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The physiological costs of being colourful: nutritional control of carotenoid utilization in the American goldfinch, *Carduelis tristis*

KEVIN J. McGRAW*, GEOFFREY E. HILL† & ROBERT S. PARKER‡
*Department of Neurobiology and Behavior, Cornell University
†Department of Biological Sciences, Auburn University
‡Division of Nutritional Sciences, Cornell University

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Many sexually selected traits are thought to be costly to produce, which ensures that they communicate information honestly to conspecifics. Carotenoid pigmentation is classically considered as a costly sexual signal in many fish and birds. It is often argued that carotenoid colours are 'condition dependent', with an individual's nutritional or health state directly determining trait expression. However, few studies have investigated precisely how a compromised nutritional state affects an animal's capacity to develop these sexually attractive colours. Here, we studied the effect of food restriction on the ability of male American goldfinches to physiologically process carotenoids during the period of feather growth. We used high-performance liquid-chromatography to determine the types and amounts of carotenoids circulating in blood during moult as well as in newly grown colourful feathers. We show that nutritional deprivation affects the degree to which male goldfinches transport carotenoids through the bloodstream. Food-restricted males circulated significantly less blood carotenoids than controls. They also incorporated less carotenoids into feathers and grew less colourful plumage, but the decrease in plumage carotenoids did not significantly exceed the depressed amounts already present in blood. These results suggest that the means by which these yellow-coloured passerines either extract carotenoids from food (e.g. via lipoidal micelles) or transport them through blood (e.g. via lipoproteins) are more sensitive to changes in nutritional/energy state than are the mechanisms for metabolizing dietary pigments or depositing metabolites into feathers.

Handicap theories of sexual selection predict that traits conferring mating advantages must be costly to produce (Zahavi 1975; Grafen 1990); otherwise, these traits would be susceptible to cheating and would not reveal reliable information to conspecifics about the quality of the signaller. There is now overwhelming support for the idea that sexually selected features, from elaborate courtship dances to exaggerated weaponry, incur certain costs (Andersson 1994; Johnstone 1995). The countless studies that have examined signal costliness in animals, however, have relied on correlational links between trait expression and an individual's 'condition' (e.g. Vehrencamp et al. 1989; Hill & Montgomerie 1994) or have employed very general manipulations of an individual's phenotype (e.g. Møller et al. 1998) or nutritional/health state (e.g. Swaddle & Witter 1994; Johnson & Rashotte 2002; Scheuber et al. 2003). In virtually all of these instances, it is unclear exactly how the trait is costly to develop. For example, what specific physiological processes govern the formation of sexual ornaments like elongate tail feathers in birds? What are the physiological 'units' or 'currency' for the development of energetically demanding, complex vocalizations? These questions are difficult to answer for most secondary sexual characters, because nothing is known of the precise molecular and biochemical pathways that control their production, and yet they factor critically into our understanding of how sexually selected traits evolve as honest advertisements of mate quality.

There are, however, ideal systems in which we know the molecular components of a signal and, by monitoring the

Correspondence: K. J. McGraw, School of Life Sciences, Arizona State University, P.O. Box 85287-4501, Tempe, AZ 854501, U.S.A. (email: kevin.mcgraw@asu.edu). G. E. Hill is at the Department of Biological Sciences, Auburn University, 331 Funchess Hall, Auburn, AL 36849, U.S.A. R. S. Parker is at the Division of Nutritional Sciences, Cornell University, Ithaca, NY 14853, U.S.A.

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abundance, types and distribution of these molecules, we can explicitly investigate the physiological demands of ornamental features. The carotenoid-based colours of many animals, particularly birds, are a classic example. Many birds acquire their brilliant patches of red, orange and yellow colour by obtaining carotenoid pigments from their diets (Fox & Vevers 1960). Carotenoids are manufactured only by plants, algae, bacteria and fungi, so birds must ingest large amounts of carotenoid-enriched plant matter or herbivorous prey and transport these pigments through the bloodstream to deposit in feathers or bare parts and become colourful (Goodwin 1984; see Figure 4 in McGraw & Hill 2001). Thus, if we trace the accumulation of carotenoids from the diet, through the body, and into brightly coloured tissues (e.g. Partali et al. 1987), we can assess if any step(s) in the carotenoid-utilization pathway are costly to complete.

