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RESEARCH ARTICLE

Dietary protein level affects iridescent coloration in Anna's hummingbirds, *Calypte anna*

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SUMMARY

Many animal displays involve colorful ornamental traits that signal an individual's quality as a mate or rival. Brilliant iridescent ornaments are common, but little is currently known about their production cost and signaling value. One potential cost of colorful ornaments is the acquisition of limited dietary resources that may be involved, directly or indirectly, in their production. Protein, the primary component of bird feathers and of many nanostructural components of iridescent traits, is naturally restricted in hummingbird diets (comprised mostly of sugars), suggesting that iridescent coloration may be especially challenging to produce in these animals. In this study, we experimentally investigated the effect of dietary protein availability during molt on iridescent color expression in male Anna's hummingbirds (*Calypte anna*). We fed captive birds either a 6% (high) or a 3% (low) protein diet and stimulated molt by plucking half the gorget and crown ornaments on each bird as well as the non-ornamental iridescent green tail feathers. We found that birds receiving more protein grew significantly more colorful crown feathers (higher red chroma and redder hue) than those fed the low-protein diet. Diet did not affect gorget coloration, but regrowth of feathers in captivity affected both gorget and crown coloration. Additionally, birds on the high-protein diet grew yellower (higher hue) green tail feathers than birds on the low-protein diet. These results indicate that iridescent ornamental feathers are sensitive to diet quality and may serve as honest signals of nutrition to mates or rivals. Further, because both ornamental and non-ornamental iridescent coloration were affected by conditions during their growth, iridescent color in these birds appears to be generally condition dependent.

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INTRODUCTION

Mate choice and agonistic interactions are often mediated by honest signals of quality, such as behavioral displays, songs or colorful body parts (Andersson, 1994). To function as honest signals, these displays must have some differential cost of production or maintenance, such that only the highest-quality individuals are able to produce or maintain the most elaborate displays (Zahavi, 1975; Kodric-Brown and Brown, 1984; Grafen, 1990; Getty, 2006).

Many studies have examined the role of coloration in honest signaling, but the majority of this work has focused on pigmentary colors, which interact with light on a molecular level to absorb certain wavelengths (Senar, 2006). Much less is known about the information contained in structural colors, which are produced by nanoscale optical structures that cause the constructive interference of certain light wavelengths (Prum, 2006). Some nanostructural arrangements appear iridescent, producing some of the most dazzling displays in nature, such as peacocks' trains, gem-like beetle elytra and neon tetra fish that seem to glow. Iridescent colors change in hue and intensity with viewing angle, light angle or the orientation of the colored surface, and their reflectance characteristics are determined by the size and arrangement of nanostructural elements (Prum, 2006).

Iridescent colors serve a variety of functions in animals and are present in a wide diversity of taxa, several of which use iridescent coloration as a sexual signal (reviewed in Doucet and Meadows, 2009). Several correlational studies have examined the ability of iridescent colors to act as honest signals of condition (e.g. Doucet, 2002; Doucet and Montgomery, 2003a; Doucet and Montgomery, 2003b; Costa and Macedo, 2005; Bitton et al., 2008; Legagneux et al., 2010; Rutowski et al., 2010). Additionally, experimental studies have causally linked the production of iridescence to nutrient and thermal stress during development in pierid butterflies [*Colias eurytheme* (Kemp and Rutowski, 2007; Kemp et al., 2006) and *Eurema hecabe* (Kemp, 2008)], to starvation in the jumping spider *Cosmophasis umbratica* (Lim and Li, 2007) and to food quantity in the damselfly *Calopteryx maculata* (Fitzstephens and Getty, 2000). In birds, two experimental studies have shown that iridescent coloration is negatively affected by food restriction in brown-headed cowbirds [*Molothrus ater* (McGraw et al., 2002)] and by parasitic infection in wild turkeys [*Meleagris gallopavo* (Hill et al., 2005)]. However, although there are many studies on the impact of dietary components on pigment-based colors (reviewed in McGraw, 2006), no studies to date have examined how iridescent colors may be affected by specific nutrients.

There are obvious costs and benefits associated with pigment-based colors [e.g. costly to obtain, cannot be produced *de novo*, boost health (McGraw, 2006)], but it has been suggested that

producing precisely spaced, sized and aligned optical nanostructures may also be expensive in terms of materials and energy (reviewed in McGraw, 2008; Prum, 2006). New research on the development of iridescent feather barbules shows that the final organization of melanosomes within a keratin matrix is the result of entropic self-assembly (Maia et al., 2012). However, the size and concentration of melanosomes and the concentration of keratin molecules as well as costs associated with 'setting the stage' for self-assembly (e.g. cellular pH, hormones and availability of materials) likely represent costs for producing iridescent colors (Maia et al., 2012).

