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A multifactorial test of the effects of carotenoid access, food intake and parasite load on the production of ornamental feathers and bill coloration in American goldfinches

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SUMMARY

It has been well established that carotenoid and melanin pigmentation are often condition-dependent traits in vertebrates. Expression of carotenoid coloration in birds has been shown to reflect pigment intake, food access and parasite load; however, the relative importance of and the potential interactions among these factors have not been previously considered. Moreover, carotenoid and melanin pigmentation have been proposed to signal fundamentally different aspects of individual condition but few data exist to test this idea. We simultaneously manipulated three environmental conditions under which American goldfinches (*Cardeulis tristis*) grew colorful feathers and developed carotenoid pigmentation of their bills. Male goldfinches were held with either high or low carotenoid supplementation, pulsed or continuous antimicrobial drug treatment, or restricted or unlimited access to food. Carotenoid supplementation had an overriding effect on yellow feather coloration. Males given more lutein and zeaxanthin grew yellow feathers with hue shifted toward orange and with higher yellow chroma than males supplemented with fewer carotenoids. Parasites and food access did not significantly affect yellow feather coloration, and there were only minor interaction effects for the three treatments. By contrast, bill coloration was significantly affected by all three treatments. Carotenoid supplementation had a significant effect on yellow chroma of bills, drug treatment and food access both had a significant effect on bill hue, and food access had a significant effect on the yellow brightness of bills. Neither the size nor blackness of the black caps of male goldfinches was affected by any treatment. These results indicate that pigment intake, food access and parasite load can have complex and variable effects on color displays, and that feather and bill coloration signal different aspects of male condition.

Key words: carotenoids, melanins, plumage color, body composition, indicator mechanism.

INTRODUCTION

Many of the ornamental traits of animals, including the antlers of deer, the calls of frogs, the courtship displays of fish and the brilliant feather colors of birds, have been shown to be condition-dependent signals of individual quality (Andersson, 1994). These so-called indicator traits are proposed to reveal specific aspects of individual condition, knowledge of which benefits a receiver such as a female choosing a mate (Andersson, 1994; Hill, 2002). While a general link between condition and ornament expression has been established for many traits, the relative importance of and the interaction between different environmental challenges in shaping the expression of ornamental traits remains essentially unstudied.

Integumentary coloration is a complex trait that seems to encode a variety of information about the condition of individuals (Hill and McGraw, 2006). Two classes of pigments are responsible for much of the coloration in plumage, bills and the bare parts of birds. Melanins produce the black, brown and rusty coloration of feathers (McGraw, 2006b) whereas carotenoid pigments are responsible for most of the yellow, orange and red coloration (Goodwin, 1984). The mechanisms of production of these different types of color displays are likely to affect the manner in which each responds to specific environmental challenges. Carotenoid pigments cannot be synthesized by birds or any vertebrates and must be ingested (McGraw, 2006a; Völker, 1934). Thus, carotenoid coloration can potentially vary with access to appropriate dietary pigments needed for coloration (Hill, 2002; Hill, 2006). Within the bodies of birds, carotenoids must be absorbed, transported and deposited, and these processes of utilization require energy and might be disrupted by various environmental perturbations (Hill, 2002; Hill, 2006). Finally, while carotenoids cannot be synthesized, they can be biochemically modified by birds once they are ingested (McGraw, 2006a). For instance, some species can convert yellow dietary pigments to red pigments before they deposit them in feathers (McGraw et al., 2001; Stradi et al., 1997).

Melanin pigments are synthesized within the bodies of birds from the amino acid tyrosine (McGraw, 2006b). Dietary tyrosine can be used directly to synthesize melanin, or phenylalanine can be converted into tyrosine, which can then be used to synthesize melanin (McGraw, 2006b). Thus, while melanin pigmentation is not dependent on dietary pigments as is carotenoid pigmentation, individuals must ingest enough of the right type of amino acids to produce maximum color expression, so nutrition has the potential to affect pigmentation. The need to ingest specific minerals during molt might also affect the expression of melanin coloration (McGraw, 2003; McGraw, 2007; McGraw, 2008).

To date, studies on the signal content of melanin and carotenoid pigmentation have focused on the singular effects of specific environmental factors on the expression of these different types of pigment coloration. These studies clearly show that the environment in which feather and bill coloration are produced can have a large impact on color expression. Access to quantities of specific types of dietary carotenoid pigments at the time of molt has been shown to have a significant effect on the expression of red, orange and yellow coloration in many species of birds in captivity (reviewed by Hill,

2006). In a study of wild house finches (*Carpodacus mexicanus*), the concentration of carotenoids in the diet of males was positively correlated with the redness of the feathers that they were growing (Hill et al., 2002). Infection by various parasites has also been shown to depress the expression of carotenoid coloration. Male house finches and American goldfinches (*Carduelis tristis*) infected with coccidia (*Isospora* spp.) had less red and less saturated plumage coloration than males kept free of coccidiosis (Brawner et al., 2000; McGraw and Hill, 2000). In addition, infection with the bacterium *Mycoplasma gallicepticum* at the time of molt caused male house finches to grow less red and less saturated plumage compared with control males (Hill et al., 2004). The same bacterial and coccidial infections that depressed the expression of carotenoid coloration had no effect on the color quality of either ornamental or non-ornamental melanin pigmentation of American goldfinches and house finches, respectively, or on the size of melanin crown patches in American goldfinches (Hill and Brawner, 1998; McGraw and Hill, 2000; McGraw et al., 2005). And finally, restricting food access during molt caused male house finches and American goldfinches to grow less red and less saturated plumage (Hill, 2000; McGraw et al., 2001) but the same food restriction had no effect on color quality of non-ornamental tail feathers of house finches (Hill, 2000; McGraw et al., 2001) or the size or color quality of house sparrow (*Passer domesticus*) badges (McGraw et al., 2002). Restricting the availability of specific amino acids in the diets of house sparrows, however, caused males to grow badges with lower achromatic brightness than controls (Poston et al., 2005). Amino acid restriction had no effect on the badge size of male house sparrows (Poston et al., 2005).

