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DIET, PLASMA CAROTENOIDS, AND SEXUAL COLORATION IN THE ZEBRA FINCH (*TAENIOPYGIA GUTTATA*)

KEVIN J. MCGRAW,[1,4] ALEXANDER J. GREGORY,[1] ROBERT S. PARKER,[2] AND ELIZABETH ADKINS-REGAN [1,3]

[1]*Department of Neurobiology and Behavior, Cornell University, Ithaca, New York 14853, USA;*
[2]*Division of Nutritional Sciences, Cornell University, Ithaca, New York 14853, USA; and*
[3]*Department of Psychology, Cornell University, Ithaca, NY 14853, USA*

ABSTRACT.—Carotenoid-based colors serve important sexual-signaling functions in many animals, but the proximate factor(s) underlying their expression has sparked controversy. In particular, the relative contributions of dietary and physiological mechanisms have been questioned of late. However, no studies have concurrently quantified levels of food intake or pigment processing in any species to examine the comparative effects of pigment acquisition and use on integumetary coloration. Here, we studied within- and between-sex patterns of food intake and plasma pigment circulation in the Zebra Finch (*Taeniopygia guttata*) to assess how sexually dichromatic, carotenoid-based bill pigmentation serves as an indicator of pigment access in the diet and carotenoid transport through the bloodstream. First, in a food-choice study, we found that males and females did not consume different types or amounts of food, despite dramatic sex differences in bill coloration. Similarly, variability in carotenoid-based bill pigmentation within each sex was uncoupled from levels of food consumption. Next, we used high-performance liquid-chromatography (HPLC) to quantify the types and amounts of carotenoids circulating through blood. Male and female Zebra Finches circulated the same four major carotenoid pigments in blood plasma (lutein, zeaxanthin, anhydrolutein, and β-cryptoxanthin), but males circulated a significantly higher concentration of plasma carotenoids than did females. Within both sexes, individuals that circulated more carotenoid pigments displayed more brightly colored bills. In sum, these results suggest that physiological factors such as pigment transport may play a more important role in shaping variability in carotenoid-based bill coloration in this species than does diet. Future studies should be aimed at identifying the proximate determinants of plasma carotenoid circulation in these birds as well as how circulated pigments are used to produce maximum color displays.
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RESUMEN.—Los colores basados en carotenoides tienen funciones importantes como señales sexuales en muchos animales, pero los factores proximales que determinan su expresión han generado controversia. En particular, la contribución relativa de mecanismos alimenticios y fisiológicos ha sido cuestionada recientemente. Sin embargo, ningún estudio ha cuantificado paralelamente los niveles de ingestión de alimento y el procesamiento de los pigmentos en una misma especie para examinar el efecto comparativo de la adquisición y uso de pigmentos sobre la coloración integumentaria. En este estudio investigamos los patrones intra e intersexuales de ingestión de alimento y circulación plasmática de pigmentos en *Taeniopygia guttata* para determinar cómo la pigmentación del pico, que es sexualmente dicromática y está basada en carotenoides, funciona como un indicador del acceso a los pigmentos en la dieta o del transporte de carotenoides a través del torrente sanguíneo. Primero, en un estudio de selección de alimento, encontramos que los machos y las hembras no consumieron diferentes tipos o cantidades de alimento a pesar de las marcadas diferencias en la coloración del pico. Del mismo modo, la variabilidad en la pigmentación del pico en cada sexo no se acopló con los niveles de consumo de alimento. Luego, utilizamos cromatografía líquida de alto rendimiento para cuantificar los tipos y cantidades de carotenoides que circulaban por la sangre. Las hembras y los machos presentaron los mismos cuatro pigmentos principales (luteína, zeaxantina, anhidroluteína y β-criptoxantina) circulando en el plasma sanguíneo, pero los machos presentaron concentraciones significativamente mayores de carotenoides plasmáticos. En ambos sexos, los individuos que circularon más pigmentos carotenoides en el plasma mostraron picos con coloración más brillante. En resumen, estos resultados sugieren que en esta especie, factores fisiológicos como el transporte de pigmentos podrían jugar un papel más importante moldeando la variabilidad en la coloración carotenoide del pico que la dieta.

[4]E-mail: kjm22@cornell.edu

Estudios futuros deberían tener como objetivo identificar los determinantes proximales de la circulación de los carotenoides en el plasma en estas aves, y determinar cómo son utilizados los pigmentos transportados para producir una máxima expresión del color.

