness or UV-chroma as response variables. We first examined possible predictor variables for evidence of multicollinearity and found that several measurements of feather microstructure were highly correlated (Supplementary Table S1). Therefore, in building the sets of candidate models, we excluded "distance between barbules" and "barb cortex thickness" for chickadees and excluded "distance between barbules," "barb cortex thickness," and "vacuole area" for zebra finches. In total, we evaluated 31 models for black-capped chickadees and 15 models for zebra finches (see Supplementary Table S2 for full list of candidate models) with different combinations of the following predictor variables: barb thickness, barbule density, melanosome density, vacuole area (chickadees only), and condition (health status for black-capped chickadees and food supply for zebra finches). We ranked the models based on Akaike's Information Criterion corrected for small sample size (AIC_c) (Burnham and Anderson 2002). When more than one candidate model showed explanatory ability as indicated by an AIC_c value that differed by less than or equal to 2 from the best model (i.e., the one with the lowest AIC_c), we model-averaged parameter

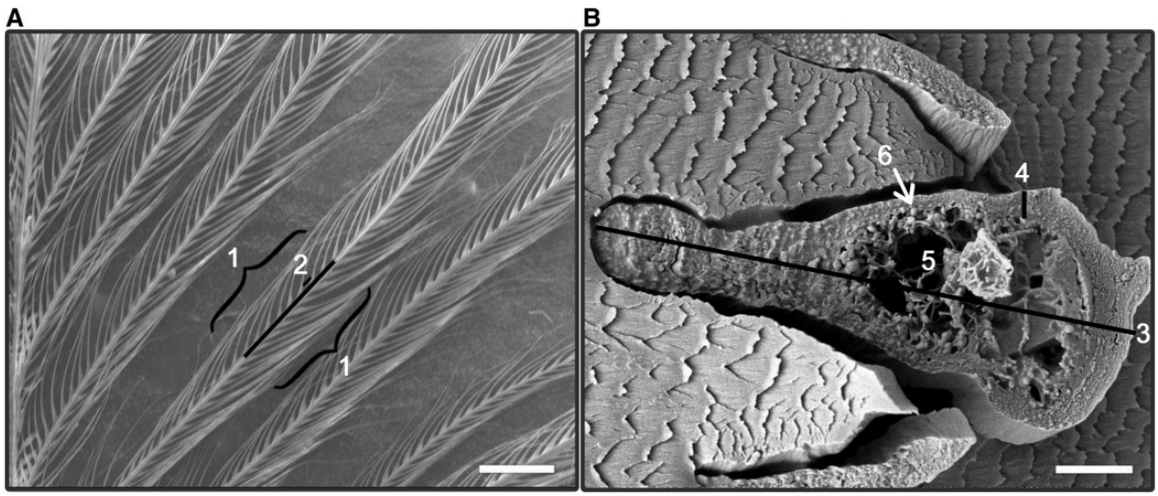

Fig. 1 SEM images of (**A**) barbs and barbules and (**B**) cross-section of a barb from a zebra finch feather, showing the microstructural variables measured in this study: (1) number of barbules (barbule density) along 500 μm of a barb transect, (2) distance between barbules, (3) barb thickness, (4) barb cortex thickness, (5) vacuole area, and (6) number of melanosomes per area (melanosome density). Scale bars are 300 μm in (**A**) and 5 μm in (**B**).

estimates to obtain effect sizes (θ) and the associated variances from the 95% confidence set of candidate models (Burnham and Anderson 2002). We used the sum of Akaike weights ($\sum w_i$) across all candidate models in which each predictor variable appeared to assess its relative importance. We calculated the evidence ratio (Burnham and Anderson 2002) as a measure of relative fit of models that included both condition (health status or food supply) and microstructural variables versus those that included only microstructural variables. Selection of models to explain brightness and UV-chroma was performed in R 3.01 (R Development Core Team 2007). All other analyses were performed in SPSS v. 21.