These single-factor experiments identified specific environmental factors that can affect the expression of ornamental coloration. From these single-factor experiments, however, it is impossible to know the relative importance of pigment access, parasite load and nutrition in determining color expression. Moreover, these environmental challenges do not act on individuals independently but rather they interact in complex manners. To gain a more comprehensive understanding of how environmental factors shape color expression, a multifactorial design is needed.

We simultaneously tested the effects of pigment access, parasite load and food access on ornamental yellow carotenoid and black eumelanin pigmentation in male American goldfinches (*Carduelis tristis* Linnaeus 1758). A primary goal of this experiment was to test for links between the expression of ornamental coloration and condition; however, condition is often loosely defined in studies of ornamental traits. Chemical analyses of body composition are the most accurate and direct measures of an individual's stored resources and muscle and organ development. Therefore, we directly measured the body composition of birds in our treatment groups and used body composition as an index of condition. We focused on both feathers, in which pigment coloration is fixed at the completion of molt, and bill color, which can respond to factors such as stress and carotenoid supplementation within days (McGraw, 2006a).

MATERIALS AND METHODS

We captured American goldfinches from large winter flocks in January and February 2005 in Lee County, Alabama, USA by trapping them at established feeding stations. As birds were captured we sorted them by sex and age (first-year or older) following Pyle and colleagues (Pyle et al., 1987). We retained only first-year males for this study, releasing females and older males. In this way, we standardized for the effect of sex and age on coloration. Birds were collected and handled according to federal (# 21661), state (#97181) and IACUC (2005–0825) permits.

General design

Within one week of capture, we placed the birds in small cages ($0.5\times0.5\times0.5$ m) in rooms with large windows that emitted abundant natural light, allowing the birds in the present study to molt on a natural light regime. We randomly assigned two birds to each cage, and we assigned each cage to a carotenoid, disease and nutritional treatment as follows. Half of the males were provided with a high dose of carotenoids in their water and half of the males were provided with a moderate dose of carotenoids in their water. Half of the males were given continuous treatment with the anti-coccidial drug sulfadimethoxine and half were given sulfadimethoxine two out of every three days. Half the males were provided with *ad libitum* food and half had the food removed from their cages periodically during molt. A total of eight treatment combinations were possible and our design called for eight replicates of each treatment combination, so we maintained 32 cages of birds housing 64 individuals (Table 1).

Carotenoid treatment

Carotenoids were provided as a 70:30 mix of lutein and zeaxanthin following Navara and Hill (Navara and Hill, 2003). Males in half of the cages were provided with carotenoids in the form of starch gel beadlets dissolved in water at a concentration of 1000 mg of beadlets per liter of drinking water, which was the high-carotenoid supplementation and half were provided with 10 mg of beadlets per liter of drinking water, which was the low-carotenoid treatment. High- and low-carotenoid levels were chosen based on the response of male goldfinches to various doses of supplemental carotenoids in Navara and Hill (Navara and Hill, 2003).

Parasite treatment

As a means to manipulate the degree to which male goldfinches were parasitized, we added to the drinking water either a constant or pulsed dose of sulfadimethoxine ($0.26\,g\,l^{-1}$), a broad-spectrum antimicrobial drug that depresses a wide range of parasitic microbes (Cates, 1986). For the pulsed dose, sulfadimethoxine was withheld every third day. Our target parasite was isoporan coccidia, which we knew from previous studies depresses feather coloration in American goldfinches (McGraw and Hill, 2000) and responds to sulfadimethoxine; however, we expected treatment with sulfadimethoxine to potentially affect a range of parasites. Sulfadimethoxine is broad-spectrum and microbiostatic rather than microbiosidal (Chambers and Jawetz, 1998), meaning that this class

Table 1. The experimental design used to test for the effects of multiple simultaneous environmental challenges on the production of bill and feather coloration in male American goldfinches

Group	Carotenoids	Food	Drug dose
1	High	*Ad libitum*	High
2	High	Restricted	High
3	Low	*Ad libitum*	High
4	Low	Restricted	High
5	High	*Ad libitum*	Low
6	High	Restricted	Low
7	Low	*Ad libitum*	Low
8	Low	Restricted	Low

Male American goldfinches received either high or low levels of lutein and zeaxanthin in their drinking water, they had access either to *ad libitum* food or their food was removed for 6 h periods scattered throughout the week and they received either continuous (high drug dose) or pulsed doses (low drug dose) of sulfadimethoxine as protection against pathogens.

of drugs depresses the biological activity of microbial pathogens but does not kill them. We assumed that the pulsed dose would allow parasites to persist at higher levels than at the constant dose. To check the effect of sulfadimethoxine treatment on coccidial infection, one month after males were assigned to a treatment, when all individuals were undergoing molt, we collected a fecal sample from each male after 15:00 h and screened the collected fecal samples for coccidial oocysts following Brawner and colleagues (Brawner et al., 2000).

Food treatment

Males were either given unlimited access to food or had all food removed from their cages during mornings or afternoons. We staggered food removal between mornings and evenings following Hill (Hill, 2000), such that birds in the food-restricted treatment group had no access to food for 38% of daylight hours during molt. On no-food mornings, food dishes were removed just before dark on the evening before and returned at the midpoint of daylight the following day. Alternatively, for afternoons with no food, food was removed at the midpoint of daylight and returned to cages just before sunrise the following morning.

Body composition and feather collection

After all of the birds had completed growth of yellow and black feathers, all food was removed from the cages on 29 April 2005 at sunset to ensure that birds would be post-absorptive the next morning. On 30 April 2005, males were removed from the cages and killed. We immediately weighed each bird and took a digital image of the bill with color references in the image. We pulled approximately 20 feathers from the crown and 20 feathers from the upper breast of each male and measured the cap size as the length of black feathering from the bill to the back of the cap. Carcasses were then placed in airtight plastic bags and frozen.