We currently know nothing about the condition dependence of iridescent colors produced in part by hollow melanosomes, which dramatically increase the reflectance of iridescent displays. Thin-film nanostructures, such as those created by hollow melanosomes, have been hypothesized to be particularly sensitive to perturbations during their development (Prum, 2006). In iridescent barbules composed of layers of air-filled melanosomes alternating with keratin protein, the nanoscale variation in arrangement and number of layers results in varied reflectance characteristics. Increasing the thickness of melanin and decreasing the amount of air inside the melanosomes, for example, generally results in a shift towards the right in hue values (Greenewalt et al., 1960). Similarly, variation in the thickness of structures affects brightness and chroma (Prum, 2006). Because light is reflected at each keratin–melanin and melanin–air interface, increasing the number of optical layers increases the brightness and chroma of iridescent colors (Dyck, 1987; Prum, 2006). Very small changes in these parameters have detectable effects on observed color; thus, any perturbation or shortage of materials during the development of feathers should have an impact on iridescent coloration.

Low protein availability may constrain the structure of the feathers a bird can produce and limit the thickness or number of layers of keratin protein in iridescent feathers. It may also affect the thickness or number of melanosomes because melanin production requires the amino acid tyrosine as a precursor (Fox, 1976). Keratin concentration and melanosome size and concentration could both affect self-assembly of iridescent nanostructures in feather barbules (Maia et al., 2012). Tyrosine can be obtained *via* protein in the diet or be synthesized from phenylalanine, which is an essential amino acid that must be derived from dietary protein. Because the optical nanostructures that produce structural colors in birds are made from protein or specific amino acids, we hypothesized that protein availability may affect the ability to produce brilliant iridescent coloration in birds. In this study, we experimentally manipulated dietary protein levels during molt and examined the effects of this manipulation on the iridescent coloration of male Anna's hummingbirds, *Calypte anna* (Lesson 1829).

Because hummingbirds are both brilliantly iridescent and have a diet that is naturally limited in protein, this taxon is ideal for testing the hypothesis that iridescent color is indicative of dietary protein level. Hummingbirds feed primarily on flower nectar, which contains only trace amounts of protein (Brice et al., 1989; Gottsberger et al., 1984). They supplement this diet to a limited degree with tiny insects, which are their only significant source of protein (Remson et al., 1986). These insects are usually captured while hovering, during which time hummingbirds have the highest mass-specific metabolism of any vertebrate (Suarez, 1992). So, hummingbirds are in general regarded as protein-limited and protein is costly to obtain. Anna's hummingbirds have iridescent magenta crown and chin feathers that they erect during both competitive and courtship displays (Stiles, 1982). The displays of these magenta ornaments are usually oriented towards the sun, especially during dive displays, which serves to maximize reflectance towards the observer (Hamilton, 1965). In addition to being conspicuously displayed during courtship, these ornamental feathers are regrown yearly in October–December directly preceding their breeding season, and have the potential to be indicative of condition just prior to breeding (Williamson, 1956).

MATERIALS AND METHODS

From 22 September to 16 December 2009, we captured 29 adult male Anna's hummingbirds in Tempe, AZ, USA using Hall traps (Russell and Russell, 2001) baited with sugar-water feeders. We housed these birds in captivity in Arizona State University animal care facilities in individual visually isolated nylon mesh cages with PVC pipe frames (60×60×60 cm). Each cage contained a 1/8 inch wooden perch and a hanging nectar feeder made from a small lidded plastic dish. We provided full-spectrum lighting *via* overhead lights with plastic covers removed (Zoo Med Reptisun 10.0 high-output UVB, San Luis Obispo, CA, USA), and photoperiod was adjusted weekly using light timers to mimic outdoor light:dark cycles. Additional small lights (two Zoo Med PowerSun UV mercury vapor lamps, 100 W, and two Energy Savers Unlimited Reptile BrightLight 150 W incandescent daylights, Carson, CA, USA) provided 20 min of twilight at dawn and dusk. Before beginning the diet manipulation, we fed the birds *ad libitum* a base diet of Roudybush Nectar 3 (Roudybush Inc., Woodland, CA, USA), containing 3% protein in the form of isolated soy protein and methionine, that was changed twice daily to prevent spoilage. Two days after capture, we measured body mass, tarsus length, wing chord and bill length and fitted the birds with metal rings for individual identification.

The day after the last bird was captured, we assigned 15 birds to a high protein diet (6%) and 14 birds to a low protein diet (3%; the same as the base diet), isocalorically formulated for the study by T.E.R. (see supplementary material Tables S1 and S2 for diet composition details). We chose 3 and 6% protein diets because Anna's hummingbirds fed these percentages of protein within a diet similar in nutrient composition to the Roudybush diet maintain mass over a period of at least 1 year and molt normally (Brice and Grau, 1989). Although Costa's hummingbirds (*Calypte costae*) fed 1.5% protein maintained mass for 10 days (Brice and Grau, 1991), our pilot work indicated that this low amount of protein was not sufficient for mass maintenance in Anna's hummingbirds, perhaps owing to the study period and the larger size of the birds. In our study, we wanted to select diets on which the birds could maintain body mass to examine the sensitivity of coloration to relatively small deviations in dietary protein in healthy animals.

