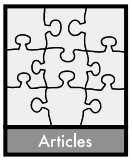

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Season-, sex-, and age-specific accumulation of plasma carotenoid pigments in free-ranging white-winged crossbills *Loxia leucoptera*

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Many birds acquire carotenoid pigments from foods and deposit these pigments into feathers and bare-parts to become sexually attractive, but little work has been done on the interindividual and temporal variability in the types and amounts of carotenoids that free-ranging individuals have available for use in coloration or other functions (e.g., in immunomodulation). To address this issue, we studied intra-annual variation in plasma carotenoid profiles of juvenile and adult white-winged crossbills *Loxia leucoptera* of both sexes. Adult male crossbills exhibit bright red carotenoid-based plumage pigmentation, whereas females uniformly display drab yellow feather coloration and juvenile males only occasionally display some orange or pink color. Yellow xanthophylls (e.g., lutein, zeaxanthin) were predominant in plasma of birds from both sexes and age classes throughout the year. Plasma xanthophylls levels tended to be highest in the summer, when crossbills increase seed consumption for breeding as well as supplement their diet with insects. Blood accumulation of three other, less common plasma carotenoids-β-cryptoxanthin, rubixanthin, and gazaniaxanthin-varied in a highly season-, sex-, and age-dependent fashion. These carotenoids were virtually absent in juvenile or adult female plasma at all times of year and were only present in male plasma, at higher concentrations in adults than juveniles, during the period of feather growth (Sept.–Nov.). These pigments have been reported as valuable precursors of the metabolically derived red pigments (e.g., 3-hydroxy-echinenone, 4-oxo-rubixanthin, and 4-oxo-gazaniaxanthin, respectively) that appear in the plumage of male crossbills. These findings suggest that male crossbills either adopt a season-specific foraging strategy to acquire foods rich in these pigments at the time they are needed to develop red coloration, or have a unique physiological ability to metabolically produce these pigments or absorb them from food during molt, in order to maximize color production.

Variation is a hallmark of sexually selected traits (Cuervo and Møller 1999, 2001), and occurs in several dimensions within and among individuals and populations over time (Dale 2006). Many studies have been aimed at identifying the evolutionary and functional significance of age, sex, morph, seasonal, and geographic variability in secondary sexual features (e.g., Butcher and Rohwer 1989), but comparatively fewer investigations have addressed the proximate mechanisms underlying such variation. Mechanistic studies conducted to date have traditionally centered on the role of physiological (e.g., hormones; Kimball and Ligon 1999) and genetic (e.g., Mundy 2006) processes in maintaining trait variability, although some have also targeted environmental variables (e.g., diet, parasites, lighting conditions; Hamilton and Zuk 1982, Hill 2002, Gomez and Thery 2005).

Certain ornamental features provide particularly valuable opportunities for assessing the proximate factors behind trait variation, because we can identify the molecular currency underlying their expression. Among these are the pigment-based colors in animals, where we can chemically track the uptake, production, processing, and/or deposition of pigment molecules and the associated conditions that might modify their accumulation. The variability and control agents of carotenoid-based coloration in birds have particularly attracted the interest of behavioral ecologists in recent years (Olson and Owens 1998, Hill 1999, Møller et al. 2000). Vertebrates derive these pigments from foods (either photosynthetic organisms or herbivorous prey), so there may be important differences in diet that regulate color expression between sexes, age classes, morphotypes, seasons, and locales (Hill 2006). Alternatively, classes of individuals may vary in their physiological abilities to accumulate or metabolize ingested pigments that contribute to color variability (e.g., Bortolotti et al. 1996, Negro et al. 2001a, McGraw 2005).

Numerous, mostly experimental studies have investigated the effects of extrinsic and intrinsic forces on the development of carotenoid-based coloration in birds (reviewed in Hill 2002, 2006). However, few investigations have documented natural variability in the accumulation of these molecules across different spatiotemporal scales and

across different classes of individuals within a species (e.g., Hill 1995, Grether et al. 1999, McGraw and Gregory 2004). Studies on this topic seem critical toward understanding the true limitations that different individuals face over time in taking up and using these pigments for bright coloration, as well as for other demonstrated physiological roles of carotenoids (e.g., immunostimulants, egg-yolk antioxidants; McGraw 2006).

Therefore, our goal was to broadly monitor the types and amounts of carotenoid pigments available to a wild bird species with carotenoid-dependent coloration. As it is characteristically difficult to quantitatively track the diet of free-ranging birds or to capture and euthanize foraging animals throughout the year to recover gut contents, we took the approach of sampling blood from wild-caught individuals, which provides a snap-shot of recently ingested food carotenoids as well as mobilized stores (e.g. from fat and liver) that are available for delivery to feathers (where they are either deposited directly or metabolized into other forms for coloration; McGraw 2004). We tracked season-, sex-, and age-specific accumulation of carotenoid pigments in the blood of white-winged crossbills *Loxia leucoptera*. This cardueline finch species inhabits coniferous forests and relies heavily on conifer seeds for food (Benkman 1987a,b, 1988, 1997). Most notably, crossbills exhibit conspicuous age and sex differences in plumage pigmentation. Adult males develop extensive rich red coloration, whereas young males tend to be more orange in appearance and females, young and old, exhibit drab yellow body color (Benkman 1992). Birds of all ages and sexes grow their colorful feathers in the autumn (Sept.–Nov.; Deviche 2000, Deviche and Sharp 2001).