Results

Plumage color in relation to health and developmental conditions

Both feather brightness and UV-chroma were influenced by the status of health (chickadees) and by the unpredictability of food (zebra finches). In black-capped chickadees, eumelanic bib feathers of healthy individuals ($n = 10$) showed higher brightness ($t = 2.14$, $P = 0.04$; Fig. 2C) and UV-chroma ($t = 2.61$, $P = 0.01$) than did feathers from individuals affected by the keratin disorder ($n = 10$; Table 1). Similarly, zebra finches that received food predictably during development (controls; $n = 16$) showed brighter cheek feathers at maturity than did experimental birds ($n = 15$), which were given food unpredictably ($t = 4.18$, $P < 0.001$; Fig. 2D and Table 1). Conversely, UV-chroma was higher in zebra finch feathers from experimental birds compared with controls ($t = 3.34$, $P < 0.01$). Size of cheek patches on zebra finches (measured from images in pixels) did not differ between control (286.88 ± 8.15 pixels^2) and experimental (273.58 ± 4.98 pixels^2) birds ($t = 1.33$, $P = 0.18$).

Microstructure of feathers in relation to health and developmental conditions

The microstructure of feathers varied with the status of health and with developmental condition. Healthy chickadees had feathers with higher densities of barbules compared with those from affected birds (Table 1 and Fig. 3A). With the exception of an affected bird whose feather showed an extremely large barb vacuole (six times larger than the interquartile range), feathers of affected birds had vacuoles only one-eighth the size of those of healthy birds ($t = 2.64$, $P = 0.01$). This difference was not significant when the single extreme value was included in the analysis (Table 1). No other microstructural parameter differed significantly between healthy and affected chickadees (Table 1). Zebra finches in the control group also grew cheek feathers with higher barbule density (Fig. 3B) and shorter inter-barbule distance compared with birds in the unpredictable food group (Table 1). None of the other microstructural variables of feathers measured varied with developmental condition for finches.

Relationships between microstructure, condition, and color characteristics of feathers

Model selection results provided strong evidence that feather color was affected not only by microstructure of the feathers but by an additional effect of condition not explained by microstructural variables alone. Among candidate models to explain brightness in black-capped chickadees, the model receiving the greatest support ($w = 0.43$) included health status, barbule density, barb thickness, and melanosome density (Table 2; Supplementary Table S2). The second-most parsimonious model ($w = 0.18$) included the same variables except melanosome density. There was much less support for any other model (all $\Delta AIC_c > 3.5$; Table 2). Across all models in the candidate set, health status had the highest importance value ($\sum w_i = 0.96$), followed by barbule density (0.92), barb thickness (0.82), melanosome density (0.63), and vacuole area (0.19). Model-averaged estimates of parameters showed that brightness decreased with barbule density (θ: −0.251, 95% CI −0.451, −0.051; Fig. 4A) and thickness of barbs (θ: −0.255, 95% CI −0.508, −0.002; Fig. 4B) and was higher among healthy birds than among those affected by avian keratin disorder (θ: 1.50, 95% CI 0.53, 2.46) but there was significant uncertainty regarding the effect of melanosome density (θ: −2.944, 95% CI −4.998, 1.406; Fig. 4C). There was strong support for the effect of health status on brightness, with an evidence ratio of 24:1 for the set of models that included both health status and microstructural variables versus the model set that included only microstructural variables.

Model-selection results were equivocal for explaining UV-chroma in black-capped chickadees (Table 2). Barbule density, health status, and vacuole area were included singly or in combination in the most parsimonious models but none of the models received strong support (all $w \leq 0.16$). Across all models in the candidate set, health status had the second-highest importance value ($\sum w_i = 0.57$) after barbule density (0.77). Model-averaged estimates of parameters suggested that UV-chroma did not vary with either barbule density (θ: 0.0017, 95% CI -2.9605×10^{-4},