There are several existing lines of correlational and experimental evidence demonstrating that carotenoid-based colour ornaments in birds are costly to produce (reviewed in Hill 2002). Most notably, Hill (2000) found that experimentally induced food deprivation decreased the expression of carotenoid-based plumage coloration in male house finches, *Carpodacus mexicanus*, independent of the amount of carotenoids acquired from the diet. This suggested in fact that certain feature(s) of carotenoid processing were compromised by nutritional state. In this study, we manipulated food access in a similar fashion and subsequently investigated particular components of carotenoid utilization to determine how plumage pigmentation is under nutritional and physiological control.

We studied the American goldfinch, which is a close relative of the house finch (subfamily Carduelinae, family Fringillidae) and has a quite tractable system of pigmentation for such a study. In contrast to the hypervariable red-to-yellow plumage coloration shown by male house finches, which contains a suite of 12 carotenoid pigments (Inouye et al. 2001), male goldfinches display brilliant lemon-yellow breeding plumage that contains two main yellow carotenoids. These pigments, canary xanthophylls A and B, are metabolic derivatives of the two major dietary and serum carotenoids present (lutein and zeaxanthin) (McGraw et al. 2001), which represents another (metabolic) mode of carotenoid use that may be difficult or demanding for birds to complete (Hill 1996).

In previous work, we found that male goldfinches develop variable carotenoid-based plumage coloration when fed a standard diet in captivity during moult (McGraw & Hill 2001), which suggested to us that some physiological aspect of carotenoid use was controlling the expression of bright plumage. In this study, we subjected a group of male goldfinches to a food-restriction regime during the period of feather growth (sensu Hill 2000) to determine which phase(s) of carotenoid utilization is/are costly to complete. We monitored carotenoid status in blood during moult and plucked feathers at the end of moult to determine the effect of food deprivation on carotenoid circulation, plumage coloration and feather-pigment composition. If aspects of carotenoid intake or transport are demanding physiological processes for goldfinches, we predicted that nutritionally stressed birds would circulate significantly less pigments through blood than control males. If carotenoid metabolism and/or deposition are costly processes, we predicted that food-deprived birds would grow yellow feathers containing disproportionately less carotenoids than would be expected from blood levels than would controls.

METHODS

In February 2002, 20 male goldfinches were trapped in basket traps at baited feeding stations in Lee County, Alabama, U.S.A. At capture, we randomly divided males into two groups of 10 and placed them into separate large outdoor aviaries (see McGraw & Hill 2000 for details). Birds were fed an ad libitum diet of sunflower hearts (ca. 50 g/cage of fresh seeds provided daily) and tap water. Water was treated with a coccidiostat throughout moult to eliminate coccidial infections (sensu McGraw & Hill 2000) that can inhibit carotenoid uptake from the diet (Allen 1987). Because sunflower seeds contain a very low concentration of carotenoids (McGraw et al. 2001), we also added lutein (9 μg/ml) and zeaxanthin (2 μg/ml) beadlets (supplied by Roche Vitamins Inc., Parsippany, New Jersey, U.S.A.) to drinking water. This amount elevated plasma-carotenoid levels (see Results) to within the natural/physiological range of values found in wild male goldfinches during their nuptial moult (10–40 μg/ml; McGraw & Gregory 2004).

Prior to the captive moult, we determined body mass for each male to the nearest 0.1 g with an electronic balance and scored carotenoid-derived plumage colour with a Colortron II reflectance spectrophotometer (sensu McGraw & Hill 2000). We took three measurements from the ventral and dorsal feathers (upper, middle and lower regions) and averaged these scores to obtain mean plumage hue, saturation and brightness values for each bird. At this time, we also drew 50–100 μl of whole blood from the alar vein, centrifuged off the red blood cells, and saved plasma at −80 °C for later carotenoid analysis (see below). We collected these data to be sure that treatment groups, despite random assignment, did not differ in carotenoid status at the start of the study.