We were interested in testing whether plasma carotenoid profiles parallel age and sex differences in coloration, and whether carotenoid status shifts seasonally to coincide with the need to deposit pigments in feathers during molt. Several biochemical studies have reported that female and young male White-winged crossbills develop their yellow plumage by acquiring two xanthophyll carotenoids from the diet-lutein and zeaxanthin-and metabolically transforming them into two other yellow carotenoids-canary xanthophylls A and B-that are deposited in feathers (Volker 1957, Weber 1953, Stradi 1998, 1999, Stradi et al. 1996). In contrast, adult male *L. leucoptera*, and occasionally some young males, develop their rich red coloration by ingesting different non-xanthophyll dietary precursors and metabolically transforming them into five red pigments that appear in feathers: 3-hydroxy-echinenone, 4-oxo-rubixanthin, 4-oxo-gazaniaxanthin, astaxanthin, and adonirubin, as well as small amounts of yellow pigments (e.g., canary xanthophylls, lutein; Volker 1957, Weber 1953, Stradi 1998, 1999, Stradi et al. 1996). Based on parsimonious chemical modifications, Stradi (1999) suspected that these dietary precursors were β-cryptoxanthin, rubixanthin, and gazaniaxanthin. Thus, another objective of the present study was to determine whether these precursor carotenoids are present in blood and whether their presence or abundance covaries with seasonal, sexual, and ontogenetic plumage patterns.

Methods

Capture and handling

Blood samples were collected as part of a previous study of molt and reproductive endocrinology in White-winged crossbills (Deviche 2000, Deviche and Sharp 2001). Crossbills (n = 183) were caught in Japanese mist nets at a single location in Fairbanks, Alaska (64°51′N; 147°54′W) between 26 April 1998 and 6 August 1999. All birds were caught between 06.30 am and 16.10 pm (AST) and the time of capture to the nearest 10 min was noted. Within minutes of removal from the net, a blood sample (maximum 250 μl/bird) was collected from an alar vein into heparinized microhematocrit tubes. Samples were kept on ice until centrifuged within hours in the laboratory. After centrifugation, plasma was transferred to fresh microcentrifuge tubes and stored at −80°C. Samples were kept at this temperature until assayed for carotenoid concentrations in 2006 (see below), except that they were briefly thawed once in 2000, when a small aliquot was taken from each sample for hormone assays (Deviche and Sharp 2001). Long-term temporal stability of carotenoids in frozen avian plasma has not been determined, but carotenoids in human plasma kept at −70°C did not degrade significantly after 4 years (Comstock et al. 1995), and after 2.3 years (Craft et al. 1988) in two separate studies. We assume that carotenoid concentrations in crossbill plasma used here either did not decrease during the storage period or, if they did, did so in relatively constant proportions in the various samples (and thus should not affect our statistical conclusions). After blood collection, the age of each bird was determined using plumage characteristics and the degree of skull pneumatization (Pyle 1997). Birds were weighed to the nearest 0.1 g and their wing chord was measured to the nearest millimeter. Birds were divided into two age classes: hatch-year (hereafter HY; Euring age 5) and after hatch-year (hereafter AHY; Euring age 6). Whenever possible, sex was determined based on plumage characteristics and, during the breeding season, presence of a developed cloacal protuberance (AHY males), or incubation patch (AHY females; Pyle 1997). Intensity of contour feather molt was determined on a scale ranging from 0 (no molt) to 3 (heavy, generalized molt). After all data were collected, birds received a U.S. Geological Survey numbered aluminum tarsal band and were released. The final sample of birds for this study consisted of 43 HY birds (16 males, 14 females, and 13 unknowns), and 140 AHY birds (75 males and 65 females). All procedures were approved by the University of Alaska Fairbanks Institutional Animal Care and Use Committee and were conducted under Scientific Collecting Permits from the Alaska Fish and Game Department, and the U.S. Fish and Wildlife Service.

Analyses of plasma carotenoid pigments

Analyses of plasma carotenoids followed previously published methods (McGraw et al. 2002), with the following modifications. Pigment extracts were injected into a Waters Alliance 2695 HPLC system (Waters Corporation, Milford, Massachusetts) 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 analyze both xanthophylls and carotenes in a single run, at a constant flow rate of 1.2 ml/min: 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 30–48 min. Data were collected from 250–600 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.