Because pilot work indicated that Anna's hummingbirds would not consistently and completely molt naturally in captivity, even given a time period of several months and when housed either outdoors or indoors, we stimulated replacement of feathers by plucking (*sensu* McGlothlin et al., 2007). After the birds had been fed their diet treatment for 1 week, we systematically plucked ornamental [based on apparent use in displays and sexual dimorphism (Hamilton, 1965; Stiles, 1982)] and non-ornamental (no apparent use in displays) iridescent feathers to stimulate their regrowth during the diet manipulation (see Cotton et al., 2004). We plucked the right half of the magenta iridescent crown (top of head) and gorget (chin patch) feathers from each bird, leaving the left side of these ornaments intact to serve as a reference. This amounted to plucking approximately 50 of the tiny (less than 1 cm in length) crown feathers and 75 gorget feathers from each bird. We also plucked the right retrix 1 (R1), which is an iridescent green tail feather that is not clearly used in any behavioral display, to examine the effects of protein manipulation on both ornamental and non-ornamental iridescent color. Plucked feathers were regrown over

the following 2.5 months (January–March 2010). Three feathers plucked from standardized locations on the front, middle and back of the crown and the top, middle and bottom of the gorget and the single plucked R1 tail feather were selected for pre-manipulation (PRE) spectrophotometric measurement (see below).

During feather regrowth, we measured body mass, food consumption rate and molt progression weekly. To measure food consumption rate, we weighed new food before it was placed in the cage in the morning to replace the previous afternoon's food and after it was removed and replaced with new food in the afternoon. The amount of food consumed during this period was divided by the time between food introduction and removal. We controlled for evaporative water loss by subtracting the mass difference in control dishes containing the two diets placed in the study room during the same periods of time but not accessible by hummingbirds. When a bird had completely finished regrowing feathers, we plucked the regrown feathers for spectrophotometric analyses. For post-experiment (POST) measurements, we sampled three feathers from the newly grown right side of each ornament (gorget and crown). We also plucked the newly grown R1 tail feather. In addition, at the end of the study, we plucked three feathers from the control side of the gorget and crown to examine differences in reference side (REF) coloration from the beginning to the end of the study. If treatment differences in REF feather color were observed, this could reflect physical degradation of feathers during the experiment (i.e. as a result of poor feather preening or maintenance).

Reflectance spectrophotometry

We used reflectance spectrophotometry to measure the color of the gorget, crown and tail feathers pre-manipulation (PRE) and after regrowth (POST). For the crown and gorget, REF feathers that were not regrown during the experiment were also measured at the end. Our methods for achieving repeatable measurements from iridescent colors that change with small deviations in viewing geometry are detailed in Meadows et al. (Meadows et al., 2011). Briefly, feathers were mounted on matte black art-quality cardstock and stored in glassine envelopes at room temperature until measurement. Spectral measurements were collected using the custom-made goniometric light table described in Meadows et al. (Meadows et al., 2011), which allows for the precise alignment of a feather, light source and spectrophotometer probe when measuring reflectance from the 2 mm iridescent portion of an Anna's hummingbird feather. All equipment and the OOIBase program are from Ocean Optics (Dunedin, FL, USA), unless otherwise noted. A PX-2 pulsed xenon light source delivered light to the surface of the feather *via* a 400 nm fiber-optic cable focused with a 74-UV collimating lens connected to a rotating arm with its center of rotation about the surface of the sample. A separate 400 nm fiber-optic cable attached to a second rotating arm and focused on the 2 mm measurement spot on the sample with a 74-UV collimating lens delivered reflected light from the feather to a USB2000 spectrophotometer. The mounted sample was placed on a translational stage adjusted in height so that the surface of the sample was at the center of rotation of the light and spectrophotometer arms, and this stage could be tilted to alter the orientation of the feather sample with respect to the angle of the light and collector. We aligned the feather, light source and collector probe to mimic an orientation similar to that observed in nature (Meadows et al., 2011), and we tilted feathers with the light and probe at constant angles, mimicking a natural orientation until maximum brightness was achieved based on real-time output from OOIBase (for details, see Meadows et al., 2011).

Using the program CLR (version 1.5) (Montgomerie, 2008), we binned spectra in 1 nm increments from 300 to 700 nm [the visual range of birds (Bennett et al., 1994)] for the calculation of mean brightness, hue and red chroma measured as proportion of reflectance from 605 to 700 nm (B2, H1 and S1R in CLR version 1.5). For green tail feathers, we measured green rather than red chroma as the proportion of reflectance from 501 to 605 nm (S1G in CLR version 1.5). In addition, we added a novel color metric to capture the iridescent quality of the feathers, which we term 'directionality'. It is a measure of how much brightness is lost when the feather is rotated a set amount away from maximum brightness. Directionality should increase when optical nanostructures and barbules are precisely oriented in the same direction within a feather. To calculate directionality, we rotated feathers 10 deg away from the position resulting in maximum brightness, and we subtracted the resulting brightness value from the maximal brightness value (Directionality=$B2_{max}$–$B2_{off}$). Mean values of each color parameter were calculated from the three measurements from separate feathers taken from each ornament or time point, or from single measurements for tail feathers [see Meadows et al. (Meadows et al., 2011) for repeatability estimates].

