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The effect of dietary carotenoid access on sexual dichromatism and plumage pigment composition in the American goldfinch

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Abstract

We investigated potential dietary and biochemical bases for carotenoid-based sexual dichromatism in American goldfinches (*Carduelis tristis*). Captive male and female finches were given access to the same type and amount of carotenoid pigments in the diet during their nuptial molt to assess differences in the degree to which the two sexes incorporated ingested pigments into their plumage. When birds were fed a uniform, plain-seed diet, or one that was supplemented with the red carotenoid canthaxanthin, we found that males grew more colorful plumage than females. HPLC analyses of feather pigments revealed that male finches incorporated a higher concentration of carotenoids into their pigmented feathers than females. Compared to females, males also deposited significantly more canary xanthophyll B into feathers when fed a plain-seed diet and a greater concentration and proportion of canthaxanthin when fed a carotenoid-supplemented diet. These results indicate that sex-specific expression of carotenoid pigmentation in American goldfinches may be affected by the means by which males and females physiologically utilize (e.g. absorb, transport, metabolize, deposit) carotenoid pigments available to them in the diet.

Keywords: Cardueline finches; *Carduelis tristis*; Carotenoid biochemistry; Carotenoid physiology; Carotenoid pigments; Diet; HPLC; Plumage coloration

1. Introduction

Birds are some of the most colorful animals in nature. Within species, male birds tend to be more brightly colored than females, and much research has been devoted to identifying the evolutionary forces underlying avian sexual dichromatism (e.g. Hamilton and Zuk, 1982; Gray, 1996; Badyaev, 1997; Owens and Hartley, 1998; Badyaev and Hill, 2000). Comparatively less emphasis has been placed on the proximate mechanisms generating sex differences in plumage color displays (Kimball and Ligon, 1999).

Because of our detailed understanding of the means by which they are produced, carotenoid-based colors (e.g. reds, oranges, yellows) provide an ideal system in which to investigate how environmental, physiological and genetic factors shape plumage color differences between the sexes. Vertebrates cannot synthesize carotenoids de novo, so birds must ingest pigments in the diet before depositing them in feathers (Palmer, 1922; Völker, 1938). This led to the hypothesis that variation in dietary access to carotenoids between males and females influences sexual dichromatism

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(Hill, 1993). Support for this idea has come from studies investigating geographic variation in plumage coloration (Hill, 1993), sex patterns of circulating plasma carotenoids (Hill et al., 1994; Hill, 1995; Figuerola and Gutierrez, 1998) and the experimental effect of dietary carotenoid manipulations in captivity on pigment displays (Hill, 1992; Hill and Benkman, 1995).

However, other research has suggested that physiological differences between the sexes, independent of diet, contribute to carotenoid-based sexual dichromatism in certain avian species (Bortolotti et al., 1996). Birds must absorb dietary carotenoid pigments through the intestine (Furr and Clark, 1997), transport them via lipoproteins through the blood (Erdman et al., 1993), and often convert them to different forms (Brush, 1990) before depositing them in the integument for display (McGraw and Hill, 2001). Males and females may differ in their pigment absorption efficiency (Williams et al., 1998), lipoprotein circulation or affinity (Trams, 1969), enzyme-catalyzed metabolism (Brush, 1967), or follicular uptake of pigments (Schlinger et al., 1989). Sex differences in hormone levels have also been proposed to influence patterns of carotenoid-based ornamental coloration (Cynx and Nottebohm, 1992; Adkins-Regan and Wade, 2001).

Clearly, more experimental studies are needed to help resolve the mechanisms controlling carotenoid-based sexual dichromatism in birds. In this study, we investigated dietary and biochemical factors affecting carotenoid-based plumage pigmentation in male and female American goldfinches (*Carduelis tristis*). Captive unisex flocks were given access to the same types and amounts of carotenoid pigments during molt to assess the degree to which the sexes grew similar plumage colors. We used HPLC techniques to identify and quantify the carotenoids present in the feathers of birds to explore potential differences in the means by which male and female finches incorporate pigments into colorful plumage.

2. Methods

2.1. Study species

American goldfinches are sexually dichromatic cardueline finches from the family Fringillidae. Although males and females display drab plumage in the non-breeding season, both sexes complete a pre-alternate molt in the spring to develop colorful breeding plumage. Males exhibit brilliant lemon-yellow carotenoid-based plumage pigmentation on the throat, breast, nape, rump and epaulettes, whereas pigmentation in females covers less of the body and is not as brightly colored (Middleton, 1993).

