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Ornamental bill color rapidly signals changing condition

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Ornamental bill color is postulated to function as a condition-dependent signal of individual quality in a variety of taxonomically distant bird families. Most red, orange, and yellow bill colors are derived from carotenoid pigments, and carotenoid deposition in ornamentation may trade off with their use as immunostimulants and antioxidants or with other physiological functions. Several studies have found that bill color changes in response to physiological perturbations, but how quickly such changes can occur remains unclear. We tested the hypothesis that carotenoid-based orange bill color of American goldfinches *Spinus tristis* responds dynamically to rapid changes in physiological stress and reflects short-term changes in condition. We captured male and female goldfinches and measured bill color in the field and again under captive conditions several hours later. The following day, the captive birds were injected with either immunostimulatory lipopolysaccharide (LPS) or a control saline and changes in bill color were measured over a five day period. Yellow saturation of the bill decreased within 6.5 h between the field and captivity measures on the first day, presumably in response to capture stress. Over the longer experimental period, bill hue and luminance decreased significantly, whereas saturation significantly increased in both LPS and control groups. Bill hue and luminance decreased significantly more in birds treated with LPS than in control birds. Among LPS treated birds, individuals expressing high bill color at the beginning of the experiment lost more color than 'low-color' birds, but still retained higher color at the end of the experiment, suggesting that birds that invest heavily in bill coloration are able to sustain high costs in the face of a challenge. Bill color change may have resulted from rapid reallocation of carotenoids from ornamentation to immune function. However, the complex shifts in bill color over time suggest that bill color may be influenced by multiple carotenoid compounds and/or changes in blood flow or chemistry in vessels just beneath the translucent keratinized outer layer of the bill. We conclude that bill color is a dynamic, condition-dependent trait that strategically and reliably signals short-term fluctuations in physiological condition.

Condition-dependent signaling fundamentally influences social interactions by linking signal strength to the physiological state of the individual (Searcy and Nowicki 2005). Condition-dependent signals reliably indicate characteristics of the signaler such as immune status (Ressel and Schall 1989, Møller 1991, Nolan et al. 2006), nutritional status (Davies and Lundberg 1984, McGraw et al. 2005, Smith et al. 2007), developmental stability (Buchanan et al. 2003), and genetic quality (Kotiaho et al. 2001). Although the concept of condition-dependence implies that traits vary with condition, few studies have examined the time span over which such changes in condition and expression of visual traits such as coloration take place (but see Velando et al. 2006, Pérez-Rodriguez et al. 2008, Ardia et al. 2010).

In contrast to static traits (e.g. bird plumage), some condition-dependent ornamental traits are dynamic – they have the capacity to fluctuate on time scales that are more likely to mirror short-term changes in physiological condition (Sullivan 1990). For example, structures such as skin, wattles, snoods, and bills are vascularized (Lucas and Stettenheim 1972), and their color therefore has the potential to be influenced by blood flow and active deposition or depletion of pigments. This means that such structures can play an important role in sexual and social communication because they can reflect short-term changes in physiological or motivational status (Lozano 1994, Torres and Velando 2003, Negro et al. 2006, Bamford et al. 2010).

Carotenoids are well-studied pigmentary compounds that have important physiological functions and contribute to colorful integumentary structures. Carotenoids affect immune function by modulating T and B lymphocyte proliferative responses, humoral immunity, antibody production (Bendich 1989, Chew 1993, Jyonouchi et al. 1994, Costantini and Møller 2009), nuclear regulation (Chew 1993), and perhaps through their action as antioxidants (Burton 1989, Krinsky 1989, McGraw and Ardia 2004, Biard et al. 2009, but see Pérez-Rodriguez et al. 2008, Costantini and Møller 2009, Pérez-Rodriguez 2009, Hõrak et al. 2010). Although the precise mechanisms for many of these actions are not clear (Pérez-Rodriguez 2009), increased levels of dietary carotenoids have been linked to increased levels of immunocompetence

(Blount et al. 2003, McGraw and Ardia 2003, Navara and Hill 2003, Fitze et al. 2007), and immune responses deplete tissue and plasma reserves of carotenoids (Koutsos et al. 2003, Pérez-Rodriguez et al. 2008, Biard et al. 2009, Toomey et al. 2010).

The role of carotenoids in coloring non-feather integument ('soft parts') has been studied in several species. Bill color, in particular, has been experimentally altered by manipulating stress, diet, or immune activity over a period of a few weeks (e.g. zebra finches *Taeniopygia guttata*: Blount et al. 2003, Alonso-Alvarez et al. 2004, McGraw and Ardia 2004, Eraud et al. 2007; European blackbirds *Turdus merula*: Faivre et al. 2003, Baeta et al. 2008, Biard et al. 2009; spotless starlings *Sturnus unicolor*: Navarro et al. 2010; ring-necked pheasants *Phasianus colchicus*: Smith et al. 2007). Because of a possible tradeoff between allocation of carotenoids to physiological function and ornamentation, bill color has the potential to reliably signal current condition. However, almost all studies of change in bill color have measured the change in physiological condition over a broad time span, on the order of several weeks (e.g. but see Rosen and Tarvin 2006 and Ardia et al. 2010 for measures of bill color change on the order of one to three days).

Bill color of male and female American goldfinches *Spinus tristis* is strongly influenced by dietary carotenoids (McGraw et al. 2004, Hill et al. 2009) and changes from dull gray-brown in the winter to bright orange prior to the breeding season (Mundinger 1972). During the breeding season, goldfinch bill color has been reported to change quickly, over a period as short as one day (Rosen and Tarvin 2006). Thus, goldfinch bill color has the potential to function as one of the most rapid dynamic condition-dependent carotenoid-based signals reported to date. Previous research has established that goldfinch bill color functions as a status signal in females (Murphy et al. 2009) and may function as a mate-choice signal in males (Johnson et al. 1993); however, it remains unclear whether short-term changes in coloration are linked to changes in condition. If they are, then the degree of bill color change itself may provide information to receivers. American goldfinches are socially monogamous and both sexes invest heavily in the care of young (McGraw and Middleton 2009). However, 25–30% of broods contain extra-pair offspring (Gissing et al. 1998, Tarvin unpubl.) and members of mated pairs sometimes pair with a new mate within a breeding season (Middleton 1988). Hence, condition-dependent dynamic signals may provide information to individuals about the current condition (and future prospects) of partners and potentially could influence decisions about parental investment or divorce.