Statistical analyses

Because we were interested in how different carotenoids may vary seasonally, sexually, and ontogenetically in crossbills, we performed all analyses separately for each carotenoid present in plasma (see Results). We analyzed seasonal changes in the concentration of each plasma carotenoid of AHY crossbills (see more below for analyses of HY birds) using two-way analysis of variance (ANOVA), with month and sex as main independent factors, followed by Student-Newman-Keuls pairwise comparisons. For these analyses, we sorted data as a function of the Julian capture date and divided them into nine consecutive blocks, each including at least five males and five females. Data are presented as a function of the median Julian date of capture of the birds in each block of data. Sample size per block ranged from 11 to 18 birds. Plasma concentrations of zeaxanthin were not normally distributed, so we log (concentration + 1) transformed them prior to analysis. Similar data transformations for β-cryptoxanthin and rubixanthin/gazaniaxanthin did not result in normal distributions due to the presence of many zero values, so these data were converted to ranks (*sensu* Conover and Iman 1981) before ANOVAs. Age was not included in this analysis because samples for HY birds spanned a different range of dates (only 9 July–26 Nov. 1998) than did those for adults and some HY birds could not be sexed. Time of day also was not entered into the model because, although plasma carotenoid circulation can increase throughout the day in some birds (e.g., great tits *Parus major*; Horak et al. 2004), there was no diel cycle in our dataset for crossbills (Pearson's product-moment correlations, all $r < 0.15$ and all $P > 0.10$ for the different carotenoid types).

We were most interested in patterns of carotenoid accumulation during molt, when carotenoids in blood are shuttled to feathers for coloration. Adult body molt spanned 21 Sept.–21 Nov. 1998. In our sample, there were 8 adult males and 2 adult females that were molting their carotenoid-pigmented contour feathers at the time that blood was collected. We first examined sex differences in plasma carotenoid titers during molt in these 10 birds using unpaired Student's t-tests or Mann-Whitney U tests either if data were not normally distributed or if group variances differed significantly. We used these same tests to compare levels of each carotenoid between non-molting and molting birds, separately for the two sexes. We also tested whether molt itself, irrespective of the time of year when it occurs, was associated with certain carotenoid profiles, so we matched our 10 molting adults against three non-molting adults (2 females, 1 male) sampled at the same time of year and examined differences in mean carotenoid levels (using Student's t- or Mann-Whitney U tests) as well as whether or not the carotenoid was present or absent in the plasma (using Fisher exact probability tests). We also used Student's t-tests to compare the body mass and body condition (body mass/wing chord ratio) of non-molting and molting adults.

To investigate age-related variation in carotenoid accumulation, we first compared carotenoid levels between HY birds and those AHY individuals that were captured in the same date range as HYs (9 July to 26 Nov. 1998). Separate unpaired t-tests or Mann-Whitney U tests were run on non-molting birds (not split by sex due to the unsexed HYs) and molting birds (the sexes were pooled here due to the low number of molting adults). Using these same statistical tests, we then examined sex differences in plasma carotenoid levels of molting HY birds; we also used a Fisher exact probability test to consider the likelihood that the plasma from molting males or females contained a particular carotenoid. We investigated seasonal differences in plasma carotenoids of HY birds by pooling the sexes during molt and comparing molting with non-molting HYs using unpaired t-tests or Mann-Whitney U tests.

Finally, we were interested in how levels of the different carotenoids covaried within samples and whether such patterns of covariation were consistent seasonally, sexually, and ontogenetically. For this, we used Pearson's correlations to examine relationships between the concentrations of the different carotenoids, and performed these correlations separately for non-molting AHY males, non-molting AHY females, molting AHY birds (pooling data for the 8 males and 2 females), non-molting HYs (for which we could not determine sex), molting HY males, and molting HY females. In all cases, unless otherwise indicated, mean ± SE is reported and α-level is set at 0.05.

Results

Carotenoid composition of plasma

We identified six carotenoids in crossbill plasma (Fig. 1): lutein, zeaxanthin, β-cryptoxanthin, rubixanthin, gazaniaxanthin, and β-carotene. Rubixanthin and its 5′-Z isomer, gazaniaxanthin, co-eluted in our analytical system (Fig. 1), which is a common problem with these two pigments (Hornero-Mendez and Minguez-Mosquera 2000), and thus are reported together as total rubixanthin/gazaniaxanthin levels.

Considering all samples together ($n = 183$), lutein (17.5 ± 0.9 μg/ml) and zeaxanthin (0.63 ± 0.06 μg/ml) were the most common and concentrated of the plasma carotenoids, occurring in 100% and 89% of all samples, respectively. β-cryptoxanthin (0.61 ± 0.21 μg/ml) and rubixanthin/gazaniaxanthin (0.35 ± 0.12 μg/ml) were more dilute and less frequently found in plasma, occurring in 32% and 19% of all samples, respectively; however, in