At a later date, carcasses were thawed to determine the fat and lean mass. Birds were weighed and then homogenized in a Waring Laboratory Blender (Torrington, CT, USA), dried to a constant mass for approximately 76 h in a laboratory oven at 60°C and then blended with a Braun coffee grinder (Proctor and Gamble, South Boston, MA, USA) to improve homogeneity. Carcasses were then dried again for an additional 76 h to determine the final dry mass. Care was taken to account for all tissue lost during homogenization. The fat content of homogenized samples (1.00±0.15 g each) (±s.e.m.) was determined in duplicate in a soxhlet apparatus (Pyrex Brand, Corning, Lowell, MA, USA). Samples were sealed in paper tea bags, with the top of the bag folded and stapled to reduce fine particulate loss. The full tea bag was then placed in an alundum extraction thimble within the soxhlet extraction tube, and lipids were extracted with petroleum ether for approximately 12 h. After all of the ether had evaporated, the bagged sample was removed from the soxhlet, the sample was air-dried overnight and dried for approximately 3 h at 60°C before determining the final fat-free dry mass. We calculated percentage fat (fat mass/body mass×100) as an indicator of relative energy reserves and lean dry mass [(total dry mass–fat mass)/(dry mass×100)] as a measure of total muscle and organ mass.

Color measurements

We measured the color quality of yellow feathers using a reflectance spectrophotometer following standard techniques as described in Shawkey and colleagues (Shawkey et al., 2006). Briefly, we took reflectance measurements with an Ocean Optics S2000 spectrometer (range 250–880 nm: Dunedin, FL, USA) using a bi-furcated micron fiber optic probe at a 90 deg. angle 5 mm from the feather surface. A 2 mm area was illuminated with both UV (deuterium bulb) and visible (tungsten–halogen bulb) light sources. Reflectance data were generated relative to a white standard (Labsphere, North Sutton, NH, USA).

We calculated color variables from spectral reflectance data between 320 and 700 nm. We calculated hue as the point of maximum inflection of the curve, brightness as the mean reflectance between 320 and 700 nm and UV chroma and yellow chroma as the percentages of total light reflected in the range of 320–400 and 575–600 nm, respectively.

We were unable to take bill color measurements with the spectrometer when birds were killed at the end of the experiment, and we suspected that bill coloration would fade in frozen birds. Therefore, we used digital images with a color standard taken of the right side of each bird within 5 min of death for color analysis. We used Adobe Photoshop color sampler tool (Adobe Photoshop CS3 extended, v. 10.0, Adobe Systems, San Jose, CA, USA) to quantify yellow hue, saturation and brightness at three points on the lateral lower mandible and medium gray to black (hereafter, black) pigmentation at three points at the tip of the upper mandible. The assistant who made these measurements did not know the treatment grouping of any of the males and was instructed to sample the most intense areas of yellow pigmentation and the darkest areas of melanin pigmentation, thereby eliminating the possibility of quantifying scuffed portions of the bill or regions with glare as may have happened if the points were chosen randomly. We averaged the color measurements from each bill to arrive at single yellow hue, chroma (saturation) and brightness and a single black brightness for each bird. For melanin pigmentation of bills, we were interested in achromatic brightness so we excluded hue and chroma measurements. We included the same yellow and gray color swatches in each image, measured the hue, chroma and brightness of each and used these measurements to standardize all photographs based on the deviation of each standard from the mean hue, chroma and brightness of each swatch. Because we used standardized lighting for all digital images, only minor adjustments between images were necessary.

Units generated with Photoshop differ from those generated from reflectance spectrophotometry values and thus spectrophotometry and Photoshop values should not be considered comparable. In Photoshop, yellow–orange hue is a measure of the rotation around a color wheel (0–360 deg.); high values are closer to green wavelengths and lower values are closer to red wavelengths. Saturation is scaled from 0 to 100% with 0% dull and 100% fully saturated. Brightness is also scaled from 0 to 100% with 0% black and 100% white (Adobe Photoshop CS3 extended, v. 10.0 Manual). Digital photographs record only human visible coloration, so no analysis of UV coloration was possible for bills.

Digital photographs were also used to compare the relative area of bills with dark melanin pigmentation. Extent of melanin pigmentation was based on the right profile of the bird. Each photograph was opened in ‘ImageJ’ software (National Institutes of Health, Bethesda, MD, USA); measurements were standardized to a 1 cm ruler in each photograph. The polygon tool was then used to determine the area of the bill and the area that had conspicuous melanin pigmentation.

Statistics

All statistical analyses were completed using SAS 9.1.3 (SAS Institute, Cary, NC, USA). We used analysis of variance [ANOVA (proc GLM)] to determine if there were differences in the number of coccidia oocytes between treatment groups. We used factorial

ANOVA (proc GLM) to examine the effect of treatment (high or low carotenoids, continuous or pulsed sulfadimethoxine and *ad libitum* or restricted food access) on breast coloration, bill coloration, cap brightness, cap size and body composition variables, with an independent test run for each dependent variable. Because birds were held as pairs in cages, we tested for a cage effect by including cage as a covariate in initial analyses. We found no significant effect of cage in any comparison (P>0.17), so we removed cage from our final analyses. All proportional data were arcsine transformed, including all feather chroma measurements, body fat and lean body mass measurements and bill coloration chroma and saturation measurements. We then used an a posteriori Eta-squared test, which quantifies the proportion of total variation within the model explained by each treatment (Olejnik and Algina, 2000; Olejnik and Algina, 2003). The relationship between body mass and body composition variables and breast coloration and bill coloration variables were examined using multiple regressions. Single-variable regressions were used to examine the relationship between body mass and body composition variables and cap size.

RESULTS

Effectiveness of drug treatment

We found no difference in the level of coccidial infection between males in the pulsed and continuous drug treatment groups ($F_{6,56}$=1.24, P=0.298). Fecal analysis showed that no birds were passing more than a few oocysts (range 0–23 oocysts) whereas cardueline finches in the wild infected by coccidia commonly pass thousands or tens of thousands oocysts (Brawner et al., 2000). Sulfadimethoxine is a broad-spectrum antimicrobial that might affect a wide range of microbial pathogens in American goldfinches (Cates, 1986), so we retained drug treatment groupings in our statistical analyses. Hereafter, we refer to the sulfadimethoxine treatment as the ‘drug treatment’ due to the non-specific nature of this drug.