Moult began in our captive group on 15 March and all birds had grown their full set of nuptial feathers by 15 May. During this time, we subjected our experimental group to a nutritional-stress regime previously employed by Hill (2000) and McGraw et al. (2002a) to reduce food intake in passerine birds. This protocol involves the removal of food (but not water) from treatment cages for randomly selected 6-h periods on 5 out of every 7 days, which amounts to approximately 40% of all daylight hours. Floors were swept clean of spilled seed in all cages prior to deprivation periods to be sure that birds had no access to food (as in Hill 2000). Also, to ensure that control cages received an equal amount of disturbance during periods of food restriction, we entered those cages and briefly lifted and replaced the food basins back onto the ground. Overall, our food-deprivation protocol should serve as only a minor stressor, since body mass did not significantly change during similar 2-month experiments with male house finches, house sparrows, *Passer domesticus*, and brown-headed cowbirds, *Molothrus ater* (Hill 2000; McGraw et al. 2002a).

Although the goal of the study was to examine the effect of food deprivation on the physiological demands of carotenoid assimilation, we realized that food-deprived (hereafter FD) males would inherently ingest less pigments than control (hereafter C) males by eating less food. The fact that we supplemented both groups with a high dose of carotenoids in the drinking water, however, made this amount very small. Since we have previously determined the amount of food and water that moulting male goldfinches consume on a daily basis in captivity (K. J. McGraw & A. J. Gregory, unpublished data), we can estimate the relative amounts of carotenoids ingested by our two treatment groups. Captive, moulting male goldfinches consume an average of 2.5 g of sunflower hearts/day and drink an average of 3 ml of water/day. As sunflower hearts contain 1 μg of total carotenoids (lutein + zeaxanthin) per gram of seed (McGraw et al. 2001) and drinking water contains 11 μg/ml, C males should have been ingesting approximately 35.5 μg of carotenoids, on average, daily. FD males consumed 22% less food than C birds but did not differ in water consumption (see Results), and thus should have acquired 35.0 μg from food and water sources, amounting to 1.5% less carotenoids/day than C goldfinches. We ran all statistical tests to determine whether the FD males showed a greater than 1.5% difference in blood- and feather-carotenoid status than C males.

To confirm that FD males in fact consumed less food overall than C males, we measured food intake on two randomly chosen food-stressed days (25 March, 23 April) during moult. Even though drinking water was continuously available, we also considered whether birds drank different amounts of water during these days. On both days, we provided birds with 30 g of fresh seed and 50 ml of fresh carotenoid-enriched water at noon, removed the food for 6 h, and returned the following day at noon to measure the remaining food and water; birds in a cage never consumed more than a total of 22 g of food or 35 ml of water during these trials. Once we began food-stressing the moulting goldfinches, we sampled blood on three separate days (1 April, 15 April, 1 May) to determine plasma-carotenoid status. Note that birds had ad libitum access to food and water for the entirity of both blood-sampling days. At the end of moult, we again determined body mass, scored plumage coloration (as above), and released the birds back into the wild at their site of capture.