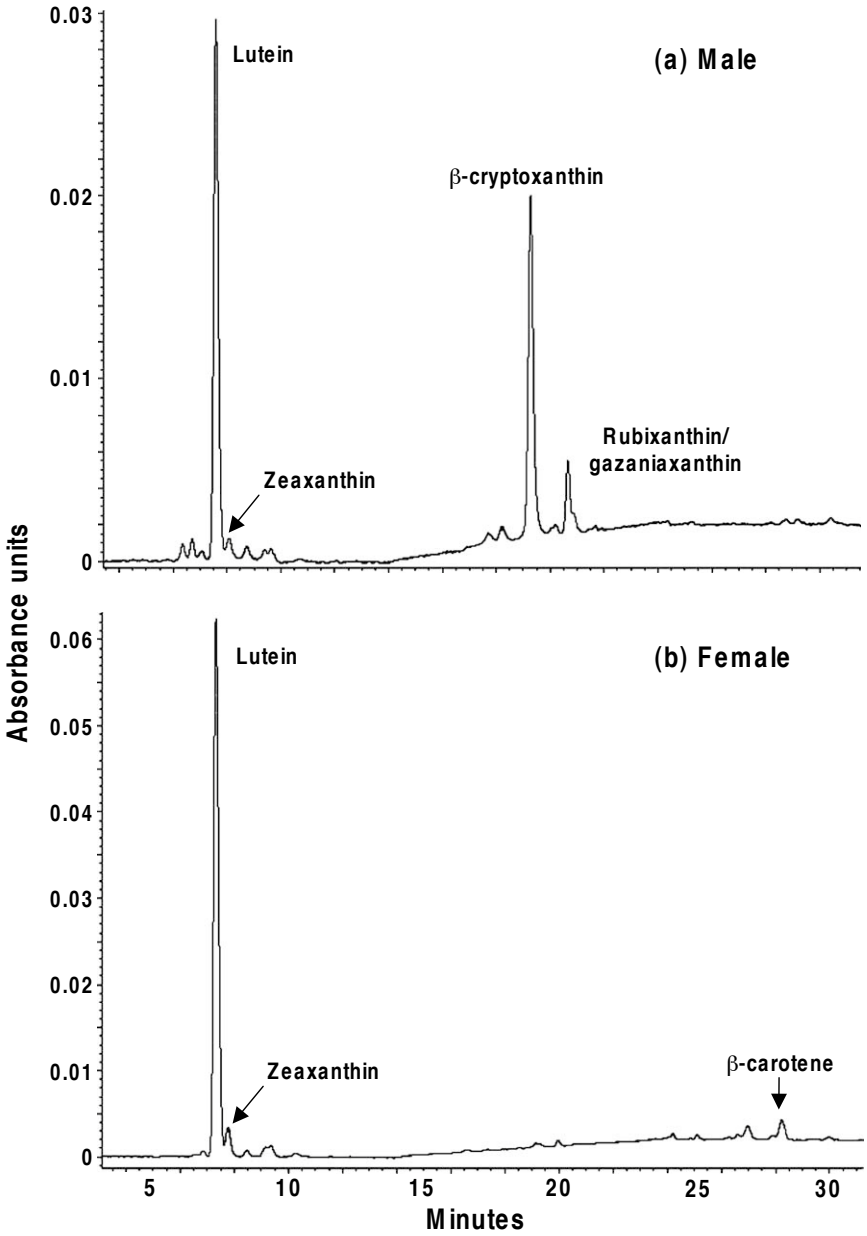

Figure 1. Representative two-dimensional HPLC chromatograms illustrating the carotenoid pigments detected in the plasma of: (a) male and (b) female white-winged crossbills (data gathered at $\lambda = 448$ nm). Note the shoulder on the right most labeled peak in (a), which signifies the incomplete resolution of rubixanthin and gazaniaxanthin peaks in our analytical procedure.

some cases, β-cryptoxanthin and rubixanthin/gazaniaxanthin did appear at high levels (e.g., up to 22 μg/ml of β-cryptoxanthin and up to 12 μg/ml rubixanthin/gazaniaxanthin). β-carotene was detected in the plasma of only 11 birds (8 AHYs and 3 HYs; 6% of all birds) from July-Sept., and at very low levels (0.02 ± 0.01 μg/ml; never higher than 1.3 μg/ml), so data for β-carotene were not analyzed statistically.

Seasonal changes in adult plasma carotenoid concentrations

Concentrations of each carotenoid in adults varied seasonally, but they did not differ between males and females and in no case was there a sex by month interaction (Fig. 2). Lutein and zeaxanthin reached maximum levels in summer (June and July). High lutein concentrations were sustained through the autumn and early winter. Zeaxanthin levels declined in autumn. Although this decline was not significant relative to peak summer concentrations, levels in autumn were higher than in early winter (January). In contrast to the xanthophylls, rubixanthin/gazaniaxanthin and β-cryptoxanthin were virtually undetectable in plasma from January to May, increased sharply in Sept.–Oct., and decreased back to low levels in Nov.–Dec. In June, there was a small secondary peak in β-cryptoxanthin levels, but not in rubixanthin/gazaniaxanthin levels.

