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Carotenoid accumulation strategies for becoming a colourful House Finch: analyses of plasma and liver pigments in wild moulting birds

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Summary

1. Male House Finches (*Carpodacus mexicanus*) colour their sexually selected plumage with carotenoid pigments, and there has been much interest in the factors that affect their ability to become bright red rather than drab yellow.
2. There is good support for the notions that health, nutritional condition and total carotenoid intake influence colour expression, but there are also suggestions that acquiring particular types of carotenoids from the diet may be important for developing red plumage.
3. We used high-performance liquid chromatography (HPLC) to analyse the types and amounts of endogenous (in plasma and liver) and integumentary (in newly grown feathers) carotenoids in a wild, native population of moulting male and female House Finches from the south-western United States to determine the carotenoid-accumulation strategies for becoming optimally colourful.
4. Four plant carotenoids – lutein, zeaxanthin, β-cryptoxanthin and β-carotene – were detected in plasma and liver. However, as was found previously, 11 carotenoids were observed in colourful plumage, with xanthophylls (e.g. lutein, dehydrolutein) predominant in yellow feathers and ketocarotenoids (e.g. adonirubin, 3-hydroxy-echinenone) in red feathers. This indicates endogenous modification of ingested carotenoids.
5. Birds that accumulated more of one type of carotenoid in plasma and liver did not necessarily accumulate more of all other types, suggesting that individuals are not employing a simple 'more is better' strategy for coloration. Instead, when forward stepwise regression was used to examine the ability of individual types of carotenoids in plasma and liver to explain variation in red plumage pigments and plumage redness, we found that the lone variable remaining in all models was β-cryptoxanthin concentration.
6. This supports the idea that, unlike some other songbirds (e.g. yellow *Carduelis* finches), there is a specialized biochemical strategy that male House Finches follow to become red and most sexually attractive – to accumulate as much β-cryptoxanthin in the body as possible. β-Cryptoxanthin is a less common dietary carotenoid than the typical xanthophylls and carotenes in grains and fruits and may be limited enough in the diet that, to become colourful, House Finches might adopt selective foraging strategies for the most β-cryptoxanthin-rich foods.

Key-words: *Carpodacus mexicanus*, ketocarotenoids, plumage coloration, sexual selection, xanthophylls

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Introduction

The variable red-to-yellow plumage of male House Finches (*Carpodacus mexicanus*) has emerged as a classic example of a sexually selected indicator of quality in birds (reviewed in Hill 2002). Numerous field and laboratory studies demonstrate female mate choice for male House Finches with the reddest plumage (e.g. Hill

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1990, 1991) and the associated reproductive benefits (e.g. earlier timing of breeding, increased young production) reaped by colourful males and their mates (Hill *et al*. 1994, 1999; McGraw *et al*. 2001). Moreover, a series of correlational and experimental studies indicate that males must ingest sufficient amounts of carotenoids from food to become colourful (Hill 1992, 1993; Hill *et al*. 2002) as well as maintain adequate overall nutrition (Hill & Montgomerie 1994; Hill 2000) and health (Thompson *et al*. 1997; Brawner *et al*. 2000; Hill *et al*. 2004) during feather growth.

There are indications from other work, however, that acquiring certain *types* of carotenoids from foods might also be critical for developing red, as opposed to yellow, coloration. Without exception, House Finches housed in captivity on a normal seed diet during moult grow yellow plumage (Hill 1992, 2002), and it was only when additional sources of non-xanthophyll carotenoids (e.g. synthetic red carotenoids, tangerine juice) were added to their diet that they grew red feathers (Hill 1992, 2000). More recent biochemical studies of the carotenoid components in House Finch feathers (Inouye *et al*. 2001) show that important red carotenoids absent from other tissues and fluids in these birds (McGraw 2004) are what determine red coloration and thus must be manufactured by birds from precursor molecules not commonly available to them. Hill (2000, 2002) speculated that it was the ability of birds to accumulate the precursors of red pigments in feathers – candidate molecules such as β-cryptoxanthin (based on purported chemical transformations) – that controls, at the pigment level, whether birds could acquire red plumage or not (also see Stradi *et al*. 1996 for similar predictions for other red-coloured cardueline finches). However, to date, there are no published studies on variation in the types of carotenoids that free-ranging House Finches ingest and accumulate in the body or on the extent to which ingested carotenoids predict the pigment composition and expression of colourful plumage.

We studied carotenoid accumulation strategies during moult in a native population of House Finches from the desert south-western United States. We collected blood from live birds (to obtain a recent record of dietary-carotenoid assimilation), liver tissue and feathers from freshly euthanized animals, and analysed carotenoid content of all samples so that we could ask the following questions about the relationships between pigment accumulation and coloration:

1. How do levels of different carotenoids covary within plasma or liver? In those bird species for which the biochemical basis of carotenoid coloration has been investigated (e.g. American Goldfinch [*Carduelis tristis*], McGraw & Gregory 2004; Greenfinch [*Carduelis chloris*], Saks *et al*. 2003), birds tended to accumulate more of all types of carotenoids within a fluid/tissue type (e.g. plasma, feathers). However, if certain dietary pigments are of special value to colour production in House Finches, then levels of internal fluid/tissue carotenoids should not uniformally covary.
2. What is the relationship between plumage redness and the concentration of particular carotenoids in feathers? As was previously done by Inouye *et al*. (2001) in House Finches from California and Mexico, we set out to confirm which suite of carotenoids in feathers generated the reddest plumage in our study population, so that we could then target the types and amounts of carotenoids in liver and plasma with which these plumage pigments are most closely correlated.
3. What profile of plasma and liver carotenoids allows birds to accumulate the most red feather pigments? Whether it shows that one or all types of plasma or liver carotenoids are significantly associated with red pigment accumulation in feathers, this analysis will ultimately illustrate the endogenous strategy for carotenoid accumulation that House Finches follow for becoming sexually attractive. We also aimed to test, as secondary confirmation, how well levels of plasma and liver carotenoids predicted plumage redness.
4. Do birds that accumulate more carotenoids in plasma also accumulate more in liver? Plasma carotenoids represent the mobile pool of pigments that are currently being delivered to peripheral tissues. The liver, in contrast, has been touted as a valuable storage site for carotenoids (Koutsos *et al*. 2003), and birds may draw on these stores during the moult period, such that the reddest birds are those with the lowest liver carotenoid levels but highest plasma levels. Alternatively, plasma and liver levels may be tightly correlated and reflect the genuine 'high quality' of red birds, who continue to ingest and mobilize sufficient amounts for coloration while still having high tissue supplies.
5. How do relationships between carotenoids and coloration compare between males and females and birds of different age? Inouye *et al*. (2001) previously demonstrated age differences in plumage colour and feather-carotenoid content in male *C. mexicanus*, with hatch-year (HY) birds growing yellower feathers with fewer red carotenoids than after-hatch-year (AHY) birds. We were interested in testing whether there were parallel age differences in endogenous carotenoid pools. Moreover, all prior studies on proximate control of carotenoid colour in House Finches have focused on males, but some females also display a splash of colour on the rump that may or may not come under different regulatory mechanisms.