2.2. Feeding experiment

In January 1999, we trapped 23 female and 58 male goldfinches at baited feeding stations using basket traps in Lee County, AL, USA. We separated birds into six unisex flocks ($n=8$ and 15 females; $n=13$, 15, 15 and 15 males) that were housed in separate outdoor cages (3.7 m long $\times$ 1.5 m wide $\times$ 2.4 m high). Throughout the study, these captive groups were provided with ad libitum access to a diet of fresh sunflower hearts and water that was replaced on a bi-daily basis.

Two cages of males ($n=15$ each) and one cage of females ($n=15$) remained on this diet for the course of the experiment. These seeds contain two primary carotenoid pigments — lutein and zeaxanthin — in low concentrations (0.7 and 0.4 μg/g, respectively; McGraw et al., in press). For 1 month prior to and for the duration of the pre-alternate molt, we supplemented the diet for the other cage of females ($n=8$) and two cages of males ($n=13$ and 15) with carotenoids. Canthaxanthin beadlets (0.001 g/ml; Roche Vitamins, Parsippany, NJ) were dissolved in the water of these groups.

2.3. Plumage color scoring

After molt (1 June), we quantified carotenoid-based plumage coloration with a Colortron™ reflectance spectrophotometer (Hill, 1998). We obtained mean tristimulus scores (hue, saturation and brightness) for each male by scoring and then averaging three ventral and three dorsal (upper, middle and lower) regions of carotenoid pigmentation. We averaged two measurements from the colorful throats of females. The Colortron™ measures hue based on a 360° color wheel, with red set at 0° and numbers increasing through orange to yellow. Saturation and brightness are measured as percentages relative to black (0% reflectance) and white (100% reflectance) standards.

2.4. Biochemical analyses

At the end of the feeding experiment, we plucked plumage patches from standardized throat regions for a random subset of males (five seed-only, seven canthaxanthin) and females (eight seed-only, three canthaxanthin) to compare the amounts and types of carotenoid pigments that were present in feathers. Plumage carotenoids were identified using HPLC and mass spectrometry methods (Stradi et al., 1995; McGraw et al., in press). To determine pigment concentration in feathers, samples were removed from storage at −80 °C and were first washed separately in 3 ml of ethanol and hexane. Pigmented barbules were trimmed and weighed to the nearest 0.00001 g with an electronic balance (Mettler Toledo AG245, Greifensee, Switzerland). We extracted carotenoid pigments from feathers using acidified pyridine (sensu Hudon and Brush, 1992). Barbules were dispersed in 1 ml of acidified pyridine in a 9-ml glass tube. We capped the tube with argon and incubated the solution at 85 °C for 3 h. After cooling to room temperature, we added 1 ml of distilled water and 5 ml of hexane and shook the mixture for 2 min. The suspension was centrifuged for 3 min at 3000 rev./min and the supernatant (hexane) was transferred to a clean tube. We evaporated this hexane phase to dryness under a stream of nitrogen and resuspended the pigments in 200 μl of HPLC mobile phase (methanol–acetonitrile, 50:50 v/v, +0.05% triethylamine) prior to HPLC analysis.

We injected 5–50 μl of each sample into a Hitachi L-6200A HPLC (Hitachi Ltd, Tokyo, Japan) fitted with a Develosil RPAqueous RP-30 HPLC column (250×4.6 mm i.d.; Nomura Chemical Co Ltd, Japan). An isocratic system, using the aforementioned mobile phase for 28 min, was used for analysis at a constant flow rate of 1.2 ml/min. Carotenoids were detected at 450 nm using a Hitachi L-4250 UV/Vis detector, and peak areas were integrated with an HP 3390A integrator. We confirmed the identity of plumage pigments by comparing their retention times to those for authentic reference carotenoids provided by Roche Vitamins Inc (canary xanthophyll B, 9.7; canary xanthophyll A, 10.7; lutein, 15.7; zeaxanthin, 18.3; and canthaxanthin, 24.8 min). The total carotenoid concentration in each sample was determined by spectrophotometry (Bausch and Lomb Spectronic 1001). We applied the following formula:

$$\frac{A \times \text{volume of extract (ml)}}{E \times \text{feather mass (g)}}$$