Treatment effects on yellow feathers

Carotenoid access had significant positive effects on the yellow hue and yellow chroma of carotenoid-based breast feathers (Fig. 1, Table 2). Based on the results of the Eta-squared analyses, carotenoid treatment accounted for 44.4% of the variation in yellow hue and 28.7% of the variation in yellow chroma (Fig. 2). Food access and drug treatment did not independently influence yellow body variables. There was, however, a significant interaction between carotenoid access and drug treatment and yellow hue (Table 2). In this interaction and in other treatment interactions that had significant effects on color expression, we observed that multiple environmental challenges do not necessarily have simple additive effects on color. For example, we predicted that the low carotenoid and low drug treatment birds would have the lowest hue among the four treatments in this interaction and that the high carotenoid and high drug treatment group would have the highest hue values. What we observed, however, was a ranking of treatment combinations from the lowest color expression to the highest as follows: (1) low carotenoids, low drug treatment [$\bar{x}$=490.4±0.1 (±s.e.m.)], (2) low carotenoids, low drug treatment [$\bar{x}$=491.4±0.4 (±s.e.m.)], (3) high carotenoids, high drug treatment [$\bar{x}$=493.3±0.6 (±s.e.m.)] and (4) high carotenoids and low drug treatment [$\bar{x}$=493.8±0.2 (±s.e.m.)].

For the UV component of breast coloration, the UV hue did not vary with treatment but treatment did significantly affect UV chroma with a significant interaction between carotenoids and food access (Table 2) {interactions ranked from lowest to highest color expression: (1) high carotenoids, *ad libitum* food [$\bar{x}$=18.7±0.9 (±s.e.m.)], (2) low carotenoids, restricted food [$\bar{x}$=19.7±0.6 (±s.e.m.)], (3) low carotenoids, *ad libitum* food [$\bar{x}$=20.4±0.8 (±s.e.m.)] and (4) high carotenoids, restricted food [$\bar{x}$=21.2±0.5 (±s.e.m.)]}. Brightness of body feathers was not impacted by carotenoids, food access or drug treatment independently; however, interactions between carotenoids and drug treatment {ranked from lowest to highest: (1) high carotenoids, low drug treatment [$\bar{x}$=12,104±745 (±s.e.m.)], (2) low carotenoids, high drug treatment [$\bar{x}$=14,072±765 (±s.e.m.)], (3) high carotenoids, high drug treatment [$\bar{x}$=14,910±1242 (±s.e.m.)] and (4) low carotenoids and low drug treatment [$\bar{x}$=15,825±1219 (±s.e.m.)]}, carotenoids and food access {ranked from lowest to highest: (1) high carotenoids, *ad libitum* food [$\bar{x}$=12,459±863 (±s.e.m.)], (2) low carotenoids, restricted food [$\bar{x}$=14,232±862 (±s.e.m.)], (3) high carotenoids, restricted food [$\bar{x}$=15,010±1320 (±s.e.m.)] and (4) low carotenoids, *ad libitum* food [$\bar{x}$=15,948±1308 (±s.e.m.)]} and drug treatment and food access were significant (Table 1) {ranked from lowest to highest: (1) high drug treatment, *ad libitum* food [$\bar{x}$=13,196±803 (±s.e.m.)], (2) low drug treatment, restricted food [$\bar{x}$=13,285±862 (±s.e.m.)], (3) low drug treatment, *ad libitum* food [$\bar{x}$=15,378±1542 (±s.e.m.)] and (4) high drug treatment, restricted food [$\bar{x}$=16,863±1151 (±s.e.m.)]}.

All significant interactions account for no more than 10.9% of the variation in color (Fig. 2).

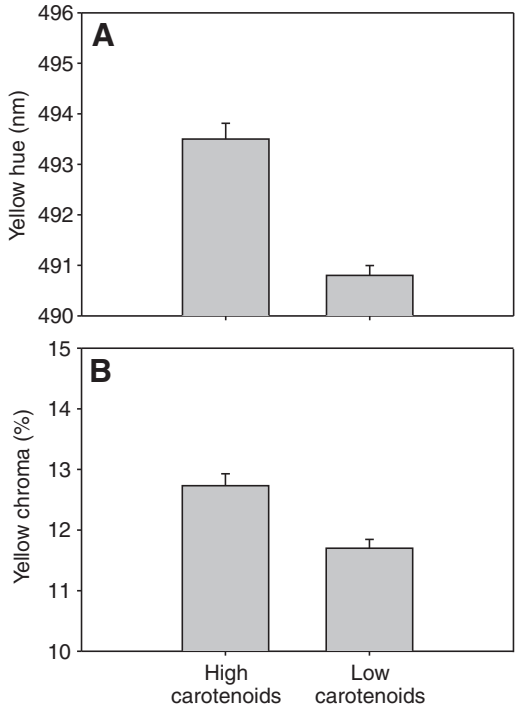

Fig. 1. Mean yellow hue (A) and yellow chroma (B) of breast feathers of male American goldfinches held on diets supplemented with either low or high doses of lutein and zeaxanthin. Lines above the bars show standard errors of the mean.

Treatment effects on bill coloration

Treatment influenced all measures of bill coloration including yellow hue and yellow chroma and both yellow and black brightness (Table 3); however, treatment did not influence the proportion of the bill with black pigmentation [$\bar{x}$=4.86±0.41% (±s.e.m.); factorial ANOVA, $F_{7,40}$=1.13, P=0.363]. Drug treatment significantly impacted yellow hue (Table 3; Fig. 3A), with birds receiving a continuous dose of sulfadimethoxine displaying a greater hue than