Statistical analyses

All models were generated using SPSS version 18 (IBM Corporation, Armonk, NY, USA). Doubly multivariate repeated-

Table 1. Between-subjects effects of diet on post-molt ornament and tail coloration in Anna's hummingbird

Color variable	Low diet	High diet	*F*	*P*
POST ornamental coloration				
Crown brightness	0.17±0.04	0.21±0.07	2.97	0.098
Crown chroma	**0.49±0.05**	**0.53±0.03**	**5.54**	**0.028**
Crown hue (nm)	**640.18±7.80**	**650.42±5.88**	**13.54**	**0.001**
Crown directionality	0.13±0.04	0.17±0.07	3.03	0.095
Gorget brightness	0.39±0.17	0.38±0.13	0.03	0.859
Gorget chroma	0.47±0.05	0.50±0.05	3.50	0.074
Gorget hue	617.04±8.80	621.24±7.41	1.65	0.212
Gorget directionality	0.35±0.16	0.33±0.12	0.09	0.770
POST tail coloration				
Tail brightness	0.05±0.01	0.05±0.02	1.91	0.179
Tail chroma	0.33±0.02	0.33±0.03	0.58	0.455
Tail hue (nm)	**542.31±19.50**	**568.15±21.82**	**10.14**	**0.004**
Tail directionality	0.02±0.01	0.01±0.01	3.33	0.080

The d.f. for POST ornamental and tail coloration were 1,23 and 1,24, respectively. Overall MANOVA: Pillai's T=0.69, $F_{8,16}$=4.43, P=0.006. Significant effects are in bold.

measures ANOVAs (RM-MANOVAs) were used to examine the effects of time point (PRE, REF and POST), diet (high and low) and time point × diet interactions on plumage color metrics. Individual MANOVA models were used to assess the effect of diet on endpoint color metrics of molted feathers, to verify that there were no differences in diet treatment groups at the beginning of the study and to test for changes in coloration of unmolted control feathers. We used RM-MANOVAs to assess the effects of diet on feeding rates and body mass throughout the study. Standard model assumptions were met or corrected for, and α was set at 0.05. We corrected for violations in sphericity, the only model assumption that was violated, using Greenhouse–Geisser-corrected *P*-values.

RESULTS

Treatment groups did not significantly differ in color metrics at the beginning of the study (PRE; Pillai's T=0.186, $F_{8,20}$=0.57, P=0.789),