EVOLUTIONARY BIOLOGISTS SINCE Darwin (1871) and Wallace (1889) have been interested in the evolution and function of colorful displays in birds. Among the three primary types of color that birds produce in their integument (carotenoid-, melanin-, and structural-based coloration) carotenoid-based colors (e.g. reds, oranges, yellows) have received the most attention (Olson and Owens 1998). Male birds typically are more brightly colored than females, and Darwin's idea that sexual selection via female mate-choice maintains carotenoid-based sexual dichromatism has withstood rigorous theoretical and empirical testing over the last few decades (Andersson 1994, Hill 1999a). Although biologists also became interested in the means by which birds grow colorful carotenoid-based structures nearly a century ago (Palmer 1922), the underlying mechanisms that control the expression of carotenoid pigmentation remain poorly resolved.

The pioneering work of Völker (1938) on European passerines and "lipochromes" established that carotenoids in vertebrates are ultimately derived from plant matter, either by obtaining fruits, seeds, and other plant parts from the diet or by consuming herbivorous prey (Brush 1978, Goodwin 1984). That has led to the hypothesis that differences in dietary access to pigments can influence carotenoid-based sexual coloration in birds, with males consuming more pigments and consequently developing brighter coloration than females (Hill 1992, 1993). However, there are a series of physiological factors that may also contribute to sexually dichromatic carotenoid pigmentation in these birds (Hill et al. 1994, Hill 1995). Animals must absorb pigments from their diet (Furr and Clark 1997), transport them through the bloodstream (Trams 1969), and often metabolize them into different forms (Brush 1990) before depositing them in the integument (McGraw and Hill 2001). Differences in circulating plasma carotenoid levels between sexes have been used to implicate physiological regulation of sexual dichromatism (Hill 1995, Bortolotti et al. 1996, Negro et al. 1998).

Despite the preponderance of studies investigating dietary and physiological control over carotenoid pigmentation in birds, few have actually observed diet preferences or food consumption of individuals in relation to carotenoid-based ornamental coloration. Instead, investigators standardize food access and assume equal consumption for all animals in the study (Bortolotti et al. 1996, McGraw and Hill 2001, Negro et al. 2001) or they supplement dietary carotenoid pigments experimentally (Hill 1992, McGraw et al. 2002a). Moreover, studies of carotenoid processing have focused on the color of blood plasma (Hill et al. 1994, Figuerola and Gutierrez 1998) or total concentration of carotenoids in the serum (Bortolotti et al. 1996, Negro et al. 1998, Tella et al. 1998), but less commonly on types and amounts of pigments present the blood as they relate to those available in the diet of the birds as well as to the color of their integument (Negro and Garrido-Fernández 2000; Negro et al. 2000, 2002). Studies that link pigment acquisition and use are imperative for elucidating the relative effects of environmental and physiological factors on carotenoid-based sexual dichromatism in birds.

In this study, we investigated the relationships between food intake, plasma carotenoid circulation, and carotenoid-based beak pigmentation in the Zebra Finch (*Taenopygia guttata*). When sexually mature, male Zebra Finches develop bright red, carotenoid-based coloration in their bill, whereas females display orange bill pigmentation (Zann 1996). First, we conducted a food-choice experiment in which we isolated individuals, provided each with the same types and amounts of food, and allowed birds to consume food over two 24-h periods. We also scored bill color of individuals with a reflectance spectrophotometer, and sampled blood to determine types and amounts of circulating plasma carotenoids using high-performance liquid-chromatography (HPLC). That allowed us to assess both within- and between-sex patterns of food intake and plasma pigment circulation in relation to bill color in this species.

METHODS

Diet study.—Twelve male–female pairs of Zebra Finches with wild-type plumage were housed in small wire cages (0.6 long × 0.4 wide × 0.4 m tall) on a 14: 10 h light–dark cycle in an animal-approved indoor

room on the campus of Cornell University. All birds had similar breeding experience, having raised two broods 12 and 9 months previously, and since that time had been fed an *ad libitum* diet of water and Kaytee® Forti-Diet™ finch blend (Kaytee Products, Chilton, Wisconsin). This mix contained 12 components in the following proportions: white millet (19%), red millet (19%), golden yellow millet (19%), canary grass seed (19%), niger seed (7%), red fortified extruded supplement (4%), green fortified extruded supplement (4%), flax (2%), plain oat groats (2%), red-colored oat groats (2%), yellow-colored oat groats (2%), and green-colored oat groats (1%).

Food intake for individual male and female Zebra Finches was measured between 23 January and 28 April 2001. Trials were conducted in a separate room that visually and acoustically isolated the focal bird from all others. Each bird participated in two trials that were spaced approximately six weeks apart. In each trial, individuals were housed in a larger wire testing cage (0.9 × 0.6 × 0.55 m) for a 24-h period (noon til noon). Before placing birds in test cages, each individual was weighed to the nearest 0.01 g with an electronic balance (Mettler-Toledo AE 166, Mettler Instrument Corp., Highstown, New Jersey). Each finch was provided with *ad libitum* access to water and 3.00 g of each of the 12 types of food present in the commercial diet, which was placed separately into plastic Rubbermaid™ containers (21 × 11 × 9 cm) that were spread around the perimeter of the cage. The order of food presentation was randomized across trials to minimize positional effects on food choice.