Here we test the hypothesis that goldfinch bill color has the potential to rapidly signal changes in physiological condition. Due to the vascular layer of the bill, we predict that bill color will change rapidly in response to stressful captive conditions (Dickens and Romero 2009, Dickens et al. 2009a, b), and that, because maintaining bill color is likely costly, birds given an immune challenge will experience greater changes in bill color than control birds (Alonso-Alvarez et al. 2004). We also evaluate the relationship between initial investment in bill color and the ability to sustain bill color in response to a challenge, as such a relationship can indicate whether high-quality individuals are able to sustain a stressor with lower costs than experienced by less ornamented individuals. If goldfinches maintain costly bill color strategically, then we predict that bill color of birds that initially invest more in bill color will be affected more strongly by immune activation, but that those birds should still sustain more colorful bills than birds that initially invest less in bill color.

Methods

We captured goldfinches in Lorain County, Ohio on 16 May, 21 May, 17 June, and 24 June, 2008, for a total of 19 females and 23 males. All females and males captured on a single day constituted an experimental group (creating 4 groups). Group size ranged from 6 to 18 birds. The birds were in breeding plumage and their bills were orange, but they had not begun nesting. Upon capture, we weighed each bird to the nearest 0.1 g, measured bill color (see below), and determined sex from plumage (Pyle 1997).

After capture, birds were held in individual cages and maintained with ad libitum access to sunflower hearts and nyjer seed, grit, and water supplemented with 0.26 g l^{-1} sulfadimethoxine (Sigma, S7007; McGraw and Hill 2001) to prevent coccidiosis, an infection that commonly develops in wild-caught seed-eating birds held in captivity (McGraw and Hill 2001). Birds were kept on a day-length schedule that matched current day-length (approx. 14 h L 10 h D). In the evening on the day of capture (day 1), we again measured mass and bill color and randomly allocated individual birds to immuno-stimulatory treatment group (n = 9 females and 12 males) or control group (10 females and 11 males). On the following morning (day 2) we again measured mass and bill color, and immediately thereafter injected treatment group birds subcutaneously with lipopolysaccharide (LPS) extracted from *E. coli* (Sigma, L4005) dissolved in 0.9% phosphate buffered saline (PBS), and control group birds with 0.9% PBS. The amount of LPS in each injection was equal to 1 mg kg^{-1} of body weight (procedure adapted from Owen-Ashley et al. 2006). Both control and experimental injections were delivered in doses of 10 µl of fluid per gram of body weight (injections were between 100–140 µl). As part of another experiment, we collected blood samples (50–80 µl) from both treatment and control birds on days 1 and 3 following mass and bill color measurements, and took spectrographic color measurements of plumage.

We used LPS, a component of the *E. coli* cell wall, to induce an immune response. In vertebrates, exposure to a pathogen initiates a generic response to pathogenic invasion, which is known as the acute phase response (APR). The APR comprises a highly coordinated collection of behavioral and physiological responses including a shift in protein output by the liver to a set of acute phase proteins that have antibody-like activity (Janeway et al. 1999), sickness behavior such as anorexia, adipsia, and lethargy, several neuroendocrinological effects (Hart 1988, Owen-Ashley and Wingfield 2006), increased sequestration of plasma iron and zinc, and fever (Hart 1988).

These responses can be triggered by a subcutaneous or intramuscular injection of LPS and effects of the treatment can be assessed behaviorally by the presence of sickness behaviors (Owen-Ashley et al. 2006). LPS is useful in experimental settings because it triggers the same response as a live bacterial pathogen, but does not cause continuing infection. Hence, it is a convenient mechanism for manipulating the immune system under experimental conditions.

We measured bill color multiple times over the course of the experiment. Birds in group 1 ($n = 6$) were measured 8 times over a 5 d period; whereas all other birds ($n = 36$) were measured 12 times over 7 d. We evaluated the rapidity of the onset of bill color change by comparing bill color between sequential measurements. Measurement 1 was taken within 20 min of capture (day 1). Measurement 2 was taken on average 6.33 h ± 10 min after measurement 1 on the day of capture, after birds were transported to the animal care facilities and allowed to settle in their individual cages. Subsequent measures were taken each morning and evening on days 2 through 5 (measurements 3–10) and in mid-afternoon on days 6 and 7 (measurements 11–12) (Table 1). Birds were released at their original capture site following the final measurement.

Color measurements

We objectively measured bill color with an Ocean Optics USB4000 reflectance spectrometer using a PX-2 pulsed xenon light source. We took four replicate spectral measures of the right side of the bill immediately anterior to the nares for each bird. All spectral measurements were taken by KAT. We used a probe holder with a conical head and a 3 mm aperture held at a 90° angle to the bill surface. The probe holder placed the probe 3 mm from the bill surface. To reduce stray light entering around the slightly curved bill surface, we held the probe holder in a standardized way so that little light from the PX-2 was visible around the edges. We took dark standard and Spectralon white standard measurements before measuring each bird to account for electronic noise and lamp drift. Spectra were processed with Ocean Optics Spectrasuite software.

We used CLR ver. 1.05 (Montgomerie 2008a) and RCLR ver. 0.9.28 (Montgomerie 2008b) to generate tristimulus variables to describe bill color. We visually checked each spectral curve for unusual spikes and erroneous measurements prior to analysis. We used spline smoothing (spline value = 0.5) and averaged the four smoothed spectra we took for each bill measure before calculating the tristimulus variables. We calculated mean luminance, yellow saturation, and hue following Montgomerie (2006). Briefly, mean luminance was calculated by dividing the sum of the reflectance levels of all measured wavelengths by the number of wavelengths for which reflectance is calculated. Saturation is the sum of the reflectance of wavelengths within a specified range (in our case, 550–625 nm) divided by the sum of reflectance at all measured wavelengths across the spectrum. Hue was calculated as the wavelength corresponding to the midpoint between the highest and lowest reflectance values. Luminance was not correlated with either saturation or hue ($r < 0.2$, $p > 0.2$), but saturation and hue were correlated ($r = -0.56$, $p < 0.001$; $n = 41$; analyses based on measurement 2).