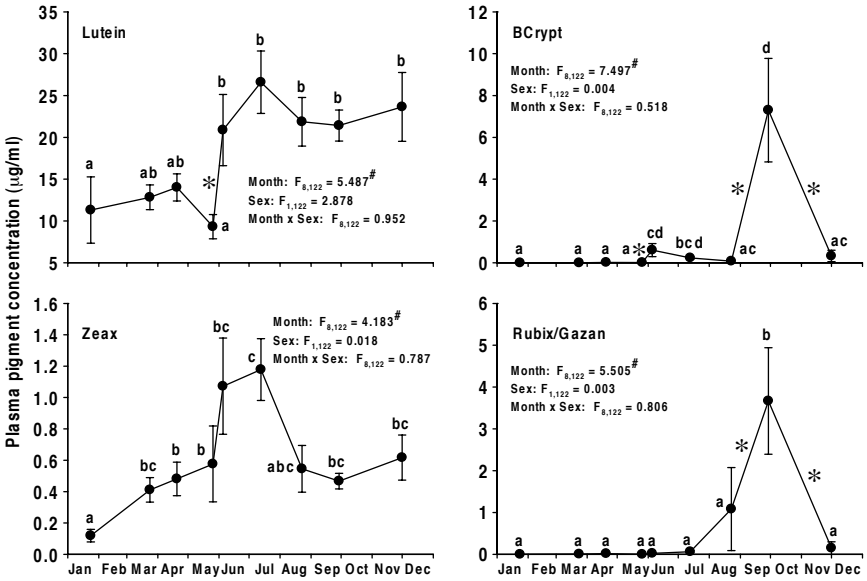

Figure 2. Seasonal changes in plasma concentrations (means ± SE) of carotenoids (lutein, zeaxanthin (Zeax), β-cryptoxanthin (BCrypt), and rubixanthin/gazaniaxanthin (Rubix/Gazan)) in adult white-winged crossbills ($n \geq 5$ individuals of each sex per data point), sampled in 1998–1999 at a single location in Fairbanks, Alaska. Each panel includes the summary of a two-way analysis of variance with month of sampling, sex, and the corresponding month by sex interaction. # indicates a significant ($P < 0.05$) influence of the factor considered. *denotes a significant difference (Student-Newman-Keuls pairwise comparison test) between consecutive sampling dates. For each carotenoid, points that share superscript letter(s) do not differ significantly.

Patterns of carotenoid circulation during molt

Molting adults ($n = 10$) in the fall had higher plasma concentrations of β-cryptoxanthin and rubixanthin/gazaniaxanthin (id.) than non-molting adults ($n = 3$) sampled during the same period (Mann-Whitney U test, $U = 6$, $P = 0.014$). However, the two groups of birds did not differ with respect to their plasma levels of lutein or zeaxanthin (Student's t-tests, $t < 1.8$, $P > 0.10$), though statistical power was low (0.05 and 0.26, respectively) for these analyses with small sample sizes.

The plasma of no non-molting adult at this time of year contained detectable β-cryptoxanthin or rubixanthin/gazaniaxanthin, whereas all molting adults had detectable levels of both carotenoids (Fisher exact probability tests, both $P = 0.003$). However, the probability of detecting lutein or zeaxanthin in plasma was not dependent upon molt status, as all 13 birds in this sample circulated some of each. Molting and non-molting adults in the above analyses did not differ in capture date ($median_{non\text{-}molting} = 23$ Sept.; $median_{molting} = 30$ Sept.; Mann-Whitney U test, $U = 17.5$, $P = 0.60$), body mass (non-molting: 26.5 ± 1.5 g; molting: 26.4 ± 0.5 g), or body condition (body mass/wing chord ratio; non-molting: 0.31 ± 0.02; molting: 0.31 ± 0.01; Student's t-tests, both $t < 0.150$, $P > 0.9$).

Molting adult males had higher plasma rubixanthin/gazaniaxanthin levels than molting adult females and tended to have higher plasma β-cryptoxanthin levels than these females (Fig. 3). These differences were likewise carotenoid-specific, as plasma concentrations of lutein or zeaxanthin did not differ between adult molting males and females.

Caution should be exercised when interpreting the above results, because sample sizes were relatively small. Nevertheless, these results are consistent with the hypothesis that the molt status and sexual phenotype of a bird are better predictors of blood accumulation of β-cryptoxanthin and rubixanthin/gazaniaxanthin, which are seasonally restricted plasma carotenoids, than is season *per se*. In contrast, the probability of detecting lutein or zeaxanthin in adult bird plasma was apparently unrelated to molt status or sex.

Age-related patterns of carotenoid circulation

Plasma carotenoids did not differ between HYs sampled before the beginning of their prebasic molt and non-molting AHYs sampled during the same time period (9 July to 13 Sept. 1998) (Table 1). Molting HY crossbills had lower levels of lutein, β-cryptoxanthin, and rubixanthin/gazaniaxanthin than molting AHYs, but levels of zeaxanthin were not age-dependent (Table 1).

Considering only molting HY birds, HY males and females had similar plasma levels of lutein, zeaxanthin, and β-cryptoxanthin, but levels of rubixanthin/gazaniaxanthin, although low in both sexes, were higher in males than females (Table 1). Furthermore, when we again focused on the probability of detecting individual carotenoids in plasma, the proportion of molting HY males that circulated rubixanthin/gazaniaxanthin through plasma (50%) was higher than the proportion of molting HY females (0%) that circulated this pigment (Fisher exact test, $P < 0.001$). These proportions did not differ for the other pigments ($P > 0.6$). As above, differences were not due to a difference in median capture date (Mann-Whitney U test: $U = 190$, $P = 0.60$).