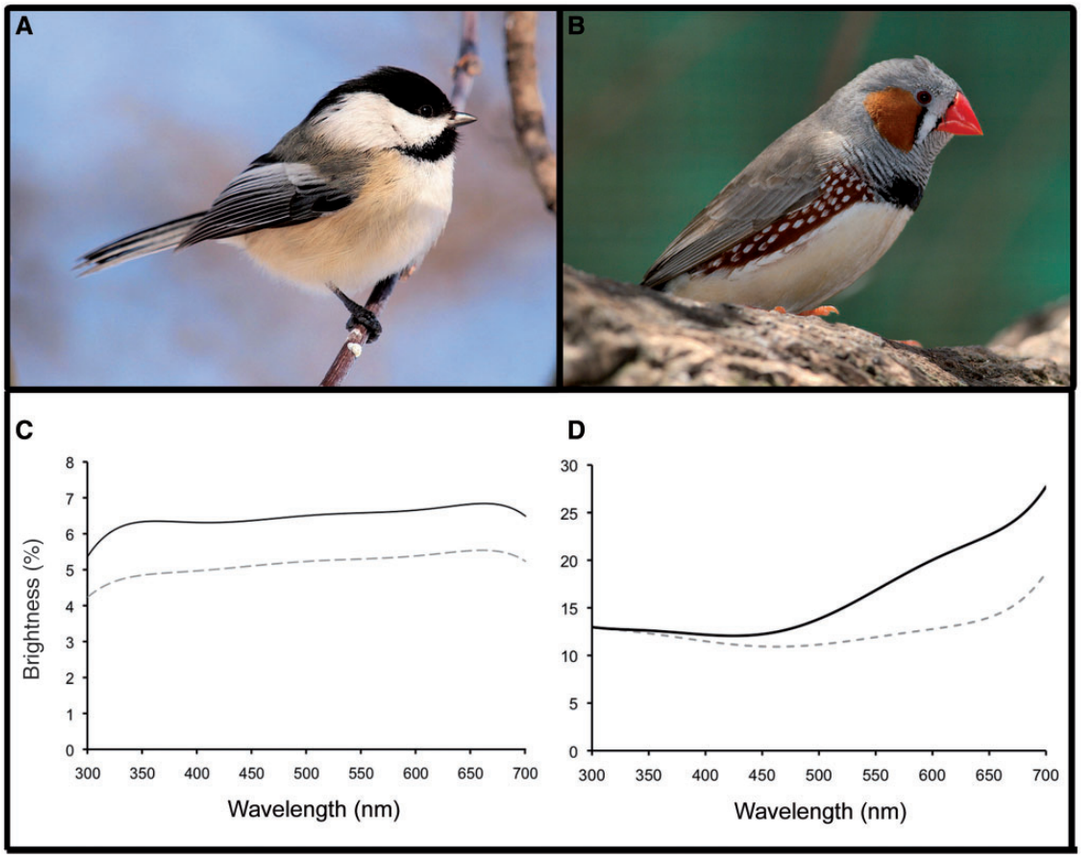

Fig. 2 Images of (**A**) black-capped chickadee and (**B**) zebra finch showing their respective black bib and orange cheek patches from which feathers were collected. (**C**) Mean reflectance of bib feathers of healthy black-capped chickadees (solid line) and those affected by avian keratin disorder (dashed line). (**D**) Mean reflectance of cheek feathers from control zebra finches (solid line) and experimental birds subjected to unpredictable food (dashed line).

Table 1 Univariate comparisons of brightness, UV-chroma, and feather microstructure variables between 10 black-capped chickadees affected by avian keratin disorder and 10 controls, and between 15 zebra finches subjected to unpredictable food and 16 controls

	Black-capped chickadee				Zebra finch			
Feather variable	**Healthy**	**Affected**	***t***	***P***	**Control**	**Unpredictable food**	***t***	***P***
Brightness	6.4 (0.39)	5.36 (0.33)	2.14	0.04	16.3 (0.75)	12.5 (0.4)	4.18	<0.001
UV-chroma	24.0 (0.19)	23.2 (0.21)	2.61	0.01	20.1 (1.05)	24.5 (0.77)	3.34	<0.01
Barbule density (number/500 μm)	21.8 (0.41)	18.9 (0.71)	3.45	0.003	24.6 (0.90)	21.5 (0.84)	2.44	0.02
Distance between barbules (μm)	32.8 (1.76)	36.9 (1.77)	1.63	0.11	18.0 (0.82)	21.6 (1.6)	2.07	0.04
Barb thickness (μm)	19.8 (0.64)	20.0 (0.70)	0.25	0.80	21.2 (2.49)	19.9 (1.83)	0.38	0.70
Barb cortex (μm)	3.5 (0.41)	3.8 (0.54)	0.26	0.79	3.7 (0.41)	4.7 (0.52)	1.34	0.19
Vacuole area (μm^2)	23.3 (6.9)	11.4 (8.4)	1.08	0.29	33.9 (10.10)	55.3 (12.99)	1.30	0.20
Melanosome density (number/μm^2)	0.18 (0.02)	0.24 (0.05)	1.23	0.23	0.15 (0.02)	0.18 (0.02)	0.87	0.39

Note: Values shown are means (SE).