As ambient light has the potential to affect spectral measures of objects with curved surfaces, we noted the environment of our spectral measures and later corrected for differences caused by ambient light. Our first measure of each bird was taken outdoors in shade, and all subsequent color measurements were made with the same equipment and under constant lighting conditions inside the Animal Care Facilities at Oberlin College. To validate our comparison of measurements taken in these two different light environments, we made a series of measurements of a color standard (orange plastic with a spectral reflectance and curved surface similar to that of a goldfinch bill; see Fig. 1 for spectrum). We measured the color standard both indoors and outdoors. The spectra from both indoors and outdoors had a similar shape (Fig. 1); however, the overall reflectance of the standard was lower indoors (ranging from 0.80 to 0.91 the value of the outdoor measure, measured at each nanometer between 320 and 700 nm). To correct for this difference, we multiplied each 1 nm-wide interval of the spectra from measurement 1 (outdoors) by the appropriate percentage difference between outdoor and indoor measures. These adjustments were performed prior to generating tristimulus scores. Thus, measures taken outdoors were directly comparable with the bill measurements taken indoors (Fig. 1).

Table 1. Schedule of color measurements. The first measurement was taken immediately following capture on day 1 of the experiment. Measurements 7, 9, 11, and 12 were not taken for birds in group 1.

Measurement	Day of experiment	Mean time of day	Time range of measurements	SE	Groups for which measurement was taken
1	1	11:06	09:36–14:49	0:10:11	1–4
2	1	17:39	16:18–19:42	0:08:05	1–4
3	2	09:18	08:25–10:42	0:06:02	1–4
4	2	17:20	16:45–18:01	0:03:06	1–4
5	3	08:40	08:13–09:17	0:02:33	1–4
6	3	17:31	16:43–18:48	0:05:07	1–4
7	4	08:47	08:08–09:33	0:03:50	2–4
8	4	17:17	16:47–17:49	0:02:37	1–4
9	5	08:39	08:14–09:08	0:02:30	2–4
10	5	16:39	13:17–17:33	0:11:33	1–4
11	6	15:01	14:11–17:12	0:08:51	2–4
12	7	14:16	13:32–15:10	0:04:08	2–4

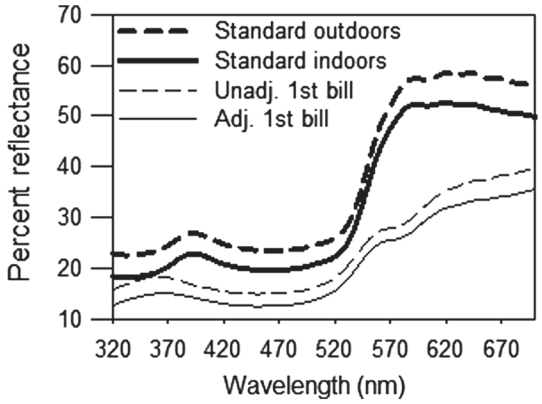

Figure 1. Mean spectra from a color standard measured outdoors versus indoors. Also shown are mean spectra from bill measurement 1 and from bill measurement 1 after adjustment by the per cent difference in outdoors versus indoors spectra of the color standard.

Statistical methods

Initial rapid change in bill color

We tested for rapid changes in bill color between measurements 1 and 2 using general linear repeated measures models with sex and group as between-subject factors. To be conservative, we analyzed the data with both the standard-corrected data and with the raw color data for measurement 1. Note that we only used the standard-corrected data for the analysis of rapidity of bill color change between the first and second bill measure, and did not use this correction for our analysis of color change due to the LPS treatment (see below), which only considered the third measurement onwards (all of which were measured indoors).

Effect of immuno-stimulation and captivity on bill color change

We analysed change in bill color from measurement 3 (taken immediately after injection of PBS or LPS on day 2) through measurement 12 in response to LPS treatment with hierarchical general linear models (HGLM; Raudenbush and Bryk 2002) using HLM (Scientific International Software). In our analyses, differences between birds were predicted from treatment, sex, and date of capture (to control for seasonal changes in bill color independent of our experiment). Changes in bill color within-birds were modeled as a function of time in captivity (in hours), treatment, sex, and date of capture, with individual bird ID included as a random variable. Treatment, sex, and date of capture were centered on the grand mean. Our primary interest is in the interaction between time in captivity and treatment, as such an effect indicates whether treatment affects the degree of color change over time. We also tested for interactions between time in captivity, treatment, and sex.

Influence of pre-experimental bill color on bill color change

To test for effects of initial bill color on the degree of color change, we divided birds into two categories for each of the three tristimulus color variables based on measurement 3, which was taken immediately before injection of LPS or PBS: 1) birds with initial bill color above the median value ('high-color') and 2) those with initial bill color below or equal to the median value ('low-color'). We categorized the sexes separately and then combined sexes for analysis. We used general linear models to test for a difference in the amount of change over time (color at measurement 12 minus color at measurement 3) with initial color category and treatment as factors (groups captured on different days were combined). In addition, we tested whether initial color differences between high-color and low-color categories persisted through the end of the experiment by comparing color between the same two categories at measurement 12 with t-tests.

Behavioural analysis

To confirm that LPS caused an immune response, we tested for the presence of the sickness behaviors lethargy, anorexia, and adipsia. The inflammatory response to an LPS injection is typically rapid in onset, appearing 2 or 3 h after injection, and is brief in duration, lasting < 24 h (Owen-Ashley et al. 2006). We video-recorded behavior of individually caged birds for 30 min: the first video was recorded approximately 5.5 h after birds were injected with either LPS or PBS, and the second video was recorded 24 h after the first video. We quantified lethargy by scan-sampling the videotaped birds every 30 s, and scored movement as 0 (no movement) or 1 (movement) (Altmann 1974). We then summed the readings to generate a movement score. To test for anorexia and adipsia, we summed the total number of visits to the food and water dishes, respectively, during each 30 min observation period. We used a Mann–Whitney U test to compare these variables at 5.5 h post-injection and 24 h later, when the effects of LPS were expected to have subsided (Owen-Ashley et al. 2006). We expected LPS-birds to be less active and eat and drink less at our first measure of behavior compared to our second.