where A is the absorbance of the sample at λ_{max} (440 for xanthophylls, 466 for canthaxanthin) and E is the extinction 1%/1 cm of the relevant carotenoids at λ_{max} (3120 for xanthophylls and 2200 for canthaxanthin; Britton, 1985). The concentration of each type of feather carotenoid was calculated by comparing their relative proportions, as reflected by HPLC peak areas, to total carotenoid content determined by spectrophotometry. We also adjusted final concentrations based on the recovery percentages from extraction procedures performed in triplicate on authentic reference carotenoids (canary xanthophyll B, 39.2 ± 1.5; canary xanthophyll A, 49.1 ± 0.8; lutein, 44.4 ± 1.5; and canthaxanthin: 39.6 ± 0.2% recovery).

2.5. Statistical analyses

When variables were normally distributed (Shapiro–Wilk W-test, $P > 0.05$) and had equal variances (equality-of-variance F-test, $P > 0.05$), we used unpaired t-tests to compare male and female plumage coloration; otherwise, we used non-parametric Mann–Whitney U-tests (Z reported). We used only Mann–Whitney U-tests for analyses of feather pigment proportions and concentrations because sample sizes were small. Same-sex flocks of birds within a diet treatment were pooled for statistical analyses. In all cases, we report two-tailed test results and means ± 1 S.D.

3. Results

3.1. Sex differences in plumage color

We found that captive male goldfinches fed a seed-only diet during molt grew significantly more yellow ($Z = 2.53$, $P = 0.01$), more saturated ($Z = 3.46$, $P = 0.0005$) and brighter plumage ($Z = 5.31$, $P < 0.0001$) than did captive females fed the same diet (Fig. 1). Similarly, males fed a canthaxanthin-supplemented diet molted into a significantly more orange (less yellow; $Z = 3.86$, $P = 0.0001$), more saturated ($t = 7.63$, $P < 0.0001$) and brighter plumage ($t = 10.17$, $P < 0.0001$) than did females fed this diet (Fig. 2).

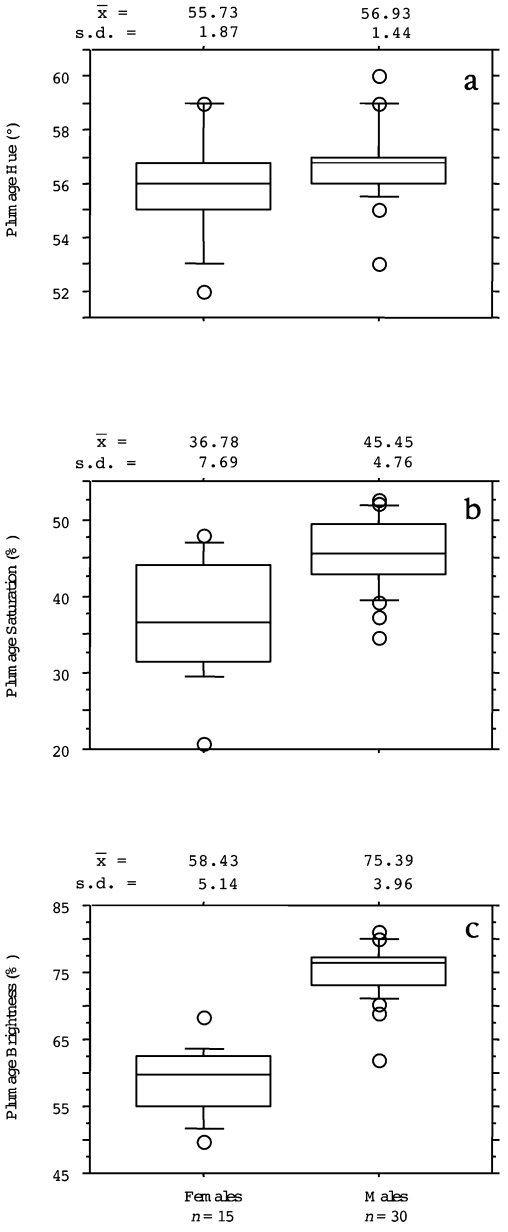

Fig. 1. Sex differences in carotenoid-based plumage (a) hue, (b) saturation and (c) brightness of captive American goldfinches fed a diet of sunflower seeds during molt. Feather patches were scored with a Colortron™ reflectance spectrophotometer after birds had completed molt. Horizontal bars in the box plot denote the 10th, 25th, 50th, 75th and 90th percentiles; points give data for individuals outside of these ranges.