Methods

Fifty-seven moulting House Finches of known age and sex (3 AHY females, 7 AHY males, 24 HY females, and 23 HY males) were captured from the wild using basket traps at baited feeding stations from 24 August

to 27 September 2004 on the campus of Arizona State University in Tempe, Arizona, USA. At capture, approximately 100 µl of blood was drawn from the brachial vein of each bird, and the plasma was centrifuged off and saved for carotenoid analysis 2–3 months later. The hue of newly moulted breast plumage in males and rump plumage in females was also scored at this time using a hand-held Colortron II reflectance spectrophotometer (see McGraw & Hill 2000 for description and justification). Birds were then rapidly euthanized, and patches of three to eight breast feathers were plucked from a standardized region on the bird and stored in the dark at room temperature prior to chemical analysis 11–12 months later. Carcasses were frozen at –80 °C for 2–4 days, at which time a small portion of the lower right lobe of the liver was excised, weighed and stored at –80 °C prior to biochemical analysis 6–7 months later.

Carotenoid analyses followed previously published methods (see McGraw *et al*. 2002 for plasma and liver, Inouye *et al*. 2001 for feathers), with the following modifications. Pigment extracts were injected into a Waters Alliance 2695 HPLC system (Waters Corporation, Milford, MA) fitted with a Waters YMC Carotenoid 5·0 µm column (4·6 mm × 250 mm) and a built-in column heater set at 30 °C. We used a three-step gradient solvent system to analyse both xanthophylls and carotenes in a single run, at a constant flow rate of 1·2 ml min^{-1}: first, isocratic elution with 42:42:16 (v/v/v) methanol : acetonitrile : dichloromethane for 11 min, followed by a linear gradient up to 42:23:35 (v/v/v) methanol : acetonitrile : dichloromethane through 21 min, held isocratically at this condition until 30 min, and finishing with a return to the initial isocratic condition from 30 to 48 min. Data were collected from 250 to 600 nm using a Waters 2996 photodiode array detector. We identified pigments by comparing their respective retention times and absorbance maxima (λ_{max}) to those of reference carotenoids run as external standards.

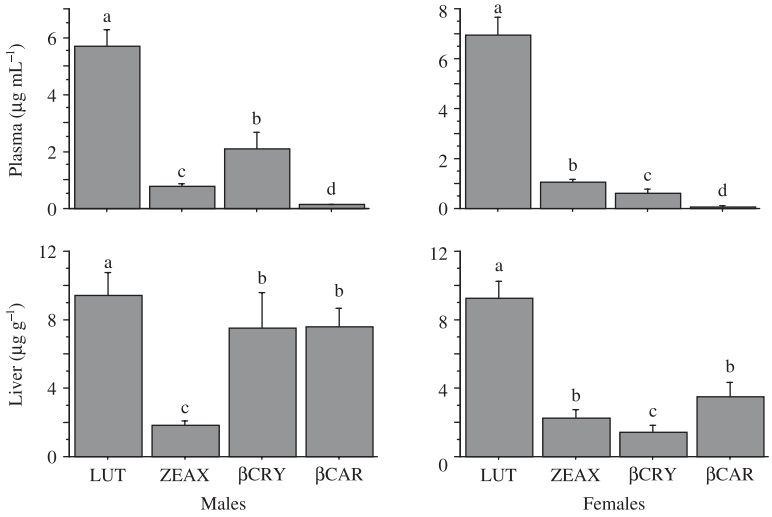

Fig. 1. Concentrations of different carotenoids in the plasma and liver of wild, moulting male and female House Finches. Letters above each bar denote statistically significant differences (Wilcoxon matched-pair signed-rank tests, $P < 0{\cdot}05$). Here and elsewhere, LUT = lutein, ZEAX = zeaxanthin, βCRY = β-cryptoxanthin, and βCAR = β-carotene.

Because data on both carotenoid concentration and hue were non-normally distributed in all cases, and in most could not be transformed to meet the assumption of normality for parametric statistics (owing to the presence of many 0-values), we ran non-parametric Spearman rank correlations to determine relationships between: (a) levels of different carotenoids within blood, liver and feather samples for individual birds, (b) levels of different carotenoids across the fluid/tissue types and (c) levels of carotenoids in plasma and liver *vs* those in feathers. We also used Wilcoxon matched-pair signed-rank tests to determine which carotenoids were most abundant in each fluid/tissue. To ascertain which set of carotenoids in plasma and liver contribute to the production of red plumage pigments, we used forward stepwise regression so that we could enter all competing variables into one model. Admittedly, the use of non-normally distributed data in this regression violates an assumption of this parametric test, so we also ran Spearman rank correlations to ensure that significant relationships still held when treated non-parametrically. Finally, we used Mann–Whitney *U*-tests to examine the effects of age and sex on plumage hue and on carotenoid content of plasma, liver and plumage. Unfortunately, we did not capture sufficient numbers of adult birds (seven males, three females) to run all of the aforementioned correlational analyses separately for the age classes, but we still show data for adults when possible (e.g. in scatterplots). Moreover, feathers were not collected from females, so we were unable to determine their plumage carotenoid content. Instead, we focus liver and carotenoid comparisons on plumage redness in females. Sample sizes vary in some instances due to insufficient material collected from the birds for carotenoid analysis.

Results

DESCRIPTION OF PLASMA AND LIVER CAROTENOIDS

Four carotenoids were detected in plasma and liver of moulting house finches: lutein, zeaxanthin, β-cryptoxanthin and β-carotene. Lutein was the major pigment in both plasma and liver, comprising 80% and 60% of the total, respectively, in females and 69% and 42% of the total, respectively, in males (Fig. 1). In female plasma, the next most concentrated pigments were: zeaxanthin (12%), β-cryptoxanthin (7%) and β-carotene (1%) (Fig. 1). In female liver, β-carotene (17%) and zeaxanthin (15%) were the next most abundant carotenoids to lutein, with β-cryptoxanthin (8%) the least concentrated (Fig. 1). In males, however, β-cryptoxanthin was the second most concentrated plasma carotenoid (20%), followed by zeaxanthin

(10%) and β-carotene (1%), and along with β-carotene was the second most concentrated pigment in liver (21% and 28%, respectively), with zeaxanthin (9%) the least abundant.