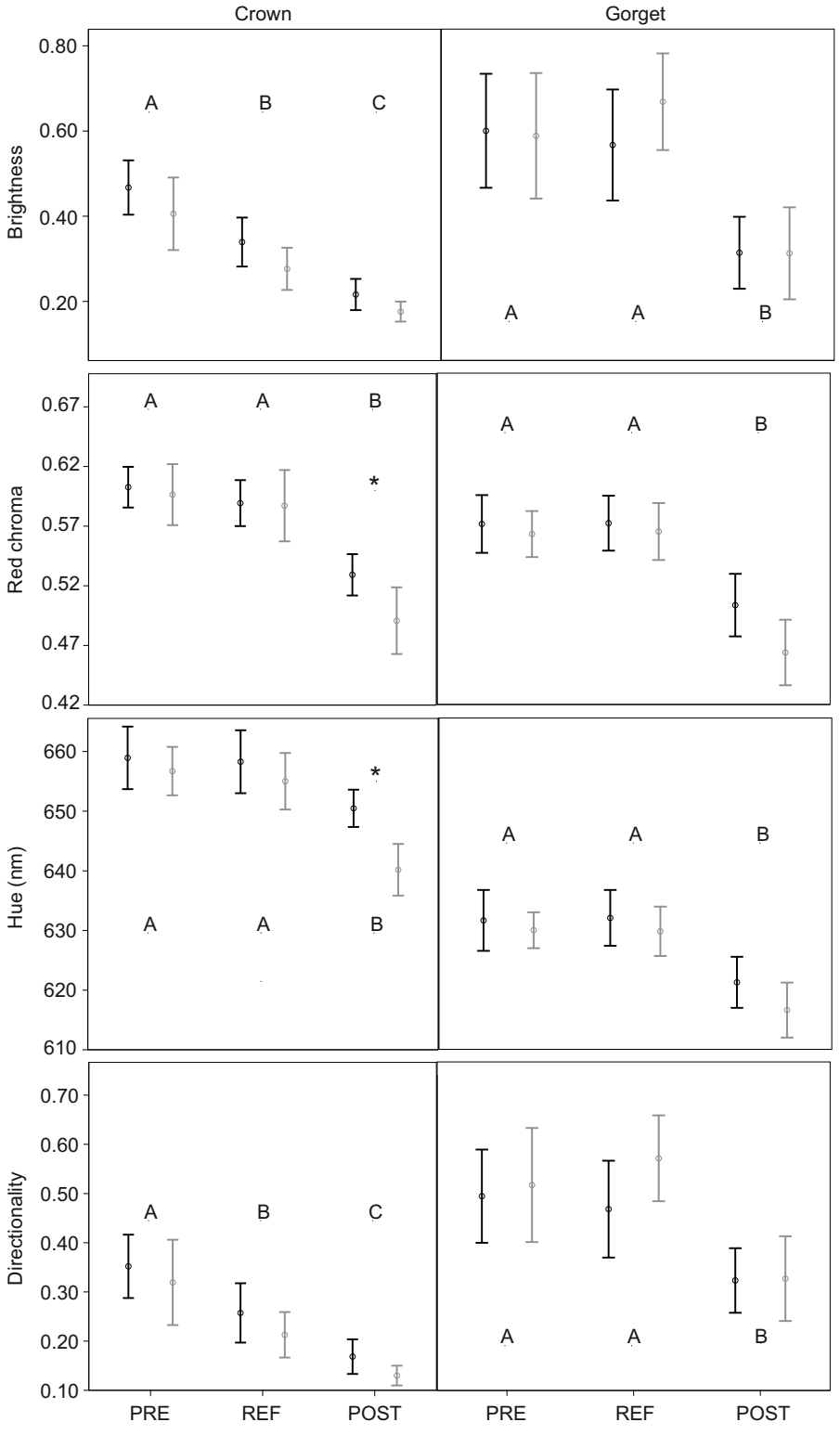

Fig. 1. Mean (±2 s.e.m.) brightness, red chroma, hue and directionality of Anna's hummingbird gorget and crown feathers pre-manipulation (PRE), from the reference unmolted patch (REF), and from the half of the ornament grown during the study (POST). Asterisks indicate significant differences between high (black) and low (gray) protein diets in POST coloration, and letters indicate significant differences between time points (PRE, REF and POST) regardless of diet.

and unmolted reference patches did not differ by diet in any color variables (REF; Pillai's T=0.42, $F_{8,19}$=1.74, P=0.153). We found a significant effect of dietary protein on two measures of post-molt ornamental coloration (POST; Pillai's T=0.69, $F_{8,16}$=4.43, P=0.006): crown red chroma ($F_{1,23}$=5.54, P=0.028) and crown hue ($F_{1,23}$=13.54, P=0.001) were significantly greater in the high-protein diet group (Table 1, Figs 1, 2). High hue values indicate that the hue is shifted toward longer, redder wavelengths, and higher chroma values indicate increased spectral purity (Fig. 2). There were no significant effects of diet treatment on gorget color variables (Table 1). There was a significant overall effect of measurement time point on plumage coloration (PRE, REF, POST; Pillai's T=0.96, $F_{16,80}$=11.94, P=0.001; Table 2); for all crown and gorget color metrics, feathers regrown during the study (POST) were less bright, directional and chromatic, and had hues shifted more towards shorter wavelengths than PRE and REF unmolted feathers (all P<0.001; Table 2, Fig. 1). In addition, unmolted REF feathers were significantly less bright and directional at the end of the study than were PRE feathers (all P<0.001; Table 2, Fig. 1).

The high and low protein groups did not differ in tail color at the beginning of the study (Pillai's T=0.23, $F_{4,22}$=1.62, P=0.205). Again, there was a significant effect of dietary protein level on post-molt (POST) tail coloration (Pillai's T=0.41, $F_{4,21}$=3.70, P=0.020); tail hue was significantly greater, or more yellow-shifted, in birds receiving a high-protein diet ($F_{1,24}$=10.14, P=0.004; Table 1, Figs 2, 3). There was a significant overall effect of time point on coloration (Pillai's T=0.74, $F_{4,19}$=13.65, P<0.001), with brightness, chroma and directionality being lower in feathers grown during the study than in those collected prior to the manipulation (all P<0.001; Table 3, Fig. 3). There was also a significant effect of the time point × diet interaction (Pillai's T=0.44, $F_{4,19}$=3.65, P=0.023) on brightness, hue, chroma and directionality (all P<0.031).

There was no effect of diet treatment on the amount of food consumed during the study ($F_{1,23}$=1.96, P=0.175) or body mass ($F_{1,27}$=1.58, P=0.219), but there was an effect of time point on food consumption (Pillai's T=0.55, $F_{6,18}$=3.74, P=0.014; Fig. 4) and body mass (Pillai's T=0.69, $F_{6,22}$=8.23, P<0.001; Fig. 4), though no directional trend was apparent.

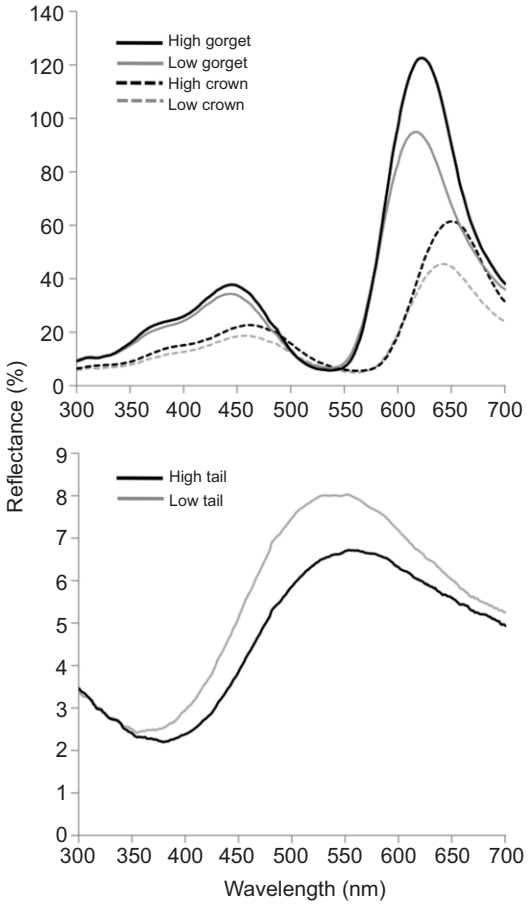

Fig. 2. Mean reflectance spectra for Anna's hummingbird feathers induced to grow during the diet manipulation for the crown and gorget (top panel) and tail (bottom panel); error bars were omitted for visual clarity. In both panels, birds fed the high-protein diet are represented by black lines and those fed a low-protein diet are represented by gray lines. In the top panel, gorget feathers are solid lines and crown feathers are dashed lines.