Table 2. Effect of treatment on breast coloration in American goldfinches

Breast coloration	Yellow		Ultra-violet		
	Hue	Chroma	Hue	Chroma	Overall brightness
Overall	**$F_{7,40}$=9.90, P<0.001**	**$F_{7,40}$=3.91, P=0.003**	**$F_{7,40}$=1.19, P=0.332**	**$F_{7,40}$=2.32, P=0.044**	**$F_{7,40}$=3.49, P=0.005**
Carotenoids	**F=48.6, P<0.001**	**F=16.3, P<0.001**	–	F=0.21, P=0.647	F=1.77, P=0.191
Drug treatment	F=0.16, P=0.683	F=1.15, P=0.291	–	F=3.69, P=0.062	F=1.03, P=0.317
Food	F=0.30, P=0.589	F=4.03, P=0.051	–	F=3.80, P=0.058	F=1.21, P=0.278
Carotenoids×drug treatment	**F=5.05, P=0.030**	F=3.08, P=0.087	–	F=2.25, P=0.141	**F=7.05, P=0.011**
Carotenoids×food	F=0.00, P=0.960	F=1.44, P=0.237	–	**F=5.75, P=0.021**	**F=4.94, P=0.032**
Food×drug treatment	F=1.01, P=0.320	F=0.51, P=0.479	–	F=0.46, P=0.503	**F=6.75, P=0.013**
Carotenoids×food×drug treatment	F=2.57, P=0.117	F=0.41, P=0.711	–	F=2.39, P=0.130	F=1.74, P=0.195

Both independent treatments and their interactions are considered. The results of factorial ANOVA's are given including the overall F, d.f. and P for each tests and partial F and P values for each variable in significant tests. Bold font indicates significant results.

those receiving a pulsed dose; drug treatment accounted for 18.0% of the variation in yellow hue (Fig. 5A). Food access also significantly impacted yellow hue and both yellow and black brightness with animals on restricted food access, displaying a bill with low yellow hue and greater yellow and black brightness (Table 3; Fig. 3B, Fig. 4B,D). This treatment accounted for 6.4% of the variation in bill hue and a large proportion of the variation in yellow (39.9%) and black brightness (27.3%) (Fig. 5A,C,D). Carotenoid access influenced the yellow chroma of the bill; animals on the high carotenoid treatment displaying greater chroma than animals on the low carotenoid treatment (Table 3; Fig. 3C);

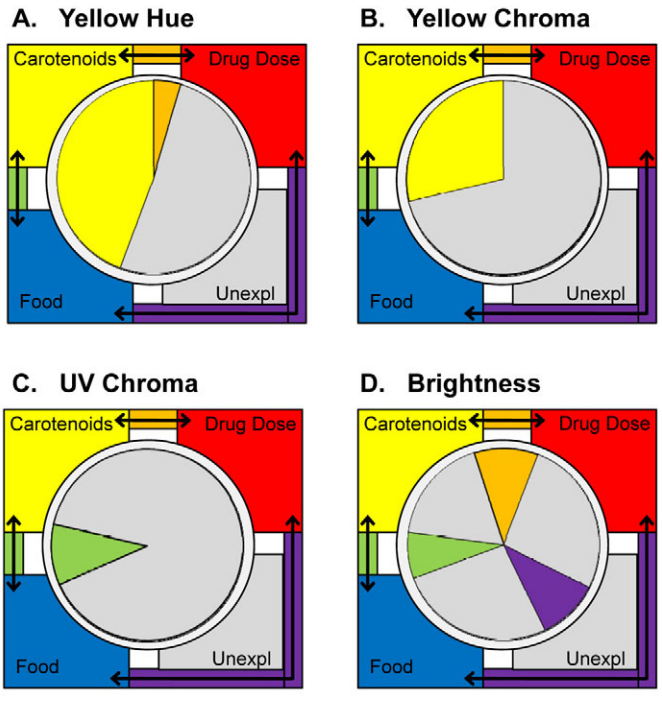

Fig. 2. Pie charts showing the results of an Eta-squared analysis describing the proportion of variation in yellow hue (A), yellow chroma (B), UV chroma (C) and brightness of yellow body plumage (D) of male American goldfinches that was explained by carotenoid intake (yellow), food access (blue) or drug treatment (i.e. drug dose; red) or that remained unexplained (unexpl; gray). Also shown is the proportion of variation explained by the interactions among treatments. Significant interactions are displayed in secondary colors as indicated by the bar under the arrow connecting the interacting variables.

carotenoid treatment accounted for 31.2% of the variation in chroma (Fig. 5B). There were also significant interactions between brightness and carotenoid access and food access {ranked from lowest to highest: (1) high carotenoids, *ad libitum* food [$\bar{x}$=12,459±863 (±s.e.m.)], (2) low carotenoids, restricted food [$\bar{x}$=14,232±862 (±s.e.m.)], (3) high carotenoids, restricted food [$\bar{x}$=15,010±1320 (±s.e.m.)] and (4) low carotenoids, *ad libitum* food [$\bar{x}$=15,948±1308 (±s.e.m.)]} and the three-way interaction between carotenoid, food access and drug treatment {ranked from lowest to highest: (1) high carotenoids, low drug treatment, *ad libitum* food [$\bar{x}$=42.9±7.1 (±s.e.m.)], (2) low carotenoids, high drug treatment, *ad libitum* food [$\bar{x}$=52.0±2.6 (±s.e.m.)], (3) low carotenoids, low drug treatment, *ad libitum* food [$\bar{x}$=57.1±4.1 (±s.e.m.)], (4) high carotenoids, high drug treatment, restricted food [$\bar{x}$=59.8±16.2 (±s.e.m.)], (5) high carotenoids, high drug treatment, *ad libitum* food [$\bar{x}$=62.4±5.3 (±s.e.m.)] and (6) high carotenoids, low drug treatment, restricted food [$\bar{x}$=76.4±2.7 (±s.e.m.)]}. Significant interaction terms accounted for no more than 6.35% of the variation in bill coloration (Fig. 5).

Treatment effects on black feathers and condition

There was no significant effect of treatment on cap brightness [$\bar{x}$=2135±100 (±s.e.m.); factorial ANOVA, $F_{7,40}$=2.09, P=0.067] or cap size [$\bar{x}$=14.15±0.44 (±s.e.m.); factorial ANOVA, $F_{7,40}$=0.74, P=0.638]. Likewise, we found no significant effect of treatment on body mass [$\bar{x}$=14.30±0.23 (±s.e.m.); factorial ANOVA, $F_{7,40}$=0.84, P=0.558], percentage body fat [$\bar{x}$=10.1±0.5 (±s.e.m.); factorial ANOVA, $F_{7,37}$=1.05, P=0.415] or percentage lean dry mass [$\bar{x}$=78.7±1.0 (±s.e.m.); factorial ANOVA, $F_{7,37}$=0.95, P=0.481].