DESCRIPTION OF PLUMAGE CAROTENOIDS

Inouye *et al*. (2001) previously characterized 13 carotenoid pigments from the colourful plumage of House Finches from California and Mexico. We found 11 of these carotenoids in freshly grown feathers from males in our Arizona population (Fig. 2); β-cryptoxanthin and β-carotene were not detected. Based on their molecular structure and thus the hue they confer, these 11 carotenoids can be categorized into yellow forms – canary xanthophylls A and B, dehydrolutein, lutein and zeaxanthin – and red forms – astaxanthin, canthaxathin, echinenone, 3-hydroxy-echinenone, adonirubin and 4-oxo-rubixanthin (Inouye *et al*. 2001). The relative abundances of these pigments closely mirrored those found by Inouye *et al*. (2001) as well. 3-Hydroxy-echinenone was the major plumage carotenoid, followed by two yellow (lutein and dehydrolutein) and three red (canthaxanthin, adonirubin and 4-oxo-rubixanthin) pigments (Fig. 2). Based on their absence from blood and liver (*sensu* McGraw 2004), it is assumed that all of the red pigments, along with dehydrolutein, are made from dietary precursors (those in blood and liver) in the maturing feather follicle.

CORRELATIONS AMONG CAROTENOID TYPES WITHIN PLASMA, LIVER, AND PLUMAGE

Concentrations of two xanthophylls – lutein and zeaxanthin – were significantly positively correlated in male plasma and in female plasma (Table 1). Despite the fact that the concentrations of all individual carotenoids were positively associated and some correlations approached statistical significance, we found no other significant correlations among the carotenoids in serum, with the exception of lutein and β-carotene in females (Table 1). In liver, lutein and zeaxanthin concentration were also significantly positively correlated in both sexes, as were (a) lutein and β-carotene and (b) the two less polar pigments – β-cryptoxanthin and β-carotene (Table 1). In male plumage, we found significant positive correlations between the concentrations of all of the yellow carotenoids and between all of the red carotenoids (except astaxanthin), but birds never accumulated significantly high levels of both a yellow carotenoid and a red carotenoid (Table 2). In fact, birds that accumulated more zeaxanthin accumulated significantly less of all of the red carotenoids (except astaxanthin).

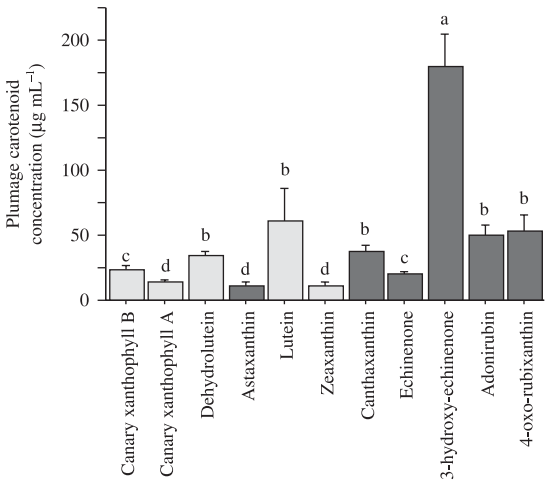

Fig. 2. Concentrations of different carotenoids in the feathers of wild, moulting male House Finches. Light bars represent yellow carotenoids, whereas, dark bars denote red carotenoids. Again, letters above each bar denote statistically significant differences (Wilcoxon matched-pair signed-rank tests, $P < 0{\cdot}05$).

CORRELATIONS BETWEEN FEATHER CAROTENOID CONTENT AND PLUMAGE HUE

We compared the types and amounts of carotenoids in feathers to plumage redness, as did Inouye *et al*. (2001), in our population so that we could then determine which types and amounts of pigments accumulated in blood and liver would produce the optimal plumage-pigment profile for attractive coloration. We found no significant links between plumage hue and concentrations of canary xanthophylls A and B, lutein, dehydrolutein and astaxanthin in feathers (Spearman rank correlations, all $P > 0{\cdot}15$). In contrast, there were

Table 1. Correlations among carotenoid concentrations (μg/ml) in the (a) plasma and (b) liver of moulting House Finches. Data in the lower left of each table are for males and in the upper right are for females. Sequential Bonferroni corrections were applied (minimum $P = 0{\cdot}017$) since all variables were used in three separate analyses. $n = 30$ males and 25 females for plasma-carotenoid comparisons and $n = 26$ males and 22 females for comparisons of liver carotenoids Statistically significant results (after Bonferroni correction) are in bold

	LUT	ZEAX	βCRY	βCAR
(a) Plasma carotenoids				
LUT		**$r_s = 0{\cdot}81$**	$r_s = 0{\cdot}41$	**$r_s = 0{\cdot}55$**
		$P < 0{\cdot}0001$	$P = 0{\cdot}04$	**$P = 0{\cdot}007$**
ZEAX	**$r_s = 0{\cdot}89$**		$r_s = 0{\cdot}43$	$r_s = 0{\cdot}37$
	$P < 0{\cdot}0001$		$P = 0{\cdot}04$	$P = 0{\cdot}07$
βCRY	$r_s = 0{\cdot}32$	$r_s = 0{\cdot}33$		$r_s = 0{\cdot}29$
	$P = 0{\cdot}08$	$P = 0{\cdot}08$		$P = 0{\cdot}16$
βCAR	$r_s = 0{\cdot}32$	$r_s = 0{\cdot}17$	$r_s = 0{\cdot}28$	
	$P = 0{\cdot}08$	$P = 0{\cdot}35$	$P = 0{\cdot}13$	
(b) Liver carotenoids				
LUT		**$r_s = 0{\cdot}72$**	$r_s = 0{\cdot}41$	**$r_s = 0{\cdot}64$**
		$P = 0{\cdot}001$	$P = 0{\cdot}06$	**$P = 0{\cdot}004$**
ZEAX	**$r_s = 0{\cdot}77$**		$r_s = 0{\cdot}15$	$r_s = 0{\cdot}40$
	$P = 0{\cdot}0001$		$P = 0{\cdot}48$	$P = 0{\cdot}07$
βCRY	$r_s = 0{\cdot}31$	$r_s = 0{\cdot}06$		**$r_s = 0{\cdot}59$**
	$P = 0{\cdot}13$	$P = 0{\cdot}76$		**$P = 0{\cdot}007$**
βCAR	**$r_s = 0{\cdot}61$**	$r_s = 0{\cdot}32$	**$r_s = 0{\cdot}58$**	
	$P = 0{\cdot}002$	$P = 0{\cdot}11$	**$P = 0{\cdot}004$**	