3.2. Sex differences in feather pigment composition

3.2.1. Basal seed diet

When fed a diet comprised of sunflower seeds only during molt, both male and female goldfinches deposited three carotenoids into their colorful feathers: canary xanthophyll A, canary xanthophyll B, and lutein (McGraw et al., in press). These three pigments are also observed in the nuptial plumage of wild goldfinches (McGraw et al., in press). Overall, the two canary xanthophylls were present in approximately equal proportions (A,

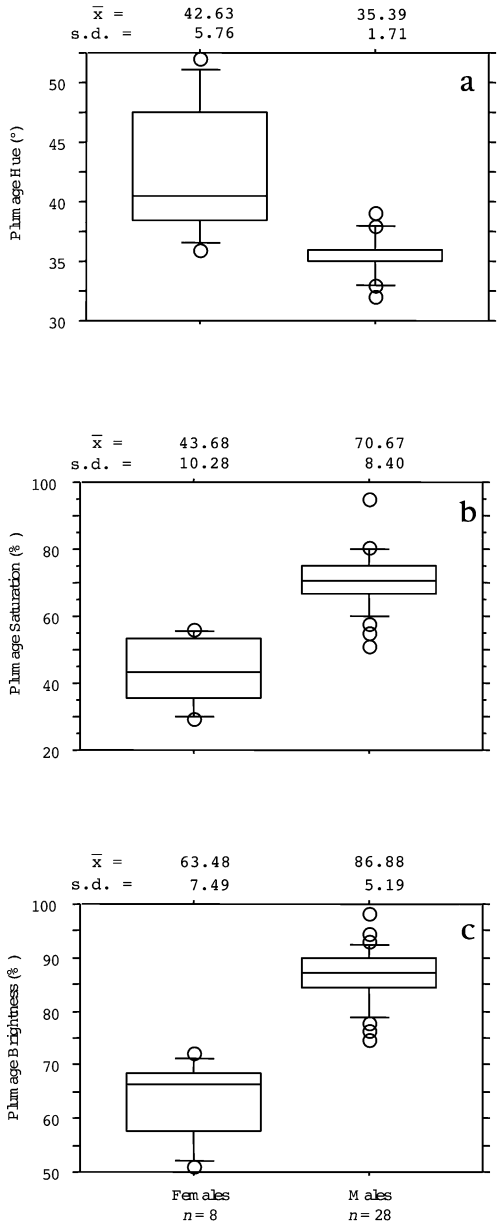

Fig. 2. Sex differences in carotenoid-based plumage (a) hue, (b) saturation and (c) brightness of captive goldfinches fed a seed diet that was supplemented with canthaxanthin during molt. See Fig. 1 for description of plumage scoring and box plot.

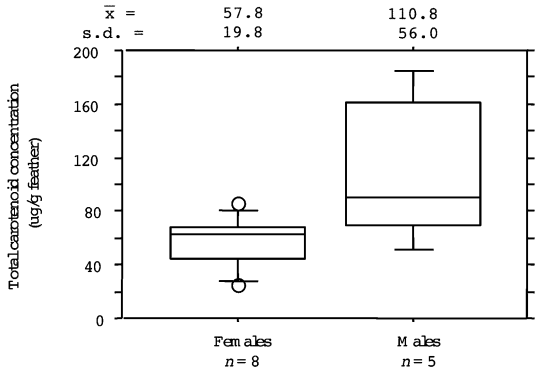

Fig. 3. Sex differences in the total concentration of carotenoids found in pigmented feather barbules from captive goldfinches fed a seed diet. Pigment concentration was determined using HPLC and spectrophotometric methods (see text for description).

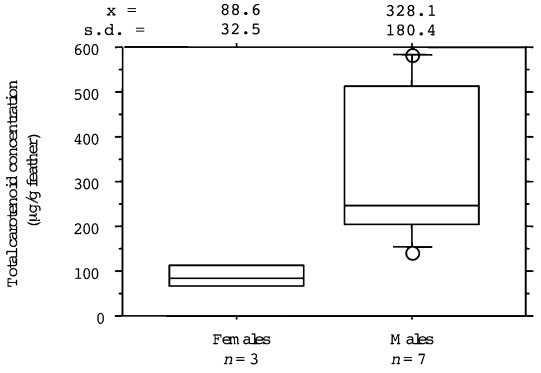

Fig. 4. Sex differences in total feather carotenoid concentrations of captive goldfinches fed a canthaxanthin-supplemented diet during molt.