Relationships among color and condition variables

Linear regressions examining the relationship between breast feather coloration and bill yellow coloration indicate that the hue and brightness of these structures are independent (hue: $F_{1,44}$=0.21, P=0.648; brightness: $F_{1,44}$=0.26, P=0.610) whereas there is a significant positive relationship between breast feather coloration and bill yellow chroma ($F_{1,44}$=27.6, P<0.001, R^2=0.386, bill chroma=(20.72×feather chroma)–1.63). Breast coloration, bill coloration and cap size were independent of body mass, percentage body fat or percentage lean dry mass (breast coloration: body mass: $F_{5,38}$=1.54, P=0.201; percentage body fat: $F_{5,38}$=0.48, P=0.792; percentage lean dry mass: $F_{5,38}$=0.40, P=0.849; bill coloration: body mass: $F_{7,36}$=0.47, P=0.853; percentage body fat: $F_{7,36}$=0.96, P=0.477; percentage lean dry mass: $F_{7,36}$=1.34, P=0.260; cap size: body mass: $F_{1,42}$=1.20, P=0.279; percentage body fat: $F_{1,42}$=1.27, P=0.267; percentage lean dry mass: $F_{1,42}$=0.57, P=0.455).

Table 3. The effect of treatment on bill coloration in American goldfinches

	Yellow		Black	
Bill coloration	Hue	Chroma	Brightness	Brightness
Overall	**$F_{7,40}$=3.81, P=0.003**	**$F_{7,40}$=5.67, P<0.001**	**$F_{7,40}$=11.0, P<0.001**	**$F_{7,40}$=5.54, P=0.001**
Carotenoids	F=0.19, P=0.663	**F=24.9, P<0.001**	**F=7.07, P=0.011**	**F=7.87, P=0.008**
Drug treatment	**F=12.0, P=0.001**	F=0.14, P=0.709	F=0.02, P=0.892	F=0.00, P=0.978
Food	**F=4.33, P=0.044**	F=0.51, P=0.478	**F=46.6, P<0.001**	**F=21.5, P<0.001**
Carotenoids×drug treatment	F=1.52, P=0.225	F=3.13, P=0.085	F=0.29, P=0.594	F=1.40, P=0.244
Carotenoids×food	F=2.20, P=0.146	F=0.19, P=0.668	**F=5.66, P=0.022**	F=3.49, P=0.069
Food×drug treatment	F=0.88, P=0.354	F=0.18, P=0.673	F=3.98, P=0.053	F=0.04, P=0.840
Carotenoids×food×drug treatment	F=0.38, P=0.539	F=1.53, P=0.223	**F=7.41, P=0.010**	F=1.85, P=0.181

Both independent treatments and their interactions are considered. The results of factorial ANOVA's are given including the overall F, d.f. and P for each tests and partial F and P values for each variable in significant tests. Bold font indicates significant results.

DISCUSSION

Previous research with various species of birds has established that parasite load, carotenoid ingestion and food access can each affect the hue, saturation and brightness of feathers and bills pigmented with carotenoids (reviewed by Hill, 2002; Hill, 2006). In wild populations, we would expect most individual birds to be subjected to all three environmental challenges during feather growth. Yet the relative importance of these factors to feather and bill coloration had not been previously studied. The three-factor experiment that we report in the present study is the first attempt to assess both the relative importance and interactive effects of pigment access, food access and parasite load on feather and bill coloration. In addition, we determined whether coloration was correlated with body condition variables, including body mass, body fat (used as an indicator of energy reserves) and total body lean mass (indicative of cumulative muscle and organ condition). Our results confirm some central themes of prior research but also reveal new patterns.

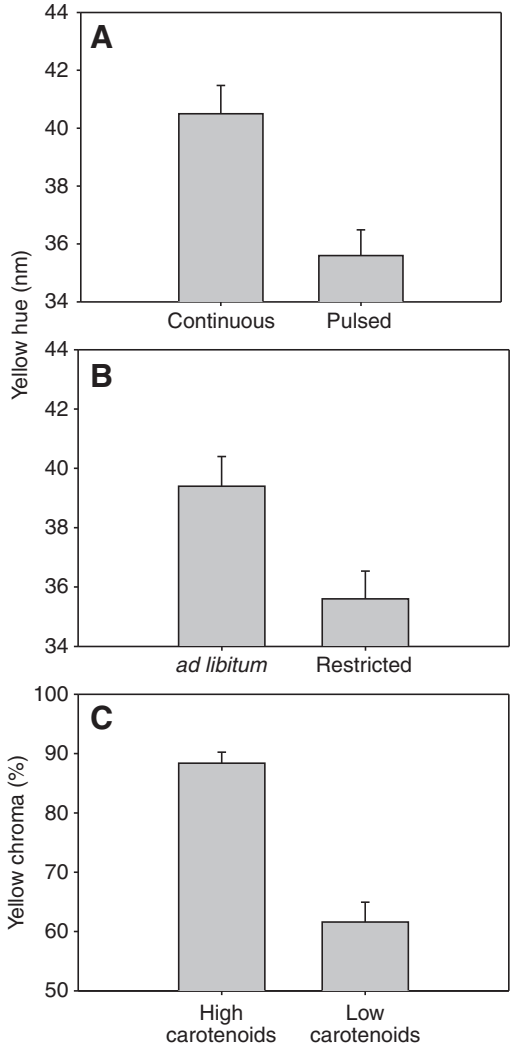

Fig. 3. Mean yellow hue or yellow chroma of bills of male American goldfinches held with continuous or pulsed treatment with sulfadimethoxine (A), with *ad libitum* or restricted food access (B) and with either low or high doses of lutein and zeaxanthin (C). Lines above bars show standard errors of the mean.