Carotenoid levels in plasma and feathers were determined following previously published methods (McGraw et al. 2002b, c). To extract carotenoids from plasma, we added 75 μl of ethanol and 75 μl of *tert*-butyl methyl ether to 10 μl of plasma. The tube was vortexed for 5 s and then centrifuged for 3 min in an Eppendorf centrifuge (model 5414). To remove carotenoids from feathers, we first washed the feathers in hexane for 10 min to remove surface lipids and blotted the feathers dry on filter paper. We then trimmed off the pigmented barbules and weighed them to the nearest 0.01 mg with an electronic balance. To the tube of feather portions, we added 1 ml of acidified pyridine and filled the headspace with argon. We placed the tube in a 95 °C water bath for 3 h and cooled it to room temperature before adding 2 ml of distilled water. We inverted the tube a few times and then added 2 ml of *tert*-butyl methyl ether. After shaking the solution vigorously for 1 min, we centrifuged the tube for 5 min at 3000 revolutions/min. At this point in both procedures, we removed the supernatant containing the carotenoids and evaporated it to dryness under a stream of nitrogen. We resuspended the pigments in 200 μl HPLC mobile phase (see below for composition) and injected 50 μl into a Waters autosampler HPLC. Due to the different polarities of plasma versus plumage carotenoids, we used slightly different mobile phases (for plasma: methanol:acetonitrile:chloroform, 46:46:8, v/v/v, for plumage: methanol: acetonitrile, 50:50, v/v). Other run conditions for both analyses were as follows: column temperature = 31 °C, flow rate = 1.2 ml/min, column pressure = ca. 800 psi (5516 kPa). Pigments were identified by comparison to authentic standards provided by R. Stradi (University of Milan, Italy) and Roche Vitamins Inc. (Parsippany, New Jersey, U.S.A.) and were quantified using an internal standard of known concentration (canthaxanthin, 1 μg/ml) that we previously determined to be absent from the blood and feathers of these birds.

All data met the assumptions of parametric statistics, so we used analyses of variance to test for differences in body mass, carotenoid status and plumage colour between treatment groups. We report means ± SE throughout.

RESULTS

Before the study, randomly assigned treatment groups did not differ significantly in plasma-carotenoid status, plumage coloration, or body mass (see Table 1). On the 2 days during moult that we measured food intake, FD birds consumed an average of 18.9 ± 0.25 g of food per day, 22% less than C birds (24.3 ± 0.3 g). On the same days,

Table 1. Comparison of plasma-carotenoid status, plumage coloration and body mass of treatment groups at the start of the study

Trait	Nutritionally deprived (*N* = 10)	Control (*N* = 10)	t_{18}	*P*
Plasma carotenoids				
Lutein concentration (μg/ml)	5.9±1.7	4.2±0.3	1.13	0.28
Zeaxanthin concentration (μg/ml)	2.5±0.5	2.0±0.2	1.08	0.30
Total concentration (μg/ml)	8.4±2.2	6.1±0.4	1.13	0.28
% Lutein	68.3±1.3	68.1±1.8	0.10	0.93
Plumage coloration				
Hue (degrees)	68.1±0.75	68.1±0.75	0.01	0.99
Saturation (%)	59.2±2.7	56.9±3.0	0.58	0.57
Brightness (%)	56.5±2.2	55.8±1.7	0.25	0.81
Body mass (g)	13.9±1.4	14.0±0.6	0.07	0.94

Means ± SE are reported throughout. We used unpaired *t* tests to evaluate potential differences between groups.

our two groups consumed nearly equal amounts of water (FD = 30.4 ± 0.46 ml; C = 29.9 ± 0.32 ml). After moult, FD and C males did not differ significantly in body mass, however (FD = 15.2 ± 0.53 g; C = 14.6 ± 0.33; $F_{1,18} = 0.94$, $P = 0.36$), indicating that this treatment served as only a mild nutritional stressor (as in other passerines; Hill 2000; McGraw et al. 2002a).

Using HPLC, we found that male American goldfinches circulate two main carotenoids through blood, lutein ($\lambda_{max} = 448$ and 477 nm) and zeaxanthin ($\lambda_{max} = 453$ and 482 nm) (Fig. 1). This was true of moulting and nonmoulting males, and is the profile observed in wild male and female goldfinches at all times of year (McGraw & Gregory 2004). During moult, FD males circulated significantly less total carotenoids (20% less, on average) than C males ($F_{1,18} = 4.6$, $P = 0.04$) (Fig. 2a). Broken down by pigment type, FD males circulated 23% less lutein ($F_{1,18} = 5.4$, $P = 0.03$) and 17% less zeaxanthin ($F_{1,18} = 2.8$, $P = 0.11$) (Fig. 2a).