0.001) or vacuole area (θ: 6.42×10^{-5}, 95% CI -6.46×10^{-5}, 1.6×10^{-4}) after accounting for the effect of health status (θ: 0.005, 95% CI −0.001, 0.01). The evidence ratio for the set of models including both health and microstructural variables versus the set including only microstructural variables was weak (1.4:1) for UV-chroma in chickadees.

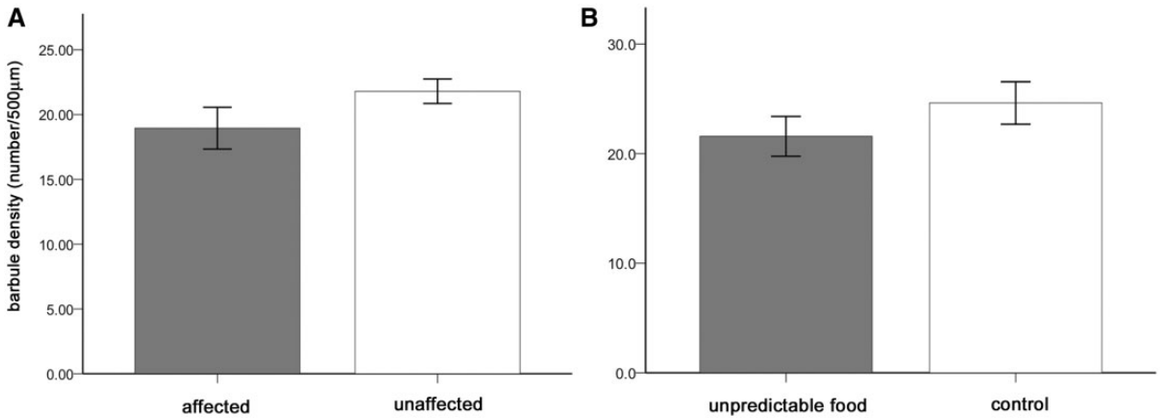

Fig. 3 Comparison of barbule density between (**A**) affected and unaffected chickadees and (**B**) experimental and control zebra finches. Values shown are means ± 95% CI.

Table 2 Top 10 general linear models for brightness and UV-chroma of black-capped chickadees ($n = 20$)

Model	*K*	Rank	ΔAIC_c	*W*
Brightness				
HEALTH + BARBULES + BTHICK + MDENSITY	6	**1**	**0.00**	**0.43**
HEALTH + BARBULES + BTHICK	5	**2**	**1.72**	**0.18**
HEALTH + BARBULES	4	3	3.52	0.07
HEALTH + BARBULES + BTHICK + VACUOLE	6	4	3.74	0.07
HEALTH + BARBULES + MDENSITY	5	5	4.13	0.05
HEALTH + BARBULES + BTHICK + MDENSITY + VACUOLE	7	6	4.14	0.05
HEALTH + BTHICK + MDENSITY	5	7	5.43	0.03
HEALTH + BARBULES + VACUOLE	5	8	5.52	0.03
HEALTH + BARBULES + MDENSITY + VACUOLE	6	9	6.33	0.02
BTHICK + MDENSITY	4	10	6.44	0.02
UV-chroma				
BARBULES	3	**1**	**0**	**0.16**
HEALTH + BARBULES	4	**2**	**0.40**	**0.13**
BARBULES + VACUOLE	4	**3**	**1.39**	**0.08**
HEALTH + BARBULES + VACUOLE	5	**4**	**1.92**	**0.06**
HEALTH + BARBULES + BTHICK	5	5	2.37	0.05
HEALTH	3	6	2.60	0.04
HEALTH + VACUOLE	4	7	2.64	0.04
BARBULES + BTHICK	4	8	2.77	0.04
BARBULES + MDENSITY + VACUOLE	5	9	3.23	0.03
BARBULES + MDENSITY	4	10	3.29	0.03

Notes: The number of estimated parameters (*K*, including the intercept and error term), rank within the candidate set, difference in AIC_c relative to the top model (ΔAIC_c), and Akaike weights (*w*) are shown for each model. Predictor variables include health status (affected vs. unaffected by avian keratin disorder; HEALTH), barbule density (number/500 μm; BARBULES), barb thickness (BTHICK), melanosome density (MDENSITY), and barb vacuole area (VACUOLE). The best-supported models within each candidate set ($\Delta AIC_c < 2$) are shown in bold. See Supplementary Table S2 for all model results.