During trials, birds remained undisturbed and were allowed to consume food *ad libitum*. Pilot studies indicated that birds ingested ~3 g of food per day, so our protocol allowed individuals to consume all of one food type if they chose. Following the 24-h period, we removed birds and measured amount of each type of seed consumed to the nearest 0.01 g. Use of deep food containers reduced the amount of seed that birds could spill outside of the dishes. We estimated that no more than 1% of seed was spilled from each dish during each trial. Thus, all food constituting this amount (0.03 g) must be considered within measurement error.

Bill color measurement.—Preliminary analyses of euthanized birds indicated that Zebra Finches deposit carotenoids into their bills, but not into the rust-colored cheek patches of males (K. McGraw, R. Parker, and R. Stradi, unpubl. data), which presumably are composed of phaeomelanin pigments. These finches also display orange, carotenoid-colored legs, but that trait was not studied here because nothing is known of its function as a variable sexual signal. Color of male and female Zebra Finch bills was scored using a Colortron™ reflectance spectrophotometer (Light Source Inc., San Rafael, California; Hill 1998). Although the Colortron does not gather data at ultraviolet wavelengths, the beaks of Zebra Finches do not exhibit discrete ultraviolet colors (Bennett et al. 1996), and thus this unit is an acceptable means of scoring bill coloration in this species. Beak color was scored at three separate times during the study: 29 December, 16 January, and 11 February. During each period, we determined hue, saturation, and brightness for males and females by taking the average of two measurements (from the right and left sides of the maxilla). Repeatability of this procedure (Lessells and Boag 1987) was high ($r = 0.90$, $F = 25.5$, df = 31 and 32, $P < 0.0001$). Hue, saturation, and brightness was averaged for each individual across the three sessions and found that those three measures were to a great extent significantly intercorrelated (all $P < 0.002$). Thus, we collapsed them into a single, composite color score using a principal components analysis. We used the first principal component (PC1) in our statistical analyses because it explained 75% of variation in color variables. Note that, because of the Colortron's scoring system, lower PC1 values correspond to redder ($r = 0.89$, $P < 0.0001$), more saturated ($r = -0.86$, $P < 0.0001$), and darker bills ($r = 0.86$, $P < 0.0001$).

Plasma carotenoid analyses.—In a previous study, we identified types and proportional amounts of carotenoid pigments present in the diet and plasma of Zebra Finches (McGraw et al. 2002b). Male and female finches circulate three main dietary carotenoids through the blood (lutein, zeaxanthin, and β-cryptoxanthin) as well as a fourth, presumed metabolic derivative (2′,3′-anhydrolutein). Here, we were interested in exploring variation in plasma carotenoid concentration between sexes and in relation to food intake and bill coloration. To determine concentration of different carotenoid types in finch plasma, blood from males and females was sampled on the three aforementioned days on which we scored bill coloration. We drew 80–100 μL of whole blood through the alar vein of each individual into heparanized microcapillary tubes. We then centrifuged the tubes at 3,000 rpm for 10 min, removed the plasma, and stored the samples at –80° C. To extract carotenoids, we thawed samples to room temperature and added 200 μL ethanol to 25 μL plasma. The mixture was vortexed and 100 μL MTBE was added. After vortexing again, we centrifuged the solution for 4 min in an Eppendorf centrifuge (model 5414). We transferred 200 μL of the supernatant to a new tube and evaporated to dryness under a stream of nitrogen. The remaining residue was dissolved in 200 μL of HPLC mobile phase (methanol-acetonitrile-chloroform, 46:46:8, v/v/v, + 0.05% triethylamine) and vortexed prior to HPLC analysis.

We analyzed 50 μL of each sample with a Waters™ 717plus Autosampler HPLC (Millipore Corp., Bedford, Massachusetts) fitted with a Develosil RPAqueous RP-30 HPLC column (250 × 4.6 mm ID; Nomura Chemical Co.). An isocratic system (HP 1050 Series Isocratic Pump), using the aforementioned mobile phase for 25 min, was used for analysis at a constant flow rate of 1.2 mL min^{-1}. We confirmed the identity of plasma

pigments by comparing their retention times to those for authentic reference carotenoids provided by Roche Vitamins Inc. (lutein, 6.0 min; zeaxanthin, 6.3 min; anhydrolutein, 13.0 min; β-cryptoxanthin, 19.3 min). Carotenoids were detected at λ_{max} for each pigment (lutein, 445 nm; zeaxanthin, 450 nm; anhydrolutein, 450 nm; β-cryptoxanthin, 450 nm) using a Waters 996 photodiode array detector (Waters Chromatography, Milford, Massachusetts). Concentration of each carotenoid type was determined by comparing peak areas (integrated by MILLENIUM™ software, version 2.0) to those for an internal standard (canthaxanthin, Roche Vitamins, 0.48 μg mL^{-1}, retention time, 9.5 min; λ_{max}, 470 nm) added to each sample prior to pigment extraction.