Table 2. Correlations among carotenoid concentrations in the feathers of male House Finches. Sequential Bonferroni corrections were applied (minimum $P = 0{\cdot}005$) since all variables were used in ten separate analyses. $n = 30$ in all comparisons. Statistically significant results are in bold

	Canary xanthophyll B	Canary xanthophyll A	Dehydrolutein	Astaxanthin	Lutein	Zeaxanthin	Canthaxanthin	Echinenone	3-Hydroxy-echinenone	Adonirubin	4-Oxo-rubixanthin
Canary xanthophyll B											
Canary xanthophyll A	**$r_s = 0{\cdot}76$**										
	$P < 0{\cdot}0001$										
Dehydrolutein	**$r_s = 0{\cdot}57$**	**$r_s = 0{\cdot}76$**									
	$P = 0{\cdot}002$	**$P < 0{\cdot}0001$**									
Astaxanthin	$r_s = 0{\cdot}06$	$r_s = 0{\cdot}05$	$r_s = -0{\cdot}01$								
	$P = 0{\cdot}76$	$P = 0{\cdot}80$	$P = 0{\cdot}96$								
Lutein	**$r_s = 0{\cdot}61$**	**$r_s = 0{\cdot}84$**	**$r_s = 0{\cdot}93$**	$r_s = -0{\cdot}12$							
	$P = 0{\cdot}001$	**$P < 0{\cdot}0001$**	**$P < 0{\cdot}0001$**	$P = 0{\cdot}53$							
Zeaxanthin	$r_s = 0{\cdot}17$	**$r_s = 0{\cdot}59$**	**$r_s = 0{\cdot}72$**	$r_s = -0{\cdot}04$	**$r_s = 0{\cdot}77$**						
	$P = 0{\cdot}36$	**$P = 0{\cdot}002$**	**$P = 0{\cdot}001$**	$P = 0{\cdot}85$	**$P < 0{\cdot}0001$**						
Canthaxanthin	$r_s = 0{\cdot}36$	$r_s = 0{\cdot}20$	$r_s = 0{\cdot}16$	$r_s = 0{\cdot}28$	$r_s = 0{\cdot}09$	$r_s = -0{\cdot}34$					
	$P = 0{\cdot}05$	$P = 0{\cdot}28$	$P = 0{\cdot}38$	$P = 0{\cdot}14$	$P = 0{\cdot}62$	$P = 0{\cdot}06$					
Echinenone	$r_s = 0{\cdot}19$	$r_s = -0{\cdot}08$	$r_s = -0{\cdot}08$	$r_s = 0{\cdot}12$	$r_s = -0{\cdot}16$	**$r_s = -0{\cdot}55$**	**$r_s = 0{\cdot}92$**				
	$P = 0{\cdot}31$	$P = 0{\cdot}65$	$P = 0{\cdot}67$	$P = 0{\cdot}50$	$P = 0{\cdot}40$	**$P = 0{\cdot}003$**	**$P < 0{\cdot}0001$**				
3′-Hydroxy-echinenone	$r_s = 0{\cdot}18$	$r_s = -0{\cdot}09$	$r_s = -0{\cdot}10$	$r_s = 0{\cdot}14$	$r_s = -0{\cdot}17$	**$r_s = -0{\cdot}55$**	**$r_s = 0{\cdot}92$**	**$r_s = 0{\cdot}99$**			
	$P = 0{\cdot}32$	$P = 0{\cdot}63$	$P = 0{\cdot}61$	$P = 0{\cdot}46$	$P = 0{\cdot}36$	**$P = 0{\cdot}003$**	**$P < 0{\cdot}0001$**	**$P < 0{\cdot}0001$**			
Adonirubin	$r_s = 0{\cdot}19$	$r_s = -0{\cdot}10$	$r_s = -0{\cdot}06$	$r_s = 0{\cdot}21$	$r_s = -0{\cdot}16$	**$r_s = -0{\cdot}55$**	**$r_s = 0{\cdot}92$**	**$r_s = 0{\cdot}98$**	**$r_s = 0{\cdot}98$**		
	$P = 0{\cdot}32$	$P = 0{\cdot}59$	$P = 0{\cdot}77$	$P = 0{\cdot}26$	$P = 0{\cdot}40$	**$P = 0{\cdot}003$**	**$P < 0{\cdot}0001$**	**$P < 0{\cdot}0001$**	**$P < 0{\cdot}0001$**		
4-Oxo-rubixanthin	$r_s = 0{\cdot}31$	$r_s = -0{\cdot}13$	$r_s = -0{\cdot}20$	$r_s = -0{\cdot}12$	$r_s = -0{\cdot}17$	**$r_s = -0{\cdot}60$**	**$r_s = 0{\cdot}71$**	**$r_s = 0{\cdot}85$**	**$r_s = 0{\cdot}85$**	**$r_s = 0{\cdot}81$**	
	$P = 0{\cdot}10$	$P = 0{\cdot}50$	$P = 0{\cdot}27$	$P = 0{\cdot}51$	$P = 0{\cdot}36$	**$P = 0{\cdot}001$**	**$P = 0{\cdot}0001$**	**$P < 0{\cdot}0001$**	**$P < 0{\cdot}0001$**	**$P < 0{\cdot}0001$**	

strong significant correlations between plumage hue and the five main red carotenoids in feathers: 3-hydroxy-echinenone, canthaxanthin, adonirubin, echinenone and 4-oxo-rubixanthin (Fig. 3). Redder males had more of all five of these carotenoids in feathers than yellower males. Redder males also had significantly less zeaxanthin in plumage than yellower males (Fig. 3). All of this amounted to a strong significant relationship between plumage hue and the red : yellow pigment ratio in feathers: redder males had a higher proportion of red pigments compared with yellow pigments in feathers ($r_s = -0{\cdot}82$, $P < 0{\cdot}0001$).