Multiple factors and feather color

In our three-treatment experiment, the amount of carotenoid pigment ingested during molt had an overriding effect on the hue and chroma of yellow feathers of male American goldfinches; males that ingested more carotenoids grew feathers that were more intensely pigmented and had hues shifted toward orange. Contrary to the results of single-variable studies, we found that access to food and protection from pathogens had no significant effect on any aspect of yellow feather coloration. Various interactions among treatments had small effects on the coloration of yellow feathers accounting for less than 11% of the variation in any color parameter. In previous single-factor studies, carotenoid access had a large effect on the expression of carotenoid-based feather coloration (Hill, 2002; Hill, 2006), and in the present study we show that the effects of pigment access on yellow feather coloration can swamp the effects of nutrition and drug treatment. These observations indicate that, at least under some conditions, pigment access can be the most important environmental factor in determining expression of carotenoid-based plumage coloration.

Interaction effects were much smaller than the effects of carotenoid supplementation alone but the interactions that we observed among treatments indicate that the relationships among environmental variables are complex. Because the response to multiple variables is not additive in predicted directions, multiple challenges in some cases may act to dampen rather than enhance coloration variation between treated and untreated individuals.

Multiple ornaments, multiple signals

The response of bill coloration to treatments was distinctly different from the response of yellow feather coloration, revealing a complex interplay among the three treatments. As with feathers, access to carotenoid pigments had a significant effect on the color of bills but the effect was essentially restricted to yellow chroma; there was only a small effect of carotenoid pigments on brightness and none

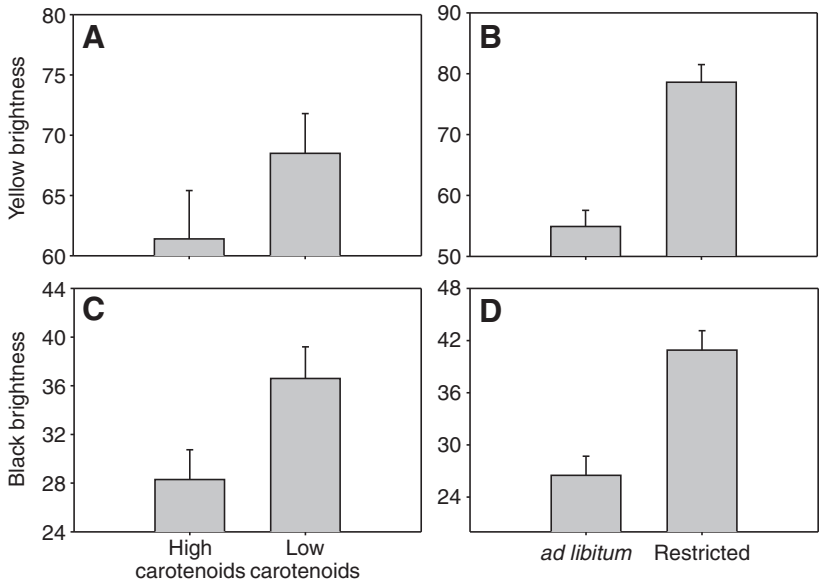

Fig. 4. Mean brightness of the dark or yellow portions of bills of male American goldfinches held with either low or high doses of lutein and zeaxanthin (A and C) or with *ad libitum* or restricted food access (B and D). Lines above bars show standard errors of the mean.

on hue. Yellow hue was affected primarily by drug treatment and secondarily by food access. Food access had the biggest effect on both yellow and black brightness with small additional effects of carotenoid pigment access. In contrast to the conclusions regarding plumage coloration, these observations indicate that carotenoid intake, food access and likely parasite load all shape expression of bill coloration in American goldfinches.

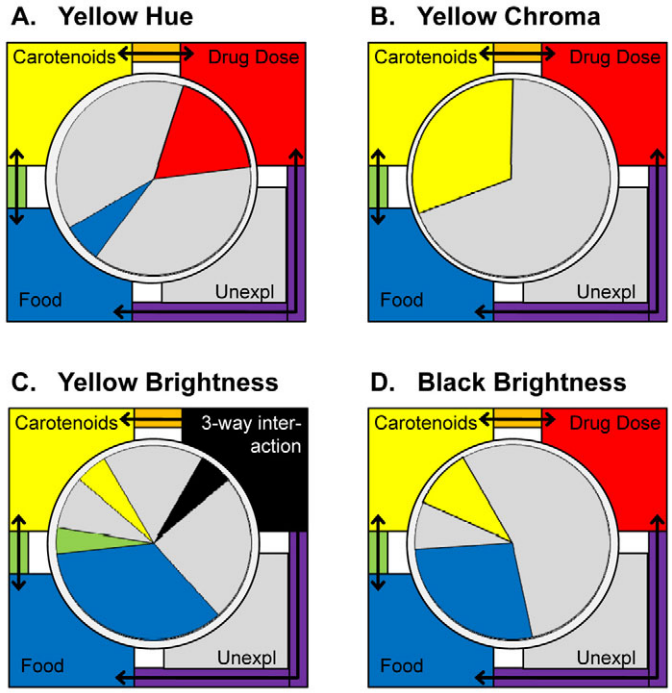

Fig. 5. Pie charts showing results of an Eta-squared analysis describing proportion of variation in yellow hue (A), yellow chroma (B) and the brightness of the yellow (C) or black portions (D) of bills of male American goldfinches that was explained by carotenoid intake (yellow), food access (blue) or drug treatment (i.e. drug dose; red) or that remained unexplained (unexpl; gray). Also shown is the proportion of variation explained by the interactions among treatments. Significant interactions are displayed in secondary colors as indicated by the bar under the arrow connecting the interacting variables. Black in C indicates a significant three-way interaction.

These effects of carotenoid supplementation and food access on bill coloration in American goldfinches are the opposite of the patterns found in a study of the coloration of the feathers of great tits (*Parus major*) by Senar and colleagues (Senar et al., 2008). In the tit study, intake of lutein affected the hue but not the chroma of feathers and body condition affected the chroma but not the hue. The observations of Senar and colleagues (Senar et al., 2008) concern feathers rather than bills and in the great tit, dietary pigments are deposited in feathers unchanged. In American goldfinches, lutein and zeaxanthin are converted into canary xanthophylls before being deposited. These differences in carotenoid processing may account for the differences in the response of coloration in the two studies but it may be a general feature of carotenoid systems that the importance of specific environmental factors to color expression differs by circumstance.