Statistical analyses.—Parametric statistics were used when data met the assumptions of normality (Shapiro-Wilk *W*-test, $P > 0.05$) and variance homogeneity (Equality-of-variance *F*-test, $P > 0.05$). In all cases, we conducted two-tailed tests and report mean ± SE. To confirm that males were more brightly colored than females during our study, we compared average bill color (PC1) for the two sexes using one-way analysis of variance (ANOVA). We also used ANOVA to examine general patterns of food intake between trial sets, among different food types, and within individuals. When ANOVA yielded significant effects, we employed Fisher's PLSD *post hoc* planned comparisons to test for specific differences between groups. Multivariate ANOVA (MANOVA) and ANCOVA (MANCOVA) were performed to evaluate potential sex differences in food selection. We conducted MANOVA (Wilks' λ reported) using all of the food-intake data as well as for only those three main food types (millet) that comprised the majority of all food eaten (see below). MANCOVA were run similarly, except that body mass was entered as a covariate because female Zebra Finches weighed significantly more than males during our study (female = 15.9 ± 0.34 g; male = 14.5 ± 0.47 g; $F = 5.8$, df =1 and 22, $P = 0.02$) and thus may have had different energetic demands. We used Spearman rank-correlations to assess within-sex relationships between food intake (overall and separately for each type) and both plasma carotenoid levels and bill coloration. Again, we examined effect of sex on overall plasma pigment concentration, specific plasma pigment concentrations, and proportion of total plasma pigments that each type constituted using ANOVA. We also used rank-correlations to investigate the relationship between plasma carotenoid composition and bill pigmentation within both sexes. Sequential Bonferroni corrections for multiple tests were applied when necessary (Rice 1989).

Results

Sex patterns of bill coloration.—While being housed in the same cage and held on the same diet during our study, males displayed significantly more colorful bills (–0.92 ± 0.28) than females (0.79 ± 0.38), with no overlap in PC1 values between sexes ($F = 12.1$, df = 1 and 22, $P = 0.002$). The repeatability of bill color expression was high for individual birds across the three sampling periods ($r = 0.63$, $F = 6.00$, df = 23 and 24, $P < 0.0001$).

General patterns of food intake.—Zebra Finches consumed an average of 2.33 ± 0.24 g of food per 24 h in the first set of feeding trials and 2.28 ± 0.20 g of food in the second set of trials (ANOVA, $F = 0.03$, df = 1 and 22, $P = 0.87$). Birds showed significant preferences for particular foods within each trial set (Kruskal-Wallis *H*-test, both $H > 130$, both $P < 0.0001$). Finches preferred white millet over all other food types in both sets of trials (*post hoc* planned comparison, Fisher's PLSD, all $P < 0.008$; Fig. 1). Red millet and golden yellow millet were the next most preferred foods (all $P < 0.02$; Fig. 1). Among the remaining nine, least-preferred food types, birds showed no distinct preferences (all $P > 0.05$; Fig. 1). It is interesting that these hierarchical preferences mimic the proportion of the commercial diet that each food type composed (see above), even though birds had access to the same amount of each food type in these trials. Individual birds consumed repeatable amounts of food across trials ($r = 0.67$, $F = 5.13$, df = 23 and 24, $P < 0.0001$), so we used mean food intake per bird per 24-h trial in subsequent statistical analyses.

Patterns of food consumption in relation to sex and body mass.—We found no significant difference between male and female Zebra Finches in overall amount of food consumed during our food-intake trials (MANOVA, Wilks' $\lambda = 1.18$, df = 12 and 11, $P = 0.39$). The sexes also did not differ in food intake for any of the 12 food types presented (Fig. 1), or when we considered only the three most preferred food types (MANOVA, Wilks' $\lambda = 0.39$, df = 3 and 20, $P = 0.29$). When we controlled for body-mass differences among individuals, there still were no significant sex differences in food consumption (all food types: MANCOVA, Wilks' $\lambda = 0.39$, df = 12 and 9, $P = 0.51$; three main food types: Wilks' $\lambda = 0.90$, df = 3 and 18, $P = 0.58$).

