Carotenoid pigments in male American goldfinches: what is the optimal biochemical strategy for becoming colourful?

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Studies of brilliant carotenoid-based coloration in birds have traditionally centred on the role that these colours play in attracting mates. More recently, biologists have begun to take a biochemical approach to understanding the types of pigments found in feathers and how these relate to the expression of ornamental coloration. Nevertheless, surprisingly few studies have assessed the types and amounts of carotenoids present in the diet or blood of animals in relation to season, sex, condition or sexually attractive colour traits, particularly for wild birds. It is conceivable not only that the total concentration of pigments available is an important predictor of sexual attractiveness and mate quality, but also that specific pigments vary among individuals and play more important physiological and pigmenting roles than others. We investigated the carotenoid content of blood and feathers in wild-caught, yellow-pigmented male American goldfinches (Carduelis tristis) throughout the year to determine the optimal biochemical strategy for becoming colourful. We found that birds acquired two main yellow hydroxycarotenoids (lutein and zeaxanthin) from the diet during both moulting and non-moulting periods. Blood concentrations of both pigments changed significantly over time, with moulting birds accumulating higher levels of both lutein and zeaxanthin, but proportionally more zeaxanthin, than non-moulting birds. Moulting birds that acquired more lutein and more zeaxanthin in blood deposited a higher concentration of carotenoid pigments (canary xanthophylls A and B) into plumage and acquired more colourful feathers. In sum, these results indicate that (a) the types of dietary carotenoids available across seasons do not change in American goldfinches, (b) seasonal fluctuations in plasma-carotenoid signatures may result from differences in dietary access or pigment processing, and (c) the best biochemical strategy for becoming a colourful, wild male goldfinch is to accumulate as many dietary/blood pigments as possible during moult. © 2004 The Linnean Society of London, Biological Journal of the Linnean Society, 2004, 83, 273–280.


INTRODUCTION

Ornamental colours in birds are some of the best-studied sexually selected traits in animals (Andersson, 1994). Hill’s (1991) pioneering work on the carotenoid coloration in male house finches (Carpodacus mexicanus) paved the way for a plethora of studies on the sexual significance of bird colours during the past decade (reviewed in Hill, 2002). It is now evident in several brightly coloured birds that, by displaying more colourful feathers, males are more successful at attracting mates (reviewed in Hill, 1999).

It is likely that carotenoid coloration has captured the attention of behavioural ecologists because of its intuitive ties to individual quality and sexual attraction. Red, orange and yellow carotenoid pigments are made only by photosynthetic organisms, so animals such as house finches must acquire them through their diet, either directly by consuming plant matter or indirectly via herbivorous prey (Brush, 1981). Thus, males demonstrate their foraging prowess by obtain-
ing and sequestering carotenoid pigments into their showy, colourful ornaments and subsequently are preferred as mates because of their superior nutritional state (Hill, Inouye & Montgomerie, 2002).

While the majority of early work centred either on the nutritional control (e.g. Brush & Power, 1976; Hill, 1992) or mating significance (e.g. Hill, 1990; Johnson, Dalton & Burley, 1993; Sundberg, 1995) of carotenoid-based plumage colours in birds, more recently there has been a surge of interest in the biochemical nature of these compounds as they relate to colour expression (Inouye et al., 2001). Modern advances in analytical techniques (e.g. high-performance liquid chromatography, or HPLC; Stradi, Celentano & Nava, 1995) offer new opportunities to characterize the types and amounts of pigments found in bird feathers. We now know that a variety of types of carotenoids are found in colourful plumage (e.g. Stradi, 1998) and that the brightness of colour patches in certain species is a direct product of the overall concentration of carotenoid pigments found within them (e.g. Saks, McGraw & Horak, 2003). In other species (e.g. the house finch), there are remarkable colour differences (e.g. red, yellow) in the types of carotenoids that appear in feathers, such that certain ones are preferable to accumulate over others for pigmentation (Inouye et al., 2001).