The different responses of feather and bill coloration support the idea that bill and feather coloration are fundamentally different traits in songbirds, even if both are produced through carotenoid pigmentation (Hill, 2002; Hill, 2006). Indeed, not only did bill and feather coloration of male goldfinches respond differently to treatments but within individuals, bill coloration was a poor predictor of feather coloration. There was no significant relationship between either the hues or brightnesses of bills and feathers. Only yellow chroma was significantly correlated between feathers and bills. The very different response of feathers and bills supports the idea that birds have multiple ornaments like colored bills and feathers because the different ornaments signal different aspects of condition (Møller and Pomiankowski, 1993).

It is particularly interesting that the hue of bill coloration was significantly affected by drug treatment (presumably through the action of some unmeasured pathogen) whereas yellow feather coloration was not. It has been proposed that birds like American goldfinches trade-off the use of carotenoid pigments for enhancement of the immune system *versus* for color display (Lozano, 1994; Moller et al., 1999; von Schantz et al., 1999). To date, all attempts to link feather coloration with oxidative stress have failed (Fitze et al., 2007; Isaksson et al., 2005; Navara and Hill, 2003). By contrast, several studies have shown that activating the

immune system or inducing oxidative stress depresses bill coloration (Bertrand et al., 2006; Blount et al., 2003; Faivre et al., 2003; McGraw and Ardia, 2003; Perez et al., 2008). It is interesting in this regard that drug treatment affected the hue of goldfinch bills but not feather coloration.

Melanins *versus* carotenoids

In contrast to the strong effects of treatment on carotenoid coloration of both feathers and bills, we found no effect of our treatments on the brightness or size of the bold black melanin caps of the male goldfinches. These observations are consistent with a previous studies of the effects of coccidiosis on feather coloration in American goldfinches in which severe coccidial infection depressed the hue and chroma of feathers but did not affect cap size or blackness (McGraw and Hill, 2000) as well as other experimental studies on other songbirds showing the melanin pigmentation is not directly affected by food access or parasite load (reviewed by Hill, 2006). A study of house sparrows (McGraw et al., 2002) showed that the size of melanin badges is mediated by social interactions, a variable not manipulated or recorded in our study.

The black cap plumage of male American goldfinches was not affected by any of the variables that we manipulated in this experiment but black melanin pigmentation in the bill was. In the spring, male American goldfinches replace dark melanin pigmentation of bills with yellow and orange carotenoid pigmentation as they come into breeding condition (Mundinger, 1972). By the end of our experiment, all birds in our study had mostly orange bills with the majority of melanin withdrawn but most birds retained some black melanin pigmentation at the tip. We found that the amount of melanin pigmentation at the tip of the bill was not related to any treatment but the brightness (best thought of as a measure of blackness in this context) of the bill, which reflects melanin pigment concentration, was significantly affected by food access. The mechanisms by which food affected bill blackness is unknown, but it seems probable that better nutrition accelerated the transition to nuptial condition in some males causing more melanin to be withdrawn.

Body condition

In response to a challenging environment, birds may change how energy and other nutritional resources are partitioned between the maintenance and the development of yellow- and black-pigmented feathers. A change in energy partitioning can affect overall body composition (Lopez and Leeson, 2008) but the interaction between body condition and coloration has largely been overlooked. Although relative carotenoid intake is unlikely to have an effect on body condition, both parasite load and food intake can independently affect body composition (Daan et al., 1990; Delahay et al., 1995; Lopez and Leeson, 2008). Interestingly, in all prior studies on the effect of food access on coloration, body mass did not differ between groups (Hill, 2000; McGraw et al., 2001; McGraw et al., 2002). This suggests that animals with restricted intake were able to metabolically compensate for periods without access to food. Body mass was only reported in one prior study on parasites and coloration. McGraw and colleagues (McGraw et al., 2005) found that parasite treatment reduced body mass in the American goldfinch, suggesting that variation in color expression may be a secondary effect of change in body condition, rather than a direct effect of the parasites.

In our experiment, the body mass and composition of birds (percentage body fat and percentage lean dry mass) did not vary with treatment and was not correlated with breast or bill coloration or cap patch size. These observations suggest that food access and drug treatment are affecting carotenoid coloration through mechanisms other than body condition. One caveat to these conclusions is that all animals were sacrificed at the end of the molt of feathers after the majority of the feathers had been replaced. It remains possible that body condition earlier in molt impacted coloration.

Aviary *versus* field studies

In studies of color production in captive animals, environmental challenges must be manipulated in an artificial and somewhat contrived manner. Parasite and food manipulations were demonstrably mild in this study. We could find no effect of our variable drug treatment on degree of coccidiosis, our target effect. We have to assume that the higher dose of sulfa drugs in one treatment reduced one or a suite of unmeasured parasites leading to the effect that we observed but we cannot rule out the possibility that the drug itself caused the effect. Our food manipulation treatment caused no significant change in body composition between birds in the two groups, showing that it was a very mild nutritional stress. In previous studies, this same food removal technique had a significant effect on carotenoid feather coloration in house finches (Hill, 2000). The greater effects of food restriction in this previous study were probably a consequence of it being conducted in outdoor aviaries where weather subjected birds to greater thermal stress. Despite what appears to have been a modest manipulation of parasite exposure and food intake, parasite exposure was the treatment with the largest effect on bill hue and food intake was the treatment with the largest effect on bill brightness. These observations underscore the value to birds of multiple ornaments and to ornaments such as bill coloration that can reflect small differences in the condition of individuals.

The insights from the present study are important but are necessarily limited to the context of birds in cages. The obvious next step in this line of investigation is to conduct a similar multifactorial study on wild birds in natural habitats. Such a study will require special circumstances because individual birds will need to be tracked over months. Some means will have to be found to sample the food intake, carotenoid intake and parasite loads of the birds during molt. Such a study would be difficult on many of the birds traditionally studied with regard to carotenoid and melanin pigmentation but there do exist populations of other species that can be more easily tracked and repeatedly sampled, and it is to these species that future studies should look.

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