Results

Initial rapid change in bill color

Within-bird changes in bill color between measurement 1 and 2 were rapid, with a significant decrease in yellow saturation occurring within 6.5 h of capture using either the adjusted or unadjusted first measures (adjusted: $F_{1,35} = 352.8$, $p < 0.001$; unadjusted: $F_{1,35} = 44.8$, $p < 0.001$; Fig. 2). We detected no significant changes in hue between measurements 1 and 2 (adjusted: $F_{1,35} = 0.807$, $p = 0.375$; unadjusted: $F_{1,35} = 3.9$, $p = 0.057$; Fig. 2). Luminance appeared to increase significantly between the first and second measurements when we used the adjusted first measures ($F_{1,35} = 31.4$, $p < 0.001$), but not when we used the unadjusted data ($F_{1,35} = 0.01$, $p = 0.908$).

Effect of immuno-stimulation and captivity on bill color change

Both treatment groups experienced significant decreases in bill hue and luminance and a significant increase in bill

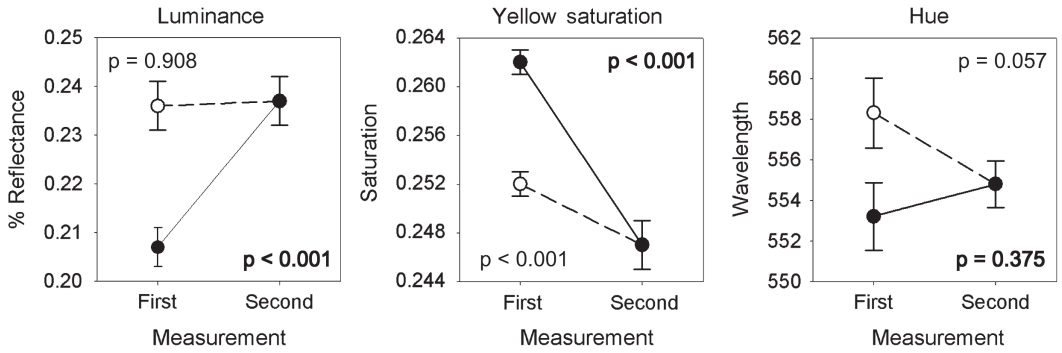

Figure 2. Change in bill color between the first (at capture) and second (approx. 6.5 h later) measurements. Filled circles and solid lines represent values for measurement 1 adjusted to account for differences in ambient light between the two measuring environments (see text for details). Open circles and dashed lines represent unadjusted values. P-values for analyses with adjusted first measures are shown in bold. Error bars represent standard error.

saturation over the course of the captive period (Table 2, Fig. 3). However, bill color change over time was significantly affected by immune system activation: birds in the immuno-stimulatory group (LPS treated) showed a significantly greater decline in luminance and hue over time compared to control group birds. We detected no difference between experimental groups for yellow saturation (Table 2, Fig. 3).

Males and females differed in all three tristimulus variables over the course of the experiment, and also differed in the change in yellow saturation and hue over time (Table 2, Fig. 3). However the change in bill color over time in response to LPS treatment did not differ between the sexes ($p > 0.17$ for all interactions).

Influence of pre-experimental bill color on bill color change

Over the course of the experiment, high-color birds (birds that started out with colorful bills, regardless of treatment group) experienced a significantly greater decrease in bill luminance ($F_{1,33} = 21.21$, $p < 0.001$) and hue

Table 2. Results of hierarchical general linear models analyses predicting bill color change from treatment with lipopolysaccharide (PBS = 0; LPS = 1), sex (male = 0; female = 1), date of capture (number of days since beginning of the experiment), and time in captivity (time in hours since the first measurement in the experiment). Unstandardized coefficients are analogous to regression coefficients; thus, negative coefficients signify decreases in the value of a color component in response to a factor. Date of capture, sex and treatment are centered on the grand mean. Initial differences in bill color between LPS and PBS groups are accounted for by the treatment variable. Background seasonal changes in bill color are accounted for by date of capture. The interaction between time in captivity and treatment reflects the effect of LPS on bill color change over time.

Color component	Comparison	Factor	Unstd. coeff.	SE	T-ratio	DF	p
Luminance	Between bird	Intercept	0.24176	0.00384	62.910	38	<0.001
		Treatment	0.01349	0.00769	1.753	38	0.088
		Sex	−0.03199	0.00809	−3.956	38	<0.001
		Date of capture	−0.00040	0.00026	−1.561	38	0.127
	Within bird	Time in captivity	−0.00347	0.00055	−6.360	350	<0.001
		Time in captivity × Treatment	−0.00234	0.00108	−2.164	350	0.031
		Time in captivity × Sex	0.00118	0.00111	1.062	350	0.289
		Time in captivity × Date	−0.00001	0.00004	−0.145	350	0.885
Yellow saturation	Between bird	Intercept	0.24811	0.00129	192.648	38	<0.001
		Treatment	−0.00153	0.00258	−0.591	38	0.558
		Sex	−0.00747	0.00271	−2.752	38	0.009
		Date of capture	0.00028	0.00009	3.271	38	0.002
	Within bird	Time in captivity	0.00163	0.00009	17.219	350	<0.001
		Time in captivity × Treatment	0.00029	0.00019	1.572	350	0.117
		Time in captivity × Sex	−0.00057	0.00019	−2.960	350	0.003
		Time in captivity × Date	0.00002	0.00001	2.568	350	0.011
Hue	Between bird	Intercept	556.545	0.80674	689.866	38	<0.001
		Treatment	3.57675	1.61479	2.215	38	0.033
		Sex	10.37102	1.69816	6.107	38	<0.001
		Date of capture	−0.18413	0.05396	−3.412	38	0.002
	Within bird	Time in captivity	−1.65043	0.10848	−15.215	350	<0.001
		Time in captivity × Treatment	−0.50485	0.21491	−2.349	350	0.019
		Time in captivity × Sex	−0.70965	0.22008	−3.224	350	0.001
		Time in captivity × Date	0.02386	0.00715	3.339	350	<0.001