Despite all of this ground-breaking research into the biochemical basis for conspicuous colours in birds, we still know very little about the actual dietary contributions to carotenoid coloration in wild birds. Birds are capable of metabolizing ingested carotenoids into alternate forms that appear in feathers (Brush, 1990), so we cannot learn much about the dietary processes underlying pigmentation by studying only feather pigments. In the best study of this sort to date, Hill et al. (2002) quantified the concentration of carotenoids present in the gut of wild male house finches and found that it correlated positively with the colour of carotenoid-containing feathers grown soon thereafter. However, even in this work we still do not know the identity of carotenoid pigments ingested. Moreover, several studies have focused on the carotenoid status of blood plasma, because carotenoids extracted from food are delivered to peripheral tissues such as feathers for pigmentation via the bloodstream. Yet in this work researchers have either scored the raw colour of plasma (Hill, 1995a, b; Figuerola & Gutierrez, 1998), which is problematic because plasma can be inadvertently coloured by red blood cells (a consequence either of coagulation during blood collection or haemolysis during centrifugation; pers. observ.), or used absorbance spectrophotometry to estimate carotenoid concentration (e.g. Saino et al., 1999; Bortolotti et al., 2000), without identifying the full complement of carotenoids found in blood.

From all of this, it is clear that further biochemical work is needed to describe dietary and plasma carotenoids in birds with carotenoid-based integumentary coloration. Such studies in wild birds will help address several important questions relevant to carotenoid biochemistry and coloration that remain unanswered. For example, how variable are the types and amounts of blood carotenoids? Do they vary among individuals, between seasons, and in relation to sexual attractiveness? Are specific carotenoids favoured during the period of feather growth and, when accumulated at the highest levels, responsible for the brightest colour patterns? Because several different carotenoid types have been documented from both plant and animal matter (e.g. xanthophylls, carotenoids; Mangels et al., 1993; Slifka et al., 1999), which vary markedly in molecular structure and hence coloration, it is reasonable to hypothesize that birds may selectively forage for or physiologically process those pigments that make them most colourful.

Here, we used HPLC to identify the types and amounts of plasma carotenoids in a yellow-coloured, carotenoid-pigmented songbird: the American goldfinch (Carduelis tristis). This cardueline finch (family Fringillidae, subfamily Carduelinae) has been extensively studied in the context of both sexual selection and ornamental pigmentation. Male goldfinches display lemon-yellow plumage during the breeding season (Middleton, 1993), and female goldfinches prefer to mate with the most brightly coloured male (Johnson et al., 1993). Male goldfinches use two main carotenoids to colour their feathers, canary xanthophylls A and B (McGraw et al., 2001, 2002). Captive studies indicate that, when fed seeds that contain two yellow carotenoid pigments (lutein and zeaxanthin), goldfinches manufacture these two canary xanthophylls from them for use in yellow plumage (McGraw et al., 2001, 2002). However, to date there is no information on the types or amounts of, or variation in, dietary or blood carotenoids in wild goldfinches.

We studied two wild populations of goldfinches during 2000–01: a pre-breeding population in Ithaca, NY, during spring 2000 and a wintering population (likely migrants from the northern and eastern portions of the USA; Middleton, 1993) in Auburn, AL, during the subsequent winter. We sampled blood from all wild-caught males and determined plasma-carotenoid signatures. For birds that were molting into their breeding plumage, we also scored the colour of newly grown yellow feathers and plucked small plumage patches for feather-carotenoid analysis. We then tested for differences in the amounts and types of plasma carotenoids across seasons and in relation to both plumage coloration and feather-carotenoid profiles.

METHODS

We captured 26 male goldfinches undergoing their spring, nuptial moult between 12 April and 8 May 2001 in Ithaca, NY. We captured 27 male goldfinches wintering in Auburn, AL, between 8 and 30 January 2002. At capture, we drew 50–100 μL blood from the alar vein of each individual, centrifuged the heparinized microcapillary tubes, and stored the plasma in 1.5 mL screw-cap Eppendorf tubes at −80 °C for later carotenoid analysis. For the Ithaca birds, we also scored plumage coloration using a hand-held Colortron II reflectance spectrophotometer (sensu McGraw & Hill, 2000, 2001). Upper, middle and lower regions of yellow plumage were measured on both the ventral and dorsal sides, and we computed mean tristimulus scores (hue, saturation and brightness) by averaging these six values for each bird. This method has been shown to be highly repeatable (McGraw & Hill, 2001). Finally, we plucked a patch of freshly grown breast feathers (5–10 feathers) from 21 of the males and stored these in the dark at room temperature for later carotenoid analysis.