DISCUSSION

We found that dietary protein levels affected the production of iridescent coloration in male Anna's hummingbirds, with birds on high-protein diets during plumage molt producing more colorful feathers than those consuming low-protein food. Birds receiving more protein grew ornamental crown feathers with higher chroma and a more red-shifted hue, and non-ornamental iridescent green tail feathers with a more red-shifted hue. However, gorget feather coloration was not sensitive to dietary protein level. This is the first study to show how a specific nutrient affects the reflectance properties of an iridescent integument in animals. Prior studies have shown that birds fed a low-protein diet developed poor-quality plumage (i.e. abnormal feathers with broken barbs) (Murphy et al., 1988; Murphy and King, 1991), smaller structurally produced white plumage areas (McGlothlin et al., 2007) and less dark melanin-colored feathers (Poston et al., 2005). Protein manipulations have also been shown to affect other types of ornaments; the wattles of ring-necked pheasants (*Phasianus colchicus*) grow to be larger and redder in birds fed a high-protein diet as chicks (Ohlsson et al., 2002).

Regardless of diet treatment, feathers regrown during the study tended to be less colorful than original feathers collected at the beginning of the study. This was true for all color metrics measured for both the gorget and the crown, as well as all tail color metrics except for hue. We note here that because chroma, directionality and brightness would all be increased by more regular organization and a greater number of layers of nanostructural elements, we consider higher values of these color metrics to indicate more elaborate coloration; it is currently unclear whether lower or higher hue values would be advantageous in this system. This reduction in color could be a result of the stress of captivity and handling (e.g. McGraw et al., 2011), generally poorer nutrition than they would receive in the wild, a lack of social interactions with other males and/or females (e.g. Karubian et al., 2011), or because the birds were completing a second molt of these feathers. Crown brightness and directionality also significantly decreased in the unmolted patch of feathers. These results suggest another potential (maintenance) cost of displaying iridescent colors. Although our results do not indicate that this decrease in non-molted feather coloration was affected by diet quality, iridescent colors may be more subject to damage by abrasion or barbule breakage than other types of colors (Fitzpatrick, 1998; Doucet and Meadows, 2009). This would affect brightness and directionality rather than hue and chroma, which is consistent with our results. Recent evidence also suggests that iridescent feathers are more subject to soiling because of decreased hydrophobicity (Eliason and Shawkey, 2011). A study similarly

Table 2. Univariate effects of time point (PRE, REF and POST) and within-subjects contrasts between time points on ornamental crown and gorget coloration in Anna's hummingbird

				Overall effect of time		Time point contrasts		
Color variable	PRE	REF	POST	$F_{2,46}$	P	Contrast	$F_{1,23}$	P
Crown brightness	0.45±0.15	0.31±0.11	0.19±0.06	54.43	<0.001	PRE–POST	74.03	<0.001
						REF–POST	28.84	<0.001
						PRE–REF	37.24	<0.001
Crown chroma	0.60±0.04	0.59±0.05	0.51±0.05	71.33	<0.001	PRE–POST	143.61	<0.001
						REF–POST	58.92	<0.001
						PRE–REF	3.95	0.059
Crown hue (nm)	658.41±9.49	657.08±10.25	645.09±8.58	34.91	<0.001	PRE–POST	88.76	<0.001
						REF–POST	33.82	<0.001
						PRE–REF	0.58	0.453
Crown directionality	0.35±0.14	0.24±0.11	0.15±0.06	34.91	<0.001	PRE–POST	49.07	<0.001
						REF–POST	20.78	<0.001
						PRE–REF	23.31	<0.001
Gorget brightness	0.62±0.23	0.64±0.19	0.39±0.15	21.45	<0.001	PRE–POST	21.46	<0.001
						REF–POST	34.11	<0.001
						PRE–REF	0.29	0.598
Gorget chroma	0.57±0.04	0.57±0.05	0.48±0.05	91.70	<0.001	PRE–POST	125.38	<0.001
						REF–POST	115.99	<0.001
						PRE–REF	0.03	0.869
Gorget hue (nm)	631.57±8.30	631.30±8.93	619.05±8.27	55.54	<0.001	PRE–POST	68.79	<0.001
						REF–POST	68.32	<0.001
						PRE–REF	0.74	0.788
Gorget directionality	0.52±0.21	0.54±0.19	0.34±0.14	14.16	<0.001	PRE–POST	14.13	<0.001
						REF–POST	23.23	<0.001
						PRE–REF	0.38	0.544

Data for PRE, REF and POST are means ± s.d. Overall repeated-measures MANOVA: Pillai's T=0.96, $F_{16,80}$=11.94, P=0.001.

found that the iridescent wing feathers of mallard (*Anas platyrhynchos*) and pintail ducks (*A. acuta*) also decrease in brightness contrast over time (Legagneux et al., 2010). Mourning dove feathers increase in brightness after experimental wetting and drying, and this result is due to reduced twisting of the base of the barbule (Shawkey et al., 2011). In sum, these results provide convincing evidence that iridescent ornamentation (1) is sensitive to the availability of specific nutrients (protein), (2) decreases in brightness and directionality over time, possibly because of maintenance costs, and (3) is affected by an unidentified factor