We also found a seasonal (and/or molt) component to age-related patterns of carotenoid accumulation: Molting HY birds ($n = 28$; admittedly caught significantly later in the year) had lower lutein concentrations than non-molting

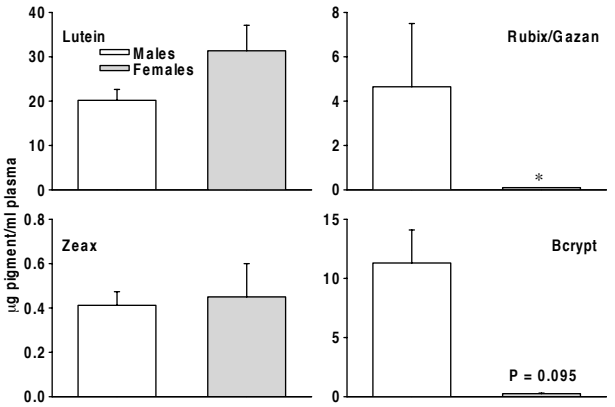

Figure 3. Plasma concentrations of carotenoids (means + SE) of adult molting male (n = 8) and female (n = 2) white-winged crossbills. *denotes a sex difference in plasma carotenoid concentration ($P < 0.05$, Student's t-test). See legend of Fig. 1 for additional details.

HY birds (n = 15; Mann-Whitney U test: U = 410; P = 0.044), but concentrations of other pigments did not differ as a function of molt status ($P > 0.10$; Fig. 4). This is likely a direct consequence of young molting birds depositing yellow (mostly lutein) carotenoids directly into growing feathers.

Correlations among carotenoid levels within a blood sample

Lutein and zeaxanthin were strongly positively correlated in plasma samples from several classes of birds: non-molting AHY males, non-molting AHY females, non-molting HY birds, and molting HY males (Table 2). Generally, we found few other significant correlations involving xanthophylls, with the exception that both lutein and zeaxanthin were positively correlated with β-cryptoxanthin in non-molting AHY females (Table 2). Among the non-xanthophyll carotenoids, we found a positive link between β-cryptoxanthin and rubixanthin/gazaniaxanthin levels, but only among molting adults (Table 2).

Discussion

We conducted the first study of annual variation in the types and concentrations of circulating carotenoids for a free-ranging passerine. We detected seasonal changes in the concentrations of two commonly occurring xanthophyll carotenoids-lutein and zeaxanthin-in the plasma of adult and juvenile white-winged crossbills. Plasma titers of lutein and zeaxanthin in adult males and females increased during summer, and in HYs were higher before (summer) than during (autumn) molt. Plasma levels of these two xanthophylls also tended to be strongly positively correlated. It is likely that the observed summer rise in circulating xanthophylls in adults is diet-driven. Crossbills increase the rate of conifer seed intake during the summer breeding season (Benkman 1987a,b), which may increase the amount of ingested xanthophylls (see below). They also consume invertebrate larvae (e.g., spruce budworm *Choristoneura occidentalis*) that are available in summer (Benkman 1992) and typically are richer sources of xanthophyll carotenoids than seeds (Goodwin 1980, 1984, Klasing 1998).

Plasma lutein or zeaxanthin levels did not differ between the sexes at any time of year. Sex differences in plasma xanthophyll concentrations are commonly reported in birds, but this is often in captive or domesticated animals (e.g., zebra finch *Taeniopygia guttata*, McGraw et al. 2003; American kestrel *Falco sparverius*, Bortolotti et al. 1996; red-legged partridge *Alectoris rufa*, Negro et al. 2001a). Instead, our study is consistent with results in two other free-ranging adult cardueline finches (American goldfinch *Carduelis tristis*, K. J. McGraw and A. J. Gregory unpubl. data; house finch *Carpodacus mexicanus*, McGraw et al. 2006) that likewise show no sex differences in plasma lutein and zeaxanthin accumulation. In captive birds, a decline in female xanthophyll levels is seen with the onset of breeding, as these females shunt lutein and zeaxanthin to egg-yolk (e.g., Negro et al. 1998, Bortolotti et al. 2003). Similar levels of yolk xanthophyll investment are expected in female crossbills, as lutein and zeaxanthin are the only carotenoids circulating through crossbills and goldfinches prior to breeding. Thus, the lack of a sex difference in plasma xanthophylls during breeding suggests either that female

Table 1. Plasma concentrations (µg/ml) of carotenoids (LUT, ZEAX, BCRYPT, and RUBIX/GAZAN) in non-molting and molting hatching-year (HY) and adult (AHY) white-winged crossbills from a wild population.

	Sample size	Median sample date	LUT	ZEAX	BCRYPT	RUBIX/GAZAN
Non-molting						
HY	12	August 10, 1998	22.5 ± 3.6[1]	0.5 ± 0.7[2]	0.10 ± 0.05[2]	0.00 ± 0.08[2]
AHY	18	August 1, 1998	28.5 ± 3.3[1]	0.8 ± 0.7[2]	0.15 ± 0.15[2]	0.00 ± 0.10[2]
Probability			0.237[a]	0.568[b]	0.408[b]	0.595[b]
Molting						
HY	28	October 27, 1998	15.2 ± 2.9[2]	0.4 ± 0.1[2]	0.00 ± 0.05[2]	0.00 ± 0.03[2]
AHY	10	September 30, 1998	21.4 ± 5.3[2]	0.4 ± 0.2[2]	8.25 ± 8.85[2]	4.25 ± 2.10[2]
Probability			0.012[b]	0.691[b]	<0.001[b]	<0.001[b]
Molting HYs						
Males	14	October 15, 1998	15.0 ± 1.4[1]	0.4 ± 0.0[1]	0.00 ± 0.05[2]	0.05 ± 0.25[2]
Females	14	October 27, 1998	16.0 ± 1.0[1]	0.4 ± 0.0[1]	0.00 ± 0.05[2]	0.00 ± 0.00[2]
Probability			0.555[a]	1.000[a]	0.474[b]	0.025[b]

[1]Mean ± SE.
[2]Median ± 0.5 interquartile interval.
[a]Student's t-test.
[b]Mann-Whitney U test.