Plasma (sensu McGraw et al., 2003a) and plumage carotenoids (sensu McGraw et al., 2002) were analysed following previously published procedures. For plasma carotenoids, we added 10 μL thawed plasma to 75 μL ethanol and 75 μL tert-butyl methyl ether, vortexed the mix for 5 s, and spun the tube 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 them dry on filter paper. We then trimmed off the yellow-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 acidified pyridine and filled the headspace with argon. We placed the tube in a 95 °C water bath for 3 h and cooled to room temperature before adding 2 mL distilled water.

We inverted the tube a few times and then added 2 mL tert-butyl methyl ether. After shaking the solution vigorously for 1 min, we centrifuged the tube for 5 min at 5000 g. 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 pigment residue in 200 μL HPLC mobile phase (see below for composition) and injected 50 μL into a Waters 717plus autosampler HPLC (Milipore Corporation, Milford, MA) fitted with a Develosil RPAquous RP-30 column (250 × 4.6 mm inner diameter; Nomura Chemical Co., Ltd, Japan) and an Eppendorf TC-50 column heater (set at 31 °C). Due to the different polarities of plasma and plumage carotenoids, we used slightly different mobile phases (for plasma, methanol: acetonitrile: chloroform, 46:46:48, v/v/v; for plumage, methanol: acetonitrile, 50:50, v/v).

An isocratic system (HP 1050 Series Isocratic Pump) was run for 25 min at a constant flow rate of 1.2 mL min⁻¹. Pigments were identified by comparison to purified standards of known carotenoids (see below) and quantified using an internal standard of known concentration (1 μg mL⁻¹ canthaxanthin) that we previously determined to be absent from blood and feathers of these birds.

Using the STATVIEW v.5.0.1 statistical software package (SAS Institute Inc., Cary, NC), we performed analyses of variance (ANOVA) to compare blood-carotenoid status between moultting males in spring and non-moultting males in winter. We used Fisher r-to-z correlation tests to examine relationships between blood carotenoids, plumage colour and feather carotenoids. Recall that N = 21 for comparisons involving plumage carotenoids; for all other comparisons, N = 27 for non-moultting birds and N = 26 for moultting birds.

RESULTS

IDENTITY OF PLASMA PIGMENTS

We identified two main carotenoid pigments in the plasma of moultting and non-moultting wild male American goldfinches. Using the aforementioned analytical system, they had retention times (tR) on the HPLC column of 6.7 min and 7.1 min and absorbance maxima (λmax) of 445 nm and 453 nm, respectively. By matching these carotenoids to authentic standards provided by Dr Riccardo Stradi (University of Milan, Italy) and Roche Vitamins Inc. (Parsippany, NJ), we determined that these pigments corresponded to lutein (β, ε-carotene-3, 3'-diol) and zeaxanthin (β, β-carotene-3, 3'-diol). Lutein was the predominant plasma pigment in both moultting and non-moultting wild males, comprising 74.9 ± 0.68% and 73.7 ± 2.1% of total pigments, respectively (Fig. 1). We found no evidence that either β-cryptoxanthin or β-carotene, two other common dietary carotenoids in bird foods (McGraw et al., 2001) and plasma (Slifka et al., 1999), were present in appreciable amounts in these birds (<1% of total pigments).

SEASONAL DIFFERENCES IN PLASMA-CAROTENOID CONTENT

Moultting and non-moultting males differed significantly in the total concentration of carotenoids found in plasma, with moultting males circulating 80% more overall (ANOVA, F1,51 = 10.6, P = 0.002; Fig. 1). Moultting males circulated significantly more of both lutein (78% more) (F1,51 = 10.0, P = 0.003; Fig. 1) and zeaxanthin (86% more) (F1,51 = 10.6, P = 0.002; Fig. 1) than non-moultting males. Interestingly, moultting males also circulated proportionally less lutein (thus, propor-
tionally more zeaxanthin) than non-moultng males ($F_{1,51} = 4.9$, $P = 0.03$; Fig. 1).