45.0±4.1%; B, 42.6±2.8%), whereas lutein was one-fourth as concentrated in feathers (12.5±1.4%). Although the carotenoid-based plumage of both sexes contained the same types of carotenoid pigments, we found a significant difference in the total concentration of carotenoids between captive males and females fed a plain-seed diet ($Z=1.92$, $P=0.05$; Fig. 3). Males deposited significantly more canary xanthophyll B into feathers than females, but there were no significant sex differences in the concentration of canary xanthophyll A or lutein in colorful plumage (Table 1). We also found no effect of sex on the relative proportions of each pigment type found in feathers (Table 1).

3.2.2. Canthaxanthin-supplemented diet

Male and female goldfinches whose basal seed diet was supplemented with canthaxanthin primarily deposited canthaxanthin (71.5±24.7%) into their feathers (McGraw et al., in press), along with smaller amounts of canary xanthophylls A (13.8±14.2%), B (10.8±6.1%), and lutein (3.9±4.7%). Again, we found a statistically significant difference in the overall concentration of carotenoid pigments that males and females displayed in colorful feathers ($Z=2.4$, $P=0.02$; Fig. 4). Males deposited a significantly higher concentration of canthaxanthin into feathers than females, but we found no other sex differences in specific pigment concentrations (Table 2). Interestingly, male plumage contained a significantly smaller proportion of canary xanthophylls and a signifi-

Table 1
Comparison of plumage pigment concentrations and compositions present in male and female American goldfinches fed a seed diet during molt in captivity

	Males ($n=5$)	Females ($n=8$)	Z	P
Concentration (μg/g feather)				
Canary xanthophyll B	53.5±30.8	25.8±8.7	2.20	0.03
Canary xanthophyll A	44.7±21.1	24.8±8.9	1.76	0.08
Lutein	12.9±6.8	7.2±2.6	1.17	0.24
Percent composition (of total plumage pigments)				
Canary xanthophyll B	45.1±6.1	44.9±3.7	0	>0.99
Canary xanthophyll A	42.3±3.9	42.7±2.6	0	>0.99
Lutein	12.7±2.1	12.4±1.2	0.41	0.68

Mann–Whitney U-tests (Z reported) were used to examine sex differences in carotenoid pigmentation. Means±S.D. are provided for each group.

Table 2
Comparison of plumage carotenoid concentrations and compositions present in male and female American goldfinches fed a seed diet supplemented with canthaxanthin during molt in captivity

	Males ($n=7$)	Females ($n=3$)	Z	P
Concentration (μg/g feather)				
Canary xanthophyll B	29.2±14.8	14.8±4.1	1.57	0.12
Canary xanthophyll A	27.0±13.4	24.5±13.6	0.28	0.78
Lutein	6.2±4.9	7.3±5.4	−0.28	0.78
Canthaxanthin	369.0±366.6	42.0±47.9	2.50	0.01
Percent composition (of total plumage pigments)				
Canary xanthophyll B	8.3±4.6	18.1±6.9	−1.94	0.05
Canary xanthophyll A	7.8±3.5	32.0±20.1	−1.94	0.05
Lutein	2.1±1.5	9.5±7.0	−1.39	0.16
Canthaxanthin	81.8±8.5	40.5±33.8	1.94	0.05

See Table 1 for additional details.

cantly higher proportion of canthaxanthin than did female feathers (Table 2).

4. Discussion

The physiology and biochemistry of carotenoid-based sexual dichromatism are virtually unstudied in birds. Nearly 35 years ago, Brush (1967) examined the chemical basis for sex differences in plumage pigmentation in the scarlet tanager (*Piranga olivacea*), a songbird species in which females display drab olive pigmentation throughout the year, whereas males exhibit a brilliant red coloration during the breeding season only. Sex-specific expression of carotenoid pigmentation in this species represents the unique metabolic conversion of a yellow xanthophyll pigment, isozeaxanthin, into canthaxanthin in males only (Brush, 1967). Since this time, despite many advances in the analytical chemistry of carotenoids (Stradi et al., 1995), remarkably little consideration has been given to potential differences in plumage pigment composition between the sexes and the dietary or physiological mechanisms that may control carotenoid displays.