PLASMA AND LIVER PIGMENTS AS PREDICTORS OF RED CAROTENOID ACCUMULATION IN FEATHERS

Clearly preferential accumulation of metabolically derived red carotenoids is the means by which House Finches become sexually attractive. This led us to test whether specific amounts of carotenoids in blood predict the concentration of red carotenoids in feathers. Because all five of the red carotenoids in plumage were so highly correlated with each other (Table 2) and with plumage redness (Fig. 3), we used a principal components analysis (PCA) to combine these into a single variable, PC1 ('plumage-pigment redness'), which explained 93% of the variation in these measures. We then entered this into stepwise multiple-regression models to test the ability of all plasma pigments and all liver pigments (in separate analyses) to predict the accumulation of red pigments in male feathers. We found that the best models explaining variation in the concentration of the red feather pigment contained only β-cryptoxanthin (Table 3). *A posteriori* Spearman rank correlational analyses confirmed that PC1 was significantly correlated with β-cryptoxanthin concentration in male plasma ($r_s = 0{\cdot}53$, $P = 0{\cdot}004$) and liver ($r_s = 0{\cdot}52$, $P = 0{\cdot}009$).

HOW CAROTENOID CONTENT OF PLASMA AND LIVER PREDICTS PLUMAGE HUE

To determine which carotenoid profiles in blood and liver had direct consequences for coloration, especially

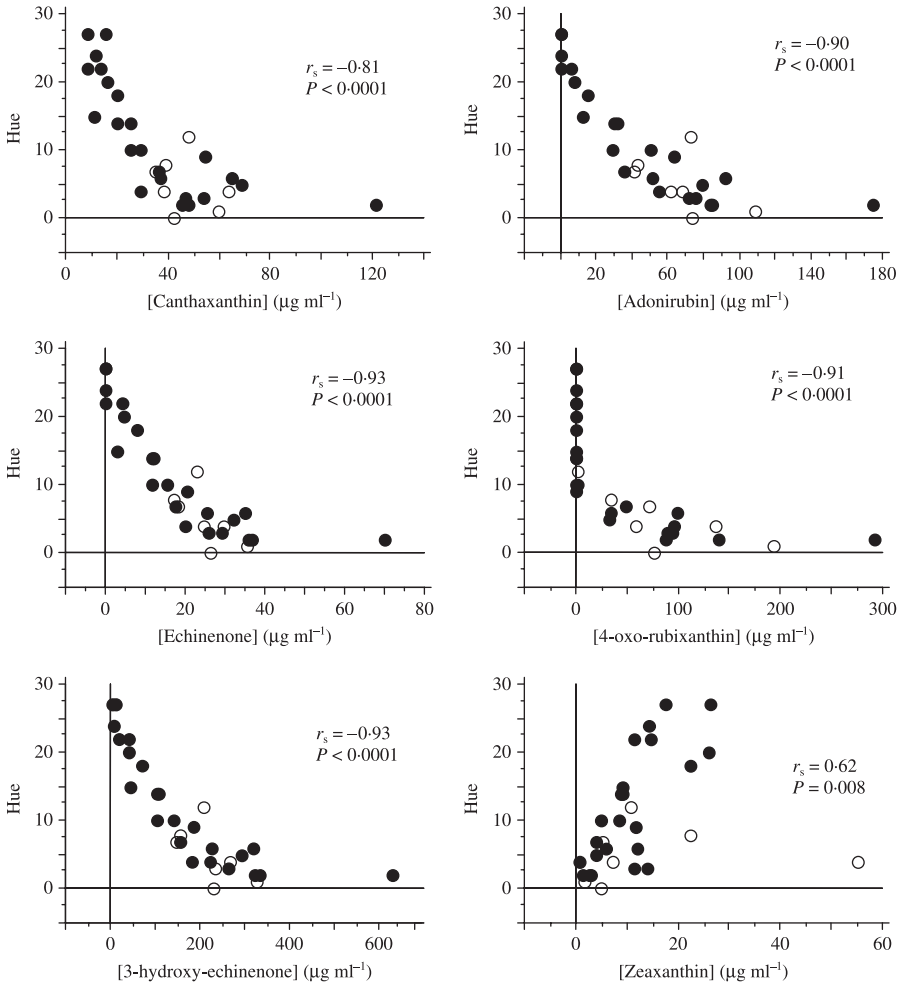

Fig. 3. Correlations between plumage redness and the concentration of six red carotenoids in the feathers of male House Finches. Recall that lower hue scores assigned by the Colortron correspond to redder plumage. These statistically significant results hold even after applying a sequential Bonferroni correction for multiple (11) tests (minimum $P = 0{\cdot}005$). Filled circles represent data for HY males; open circles signify AHY males.

Table 3. Forward stepwise regression models examining the ability of plasma pigments (in μg ml^{-1}) and liver pigments (in μg g^{-1}) to predict red plumage-pigment concentration (measured as PC1; see text) in male House Finches. Bold variables are those that remained in the final model (plasma pigments: $n = 30$, $r^2 = 0{\cdot}51$; liver pigments: $n = 26$, $r^2 = 0{\cdot}28$)

Model	Variable	*F*-ratio	Coefficient	*P*
Plasma pigments	Lutein	3·35	0·33	0·10
	Zeaxanthin	3·88	0·36	0·08
	β-cryptoxanthin	**28·9**	**0·71**	**<0·0001**
	β-carotene	3·24	0·33	0·10
Liver pigments	Lutein	1·07	0·21	0·27
	Zeaxanthin	0·44	0·14	0·52
	β-cryptoxanthin	**9·3**	**0·53**	**0·006**
	β-carotene	2·27	0·30	0·18

Table 4. Forward stepwise regression models examining the ability of plasma pigments (in μg ml^{-1}) and liver pigments (in μg g^{-1}) to predict plumage hue in male and female House Finches. Bold variables are those that remained in the final model (male plasma pigments: $n = 30$, $r^2 = 0{\cdot}15$; male liver pigments: $n = 26$, $r^2 = 0{\cdot}28$; female plasma pigments: $n = 17$, $r^2 = 0{\cdot}60$; female liver pigments: $n = 12$, $r^2 = 0{\cdot}55$)