Correlations between plasma carotenoids, plumage colour and feather carotenoids

Within seasons, we examined the extent to which birds circulated similar amounts of the two pigments through blood. In both the spring ($r = 0.97$, $P < 0.0001$) and the winter ($r = 0.73$, $P < 0.0001$), males that accumulated more lutein in blood also acquired more zeaxanthin (Fig. 2). Note that the correlation was stronger in spring than in winter. Birds that accumulated the highest overall concentration of pigments, however, did not adopt a specific strategy by selectively accumulating proportionally more of one pigment than another [winter correlation ($r$) between total carotenoids and lutein:zeaxanthin ratio $= -0.16$, $P = 0.43$; spring $r = -0.03$, $P = 0.9$].

During moult, male goldfinches that circulated the highest concentration of carotenoids through blood developed the most saturated yellow plumage ($r = 0.40$, $P = 0.04$, Fig. 3A). There was no significant relationship between total plasma-carotenoid concent-

Figure 1. Differences in plasma-carotenoid status (mean + SEM) between wild male American goldfinches during the spring moult and during winter (non-moult). We analysed plasma for both polar and non-polar carotenoids using high-performance liquid chromatography (sensu McGraw et al., 2003a). Two main carotenoids were detected: lutein and zeaxanthin. Here, we show lutein and zeaxanthin concentration in addition to total concentration and lutein:zeaxanthin ratio.

Figure 2. Correlation between the levels of the two main carotenoids found in goldfinch plasma (lutein and zeaxanthin) at two times of year: during the spring moult and during winter.

Figure 3. Relationship between the colour of yellow goldfinch plumage and total concentration of carotenoids found in blood plasma (A) or total concentration of carotenoids found in feathers (B). Colour was measured by richness or saturation (calculated as a percentage relative to absolute white and black standards provided with the Colortron). See text for the correlations between individual pigment types and plumage coloration.
tration and either plumage hue ($r = -0.29$, $P = 0.15$) or plumage brightness ($r = 0.18$, $P = 0.38$). Specifically for plumage saturation, it was the concentration of lutein that was most related to feather colour ($r = 0.41$, $P = 0.03$); neither zeaxanthin concentration nor lutein:zeaxanthin ratio were significantly related to carotenoid-based plumage saturation ($r = 0.34$, $P = 0.09$; $r = 0.33$, $P = 0.11$, respectively).

We confirmed that our primary colour score for feathers is an accurate reflection of the carotenoid content found within them. Plumage saturation ($r = 0.79$, $P < 0.0001$; Fig. 3B), but not hue ($r = -0.01$, $P = 0.97$) or brightness ($r = 0.39$, $P = 0.08$), explained a statistically significant proportion of the variation in feather-carotenoid concentration in our sample of birds. Plumage saturation (but not hue or brightness; data not shown) was also significantly correlated with amounts of individual carotenoids in feathers: canary xanthophyll A ($r = 0.76$, $P < 0.0001$) and canary xanthophyll B ($r = 0.80$, $P < 0.0001$) ($P > 0.8$ for A:B ratio).

Total feather-carotenoid concentration, in turn, was also correlated with the overall concentration of plasma carotenoids ($r = 0.47$, $P = 0.02$; Fig. 4), and specifically with plasma lutein concentration ($r = 0.50$, $P = 0.01$) and lutein:zeaxanthin ratio ($r = 0.46$, $P = 0.03$), but not plasma zeaxanthin concentration ($r = 0.40$, $P = 0.07$). When comparing concentrations of individual plasma- and plumage-carotenoids, we found general relationships between nearly all of the carotenoids (with the exception of zeaxanthin and canary xanthophyll A); higher levels of each plasma carotenoid were associated with higher levels of each plumage carotenoid (Table 1). Levels of canary xanthophylls A and B in feathers were also highly intercorrelated ($r = 0.91$, $P < 0.0001$). The only ratios of blood or plumage carotenoids that were significantly linked were: lutein:zeaxanthin ratio with canary xanthophyll A concentration and with the A:B ratio (Table 1).