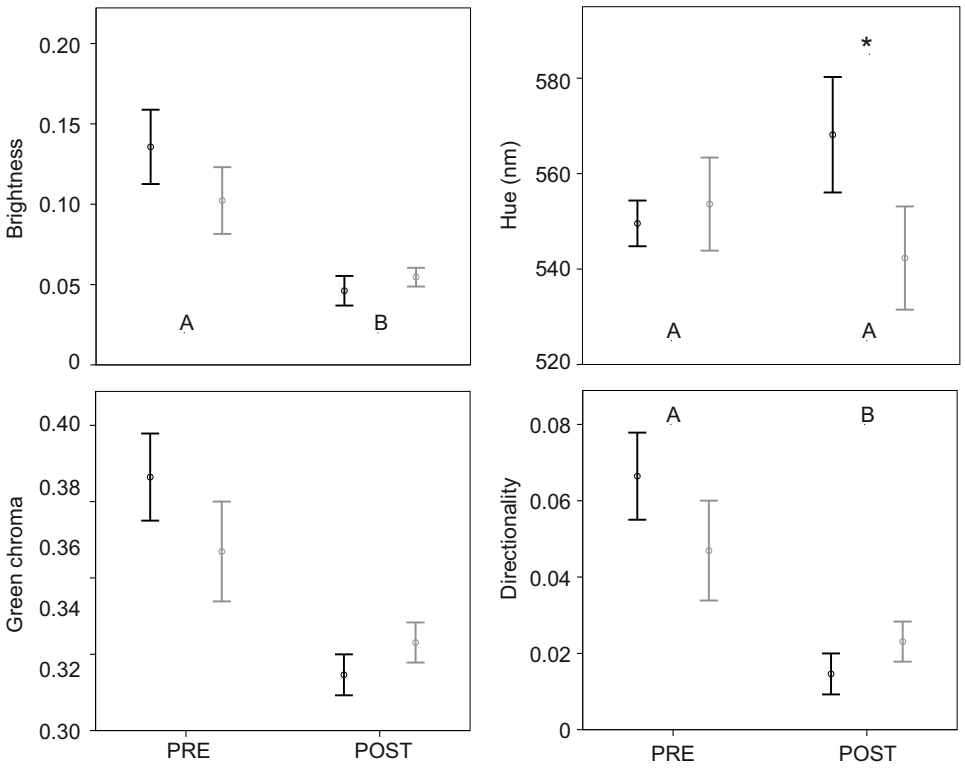

Fig. 3. Mean (±2 s.e.m.) brightness, green chroma, hue and directionality of Anna's hummingbird tail feathers from prior to diet manipulation (PRE) and from the feathers grown during the study (POST). Asterisks indicate significant differences between high (black) and low (gray) protein diets in POST coloration, and letters indicate significant differences between time points (PRE and POST) regardless of diet.

Table 3. Univariate effects of time point (PRE and POST) on tail coloration in Anna's hummingbird

Color variable	PRE	POST	Effect of time point	
			$F_{1,23}$	P
Tail brightness	0.12±0.04	0.05±0.01	49.06	<0.001
Tail chroma	0.37±0.02	0.33±0.02	54.09	<0.001
Tail hue (nm)	552.46±14.14	554.25±24.95	0.16	0.692
Tail directionality	0.05±0.02	0.02±0.01	47.05	<0.001

Data are means ± s.d. Overall repeated-measures MANOVA: Pillai's T=0.74, $F_{4,19}$=3.65, P=0.023.

associated with captivity. Although gorget coloration was not indicative of dietary protein level, it was affected by feather regrowth during the study and could be sensitive to other types of environmental conditions, a point that requires further study.

Because iridescent crown feather coloration, especially its hue and chroma, was affected by protein availability, it might serve as a sexually selected honest signal of quality during mate choice or agonistic interactions in Anna's hummingbirds. Crown hue and chroma were increased by higher levels of dietary protein and could be indicators of a male's diet quality, foraging ability, or even hovering ability and flight stamina. Hummingbirds hover while hawking for protein-rich insects, and this is the most energetically expensive activity known among vertebrates (Suarez, 1992). Colorful males with a protein-rich diet may have better genes for foraging ability or stamina, which would be passed to the offspring of choosy females. Previous studies have demonstrated that foraging ability can be a heritable trait (Owen and Harder, 1995; Karino et al., 2005; Missoweit et al., 2007), which could be particularly important in Anna's hummingbirds because male hummingbirds do not provide any parental care or resources after mating (Lack, 1968). Thus, if females choose more colorful males, they would benefit by selecting males whose diet is more protein rich and who may have good genes for foraging. Crown brightness and directionality may be difficult to maintain, requiring care (i.e. preening, increased uropygial oil application, etc.) to prevent damage to delicate structures, and all crown and gorget color metrics measured were affected by captivity. Further study is required to understand the specific maintenance costs of this coloration. Females may also benefit from choosing males better able to maintain their feathers or that have lower stress. The fact that gorget coloration was affected by feather regrowth during the study but not by dietary protein indicates that gorget coloration may be indicative of aspects of male quality other than protein in the diet. Although we demonstrate here that ornamental iridescent colors are sensitive to conditions during their development, it remains possible within this system that these traits are not meaningful within a sexual signaling context because we have not established genetic connections between increased trait expression and increased viability, a relatively higher cost of trait expression for lower-quality individuals, or mate preference for this trait (Prum, 2010). However, these were not the aims of the present study, which should instead be viewed as a first step in our understanding of the control mechanisms of color signals in hummingbirds.