For zebra finches, we found that brightness of cheek patches was best explained by a combination of the following variables: predictability of food supply, barbule density, and thickness of barbs (Table 3 and Supplementary Table S2); however, no single model received strong support (all $w \leq 0.32$). Across all candidate models, food supply had the highest importance value ($\sum w_i = 0.99$),

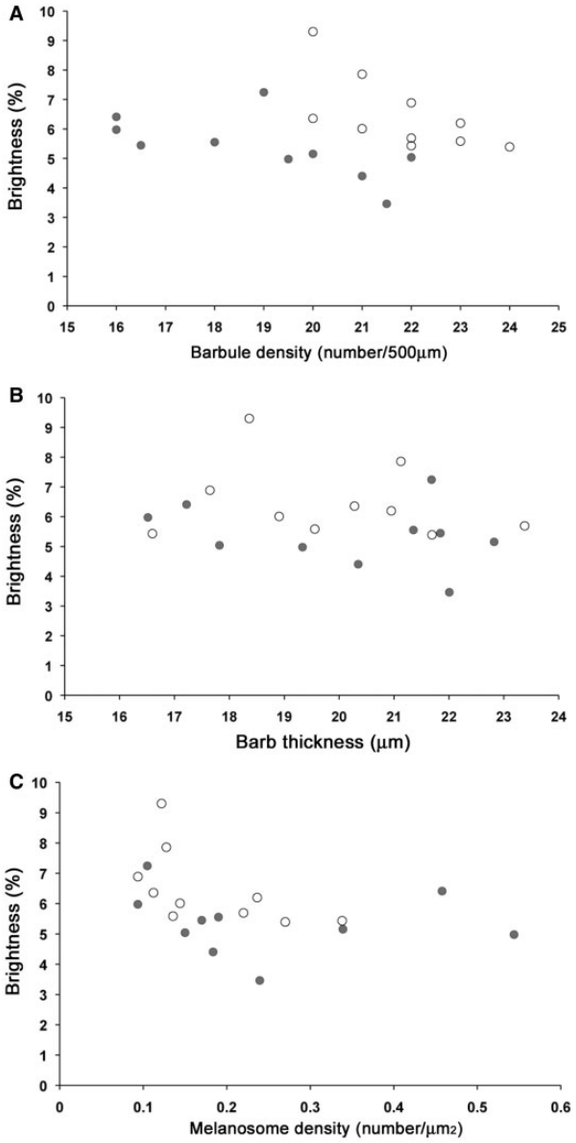

Fig. 4 Relationship between brightness and (**A**) barbule density, (**B**) barb thickness, and (**C**) melanosome density in bib feathers of black-capped chickadees. Open symbols represent control birds and closed symbols represent chickadees affected by avian keratin disorder.

followed by barbule density (0.62) and barb thickness (0.34). Brightness significantly increased with barbule density (θ: 0.004, 95% CI 0.001, 0.006; Fig. 5) and was higher among birds with a predictable food supply (θ: 0.036, 95% CI 0.01, 0.05) but did not significantly vary with thickness of barbs (θ: 0.0003, 95% CI −0.0009, 0.0015). There was overwhelming support for the effect of food supply on brightness in finches, with an evidence ratio of 110:1 for the set of models that included both food supply and microstructural variables versus the model set that included only microstructural variables.