**DISCUSSION**

We used sophisticated biochemical tools to examine seasonal and sexual patterns of carotenoid circulation and pigmentation in male American goldfinches. With the knowledge that sexual selection favours the acquisition of brightly coloured plumage in males of this species (Johnson et al., 1993), we investigated the optimal biochemical strategy a male can use to become colourful. The opportunity for such a detailed molecular investigation into the production of a sexually selected trait is rare, because we typically know so little about the exact physiological processes that generate extravagant features (e.g. behavioural displays, weaponry, elaborate vocalizations).

HPLC analyses of blood plasma from goldfinches at different times of year (spring and winter) yielded two main carotenoid pigments: lutein and zeaxanthin. These yellow hydroxycarotenoids are commonly

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<th>Canary xanthophyll A</th>
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<td>Lutein</td>
<td>$r = 0.46$</td>
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<td>Zeaxanthin</td>
<td>$r = 0.35$</td>
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<td>Lutein:zeaxanthin</td>
<td>$r = 0.49$</td>
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reported from bird blood (Slifka et al., 1999) and bird seeds (McGraw et al., 2001), and are thought to be major carotenoid components of many avian herbivore, granivore and frugivore diets (Goodwin, 1980, 1984). Goldfinches consume a diet that is predominated by seeds throughout the year (Middleton, 1993), and we have isolated these two pigments from the gut contents of wild male goldfinches during moult (KJM, unpubl. data). Thus, it seems that goldfinches acquire a rather simple mix of carotenoids from their diet throughout the year, and that there does not appear to be a unique carotenoid or suite of carotenoids ingested for pigmentation specifically during feather growth.

Two measures of plasma carotenoids did vary seasonally, however: total concentration and lutein: zeaxanthin ratio. Hill (1995a) previously demonstrated that plasma colour in house finches was significantly richer during moult than other times of year. He hypothesized that, given the seasonal need for plumage carotenoids, birds would ingest the highest volume of pigments during feather growth. He also noted that house finches shift their diet during moult and consume more fruits (from Beal, 1907), which are potentially more carotenoid-rich. Goldfinches acquire their breeding plumage at a different time of year (spring) than house finches, and we presently lack a comprehensive study of diet throughout the year in this species to address the potential for a temporal shift. However, it is worth noting that these birds do consume large amounts of newly emerging buds and flowers (e.g. dandelions) in spring (Middleton, 1993). Thus, while it is conceivable that a diet shift may also explain increased plasma-carotenoid levels in molting male goldfinches, we cannot rule out the possibility that their physiological capabilities for processing ingested carotenoids or mobilizing stored pigments (e.g. from body fat; Negro et al., 2001) are highest at the time of feather development.

It is curious that zeaxanthin was proportionally more abundant in plasma than lutein during the moult period in male goldfinches. For a given concentration, zeaxanthin is the stronger colourant of the two carotenoids, as its chromophore has more double bonds in conjugation (Stradi, 1998). Thus, given the potential coloration benefits of acquiring more zeaxanthin, it may be (a) that male goldfinches forage for foods particularly enriched with zeaxanthin in spring, (b) that the physiological systems for processing pigments in these birds is optimally tuned to assimilating zeaxanthin from foods, or simply (c) that more zeaxanthin-rich foods are available to male goldfinches in spring. We have not yet conducted diet studies to examine seasonal changes in the zeaxanthin concentration of gut contents. We have performed feeding trials, however, and found that molting males do not accumulate higher levels of zeaxanthin than lutein in blood when fed identical dietary concentrations of the two (McGraw et al., 2004). Hence, it is likely that dietary factors regulate this seasonal pattern of carotenoid circulation in male goldfinches. In most foodstuffs and animal tissues studied to date, lutein and zeaxanthin are found in combination (Goodwin, 1980, 1984; Mangels et al., 1993; Slifka et al., 1999; but see McGraw et al., 2003b), so if goldfinches do have the ability to select or discriminate zeaxanthin-rich foods it may be highly refined. Other carotenoid-pigmented birds (Shields, 1997) and fish (Rodd et al., 2002) are known to prefer more colourful foods to drab ones, but we await studies of actual carotenoid-specific visual or gustatory dietary preferences in animals.