Model	Variable	*F*-ratio	Coefficient	*P*
Male				
Plasma pigments	Lutein	2·51	–0·29	0·19
	Zeaxanthin	0·12	0·07	0·59
	β-cryptoxanthin	**5·07**	**–0·39**	**0·03**
	β-carotene	1·98	–0·26	0·27
Liver pigments	Lutein	0·29	–0·11	0·54
	Zeaxanthin	0·22	–0·10	0·56
	β-cryptoxanthin	**9·21**	**–0·53**	**0·006**
	β-carotene	2·97	–0·34	0·15
Female				
Plasma pigments	Lutein	2·44	–0·39	0·25
	Zeaxanthin	0·27	–0·14	0·59
	β-cryptoxanthin	**22·6**	**–0·78**	**0·0003**
	β-carotene	0·27	–0·14	0·59
Liver pigments	Lutein	0·88	–0·30	0·37
	Zeaxanthin	0·09	0·10	0·68
	β-cryptoxanthin	**12·2**	**–0·74**	**0·006**
	β-carotene	0·45	0·22	0·48

in females (for which we had no data on feather-pigment composition), we performed the same stepwise regression analyses using plumage hue as the dependent variable. We found that males and females that had higher amounts of β-cryptoxanthin in liver or plasma developed redder plumage (Table 4). *Post-hoc* Spearman rank correlational tests also indicated that plumage redness was significantly correlated with β-cryptoxanthin concentration in male plasma, male liver and female plasma, but not female liver (Fig. 4).

DO BIRDS THAT ACCUMULATE MORE CAROTENOIDS IN PLASMA ALSO HAVE MORE IN LIVER?

Males and females that circulated higher carotenoid levels through blood had higher levels in liver, but this was only true for each specific carotenoid type (Table 5). Thus, for example, birds with more lutein in blood had more lutein in liver, but did not necessarily have more of any other type of carotenoid in liver. This indicates that House Finches do not necessarily deplete stores during moult for feather pigmentation, but instead that those birds that deliver more to feathers via plasma also have higher carotenoid depots in liver. Also, it is noteworthy that the strongest correlations in each sex were between levels of β-cryptoxanthin in plasma and liver.

SEX AND AGE DIFFERENCES IN CAROTENOID ACCUMULATION IN BLOOD, LIVER, AND FEATHERS

For analyses of sex, we pooled birds of all ages. We found that males and females differed only in the accumulation of plasma and liver β-cryptoxanthin and in liver β-carotene (Fig. 5; recall that we could not test for sex differences in feather carotenoid content owing to the absence of data for females). In males, we found that AHY birds circulated more lutein and zeaxanthin in plasma than HY birds, but not more plasma β-cryptoxanthin or β-carotene or any liver carotenoid (Fig. 6). There were no significant age differences in plumage hue or any plumage carotenoid in males, although in most cases HY birds tended to have fewer carotenoids (and be less colourful) than adults (Fig. 6), as was found previously (Inouye *et al.* 2001). We did not conduct age analyses in females because there were only three adults for which we measured plasma carotenoids and none for which we measured liver carotenoids.

Discussion

Studies of pigment-based coloration in animals offer unique opportunities for investigating the honesty-reinforcing mechanisms of sexual traits at the molecular level. We can first deconstruct coloration into its pigmentary components and then track the accumulation and production of those pigments to understand how various environmental and physiological perturbations influence pigment accumulation. House Finches have been the subject of a large majority of studies on the proximate control of carotenoid coloration in birds, but ours is the first to track molecules internally and externally to reveal their full biochemical blueprint for coloration.

It is clear from our work and that of Inouye *et al.* (2001) that House Finches deposit many red pigments, as opposed to yellow forms, into feathers to become sexually attractive. In this study, we were interested in determining what cocktail of carotenoids accumulated in the body allows males to incorporate high concentrations of red carotenoids in feathers. We found several lines of evidence that point to a valuable role for endogenous sources of β-cryptoxanthin in the production of red plumage in House Finches: (1) unlike some of the other carotenoids in the body (e.g. lutein

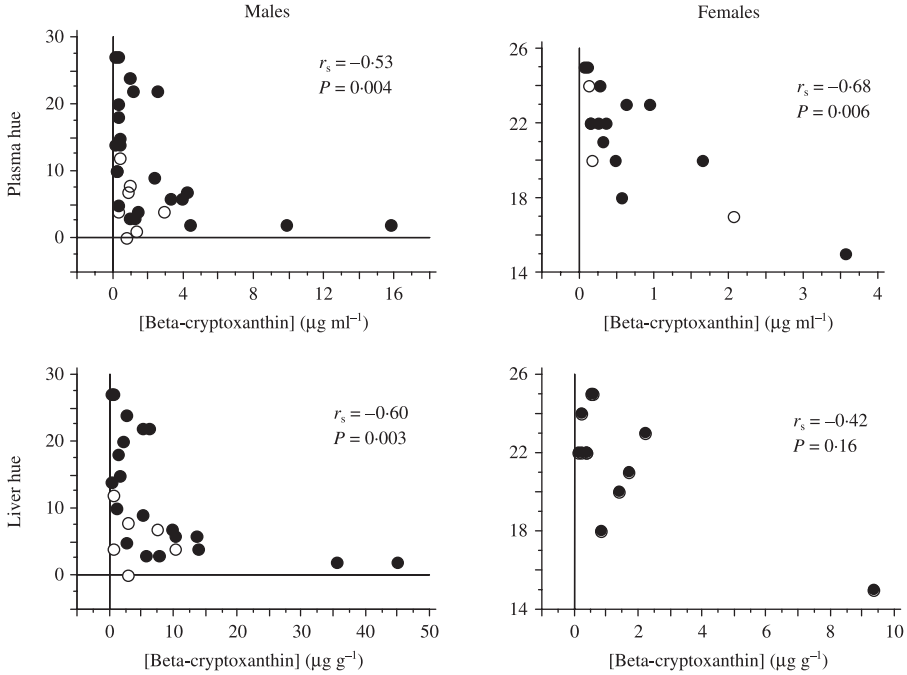

Fig. 4. Correlations between plumage redness and the concentration of β-cryptoxanthin in plasma and liver of wild, moulting male and female House Finches. The depicted statistically significant results hold even after applying a sequential Bonferroni correction for multiple (four) tests (minimum $P = 0{\cdot}01$). Filled circles represent data for HY birds; open circles signify AHY birds. We did not measure liver carotenoids in any of the three adult females.

and zeaxanthin), β-cryptoxanthin varied independently of other carotenoids within blood and liver samples; (2) β-cryptoxanthin was strongly correlated with plumage redness and, when pitted against other carotenoids, it alone (in both liver and plasma) explained significant portions of the variability in red-plumage-carotenoid concentration; and (3) males, which develop more extensive and redder plumage than females (Hill 2002), have higher levels of β-cryptoxanthin (and no other carotenoid) than females. Hill (2000) has also shown that birds fed diets rich in lutein and zeaxanthin turn only yellow, but grow red feathers when fed β-cryptoxanthin-rich foods (e.g. tangerine juice). Such a specialized biochemical strategy for developing elaborate coloration – the preferential accumulation of one type of carotenoid – is rare among songbirds (also see Stradi *et al.* 2001 for other hypothesized finch species), as prior studies have shown that the most colorful birds accumulate more of all types of carotenoids in the body and feathers (e.g. Saks *et al.* 2003; McGraw & Gregory 2004).