By comparing the carotenoid-based plumage coloration and pigment composition of male and female American goldfinches that were fed diets of similar carotenoid content in captivity during molt, we evaluated the influence of carotenoid access on the maintenance of sexual dichromatism. Under two separate carotenoid regimes, we found that male goldfinches grew significantly more colorful, saturated and brighter plumage than did females fed the same diet. Bortolotti et al. (1996) similarly found that sexual dichromatism in American kestrels (*Falco sparverius*), a species in which males display more colorful carotenoid-based fleshy parts (e.g. ceres, lores and tarsi) than females, is maintained when the sexes are fed a uniform diet in captivity. These results suggest that diet alone does not regulate sex differences in carotenoid pigmentation. We detected no sexual dimorphism in body mass among captive goldfinches (K. McGraw, unpublished data), and because birds were housed under non-breeding conditions, it is unlikely that the sexes differed in energy expenditure or resource demand. Moreover, all flocks were treated for endoparasitic infections that are known to inhibit carotenoid expression in these birds (McGraw and Hill, 2000). In all, our findings suggest that factors other than carotenoid access and parasite levels play a role in mediating sex-specific color expression in this species.

We further investigated sex differences in carotenoid display by isolating the specific carotenoid pigments that captive goldfinches deposited in plumage. Males and females deposited the same types of carotenoids into feathers when fed the same diet, which is consistent with biochemical analyses of yellow feathers from wild birds (McGraw et al., in press). This indicates that carotenoid-based sexual dichromatism in *C. tristis* cannot be attributed to unique pigments appearing in the plumage of one sex only. However, we did find that when fed the same diet, male goldfinches deposited significantly more plumage carotenoids overall than females. Stradi (1998) also noted that color differences between the sexes in Asian and European members of the genus *Carduelis* are due

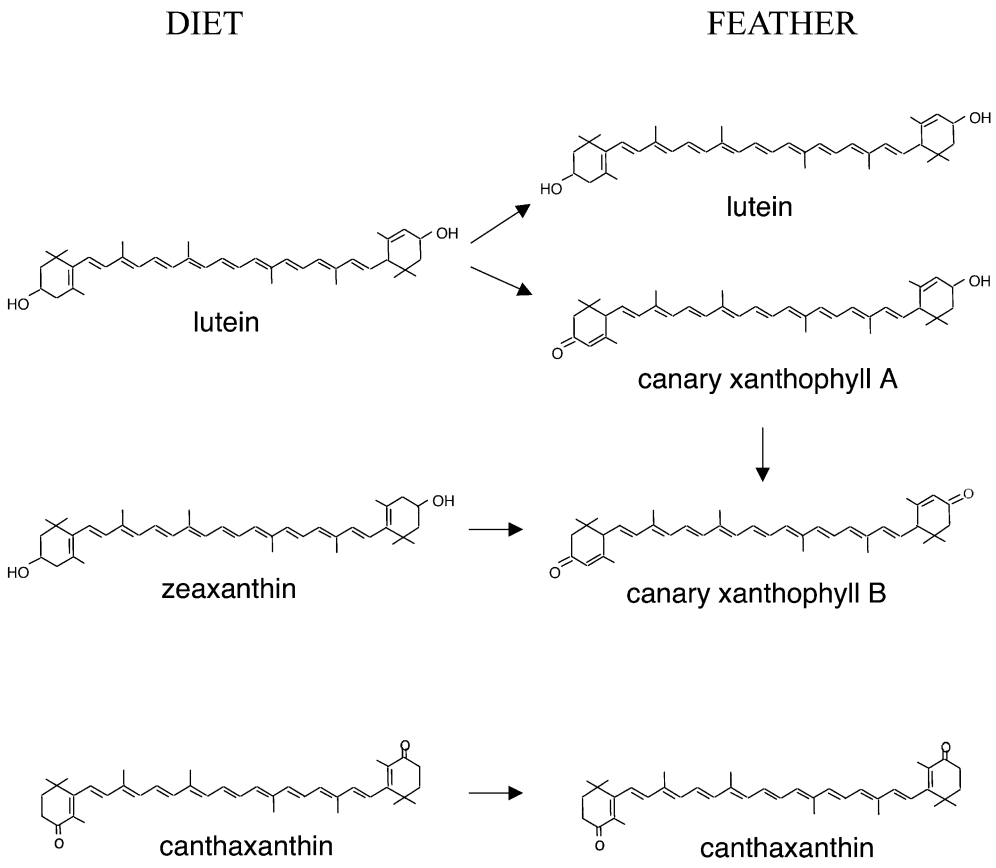

Fig. 5. Hypothetical conversion and deposition pathways of dietary carotenoids in goldfinches from this study (sensu Stradi, 1998).

to varying concentrations of identical feather pigments.