Table 3 Top 10 general linear models for brightness and UV-chroma of zebra finches ($n = 31$)

Model	*K*	Rank	ΔAIC_c	*w*
Brightness				
FOOD + BARBULES	4	**1**	**0.00**	**0.32**
FOOD	3	**2**	**1.08**	**0.19**
FOOD + BARBULES + BTHICK	5	**3**	**1.74**	**0.14**
FOOD + BARBULES + MDENSITY	5	4	2.50	0.09
FOOD + BTHICK	4	5	2.52	0.09
FOOD + BARBULES + BTHICK + MDENSITY	5	6	3.63	0.05
FOOD + BTHICK + MDENSITY	5	7	3.66	0.05
FOOD + MDENSITY	4	8	3.70	0.05
BARBULES	3	9	9.04	0.00
BARBULES + BTHICK	3	10	10.31	0.00
UV-chroma				
FOOD	3	**1**	0.00	**0.38**
FOOD + MDENSITY	4	2	2.50	0.11
FOOD + BARBULES	4	3	2.70	0.10
FOOD + BARBULES + MDENSITY	5	4	2.73	0.10
FOOD + BTHICK	4	5	2.79	0.09
FOOD + BARBULES + BTHICK	5	6	2.80	0.09
FOOD + BTHICK + MDENSITY	5	7	3.79	0.06
FOOD + BARBULES + BTHICK + MDENSITY	6	8	4.89	0.03
BARBULES	3	9	6.11	0.02
BARBULES + BTHICK	4	10	7.01	0.01

Notes: The number of estimated parameters (*K*, including the intercept and error term), rank within the candidate set, difference in AIC_c relative to the top model (ΔAIC_c), and Akaike weights (*w*) are shown for each model. Predictor variables include developmental conditions (FOOD), barbule density (number/500 μm; BARBULES), barb thickness (BTHICK), and melanosome density (MDENSITY). The best-supported models within each candidate set ($\Delta AIC_c < 2$) are indicated in bold. See Supplementary Table S2 for all model results.

The single-best model ($w = 0.38$) explaining UV-chroma in finches included only the predictability of food supply (Table 3); other models had much less support (all $w \leq 0.11$). Across all candidate models, food supply had the highest importance value ($\sum w_i = 0.95$) and there was much less support for the three microstructural variables (0.30–0.36). The evidence ratio for the model set that included food supply plus microstructural variables versus the set that included only microstructural variables was high (20:1). None of the microstructural variables produced a model with high explanatory power for this color parameter (Supplementary Table S2).

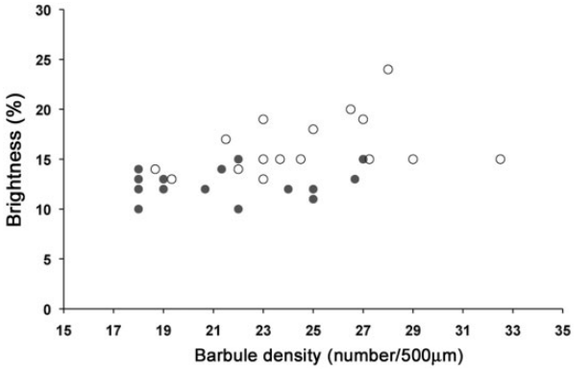

Fig. 5 Relationship between brightness and barbule density in cheek feathers of zebra finches. Open symbols represent control birds and closed symbols represent birds subjected to unpredictable food supply.

Discussion

In both experiments, control birds had brighter patches of plumage than did experimental birds, suggesting a consistent effect of health and developmental conditions on total reflectance of both eumelanin-based and pheomelanin-based plumage color. UV-chroma, however, was higher in control chickadees and lower in control finches. In both cases, density of melanosomes did not differ between control and experimental birds whereas barbule density was consistently higher in control groups. Surprisingly, barbule density had a stronger effect on color variation than did density of melanosomes. These results clearly indicate that feather microstructure can play a significant role in variation of melanin-based color production, and may be influenced by condition-dependent factors.

If production or deposition of melanin is physiologically costly, then the amount present in feathers should decrease under conditions of hardship or stress. We would expect this to be true for both eumelanin and pheomelanin, although a recent paper has argued that the amount of pheomelanin is more likely than that of eumelanin to vary with condition (Galván and Solano 2009). In both of our experiments, feathers from control and experimental birds contained similar densities of melanosomes despite differences in color. Thus, the production of melanin in the barbs of the feathers did not seem to vary depending on health or stress level.