β-Cryptoxanthin is a relatively scarce pigment in nature compared with other potential dietary-precursor carotenoids such as lutein, zeaxanthin and β-carotene (Goodwin 1980, 1984), and its distribution in both space and time may explain why not all House Finches accumulate large amounts to become red. House Finches moult at the end of and after the monsoon rains in the desert south-west, at which point several localized desert plants, including prickly pear (*Opuntia* sp.) and Saguaro (*Carnegeia gigantean*) cacti (Steenburgh & Lowe 1977), produce colourful red fruits. Moulting House Finches in Arizona have been observed foraging on these fruits in late summer, and preliminary chemical analyses of these fruits indicate that they contain β-cryptoxanthin (K. J. McGraw, P. M. Nolan & O. L. Crino, unpublished data). It is thus conceivable that locating and foraging on such fruits is a major dietary challenge that native populations of house finches face for becoming colourful. Those birds that do not acquire sufficient amounts of β-cryptoxanthin

Table 5. Correlations among carotenoid concentrations within plasma (in μg ml^{-1}) and liver (μg g^{-1}) samples of wild moulting House Finches

	Liver LUT	Liver ZEAX	Liver βCRY	Liver βCAR
Males				
Plasma LUT	**$r_s = 0{\cdot}75$**	**$r_s = 0{\cdot}55$**	$r_s = 0{\cdot}19$	$r_s = 0{\cdot}24$
	$P = 0{\cdot}0002$	**$P = 0{\cdot}006$**	$P = 0{\cdot}36$	$P = 0{\cdot}24$
Plasma ZEAX	**$r_s = 0{\cdot}61$**	**$r_s = 0{\cdot}47$**	$r_s = 0{\cdot}11$	$r_s = 0{\cdot}44$
	$P = 0{\cdot}002$	**$P = 0{\cdot}02$**	$P = 0{\cdot}58$	$P = 0{\cdot}66$
Plasma βCRY	$r_s = 0{\cdot}23$	$r_s = -0{\cdot}02$	**$r_s = 0{\cdot}91$**	$r_s = 0{\cdot}38$
	$P = 0{\cdot}26$	$P = 0{\cdot}94$	**$P < 0{\cdot}0001$**	$P = 0{\cdot}06$
Plasma βCAR	$r_s = 0{\cdot}39$	$r_s = 0{\cdot}19$	$r_s = 0{\cdot}14$	**$r_s = 0{\cdot}41$**
	$P = 0{\cdot}05$	$P = 0{\cdot}34$	$P = 0{\cdot}49$	**$P = 0{\cdot}04$**
Females				
Plasma LUT	**$r_s = 0{\cdot}57$**	$r_s = 0{\cdot}41$	$r_s = 0{\cdot}41$	$r_s = 0{\cdot}32$
	$P = 0{\cdot}01$	$P = 0{\cdot}07$	$P = 0{\cdot}07$	$P = 0{\cdot}17$
Plasma ZEAX	$r_s = 0{\cdot}43$	**$r_s = 0{\cdot}47$**	$r_s = 0{\cdot}17$	$r_s = 0{\cdot}21$
	$P = 0{\cdot}06$	**$P = 0{\cdot}04$**	$P = 0{\cdot}45$	$P = 0{\cdot}35$
Plasma βCRY	$r_s = 0{\cdot}21$	$r_s = -0{\cdot}02$	**$r_s = 0{\cdot}60$**	$r_s = 0{\cdot}33$
	$P = 0{\cdot}35$	$P = 0{\cdot}93$	**$P = 0{\cdot}009$**	$P = 0{\cdot}15$
Plasma βCAR	**$r_s = 0{\cdot}49$**	$r_s = 0{\cdot}37$	**$r_s = 0{\cdot}51$**	**$r_s = 0{\cdot}59$**
	$P = 0{\cdot}03$	$P = 0{\cdot}11$	**$P = 0{\cdot}03$**	**$P = 0{\cdot}01$**

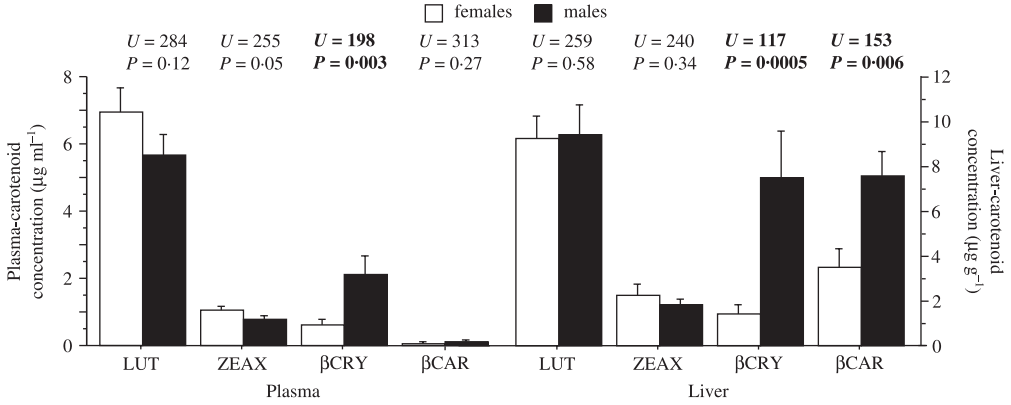

Fig. 5. Sex differences in plasma and liver carotenoids in moulting wild House Finches. Non-parametric Mann–Whitney *U*-tests were used to compare concentrations of each carotenoid between males and females; statistically significant results are in bold. $n = 22$ females and 26 males in analyses of liver, $n = 25$ females and 30 males in analyses of plasma.