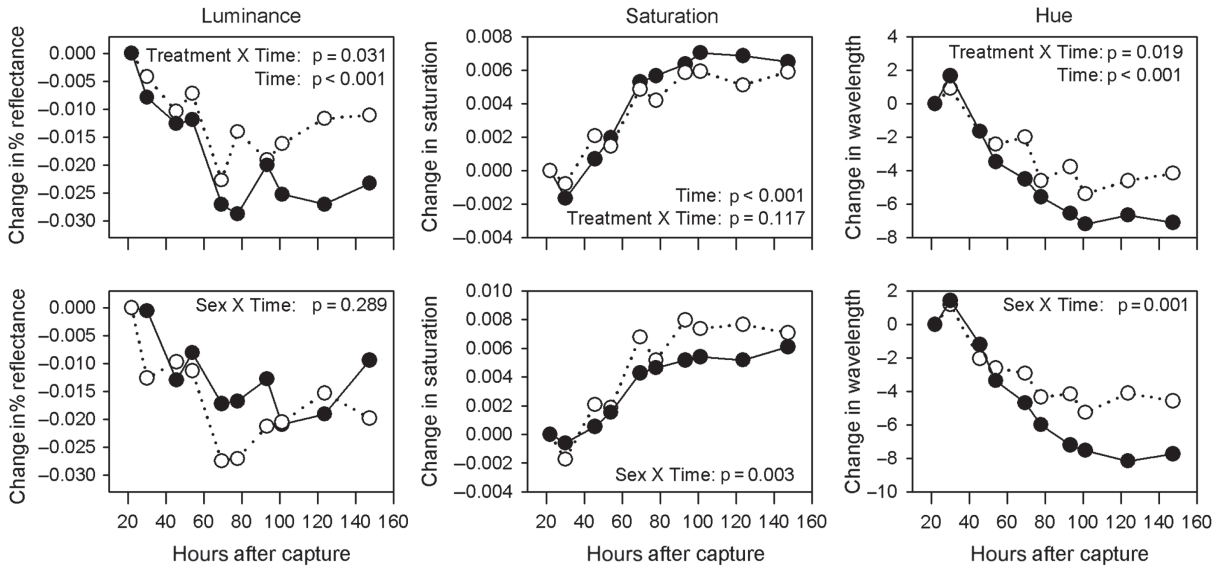

Figure 3. Change in bill color over time in response to LPS treatment and between the sexes. To facilitate visualization of temporal changes relative to starting values, the value at measurement 3 was subtracted from each subsequent data point (statistical analyses were conducted on unstandardized data). Thus, the graph shows the deviation from the initial color values over time for both LPS (filled circles, solid lines) and PBS (open circles, dotted lines) birds in the top panel, and for females (filled circles, solid lines) and males (open circles, dotted lines) in the bottom panel. Interactions between sex and LPS treatment were not significant for any tristimulus variable.

($F_{1,33} = 8.14$, $p = 0.007$) compared to low-color birds (birds that initially had dull bills), whereas the difference between these groups in the change in saturation was only marginally significant ($F_{1,33} = 3.85$, $p = 0.058$; Fig. 4). The results were similar when we analyzed the proportion of color change, rather than the absolute amount of change (luminance: $F_{1,33} = 16.16$, $p < 0.001$; hue: $F_{1,33} = 8.12$, $p = 0.007$), though the difference in the change in saturation was then significant ($F_{1,33} = 4.95$, $p = 0.033$). The decrease in bill luminance was marginally greater in the LPS group compared to control birds ($F_{1,33} = 4.03$, $p = 0.053$), but the immunostimulatory treatment groups did not differ in change in saturation ($F_{1,33} = 0.051$, $p = 0.822$) or hue ($F_{1,33} = 0.563$, $p = 0.458$; Fig. 4). The results were similar when using the proportion instead of the absolute amount of color change (luminance: $F_{1,33} = 3.14$, $p = 0.086$; hue: $F_{1,33} = 0.52$, $p = 0.477$; saturation: $F_{1,33} = 0.09$, $p = 0.765$). We also tested for interactions between color category and LPS treatment. None was significant, though the interaction between treatment and hue was nearly so (luminance × treatment: $F_{1,32} = 1.54$, $p = 0.224$; saturation × treatment: $F_{1,32} = 0.51$, $p = 0.479$; hue × treatment: $F_{1,32} = 3.57$, $p = 0.068$).

Because dividing birds into high- and low-color categories based on the median color value is somewhat arbitrary, we re-ran each analysis using color value at measurement 3 as a covariate in the model, rather than using color category as a factor. The effects of initial color value on change over time using this approach were qualitatively identical to that using color category for luminance ($F_{1,33} = 8.37$, $p = 0.007$) and hue ($F_{1,33} = 54.61$, $p < 0.001$), though the change in saturation was no longer marginally significant ($F_{1,33} = 1.31$, $p = 0.261$). The interaction between initial color change and treatment was not significant for luminance ($F_{1,32} = 0.68$, $p = 0.415$) or saturation ($F_{1,32} = 1.31$, $p = 0.262$) but was for hue ($F_{1,32} = 11.45$, $p = 0.002$).