In a previous study, we offered the hypothesis that a particular carotenoid signature in feathers was most desirable for male goldfinches to acquire the brightest coloration (McGraw et al., 2002). In this feeding experiment with captive male and female goldfinches, we found that males, which grow more colourful plumage than females, deposited significantly more canary xanthophyll B into feathers (McGraw et al., 2002). This observation led us to conduct this research with wild birds in order to determine whether particular recipes of blood or plumage carotenoids best predicted the acquisition of colourful feathers. Instead, we found remarkably general patterns of carotenoid accumulation that underlie brilliant male coloration. Birds that circulated more of one plasma pigment circulated more of the other. Birds with more blood- and plumage-carotenoids grew feathers with the deepest (most saturated) yellow colour. Thus, rather than targeting specific precursor carotenoids for the formation of specific plumage colourants, these birds seemed to be following a very straightforward, ‘more-is-better’ decision rule for pigmentation. This result is not consistent with the hypothesis above that foraging for zeaxanthin-rich foods has important consequences for plumage-colour development in goldfinches. Instead, these birds may seek foods generally rich in carotenoid concentration, which may also contain higher proportions of zeaxanthin.

In order to better understand these relationships between plasma- and plumage-carotenoids and their implications for feather pigmentation, it is valuable to consider the pathways by which the plumage colourants are formed from dietary precursors in goldfinches. In prior work, we posed hypothetical metabolic links between the two dietary carotenoids and the two plumage carotenoids based on the likely series of oxidation steps that occur in songbirds (Stradi, 1998). We speculated that lutein is the primary, probable substrate for canary xanthophyll A formation, and zeaxanthin the precursor of canary xanthophyll B.
(Stradi, 1998; fig. 7 in McGraw et al., 2001). However, we also pointed out that lutein may also be converted into canary xanthophyll B via canary xanthophyll A. Thus, in the few instances where we see lutein or a particular lutein:zeaxanthin ratio better explaining feather-colour and -pigment patterns than zeaxanthin, it may be because lutein is in higher absolute concentration in the blood than zeaxanthin or because lutein can serve as the metabolic substrate for both plumage carotenoids. Follow-up experiments in which dietary or blood pigments are radiolabelled will strengthen our biochemical studies of carotenoids in goldfinches, specifically by identifying the paths down which these different metabolic transformations are most likely to proceed.

One of the fundamental assumptions about pigments as colourants of animal tissues is that the colour of a pigment patch truly captures the variation in the pigments contained within. This idea, to our knowledge, has been tested only twice in colourful birds: in house finches (Inouye et al., 2001) and in greenfinches (Carduelis chloris; Saks et al., 2003), a yellow-feathered congener of the goldfinch. Here, we show that plumage saturation is a reliable colour measure for estimating pigment concentration in yellow goldfinch feathers. Saks et al. (2003) also found saturation to be the best tristimulus predictor of feather-pigment composition in greenfinches. Interestingly, our work with plumage pigments (also canary xanthophylls A and B) in C. chloris has also shown that the most yellow feathers are created not by any particular carotenoid combination, but instead simply by a higher concentration of both canary xanthophylls (Saks et al., 2003). Thus, the optimal pigmentation strategy we have detailed here seems to be a conserved feature in two Carduelis finches with relatively simple coloration systems (e.g. few pigments present).

The house finch, however, despite being in the same subfamily as the Carduelis finches, exhibits remarkable variation in plumage coloration (red to yellow) and in the types of carotenoids that appear in feathers (a range of 12, with some yellow and some red in colour; Inouye et al., 2001). Perhaps this species would be an ideal candidate to consider how different carotenoids may vary seasonally in abundance and differentially contribute to ornamental coloration in birds.

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