Relationship between bill color and food intake.—Total food intake did not predict expression of bill color in either sex (both $r^2 = 0.01$, both $P > 0.7$), nor did any of the individual components of the diet (Table 1). Even if we consider only the

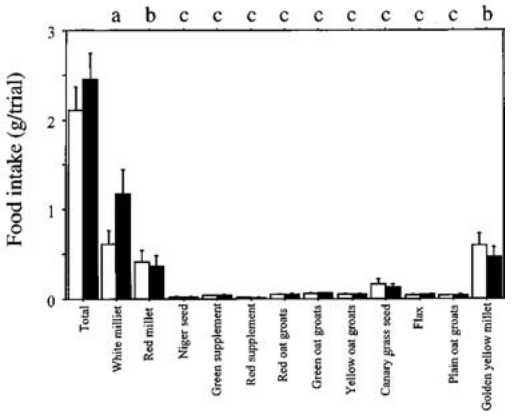

FIG. 1. Amount of each food type consumed by captive male (dark bars) and female (open bars) Zebra Finches during our diet study. Standardized amounts of all foods (3 g) were presented to individually housed birds, and birds were weighed after 24 h periods to the nearest 0.01 g. Bars show mean ± SE; letters denote significant differences among food types after correcting for multiple tests.

direction of the relationships, we still found no significant tendencies within either sex (male: sign-test, $P = 0.71$; female: $P = 0.27$) for correlations to be consistently positive or negative.

Patterns of circulating plasma carotenoids.—On average, Zebra Finches circulated 4.89 ± 0.59 μg carotenoids per milliliter plasma. Anhydrolutein was present in the highest concentration (1.81 ± 0.19 μg mL^{-1}; 37.8 ± 1.0% of total carotenoids), followed by lutein (1.41 ± 0.20 μg mL^{-1}; 28.2 ± 1.1%), zeaxanthin (1.28 ± 0.16 μg mL^{-1}; 25.9 ± 0.5%), and β-cryptoxanthin (0.39 ± 0.05 μg mL^{-1}; 8.2 ± 0.4%). Total ($r = 0.57$, $F = 5.04$, df = 23 and 24, $P < 0.0001$) and individual (lutein: $r = 0.62$, $F = 5.90$, df = 23 and 24, $P < 0.0001$; zeaxanthin: $r = 0.56$, $F = 4.81$, df = 23 and 24, $P < 0.0001$; anhydrolutein: $r = 0.54$, $F = 4.53$, df = 23 and 24, $P < 0.0001$; β-cryptoxanthin: $r = 0.64$, $F = 6.41$, df = 23 and 24, $P < 0.0001$) plasma carotenoid concentrations were repeatable for birds across the three sampling periods, so we use mean values in all subsequent analyses.

Within individuals, we found highly significant intercorrelations among concentrations of the four plasma carotenoid types (all $r > 0.8$, all $P < 0.005$). Thus, both male and female finches that circulated more of one type of carotenoid in plasma also circulated more of the others. Interestingly, correlations among carotenoid types were significantly stronger in males than in females (Wilcoxon matched-pair signed-rank test, $Z = 2.2$, $P = 0.03$), although this may be explained simply by the higher overall levels found in males. We also examined correlations among relative proportions of each pigment within individual birds. In both sexes, there were significant proportional relationships between lutein and anhydrolutein, and lutein and β-cryptoxanthin (all other $P > 0.20$). Finches whose plasma contained proportionally more lutein circulated proportionally less anhydrolutein ($P < 0.006$ in both sexes) and β-cryptoxanthin (both $P < 0.05$).

Sex differences in plasma carotenoid content.—Overall, male Zebra Finches circulated a significantly higher concentration of carotenoid pigments in their plasma than did females (Fig. 2). Broken down by specific pigments, the plasma of males contained significantly higher concentrations of zeaxanthin, anhydrolutein, and β-cryptoxanthin than females (Fig. 2). The tendency for males to circulate more plasma lutein was not statistically significant, but was in the same direction. Male plasma carotenoids also were made up of a significantly higher proportion of β-cryptoxanthin than those of females (Fig. 2). There was a nonsignificant trend for females to circulate a higher percentage of lutein in their plasma. We found no sex differ-

TABLE 1. Relationship between carotenoid-based beak coloration (PC1) and food intake (grams consumed per trial) in captive Zebra Finches. Nonparametric correlations are reported separately for each sex and each type of food presented. For each sex-specific comparison, $n = 12$. A sequential Bonferroni adjustment (Rice 1989) was applied to correct for running multiple correlations with the same bill-color data set.