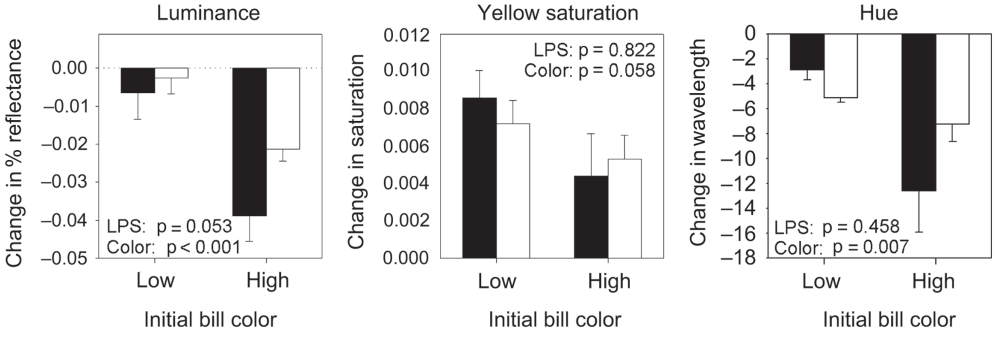

Figure 4. Change in bill color over time as a function of initial bill color at the beginning of the main experiment (measurement 3) and immunostimulatory treatment. Bill color change is calculated as measurement 12 minus measurement 3. Filled bars represent birds injected with LPS immediately following measurement 3; open bars represent control birds injected with PBS. Error bars represent standard error.

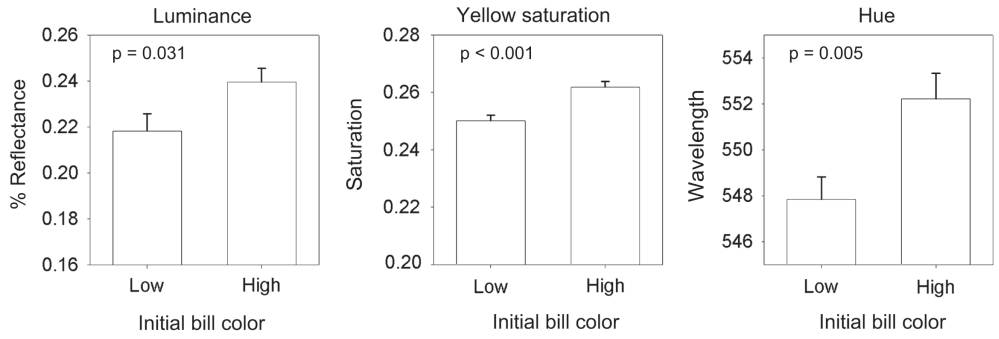

Figure 5. Difference in final bill color at the end of the main experiment (measurement 12) between birds that initially had high versus low bill-color at the beginning of the experiment. Error bars represent standard error.

High-color birds retained higher values of each tristimulus variable than low-color birds at the end of the experiment (luminance: $t_{34} = -2.25$, $p = 0.031$; saturation: $t_{34} = -4.25$, $p < 0.001$; hue: $t_{34} = -2.98$, $p = 0.005$), even though high-color birds lost more color value during the experiment (Fig. 5).

Behavior

LPS-induced sickness behavior was consistent with an acute immune response. Birds treated with LPS moved, ate, and drank significantly less than PBS birds 5.5 h after injection (movement: $Z = -5.637$, $p < 0.001$; eating: $Z = -4.365$, $p < 0.001$; drinking: $Z = -3.465$, $p = 0.001$; Fig. 6). There was no significant difference in movement, eating, or drinking between LPS and PBS birds 29.5 h after injection ($p > 0.4$ in all analyses), after the LPS presumably had been cleared by the immune system (Fig. 6).

Discussion

Here we show that carotenoid-based bill coloration of female and male American goldfinches can change on an extremely rapid time scale, spanning only a few hours. Furthermore, we found that longer-term decreases in bill hue and luminance are more pronounced in birds with challenged immune systems. These findings support the hypothesis that goldfinch bill color is a dynamic, yet reliable, signal of current physiological condition. Likewise, these results support previous work showing that bill coloration conveys reliable information (McGraw and Hill 2000, Hill et al. 2009) and is evaluated by receivers when assessing social competitors (Murphy et al. 2009).

We know of no other studies that have detected such rapid changes in a carotenoid-based trait. Several studies have shown that bill color of some species reflects condition when condition is experimentally manipulated over periods of several weeks (Blount et al. 2003, Alonso-Alvarez et al. 2004, McGraw and Ardia 2004, Eraud et al. 2007, Baeta et al. 2008, Biard et al. 2009, Navarro et al. 2010). Relatively more rapid changes on the order of 36–48 h have been reported in zebra finch (Ardia et al. 2010) and red partridge bill color (Pérez-Rodriguez et al. 2008) and in blue-footed booby foot color (Velando et al. 2006), and we previously reported (Rosen and Tarvin

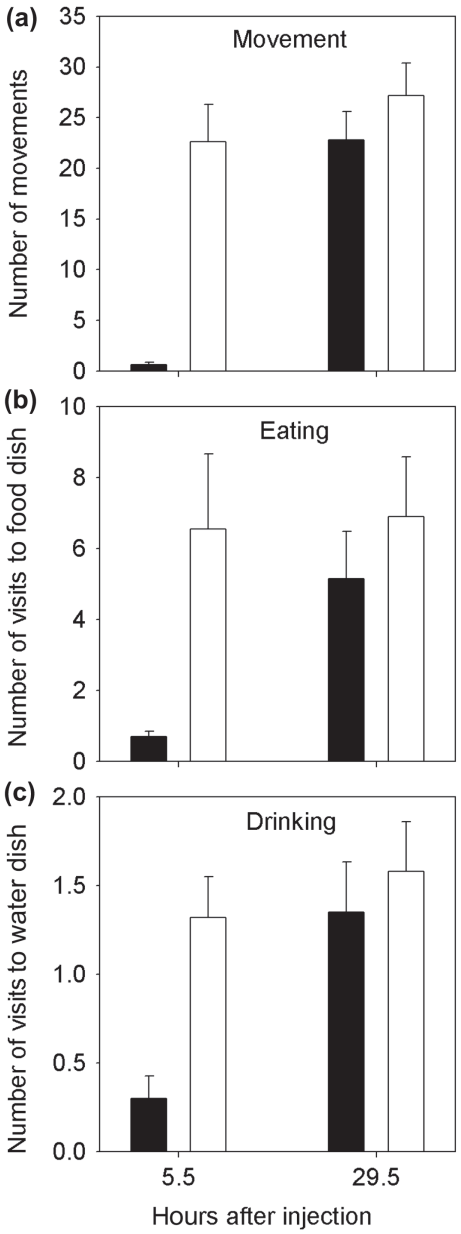

Figure 6. Activity of American goldfinches 5.5 h after injection with LPS (filled bars) or PBS (open bars) and 24 h later. N = 21 in each group for (a) movement and 20 in each group for (b) eating and (c) drinking. Error bars represent standard error.