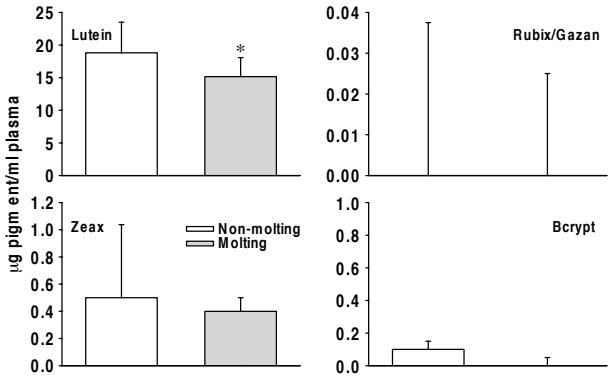

Figure 4. Plasma concentrations of carotenoids (median + 0.5 interquartile interval) of non-molting (n = 15) and molting (n = 28) hatch-year white-winged crossbills. *indicates a significant difference between non-molting and molting birds ($P < 0.05$, Mann-Whitney U test). See Fig. 1 legend for additional information.

crossbills increase food (and thus carotenoid) intake sufficiently to maintain plasma carotenoid concentrations at levels compared to males (i.e., dietary carotenoids are not limiting in either sex), that absorption of carotenoids from food increases during this time, or that females mobilize body (e.g., adipose tissue) stores of carotenoids to sustain plasma levels (e.g., Negro et al. 2001b). Seed consumption data were not reported separately for the sexes by Benkman (1990), so we cannot evaluate breeding season sex differences in food intake.

One objective of our study was to investigate variation in the types of carotenoids that are valuable for sexual coloration, so we specifically targeted carotenoid profiles during molt. In captivity, white-winged crossbills, like many other cardueline finches, will grow yellow plumage when lutein and zeaxanthin are the only carotenoids available in the diet (Stradi et al. 1996, 2001). Only when other non-xanthophyll carotenoids are fed to crossbills during molt will these birds grow red plumage (Volker 1957, Hill and Benkman 1995, Stradi et al. 2001). We discovered that three non-xanthophyll carotenoids-β-cryptoxanthin, rubixanthin, and gazaniaxanthin-were highly seasonally variable in crossbill plasma and occurred around the time of molt. These carotenoids are hypothesized to be the precursors for the metabolic formation of the three red carotenoid pigments (3-hydroxy-echinenone, 4-oxo-rubixanthin, and 4-oxo-gazaniaxanthin, respectively) that appear in the red feathers of male *L. leucoptera* (Stradi 1998, Stradi et al. 1996).

Beyond this general seasonal profile, several patterns emerged regarding the specificity with which crossbills circulated these pigments through blood. We found molting males to circulate more rubixanthin/gazaniaxanthin through blood than molting females. In fact, every adult molting male had some plasma β-cryptoxanthin and rubixanthin/gazaniaxanthin, but only two of 16 molting females were ever found to circulate any rubixanthin/gazaniaxanthin, and these pigments were present at barely detectable levels (0.1 µg/ml). Furthermore, levels of rubixanthin/gazaniaxanthin in molting HY birds, although low relative to levels in molting adults, were higher in males than females. Finally, a comparison of AHY birds that were or were not molting at the same time of year revealed that β-cryptoxanthin and rubixanthin/gazaniaxanthin were always present in molting birds and never in non-molting birds. Admittedly, these patterns emerged from data with relatively small sample sizes (especially among molting birds) and thus warrant further study and confirmation. However, taken together, these findings suggest male-specific and molt-specific accumulation of carotenoid precursors that are responsible for red coloration. The fact that some juveniles males and even some females circulated small concentrations of β-cryptoxanthin and rubixanthin/gazaniaxanthin is consistent with the idea that some young male white-winged crossbills develop orange to pink body coloration (Benkman 1992) and that females can show a

Table 2. Pearson product moment coefficients showing the association between plasma concentrations of carotenoids (LUT, ZEAX, BCRYPT, and RUBIX/GAZAN) in after hatch-year (AHY), and hatch-year (HY) white-winged crossbills from a wild population. Sample sizes are shown at the top of columns. Each cell shows the correlation coefficient between levels of two carotenoids and the corresponding probability. See text for additional details.