Interestingly, non-ornamental iridescent tail coloration was affected by dietary protein and regrowth in captivity in a similar way to ornamental crown and gorget feather iridescent coloration in these birds. This suggests that all iridescent colors are affected by condition during feather growth in Anna's hummingbirds and perhaps other birds, rather than just iridescent colors that are involved in courtship and agonistic displays. Tail color may be a cryptic sexual signal that is not obviously displayed. Alternatively, iridescent green tail and body coloration may be under strong natural selection, perhaps to aid in crypsis while perched in green plants, thus justifying the cost of production. Green coloration in many other birds is produced by a combination of non-iridescent structural blue color produced by quasi-ordered structural arrays in feather barbs and yellow produced by pigments, usually carotenoids (Prum, 2006). Because hummingbird diets of nectar and small insects do not contain a significant source of carotenoids, it is probable that hummingbirds do not produce green in this way. Thus, producing green coloration for crypsis may be limited to developing iridescent green feathers in hummingbirds.

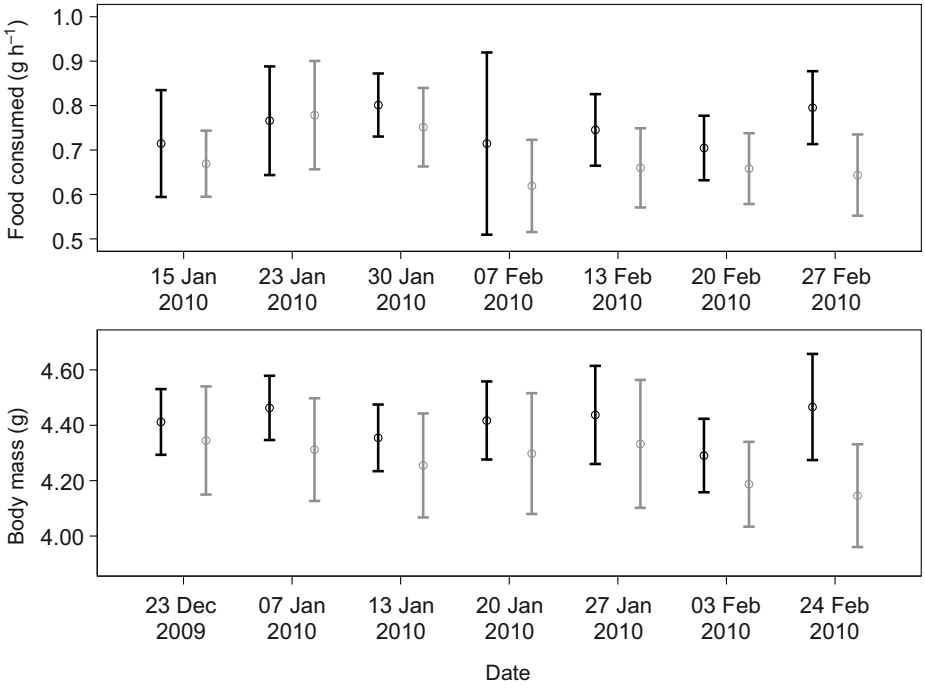

Fig. 4. Mean (±2 s.e.m.) food consumed (top panel) and body mass (bottom panel) throughout the study for Anna's hummingbirds fed high (black) and low (gray) protein diets. There was no effect of diet on food consumption or body mass.

Currently, we do not know how the iridescent nanostructures themselves were affected by protein level in the diet and regrowth during the study, and future microscopic work is needed to examine the mechanisms by which different levels of dietary protein affected the organization or size of melanin or keratin nanostructural elements that produce the iridescent color that we measured spectrophotometrically. Higher concentrations of keratin or melanosomes or larger-sized melanosomes could lead to enhanced self-assembly and greater nanostructural organization (Maia et al., 2012). Spacing of certain nanostructural elements have been correlated with color differences among feathers in satin bowerbirds [*Ptilonorhynchus violaceus* (Doucet et al., 2006)], abdomens in damselflies (Fitzstephens and Getty, 2000) and wing scales in butterflies (Kemp et al., 2006), but changes in nanostructural organization as a result of experimental manipulation of a key component of feather nanostructure, i.e. protein, have never been explored. In both crown and tail feathers, hue values were more shifted towards longer wavelengths in birds fed a high-protein diet, and this may have been the result of increasing the thickness of melanin and decreasing the air content of melanosomes (Greenewalt et al., 1960). Increased protein may also have allowed high-protein birds to increase the number or thickness of layers of melanosomes or keratin concentration, which may explain the observed increase in chroma in this group as well (Prum, 2006). Ongoing work will also examine how iridescent color in these birds is used as a signal in mate choice and agonistic interactions.

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