FD males moulted into significantly less saturated plumage than C birds (Fig. 2b). Saturation is the colour measure that captures most of the variation in carotenoid-based

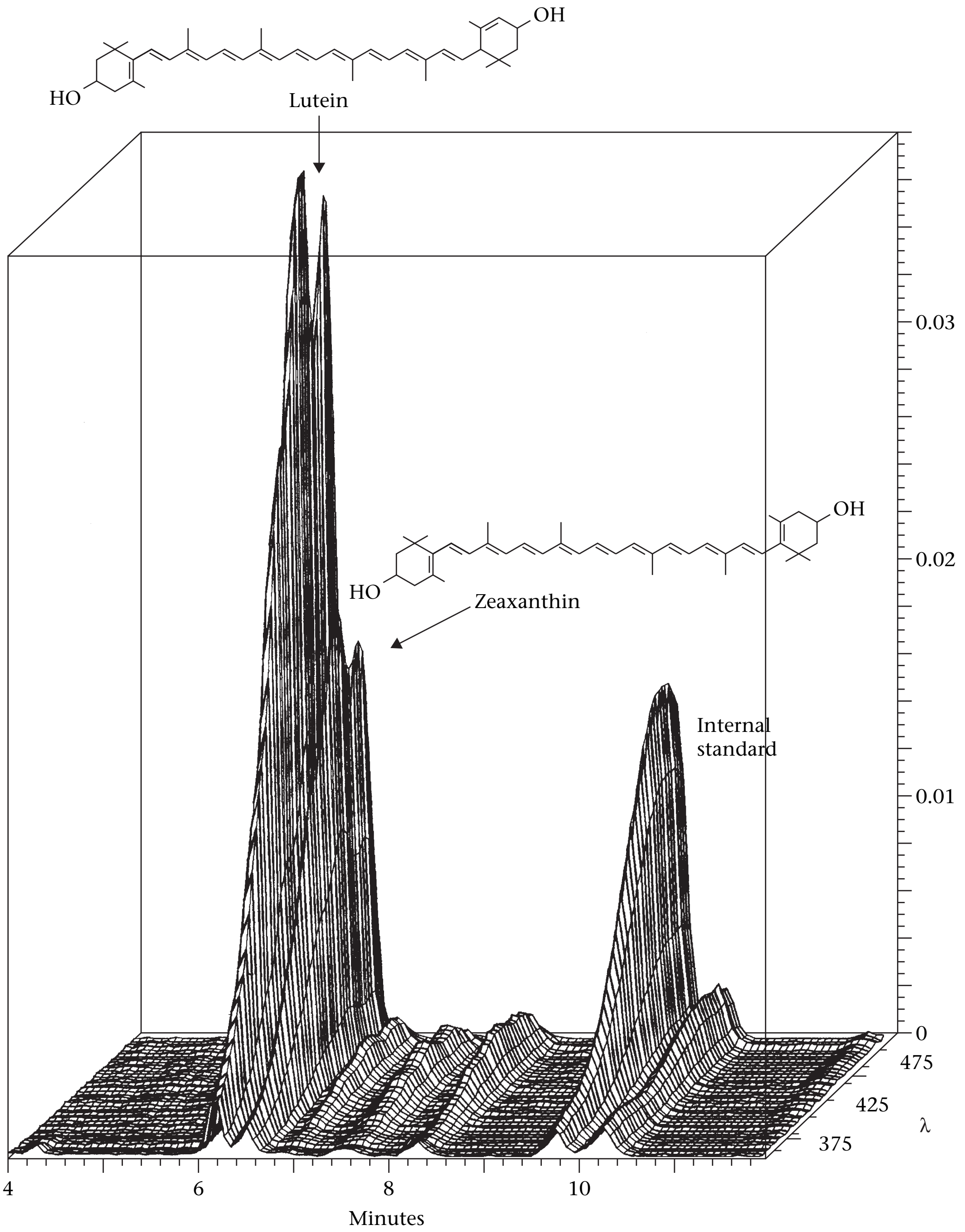

Figure 1. Representative three-dimensional HPLC chromatogram illustrating the two main carotenoid pigments found in the blood of male American goldfinches.