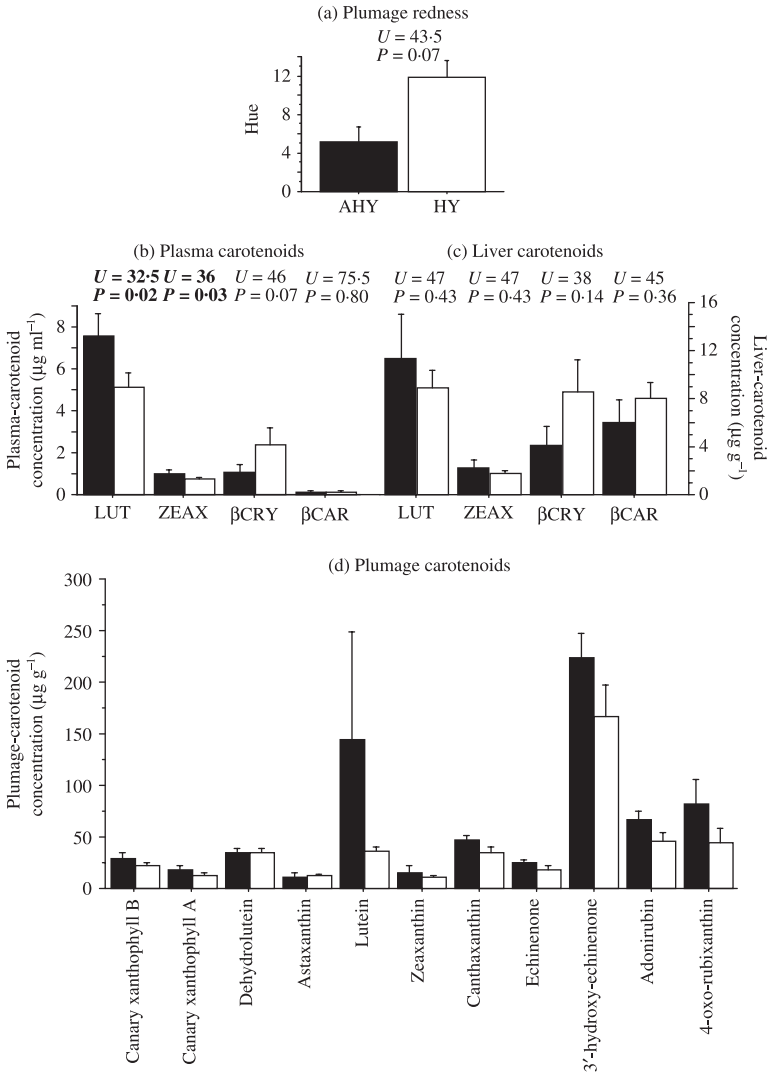

Fig. 6. Age differences in plumage colour as well as plasma, liver, and plumage carotenoids in moulting wild male House Finches. Mann–Whitney *U*-tests were used to compare carotenoid concentrations between AHY and HY males; statistically significant results are in bold. $n = 7$ AHY and 23 HY males in analyses of plumage hue and plasma and plumage carotenoids; $n = 6$ AHY and 20 HY males in analyses of liver carotenoids.

ingest foods containing yellow xanthophylls, which cannot be converted into red plumage pigments (Hill 2002) and when deposited into feathers (or metabolized into other yellow carotenoids that appear in feathers) result in a drab yellow appearance.

Still, it is apparent that some males do not require high levels of β-cryptoxanthin for becoming red. This is most evident in Fig. 4, where an L-shaped curve indicates that, while yellow plumage is always the product of low β-cryptoxanthin supplies, some birds become red with either low or high β-cryptoxanthin levels. This was especially true among older birds, which (albeit in a limited sample) all had relatively low cryptoxanthin concentrations (Figs 4 and 6b) but still grew red plumage. Inouye *et al.* (2001) similarly found that correlations between red carotenoids and plumage colour broke down among older males. Recall that β-cryptoxanthin is the metabolic precursor to the important red carotenoid pigments (e.g. 3-hydroxy-echinenone) in plumage, and as suggested by several prior studies some males better than others must also complete these important metabolic, condition-dependent conversions en route to becoming sexually attractive (e.g. Hill 2000). Our results hint at a potential age difference in proximate control of male colour, whereby the variability in HY plumage colour is controlled more by diet and the variability among adults controlled more by metabolic activity.

This is the first biochemical study of the carotenoid coloration strategies of wild female House Finches. The incidence of red coloration in females is much lower than in males (e.g. < 5% of all females in our population; K. J. McGraw, P. M. Nolan & O. L. Crino, unpublished data), and the fact that females had dramatically less plasma and liver β-cryptoxanthin than males may explain this. Still, as with males, we show that β-cryptoxanthin concentrations in liver and plasma also strongly predict plumage redness in females. In fact, the relationship between β-cryptoxanthin and colour appeared to be even more linear in females than

in males (Fig. 4), pointing to even tighter dependence on the dietary precursor, perhaps with less influence of metabolism or general nutrition. Note that this analysis was conducted largely among HY females, who are redder and potentially have a greater need for mate attraction than already-mated AHY females (which often retain mates across seasons; Hill 2002). We await future studies to determine whether AHY females are less red in colour owing to their levels of dietary precursor or enzyme activity.

Although primacy is given to β-cryptoxanthin for coloration in House Finches here, this should by no means undermine the importance of accumulating other carotenoids in the body for other functions. Different carotenoids can serve a diverse suite of endogenous biological roles, including antioxidant defence/immunoregulation, UV protection, visual filtering and vitamin synthesis in offspring (from egg yolk) and adults (Vershinin 1999). For example, lutein and zeaxanthin can act as immunostimulants in songbirds (e.g. Blount *et al*. 2003; McGraw & Ardia 2003; Saino *et al*. 2003; but see Navara & Hill 2003), whereas the cryptoxanthins and carotenes, but not xanthophylls, have pro-vitamin-A activity (Bauernfeind 1981). Investigating these various functions of carotenoids, both during moult and at other times of year, will shed additional light on how and why birds displayed such high variability in their fluid- and tissue-carotenoid profiles in this study.

Ultimately, several environmental and physiological factors influence colour expression in House Finches (Hill 2002). These include parasites (Brawner *et al*. 2000), general nutrition (Hill & Montgomerie 1994; Hill 2000) and hormone titres (Stoehr & Hill 2001), in addition to the amounts (Hill *et al*. 2002) and types of dietary carotenoids. Because we did not gather data on health status or other measures of individual quality in these birds, future studies should be geared at disentangling the relative effects of these different variables on carotenoid accumulation and coloration in both captive and wild settings (Hill 2006).

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