	Females		Males	
Food type	r_s	P	r_s	P
White millet	0.09	0.76	–0.34	0.29
Red millet	–0.09	0.75	0.69	0.03
Niger seed	0.39	0.16	–0.08	0.79
Green supplement	0.23	0.40	0.04	0.91
Red supplement	0.20	0.48	0.03	0.93
Red oat groats	–0.09	0.75	0.07	0.82
Green oat groats	0.39	0.16	–0.05	0.89
Yellow oat groats	0.33	0.23	0.04	0.91
Canary grass seed	0.15	0.58	0.60	0.06
Flax	0.11	0.69	0.01	0.98
Plain oat groats	0.29	0.30	–0.13	0.69
Golden yellow millet	–0.34	0.23	–0.05	0.89

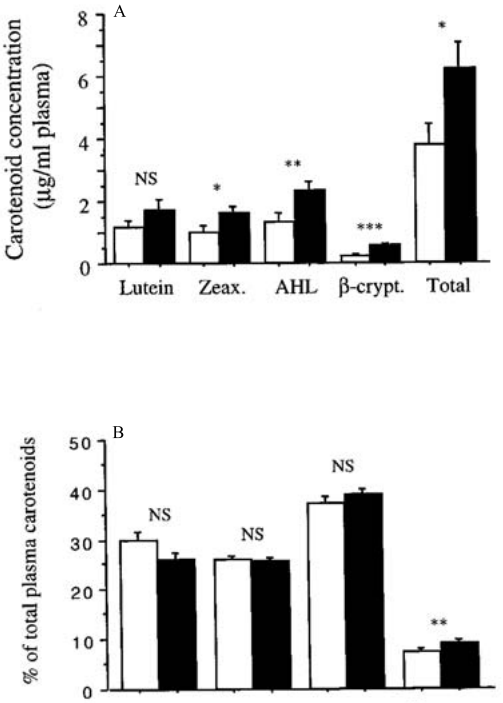

FIG. 2. Sex differences in circulating plasma carotenoids in Zebra Finches: (A) pigment concentrations, (B) relative abundance. Open bars = values for females; dark bars = males. Pigment concentrations and proportions were determined by comparing peak areas produced by HPLC analyses to a known internal standard. Bonferroni corrections for multiple tests (minimum P = 0.01) were applied. NS = $P > 0.05$; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

ences in the proportion of plasma zeaxanthin or anhydrolutein.

Plasma pigments in relation to food intake.—Separately for both males and females, we compared total and specific plasma carotenoid concentrations to total and specific amounts of food eaten. Total food intake did not predict total plasma carotenoid concentration in either sex (both $r^2 < 0.15$, $P > 0.25$). We then compared each of the 12 food types to overall plasma carotenoid concentration separately for males and females, both to avoid running 48 correlational analyses (4 pigment types × 12 food types) for each sex and also because of the extremely high intercorrelations between the pigment types within individual birds. Here, we also found no significant relationships between plasma pigments and food consumption (Table 2).

Plasma pigments as a predictor of bill color.—Within both sexes, finches that circulated a higher concentration of carotenoids displayed significantly more brightly colored bills (Fig. 3). That was true both for overall plasma carotenoid concentration (Fig. 3) and for the concentration of each of the four major serum pigments (Table 3), with the exception of zeaxanthin and β-cryptoxanthin in males, which exhibited near-significant relationships with bill color in the same, predicted direction. The proportion of total plasma carotenoids made up by each pigment type was not, however, significantly related to bill color in either males or females (all $P > 0.20$), although there was a tendency for males that circulated proportionally more lutein ($P = 0.05$) and β-cryptoxanthin ($P = 0.09$) and proportionally less zeaxanthin ($P = 0.09$) to exhibit more brightly colored bills.

DISCUSSION

Several different approaches have been used to demonstrate an effect of diet on carotenoid pigmentation in animals (reviewed in McGraw et al. 2001, Hill 2002). Early evidence came from pets and zoo animals that faded in color when held in captivity (reviewed in Brush 1981). Since that time, biologists have linked variation in carotenoid coloration among wild animals to changes in environmental carotenoid availability (e.g. Partali et al. 1987, Linville and Breitwisch 1997), or performed feeding experiments with captive animals to investigate how dietary carotenoid access can influence color expression (e.g. Hill 1992, 2000). The problem with that is that several other, often-ignored environmental, physiological, and social factors (e.g. parasites, social stress, hormones) can concomittantly affect carotenoid displays (e.g. Hudon 1994, Hill 2002). Only recently have studies begun to measure aspects of diet for direct comparison with integumentary carotenoid pigmentation (Grether et al. 1999, Hill et al. 2002).

In this study, we measured food intake among captive male and female Zebra Finches to test the idea that variability in carotenoid-based beak coloration may be attributed to within- and between-sex differences in dietary intake of food and the associated carotenoid pigments. A series of results suggest that diet alone cannot explain variation in bill color that Zebra Finches exhibit. With access to the same diet in the same cage, males displayed

TABLE 2. Correlations between total plasma carotenoid concentration (micrograms pigment per milliliter plasma) and food intake (grams consumed per trial) in captive Zebra Finches. Again, $n = 12$ for each comparison, we report nonparametric relationships separately for each sex and for each type of food presented, and a sequential Bonferroni correction was applied for running multiple, nonindependent tests.