On the plain-seed diet, males incorporated significantly more canary xanthophyll B into plumage than females. There also was a tendency for males to accumulate more canary xanthophyll A. It is hypothesized, based on oxidation reactions that appear common in birds (Stradi, 1998), that goldfinches convert dietary lutein into canary xanthophyll A and dietary zeaxanthin into canary xanthophyll B (Fig. 5); further dehydrogenation of A can result in the formation of B as well. Thus, it is possible that males convert dietary pigments more efficiently than females. Specific enzyme systems control these metabolic conversions (Brush, 1990); in scarlet tanagers, for example, presumably only males possess the enzyme(s) capable of forming red plumage pigments (Brush, 1967). By completing these energy-demanding chemical reactions and using metabolic derivatives to color their feathers, males may communicate useful information to females about their health and condition, and thus their value as a potential mate (Hill, 2000). On this note, it is interesting that the more extended, multi-step conversion to canary xanthophyll B proved to be the primary sex difference in pigment displays of goldfinches in our study. However, until we identify the sites at which these metabolic conversions occur, the enzymes that catalyze oxidation reactions and the specific pathways that dietary carotenoids follow (Brush, 1990), we cannot completely assess the role of pigment metabolism in shaping sex patterns of carotenoid pigmentation.

Our second diet experiment, in which we supplemented birds with the red carotenoid pigment canthaxanthin, allowed us to investigate the means by which male and female goldfinches assimilate ingested carotenoids, independent of the role of carotenoid metabolism. Canthaxanthin was deposited into plumage unmodified in both sexes (Fig. 5), yet male finches incorporated more canthax-

anthin and a greater proportion relative to other pigments into carotenoid-based plumage than females. This suggests that compared to other pigments available in the diet, canthaxanthin was physiologically processed more efficiently by males than females. There are a number of potential levels at which the sexes may have differed in utilizing this carotenoid. Some have noted sex differences in the color and pigment concentration of circulating serum in certain avian species (Hill et al., 1994; Hill, 1995; Bortolotti et al., 1996; Figuerola and Gutierrez, 1998), which suggests that males and females may differ in the degree to which they absorb pigments acquired from the diet, or in their ability to transport pigments through the blood. Although nothing is known of sex differences in carotenoid uptake in birds, it is clear that absorption requires a certain degree of specificity; for example, in chickens, different intestinal regions absorb different carotenoid types (Tyczkowski and Hamilton, 1986). Similarly, only Trams (1969), in his study of ibis species, has considered the lipoprotein composition of blood plasma; in these birds, he found that LDL and HDL had unique affinities for dietary and metabolically derived pigments, respectively. Lastly, carotenoids may be deposited into growing feathers differentially by the sexes. Steroids bind to feather follicles (Kovacs et al., 1986), and either binding capacity (Peczely, 1992) or androgen metabolism (Schlinger et al., 1989) may be sex-specific during molt. Accordingly, in the zebra finch (*Taeniopygia guttata*), castration results in decreased coloration of carotenoid-based bills in males (Cynx and Nottebohm, 1992), whereas masculinization of females induces the expression of male-typical bill pigmentation (Adkins-Regan and Wade, 2001). Clearly, future studies must be aimed at identifying the types and amounts of pigments that travel from the intestinal mucosa into the bloodstream to the site of deposition to determine how carotenoid absorption, transport, and deposition processes facilitate sex patterns of nuptial coloration in birds.

Although this study has demonstrated that dietary access to carotenoids alone cannot explain differences in plumage color and pigment composition between male and female goldfinches, it does not rule out the possibility that foraging differences between the sexes may contribute to sexual dichromatism in nature. For example, sex differences in the canary xanthophyll B concentration of feathers may result from males more actively foraging for zeaxanthin-rich foods. This pigment is typically rarer in seeds than is lutein (Goodwin, 1980), and we found no evidence that zeaxanthin was deposited unmodified into feathers in either sex, suggesting that it is, in fact, limiting. Certainly, we need a better understanding of the feeding behaviors of birds in the wild to elucidate the true environmental limitation of carotenoid pigments (Grether et al., 1999) and its effects on carotenoid-based sexual dichromatism.

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