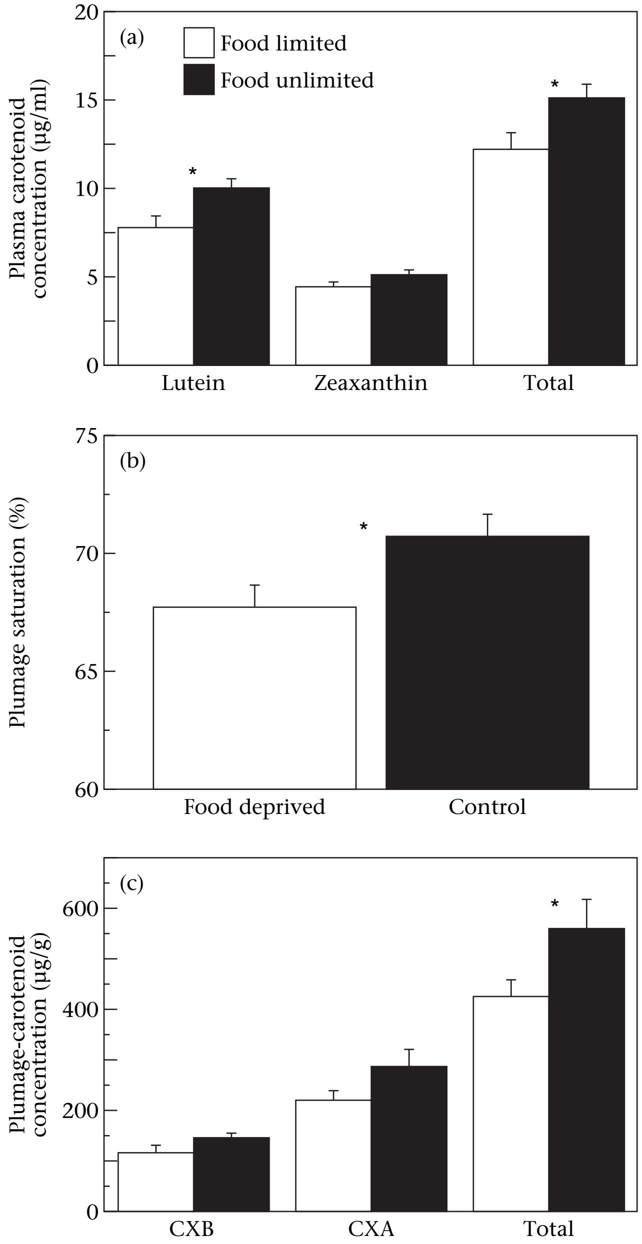

Figure 2. The effect of mild food deprivation during the nuptial plumage moult on (a) the concentration of carotenoid pigments found in blood, (b) the saturation of newly grown, carotenoid-containing yellow plumage, and (c) the carotenoid content of these yellow feathers in male American goldfinches. $*P < 0.05$. Abbreviations in (c) are: CXB = canary xanthophyll B, CXA = canary xanthophyll A.

plumage pigmentation in this species and is known to reflect other aspects of individual quality, such as incidence of parasitism (McGraw & Hill 2000). Treatment groups did not differ significantly in plumage hue (FD males = 71.8 ± 0.6°, C males = 70.8 ± 0.5°; $F_{1,18} = 1.8$, $P = 0.20$) or plumage brightness (FD = 86.3 ± 2.4%; C = 87.6 ± 1.4%; $F_{1,18} = 0.23$, $P = 0.64$).