2006) that male goldfinch bill hue faded from orange toward yellow over a 24 h period. Though the objective of these studies was not to determine how fast a dynamic ornament could change color, our present results suggest that carotenoid-based 'soft part' integumentary structures have the capacity to change color faster than researchers may have realized. Such rapid changes in dynamic signals in response to rapid changes in physiological condition have important implications for the kinds of information that dynamic signals may transmit (Sullivan 1990, 1994b, Bamford et al. 2010), and suggest that dynamic traits may signal physiological changes in 'real time'.

Although the initial reduction in bill saturation in response to capture was rapid and strong, we do not know the mechanism underlying the change. Rapid carotenoid mobilization is well-known in crustacean chromatophores (Auerswald et al. 2008), but whether substantial amounts of carotenoids can be mobilized from tissue within a few hours in birds is unclear. Studies of poultry on low-carotenoid diets have detected depletion of carotenoids in non-ornamental tissues within a few days (Tyczkowski et al. 1991, Wang et al. 2007), but we are unaware of studies focusing on shorter time scales. An alternative possibility is that some of the color change was due to changes in blood flow or composition in the vascularized tissue beneath the rhamphotheca, as flushing ornaments such as the head and facial skin of lappet-faced vultures *Aegypius tracheliotos* (Bamford et al. 2010) and other species are known to change very rapidly (Negro et al. 2006), and blood in the living tissue beneath the translucent keratin rhamphotheca could potentially influence bill color.

Our finding that immune system activation is reflected by changes in bill color parallels other studies that have demonstrated a change in carotenoid-based bill or soft integument color in response to an immune challenge (Blount et al. 2003, Faivre et al. 2003, Alonso-Alvarez et al. 2004, McGraw and Ardia 2004, Peters et al. 2004, Velando et al. 2006, Smith et al. 2007, Baeta et al. 2008, Gautier et al. 2008). Our conclusion that bill color has the potential to signal immune status is further supported by McGraw and Hill's (2000) finding that seasonal development of bill color in American goldfinches is affected by coccidia infection. However, although our experimental effect was robust, we do not know the mechanism by which the immune activation induced the decrease in hue and luminance. One possibility is that immune system activity utilizes circulating carotenoids (perhaps because it generates high levels of reactive oxygen species, which then may be neutralized by carotenoids; Costantini and Møller 2009), and the allocation of carotenoids to immune function concomitantly reduces carotenoid-based coloration of ornaments. Another possibility is that reduced foraging brought about by immune-stimulation led to a reduction in carotenoid intake, and this reduction in ingested carotenoids was directly reflected in bill color. However, we provided all of our experimental birds with a diet that was much lower in carotenoids than the natural diet (McGraw et al. 2001, 2004), so it seems unlikely that there was a substantial difference in carotenoid intake between treatments. Yet another possibility is that captive conditions and immunostimulation adversely affected the production of uropygial secretions and that the consequent diminished application of preen waxes to the bill altered its color (the 'make-up' hypothesis; Piault et al. 2008). Piault et al. (2008) found that preen waxes decreased bill luminance ('brightness') and that LPS injections reduced the production of preen waxes in nestling tawny owls *Strix aluco*. Lopez-Rull et al. (2010) and Amat et al. (2011) found that the application of uropygial secretions to plumage increased saturation of carotenoid-based coloration in house finches *Carpodacus mexicanus* and greater flamingos *Phoenicopterus roseus*, respectively. However, we found that bill luminance decreased and saturation increased during the course of our experiment, indicating that a reduction in the cosmetic application of preen wax to the bill is unlikely to account for the patterns we observed.

In addition to responding to immune system activation, bill color changed over time in both immune-stimulatory and control treatment groups. Because these color changes were independent of background seasonal changes (we controlled for seasonal change in our analysis), some other factor associated with captivity or experimental design must have induced the color changes. The stress of captivity, mediated by elevated corticosterone (Bortolotti et al. 2009, Mougeot et al. 2010), likely played an important role in the color change, although we did not measure levels of this 'stress hormone' and thus cannot draw firm conclusions about its role. Both immune and stress responses lead to oxidative stress (i.e. the build-up of harmful free radicals – Costantini et al. 2008), and bringing free-ranging adult birds into captivity for a week certainly induces both acute and chronic stress and increases in corticosterone levels (Dickens and Romero 2009, Dickens et al. 2009a, b). Any increase in free radicals should deplete reactive carotenoids, or alternatively may reflect stressed physiological pathways that are linked to carotenoid production or maintenance (Hill 2011). Indeed, response to the stress of captivity may have obfuscated some of the effects of LPS in our study (Dhabhar and McEwen 1997). However, we did not experimentally manipulate stress in a controlled fashion (all birds in the experiment experienced the stress of captivity, and all birds were handled in the same way), and we therefore are unable to exclude other possible causes of bill color change. For example, it is possible that bill color changed as circulating carotenoids were depleted over time without being replenished by the low-carotenoid diet (McGraw et al. 2001, 2004) available to the birds in captivity. Other studies have found dramatic decreases in tissue carotenoids over similar time periods in birds fed a low-carotenoid diet (Wang et al. 2007). Note, however, that under each of these alternative hypotheses for the proximate basis of color change, the change in bill color appears to reliably reflect changes in physiological condition.