	AHY birds			HY birds		
	Non-molting		Molting (10)	Non-molting (15)	Molting	
	Males (66)	Females (64)			Males (14)	Females (14)
LUT						
vs. ZEAX	0.702	0.781	0.279	0.950	0.818	0.569
	<0.001	<0.001	0.436	<0.001	<0.001	0.034
vs. BCRYPT	−0.037	0.563	−0.564	0.566	0.458	0.326
	0.769	<0.001	0.090	0.028	0.100	0.255
vs. RUBIX/GAZAN	0.189	0.281	−0.609	−0.025	−0.110	–
	0.129	0.024	0.061	0.930	0.708	–
ZEAX						
vs. BCRYPT	0.013	0.750	0.544	0.604	0.304	−0.187
	0.917	<0.001	0.104	0.017	0.291	0.523
vs. RUBIX/GAZAN	0.056	0.300	0.524	−0.054	0.000	–
	0.657	0.016	0.120	0.849	1.000	–
BCRYPT						
vs. RUBIX/GAZAN	0.052	0.085	0.895	−0.219	0.104	–
	0.681	0.507	<0.001	0.434	0.722	–

tinge of orange plumage color as well (pers. obs.; see also Jardine 1994 for similar phenomenon in red crossbill *Loxia curvirostra*).

Ornithologists have long been interested in the carotenoids that make birds colorful (e.g., Brush 1978), as well as in the particular seasonal, sexual, and ontogenetic factors that determine color variation (e.g., Brush 1967, Hill 1995), but never before have any data suggested, from the perspective of dietary carotenoids, that the precursors needed for coloration are so seasonally, sexually, and ontogenetically restricted. Our observations beg an obvious question: From where do crossbills get β-cryptoxanthin, rubixanthin, and gazaniaxanthin? Spruce seeds lack these three non-xanthophyll carotenoids (unpubl. data), and plant seeds generally are not thought of as rich in non-xanthophyll carotenoids (Goodwin 1980, McGraw et al. 2001). Instead, fruits and vegetables are thought to be the source of most non-xanthophyll carotenoids in the diets of land animals (Goodwin 1984, Rodriguez-Amaya 2001). White-winged crossbills are not commonly reported to ingest fruits (e.g., occasionally juniper berries during irruptions; Benkman 1992) and Volker (1957), considered it unlikely that the diet of common crossbills *Loxia curvirostra* contains any red carotenoid. Yet, a unique feature of the carotenoid profile of white-winged crossbills during molt suggests that they acquire fruit-derived carotenoids. Rubixanthin and gazaniaxanthin are such scarce carotenoids that, among plants, they have only been described from the fruits of *Rosa* species (Hornero-Mendez and Minguez-Mosquera 2000) and from Surinam cherry (*Eugenia uniflora*; Cavalcante and Rodriguez-Amaya 1992). The prickly rose *R. acicularis* is a common Interior Alaska plant that has a circumpolar distribution in boreal forests throughout the North American range of white-winged crossbills (NatureServe Explorer 2006; USDA Natural Resources Conservation Service 2007). Its fruits (hips) are available for consumption by birds from July until full snow cover in November (Schofield 1990). Thus, based on their blood carotenoid signatures, we suggest that crossbills acquire carotenoids from rose hips during molt to become colorful. Consistent with this hypothesis, preliminary analysis of Alaska *R. acucularis* hips revealed high concentrations of β-cryptoxanthin, rubixanthin, and gazaniaxanthin (unpubl. data). Furthermore, a close relative of *L. leucoptera,* the pine grosbeak *Pinicola enucleator* has been reported to eat rose hips in Alaska during late winter (Sutton 1945), and captive white-winged crossbills will eat rose hips if available (Stradi 1998, pers. comm.).

Without evidence of rose hip consumption in the wild, however, several alternative hypotheses may account for the observed patterns of cryptoxanthin and rubixanthin/gazaniaxanthin accumulation in crossbills. First, crossbills may eat insects that ingested these carotenoids from rose hips. Second, other species of berries found in Alaska (e.g., highbush cranberries *Viburnum edule* and blueberries *Vaccinium alaskensis*) also contain some β-cryptoxanthin (unpubl. data), and if crossbills consume these it may help explain the fact that β-cryptoxanthin and rubixanthin/gazaniaxanthin levels did not always co-vary in our study. This absence of co-variation could also result from rose hips varying in the relative amounts of carotenoids from fruit to fruit or plant to plant. Third, birds of both sexes and age classes may ingest these foods and carotenoids, but adult males and only some females and juveniles males have the physiological machinery (e.g., lipoproteins of proper affinity) to accumulate these pigments at high levels from food. The fact that female red crossbills, which like female white-winged crossbills are yellow in color, exhibited male-typical red coloration when fed an unusually high dose of a red carotenoid (canthaxanthin) in captivity (Hill and Benkman 1995), suggests that female *Loxia* have the physiological capacity to become red. Last, β-cryptoxanthin, rubixanthin, or gazaniaxanthin may not be directly acquired through the diet, but are metabolically formed products of other dietary carotenoids (e.g., lutein, zeaxanthin). The idea that β-cryptoxanthin may be a derivative of ingested lutein was previously advanced for zebra finches (McGraw et al. 2002). An immediate goal of our upcoming work is to document patterns of foraging and carotenoid assimilation in molting crossbills so that firm conclusions can be drawn about the extrinsic and intrinsic factor(s) underlying this sexual dichromatism.

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