Density of barbules, however, was consistently higher in control groups, suggesting that structural feather growth may have been affected by condition. Recent experiments have shown that structure of feathers can vary depending upon the conditions under which they are grown, and differences may reflect trade-offs or constraints in terms of time and energy (cf. Nilsson and Svensson 1996). Birds molting at more northern latitudes (Broggi et al. 2011) or under experimentally accelerated molt conditions (Vagasi et al. 2012) grew body feathers that were shorter, had a smaller proportion of plumulaceous barbs, and had higher densities of barbules than feathers grown under more benign molt conditions. Since accelerated molt is likely a stressful condition, these studies suggest that stress may induce growth of more barbules, contrary to our findings. DesRochers et al. (2009), however, showed that, similar to our results, European starlings (*Sturnus vulgaris*) subjected to experimentally restricted food and elevated levels of circulating corticosterone grew feathers with fewer barbules and greater distance between barbules compared with controls. In our study, experimental birds may not have been able to allocate energy toward the production of barbules (Lucas and Stettenheim 1972) during development of feathers. Therefore, although stress appears to influence structural characteristics of feathers, such as barbule density, the specific patterns, and mechanisms by which this occurs is not yet clear. More detailed measurements of the size, microstructure, and total mass of feathers grown by birds under controlled levels and durations of stress will help to address this uncertainty.

What caused the observed differences in coloration between control and experimental groups? Density of melanosomes and color was only weakly related in our results, and only in eumelanic feathers. This pattern contradicts a study of Barn Swallows (*Hirundo rustica*) showing strong negative correlations between brightness of plumage and its melanin content (McGraw et al. 2005). However, while significant, these aforementioned correlations were not overwhelmingly strong (e.g., r^2 ranges from 0.18 to 0.64 in McGraw et al. [2005]), suggesting that other factors also play a role in variation of color. Furthermore, the swallow study was based on brown colors caused by a mixture of pheomelanin and eumelanin, whereas the plumage patches in our study are overwhelmingly composed of either eumelanin or pheomelanin. Although it is difficult to believe that melanin content never affects color, a threshold density of melanin may exist over which slight variations make no difference to color. In other words, melanin may become saturated in the feather and addition or subtraction of a few melanosomes no longer affects color. Alternatively, these results could be explained, at least in part, methodologically. Although melanosome density has been shown to broadly correlate with color (Field et al.

2013), it may mask important chemical variation. Overall concentration of melanin within individual melanosomes varies 10-fold (Jaques and McAuliffe 1991; Jacques et al. 1996), so number or density of melanosomes may not completely reflect total content of melanin. Future studies examining the relationship between density, shape, and size of melanosomes versus chemistry and color will help to clarify these issues.

The microstructure of feathers contributes to variation in color. A previous study (Galván 2011) found that black feathers with more barbules and thicker barbs were darker, perhaps due to enhanced surface area for absorption. However, in our study results were less clear. In chickadee feathers, we found that brightness similarly declined with higher barbule density and thicker barbs; however, feathers of healthy chickadees, which had higher barbule densities, were relatively brighter than those of individuals affected by avian keratin disorder. The unexpected differential feather brightness of chickadees affected and unaffected by avian keratin disorder may have been a function of other, unmeasured microstructural or chemical differences related to the disorder.

In contrast to the pattern found in chickadees, brightness of finch feathers increased with barbule density, and control finches were brighter and had more barbules than finches under food stress. A larger number of barbules in finches may enable barbs to lock together more tightly, potentially creating a smoother and more continuous surface for reflectance comparable to that created by flattening of barbules in iridescent feathers (Durrer 1986; Prum 2006; Eliason and Shawkey 2011). Feathers of zebra finches have fewer melanosomes in their barbules than do feathers of chickadees, so adding barbules may also increase the amount of unpigmented keratin and thereby increase overall brightness. However, these potential mechanisms need further elucidation. The lack of consistent relationships between UV-chroma and melanin or structure makes both a mechanistic explanation for its variation and its dependence on condition difficult to interpret.

Our experiments suggest that melanin-based colors may reflect condition during the growth of feathers, and that microstructure of feathers is partly responsible. Researchers should be cautious when assigning variation in melanin-based color to melanin alone, and should take microstructure into account.

Ethical note: All zebra finch research was carried out under Home Office Project License 60/3447. Black-capped chickadee research was conducted under the guidance of the University of Alaska Fairbanks (UAF) and the USGS Alaska Science Center Institutional Animal Use and Care committees (Assurance 08-57). Any use of trade names is for descriptive purposes only and does not imply endorsement by the authors' institutions.

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Supplementary data

Supplementary Data available at *ICB* online.

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