Plumage-colour differences could be attributed to the significantly lower overall concentration of carotenoids found in the feathers of FD than C goldfinches ($F_{1,18} = 4.3$, $P = 0.05$) (Fig. 2c). FD males deposited 19% less canary xanthophyll B ($F_{1,18} = 3.0$, $P = 0.10$) and 23% less canary xanthophyll A ($F_{1,18} = 3.0$, $P = 0.10$) into feathers, amounting to a 21% overall difference in plumage-carotenoid levels between the groups. Note, however, that this value does not significantly exceed, and instead closely approximates, the 20% difference in carotenoids already present in serum that are delivered to maturing feather follicles for deposition into growing plumage.

DISCUSSION

A fundamental tenet of honest-signalling theory is that sexual advertisements are costly to produce or maintain, yet in very few cases do we know the precise physiological expenses incurred by extravagant traits. We investigated the specific physiological effects that food limitation had on the ability of male American goldfinches to develop bright, sexually selected plumage coloration. Carotenoid pigments colour the yellow nuptial plumage of male goldfinches, and once acquired in the diet, these pigments must be processed within the body of the animals before being incorporated into feathers. We found that nutritional deprivation depressed plumage colour in these finches, and did so by compromising the extent to which birds accumulated these pigments in blood. FD males circulated less carotenoids in serum at the time of feather growth and subsequently deposited less pigments into feathers. However, the relative decline in pigment concentration found in the feathers matched that in the blood, indicating that it was prior to the point at which carotenoids were removed from circulation and deposited into feathers that the utilization mechanisms for these pigments were impaired.

Ultimately, these results suggest that the ability of male goldfinches to assimilate carotenoid pigments from the diet and into blood circulation is more sensitive to nutritional state than is the metabolic transformation of carotenoids and the eventual incorporation of these colourants into feathers. We include carotenoid metabolism as a utilization step that occurs after pigment circulation because only dietary carotenoids are found in blood and liver in these birds and plumage pigments are present nowhere else in the body but the integument (McGraw 2004). Previous studies have hypothesized that carotenoid metabolism should be one or the primary cost of acquiring brilliant carotenoid-based sexual coloration (Hill 1996, 2000; Olson & Owens 1998). The metabolic conversions of dietary carotenoids into feather pigments in songbirds are a class of oxidation reactions, where oxygen functional groups (e.g. hydroxyl, carbonyl) are added to or modified at the end-rings of carotenoid molecules (Brush 1990; Stradi 1998). This process has been thought to demand energy, not only for the synthesis of structurally specific carotenoid-metabolizing enzymes but also to drive the series of endothermic oxidation steps (Britton 1976; Brush 1981; Hill 1996). In goldfinches, however, pigment modification proceeds down a very simple pathway, where the yellow dietary carotenoids lutein and zeaxanthin are converted into yellow plumage carotenoids (the canary xanthophylls; McGraw et al. 2001, 2002c) by the removal of two hydrogen atoms (the process of dehydrogenation) that likely occurs via a single step and enzyme (McGraw et al. 2003a). Thus, one may not suspect high energetic demands for such a basic chemical modification in goldfinches, and instead might predict that red, carotenoid-coloured animals having to metabolize these same dietary components into more oxidized, red ketocarotenoids (like astaxanthin) via a multistep conversion process (Fox & Hopkins 1966; Fox et al. 1967) would incur substantial metabolic costs to pigmentation.