	Females		Males	
Food type	r_s	P	r_s	P
White millet	–0.22	0.44	0.57	0.07
Red millet	–0.07	0.79	–0.46	0.14
Niger seed	–0.56	0.04	–0.35	0.27
Green supplement	–0.40	0.15	–0.16	0.61
Red supplement	–0.51	0.07	–0.11	0.72
Red oat groats	–0.19	0.49	0.22	0.48
Green oat groats	–0.54	0.05	0.21	0.51
Yellow oat groats	–0.33	0.24	–0.24	0.45
Canary grass seed	–0.30	0.28	–0.69	0.03
Flax	–0.14	0.60	–0.22	0.49
Plain oat groats	–0.28	0.32	0.17	0.60
Golden yellow millet	0.37	0.19	0.41	0.20

significantly redder bills than females. Male Zebra Finches are also more colorful than females when housed in large, same-sex flocks (K. McGraw unpubl. data). To determine actual patterns of food intake, however, we isolated individual birds and found that males did not consume significantly more food, or more of different types of food, than females. That was also true when we controlled for sex differences in body mass. Moreover, within a sex, neither an individual's bill color nor its levels of circulating plasma carotenoids (see below) was related to amounts or types of food ingested. These findings are consistent with eight correlative studies that show no sex differences in diet selection among wild Zebra Finches (reviewed in Zann 1996), and with recent studies of carotenoid pigmentation in American Kestrels (*Falco sparverius*; Bortolotti et al. 1996), Red-legged Partridge (*Alectoris rufa*; Negro et al. 2001), American Goldfinches (*Carduelis tristis*; McGraw and Hill 2001, McGraw et al. 2002a), and Northern Cardinals (*Cardinalis cardinalis*; McGraw and Hill 2001), in which diet seems to play a minor role in mediating color variation either within or between the sexes. Certainly, all birds must ultimately obtain carotenoid pigments from the diet in order to become colorful, but it does not seem that variation in levels of food intake can adequately explain patterns of ornamental coloration in Zebra Finches.

In parallel with our studies of diet, we also explored the physiological processes of carotenoid use in Zebra Finches, as measured by the circulation of pigments through the blood. There is a large body of literature on avian blood carotenoids, in species such as domestic chickens (*Gallus gallus domesticus*; Surai 2001) and several wading birds (e.g. flamingos, ibis, spoonbills; Brush 1981). However, only within the last few years have plasma-carotenoid levels been related to intraspecific variation in the carotenoid colors of feathers or bare parts. In those studies, relationships between plasma carotenoid circulation (or plasma color) and colorful integumentary displays have been reported in American Kestrels (Bortolotti et al. 1996), Cirl Buntings

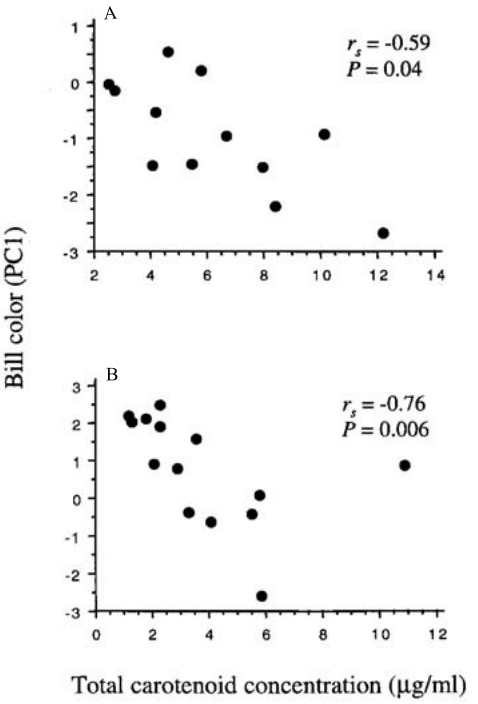

FIG. 3. Relationship between bill coloration and plasma carotenoid concentration in (A) male and (B) female Zebra Finches. Bill color represents the first principal component (PC1) of tristimulus scores (hue, saturation, and brightness) measured with a hand-held Colortron reflectance spectrophotometer. Recall that lower PC1 values correspond to redder beaks. Males and females with redder bills circulated significantly more blood carotenoids overall (see Table 3 for comparisons with levels of individual types of blood carotenoids).

TABLE 3. Spearman-rank correlations between plasma carotenoid concentrations and beak coloration in captive male and female Zebra Finches. In all comparisons $n = 12$, and sequential Bonferroni adjustments were applied to correct for multiple comparisons (carotenoid types) within each sex.