This study clearly illustrates three important patterns, namely that bill color can change rapidly, that it continues to do so in response to protracted stressful conditions, and that it also reflects specific physiological attributes such as immune activity. However, a fourth major pattern in our study is compelling, yet puzzling, and as such demands careful analysis and urges further study. We found that bill saturation quickly decreases in response to

capture, yet then increases slowly during continued captive conditions. In contrast, hue did not change immediately, but decreased in response to captive conditions over the longer term and even more so in response to immunostimulation (the possible change in luminance was equivocal, depending on whether we used adjusted or unadjusted bill measures). The reversal in the direction of change in saturation between initial capture and later captive conditions suggests a complex proximate basis for bill color change. Specific forms of carotenoids contributing to goldfinch bill color have not been identified, so we do not know whether the orange color derives from metabolically processed carotenoids or their derivatives that differ from the yellow carotenoids that occur in feathers and plasma (canary xanthophylls A and B; McGraw et al. 2001, 2002). However, the red-orange bill color of zebra finches primarily results from high concentrations of metabolically derived red ketocarotenoids that are not present in other tissues or plasma (even though higher concentrations of less modified xanthophylls are present in plasma, liver, and adipose; McGraw and Toomey 2010), and it seems possible that goldfinches also deposit or metabolically produce red or orange carotenoids in bill tissue. If so, the pattern we observed is consistent with an initial rapid mobilization of yellow carotenoids (i.e. xanthophylls) out of the bill in response to an acute stressor, as an initial depletion of yellow carotenoids would account for the rapid drop in yellow saturation. This reduction of yellow carotenoids could have been followed by a slower, longer-term reduction of redder pigments in the bill (perhaps resulting either from mobilization or breakdown of ketocarotenoids or from a reduction in hemoglobin due to increasing anemia), which could unmask underlying residual yellow carotenoids that remain in bill tissue (e.g. in the 'dead' keratin of the rhamphotheca), thus accounting for the decrease in hue (away from red) and increase in yellow saturation. The hypothesis that the bill may serve as a reserve of metabolically useful xanthophylls that can be quickly sequestered during periods of physiological stress is consistent with work by Toomey et al. (2010), who found that immunostimulation depleted carotenoids specifically from retinal tissue of zebra finches. On the other hand, although the orange color of goldfinch bills is certainly influenced by carotenoids (McGraw and Hill 2000, Hill et al. 2009), some of the variation in goldfinch bill color stems from variation in melanin pigmentation (Mundinger 1972), and, additionally, the translucent keratin rhamphotheca may allow the vascular tissue beneath it to influence bill color as well. Regardless of the specific mechanism, our results indicate that bill color reliably signals short-term changes in physiological condition. Importantly, they also suggest that temporal patterns in color change may themselves be informative, a hypothesis that has been overlooked in the study of dynamic signals.

In other work, we have found that goldfinch bill color upon capture is correlated with immunocompetence (Kelly et al. 2012). Here we show that the dynamic component of bill color also has the potential to signal condition (sensu Sullivan 1990, 1994b). Birds that began the experiment with high bill color values experienced a greater degree of bill color change over the course of the experiment than did birds that began with low color. The fact that high-color birds lost proportionately more bill color indicates the result was not simply an artifact of the difference at the beginning of the experiment in color between the two groups. That is, if both high- and low-color groups lost an equal proportion of their initial bill color, the absolute amount lost by those in the high-color group would be greater than that lost by low-color birds. Even though high-color birds lost more bill color during the experiment, their bills still were more colorful than those of low-color birds at the end of the experiment, indicating that they are better able to produce a relatively strong signal (bill color) in the face of physiological challenges. Moreover, the fact that high-color birds lose more color, yet remain more colorful afterwards suggests that a colorful bill is a strategic signal that is costly to maintain, rather than simply a passive 'window' to circulating carotenoids. We hypothesize that high-quality individuals are able to both sustain higher allocation of carotenoids to ornamentation, and sustain higher physiological and/or immunological function compared to low-quality individuals. This interpretation is consistent with work by Pérez-Rodriguez (2008), who found that individual differences in the dynamic bill color of red-legged partridges *Alectoris rufa* were consistent across seasons, even though bill color fluctuated within individuals. Our interpretation is also consistent with models of sexual signaling indicating that higher-quality individuals may pay higher marginal costs of signaling (Getty 2006). Thus, in addition to reflecting current condition, dynamic signals such as bill color may also signal long-term information about individual quality.

Our recent research on female–female interactions in American goldfinches has shown that bill color is an important signal of intrasexual dominance (Murphy et al. 2009), and other researchers have suggested that female goldfinches prefer to court males with more colorful bills (Johnson et al. 1993). In the present study we found that bill color of both males and females changes in response to captivity and immunostimulation, although the nature of the change differed between the sexes (although interactions between sex and immunostimulatory treatment were not significant). Bill saturation increased more slowly while bill hue decreased to a greater degree in females than in males, suggesting that either the sexes differ in their response to captive conditions or that bill color change reflects sex-specific processes and therefore provides sex-specific information. Although we are unable to distinguish these possibilities at present, we have shown elsewhere that bill color measured immediately upon capture reflects both similar and different components of condition in males and females, with bill color reflecting heterophil:lymphocyte ratio (a measure of stress) in both sexes and reflecting levels of immunoglobulin Y (a measure of constitutive adaptive immunity) only in females (Kelly et al. 2012). Regardless of the precise sex-specific information conveyed by changes in bill color, the present study suggests that dynamic bill color could be an important mediator of both intra- and inter-sexual interactions, relevant in conflicts over food and access to mates or nesting sites in both sexes, especially when the competitive ability of individuals may change as

physiological condition changes over time. Here, we have shown that carotenoid-based bill color of female and male American goldfinches fluctuates over a matter of hours, and that these fluctuations mirror short-term changes in physiological condition. Likewise, we suggest that in response to physiological challenges, the change in bill color reflects individual quality, as bills of birds with high initial investment in bill color remain more colorful than low-color birds following a similar challenge. Thus, goldfinch bill color has the potential to mediate behavioral interactions over both the short and long term. Rapid and informative changes such as these may be widespread among species that have carotenoid-pigmented soft parts, although few studies have tested for changes in signals on such a short time frame. Moreover, although dynamic signals have garnered substantial recent attention, few studies have addressed the responses of receivers to short-term changes in color-based signals (but see Sullivan 1994a, Torres and Velando 2003, Velando et al. 2006). Clearly, further research is necessary to evaluate whether receivers derive unique information about individual quality or condition from short term changes in integument color.

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