Instead, in goldfinches, we must look to the means by which these birds extract carotenoid pigments from the foods they ingest and transport them through the bloodstream in order to isolate the nutritionally or energetically demanding steps of pigment use and display. Animals are thought to absorb carotenoids and other dietary lipids (e.g. fats, oils) through the gut lining via passive diffusion (Parker 1996; but see During et al. 2002). There, they mix with bile salts and fatty acids to form micelles that migrate through the intestinal mucosa and are incorporated into chylomicrons, which are then secreted into lymph and enter blood circulation (Furr & Clark 1997). There appear to be particular dietary and physiological requirements for optimal carotenoid absorption (reviewed in Solomon & Bulux 1993). In humans (Williams et al. 1998) and in chickens (Tyczkowski et al. 1989), high concentrations of dietary fats maximize carotenoid uptake by helping to solubilize these pigments in the lumen of the small intestine, promoting bile secretion for the formation of micelles, and stimulating chylomicron formation. Thus, in our study, FD males ate less food (and thus lipid) and may consequently have been less efficient at extracting ingested carotenoids from the diet. On this note, it is curious that the relative difference in food intake between treatment groups (22%) closely mirrors overall plasma-pigment differences (20%) between FD and C males. Others, however, have suggested that the digestive efficiency of carotenoids hinges on the action of plasma membrane or intracellular transport proteins in enterocytes that aid in the delivery of carotenoids to chylomicrons (Erdman et al. 1993; Furr & Clark 1997). Because only very basic information is currently available on carotenoid uptake in birds (and only in chickens), we encourage future investigations aimed at understanding the costliness of synthesizing these enterocyte proteins along with the role of these other digestive processes.

Alternatively, the process(es) by which carotenoids are taken up and retained in blood circulation may be a limiting factor for integumentary pigmentation. Chylomicrons transfer carotenoids into lipoprotein particles in plasma (Parker 1996). These lipid carriers exist in a variety of forms (e.g. high-density, or HDL; low-density, or LDL) and, among other things, deliver lipid-soluble pigments to peripheral tissues for coloration. Lipoproteins are assembled endogenously from synthesized components that include proteins (apoproteins) embedded into layers of surrounding lipids (triglycerides, cholesterol, phospholipids) (Davis 1997). It is plausible then that the production of lipoprotein components and the amalgamation of lipoprotein particles may be costly physiological processes. Both lipogenesis and apolipoprotein synthesis are known to proliferate when carbohydrate fuels (e.g. Boogaerts et al. 1984) and calcium levels (e.g. Hughes et al. 1988) are in large dietary supplies. The fact that there are strong correlations between plasma-carotenoid content and integumentary coloration (independent of dietary intake) in several bird species (e.g. kestrels, partridges, zebra finches; Bortolotti et al. 1996; Negro et al. 2001; McGraw et al. 2003b) adds support to the notion that some aspect of carotenoid transport factors critically into the control of pigmentation. Since lipoprotein constituents can be assayed in a rather straightforward fashion from serum, we are now conducting studies to determine how sensitive lipoprotein levels are to changes in nutritional state and how they are subsequently correlated with and control plasma-carotenoid status and integumentary coloration.

There are still other, less direct mechanisms of carotenoid utilization that may explain the depressed serum-carotenoid status and plumage colour of food-deprived birds. Carotenoids are touted as potent antioxidants and immunostimulants in many animals, and there is now good experimental support for this notion in colourful songbirds (Blount et al. 2003; McGraw & Ardia 2003). Thus, if food-restricted birds were immunocompromised due to malnutrition, then they may have allocated more carotenoids to fighting foreign pathogens and parasites. However, there are several lines of evidence that argue against this explanation for treatment differences in carotenoid use in our study: (1) in an earlier experiment on captive goldfinches, prophylactic treatment effectively eliminated coccidial infections during moult (McGraw & Hill 2000), (2) clinical signs of avian parasite infection include weight loss, emaciation, feather-ruffling, and lethargy, none of which was observed in our birds, (3) there is overwhelming support from the nutritional-immunology literature that mild energetic restriction like that in our study results in elevated, not reduced, immune performance in many animals (Fernandes et al. 1995; Nir et al. 1996; Frame et al. 1998), and (4) the lone test of the immunoenhancing properties of carotenoids in goldfinches found no differences in several immune parameters between carotenoid-supplemented and -unsupplemented captive, moulting males (Navara & Hill 2003). Nevertheless, there clearly are numerous, complex ways that carotenoids are handled physiologically, so future studies should target these pathways simultaneously to best elucidate the links between carotenoid status and sexually attractive coloration in birds and other animals.

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