Sex	Plasma carotenoid	r_s	P
Male	Lutein	–0.69	0.01
	Zeaxanthin	–0.54	0.07
	Anhydrolutein	–0.64	0.02
	β-cryptoxanthin	–0.42	0.16
Female	Lutein	–0.71	0.01
	Zeaxanthin	–0.74	0.008
	Anhydrolutein	–0.74	0.008
	β-cryptoxanthin	–0.59	0.03

(*Emberiza cirlus*; Figuerola and Gutierrez 1998), Barn Swallows (*Hirundo rustica*; Saino et al. 1999), Red-legged Partridges (Villafuerte and Negro 1998), and House Finches (*Carpodacus mexicanus*; Hill et al. 1994). Here, we examined the degree to which the types and amounts of carotenoids present in the blood of Zebra Finches could predict within- and between-sex variability in bill color expression. We found that, although males and females circulated the same four main types of carotenoids through the blood, males circulated a significantly higher concentration of plasma carotenoids overall than females. That was true for three of the four main plasma carotenoid types as well, with the exception of lutein. Moreover, within each sex, more brightly colored birds circulated significantly more blood carotenoids. There were few proportional differences in plasma pigment composition between sexes and in direct relation to bill color, suggesting that there may not be particular carotenoids or particular mixtures of carotenoids that are most critical for attaining maximum integumentary coloration. Instead, the most colorful Zebra Finches generally circulate more of all blood carotenoids.

What factors might regulate these within- and between-sex patterns of carotenoid circulation? Animals first absorb dietary carotenoid pigments through the intestinal mucosa (Erdman et al. 1993), and conditions such as gut parasitism are known to inhibit pigment uptake and transport in birds (Allen 1987). Carotenoids circulate through the bloodstream in association with lipoproteins, particularly via high- (HDL) and low-density (LDL) lipoproteins (Parker 1996). Yet to be tested are the limitations and binding affinities of lipoproteins as they pertain to variation in carotenoid use in birds. Binding capacity does not appear to limit pigment transport in juvenile rainbow trout (*Oncorhynchus mykiss*; Chavez et al. 1998). However, lipoprotein production may be affected by gonadal activity (Fremont and Marion 1982), particularly via the action of steroid hormones (Schejf et al. 1993). Interestingly, bill color in Zebra Finches, like other secondary sex traits in animals, appears to be sensitive to changes in androgen levels. Cynx and Nottebohm (1992) found that bills of castrated male Zebra Finches fade in color, but are restored by administering exogenous sources of testosterone. Adkins-Regan and Wade (2001) also found that masculinized female Zebra Finches (with sex-reversed gonads) exhibit male-typical bill coloration. We are currently investigating the influence of circulating hormone levels on carotenoid transport and bill pigmentation in this species.

Although the physiological focus of this study has been on carotenoid transport, it is important to emphasize that this is merely one of the ways in which birds physiologically process ingested pigments. Carotenoids are taken up from blood circulation by rhamphothecal keratinocytes in the bill, accumulated in lipoid droplets, and distributed diffusely through the horny, keratinized beak tissue (Lucas and Stettenheim 1972). Moreover, many species transform dietary carotenoids into more oxidized forms that are then deposited into the integument for display (Brush 1990). That appears to be true in Zebra Finches at two levels: (1) birds manufacture anhydrolutein from dietary sources of lutein (McGraw et al. 2002b), and (2) we have preliminarily identified four red ketocarotenoids in the bills of both males and females that differ from those in the diet and plasma (K. McGraw and R. Stradi unpubl. data). Those metabolic conversions may incur energetic costs that translate into patterns of differential color expression among individuals (Hill 2000). Birds also may use carotenoids as antioxidants or immunostimulants (Møller et al. 2000). Whether the immune systems of birds are carotenoid-limited (Hill 1999b) and whether male birds have greater immunological needs for carotenoids than females should be intriguing avenues for future research. Because bill color expression does appear to have a strong genetic component in Zebra Finches (Price and Burley 1993, Price 1996), it is likely that some

or all of those physiological processes serve as more powerful control agents than environmental factors (e.g. diet).

In light of these proposed control mechanisms of sexual coloration in Zebra Finches, it is important to consider the information content and ultimately the signaling function of that carotenoid-based ornamental trait. Females from a variety of avian species prefer to mate with males displaying the most brightly colored carotenoid pigmentation (Hill 1999a). Female choice of bright bill coloration has been demonstrated in some (Burley and Coopersmith 1987, Houtman 1992, Zann 1996) but not all studies (Collins et al. 1994, Collins and ten Cate 1996) of Zebra Finches. Bill color in male finches may also act as a signal of aggressiveness to other males (Etman et al. 2001). In either case, males may be communicating information to conspecifics about their physiological state, whether it is their general health or their ability to compete aggressively (with elevated levels of testosterone). As opposed to male coloration, there is no conclusive evidence that male Zebra Finches prefer to mate with the most brightly colored females (Burley and Coopersmith 1987). Mate-preference for and hormonal control of female coloration demands more rigorous testing. An interesting potential function of carotenoid color in females is that it serves as a signal of maternal investment in developing embryos, because females deposit considerable quantities of carotenoids into their egg yolks (Blount et al. 2000).

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