A Comparison of Spectrophotometry and Color Charts for Evaluating Total Plasma Carotenoids in Wild Birds

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ABSTRACT

The study of the role of carotenoids on the physiology and evolutionary ecology of birds demands methods for their quantification in the bloodstream. We compared color-chart scores of plasma hue with the actual concentration of plasma carotenoids obtained by spectrophotometry in 356 wild birds from 26 species. Repeatability of chart scores between three independent observers was high. However, color scores did not correlate with the spectrophotometric results in interspecific analyses. Within species (n Å 3), one showed no relationship and two showed weak but significant positive correlations. Hemoglobin, and probably other substances, may mask the color of carotenoids, making the accurate use of color charts difficult. Spectrophotometry should be the method of choice as it permits precise quantifications of total plasma carotenoids and objective comparisons among studies.

Introduction

The role of carotenoids in the physiology and sexual selection of birds is the focus of an increasing forum in evolutionary ecology. These pigments are responsible for some of the brightest colors in the plumage and ornaments of birds (Brush 1990). There is also evidence that carotenoids have significant health benefits (see, e.g., Rock et al. 1996). Therefore, individuals may take positions concerning the allocation of carotenoids for ''show'' or ''health'' functions (Shykoff and Widmer 1996; Negro et al. 1998).

This theoretical framework demands tools for quantification of carotenoids in birds. Dietary carotenoids are transported in the blood until they are deposited in integuments or stored in organs such as the liver (Hill et al. 1994). While high-resolution separation techniques (i.e., high-pressure liquid chromatography [HPLC]) have been used to identify different types of carotenoids in feathers (Hudon and Brush 1992), few attempts have been made to quantify circulating carotenoids. Recently, total plasma carotenoids of American kestrels (*Falco sparverius*) have been measured by spectrophotometry (Bortolotti et al. 1996; Negro et al. 1998), a method used by poultry researchers (Allen 1987; Conway et al. 1993). Alternatively, color charts have been used to visually estimate the concentration of plasma carotenoids (Hill et al. 1994; Hill 1995*a*, 1995*b;* Figuero and Gutiérrez 1998). Because of its simplicity, color-chart matching was proposed as a quick assay for field biologists (Hill 1995*a*). However, no study has compared the scores derived from color charts with the actual carotenoid concentrations objectively measured by spectrophotometry. Our aim is to assess the validity and accuracy of color charts for quantifying total plasma carotenoids in both intra- and interspecific studies of wild birds.

Material and Methods

The study was conducted in the surroundings of La Paz (Baja California Sur, México) between November 15 and December 9, 1996. Wild birds were trapped and blood samples (0.1–0.5 mL) were taken from the brachial or jugular veins. Heparinized syringes or microcapillary tubes were used for collecting the blood. All individuals were freed after blood collection. The samples were transported to the laboratory (Centro de Investigaciones Biológicas del Noroeste, La Paz) within 8 h after blood withdrawal, and they were centrifuged at 1,500 g for 10 min. The plasma was frozen at **O**70°C until analysis 1–6 d later.

The concentration of plasma carotenoids was first estimated visually. Just after thawing the plasma samples, plasma hue was independently scored by three of the authors, using a six-option color chart derived from paint samples. The extremes (1 Å pale yellow, 6 Å red-orange) were determined on the

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basis of our experience after previously sampling the plasma of thousands of birds (J. L. Tella et al., unpublished data) and correspond to the following Munsell's color chips: 1 Å 5Y 9/6, 2 Å 5Y 8.5/10, 3 Å 2.5Y 8/12, 4 Å 8.75YR 7/14, 5 Å 3.75YR 6/12, 6 Å 10R 5/12 (see Munsell Color Company 1976). Second, the concentration of total plasma carotenoids was measured spectrophotometrically (see, e.g., Allen 1987; Bortolotti et al. 1996; Negro et al. 1998). We diluted a variable amount of plasma (20–100 mL) in acetone obtaining dilutions ranging from 1 : 10 to 1 : 40. After the plasma was well mixed with 100% acetone, the flocculent protein was precipitated by centrifuging the sample at 1,500 *g* for 10 min. We examined the supernatant in a Beckman Du-70 spectrophotometer and determined the optical density of the carotenoid peak at 476 nm. Xantophylls, the most common carotenoids in birds (Brush 1990) show two absorption maxima in acetone, at 445–450 nm and 474–478 nm, respectively. We choose the upper peak following previous researchers (e.g., Allen 1987). Carotenoid concentration (mg/mL plasma) was derived from a standard curve of lutein (alpha-carotene-3,3Ì-diol, SIGMA).

Results

A total of 356 birds from 26 species were sampled for plasma carotenoids, representing seven orders and 14 families of birds (see Appendix). The repeatability (Lessells and Boag 1987) of plasma-hue scores obtained from the color charts was high between the three independent observers (r Å 0.88, F Å 33.20, P õ 0.00001). The average of the three scores was used in further analyses. Plasma hues ranged from 1 to 6, with a median of 4.7 (n Å 341). A number of samples (especially from common ground doves, *Columbina passerina*) were highly hemolyzed and showed an intense red coloration exceeding the maximum score of 6. Excluding those samples, the median score was 4 (n Å 266).

Concentration of total plasma carotenoids in blood evaluated through spectrophotometry ranged between 0.43 mg/mL and 74.16 mg/mL (X Å 9.38, SD Å 9.37, n Å 356). Hemolysis did not seem to influence the spectrophotometric measurements, since values of hemolyzed samples of common ground doves (median 3.45 mg/mL, n Å 65) did not differ from those of the rest of doves (median 3.50 mg/mL, n Å 67; Mann-Whitney U-test, Z Å O0.75, P Å 0.45). It is important to remark, however, that median color scores were 6 and 4, respectively (Mann-Whitney U-test, Z Å O10.57, P õ 0.0001).

The median color scores of each species were not correlated with their median concentrations of plasma carotenoids (r_s Å 0.04, P Å 0.84, n Å 26), even when clearly hemolyzed samples were excluded (r_s Å 0.21, P Å 0.30, n Å 26; Fig. 1). These results do not seem to be influenced by the different sample sizes for each species. A linear regression with weighed sample sizes of median color scores on median concentrations of plasma carotenoids (excluding clearly hemolyzed samples and both variables log-transformed) did not show a significant relationship (r Å 0.04, P Å 0.50).

Intraspecific correlations were also obtained for the three species with larger sample sizes, excluding the clearly hemolyzed samples. The results differed between species: while in American kestrels and white-crowned sparrows (*Zonotrichia leucophrys*) plasma-hue scores correlated significantly with actual plasma concentrations (r_s Å 0.49, P Å 0.0001, n Å 62 and r_s Å 0.31, P Å 0.03, n Å 50, respectively), there was no correlation in common ground doves (r_s Å 0.042, P Å 0.73, n Å 67; Fig. 2).

Discussion

Our color chart covered all natural variability found in the plasma of a wide phylogenetic range of wild birds, and the scores of independent observers were highly repeatable. However, color scores did not accurately reflect the actual concentration of total carotenoids in plasma.

A critical assumption for validating the use of color charts is that the redness of plasma is a function of the type and quantities of carotenoids contained (Hill et al. 1994; Hill 1995*a*, 1995*b*). This assumption was violated when hemolysis occurred, since hemoglobin gave a red coloration to the plasma unrelated to carotenoids. On the one hand, using median values for species, no correlations were found between visual estimations by color charts and actual plasma concentrations, even after removing the clearly hemolyzed samples. On the other hand, the intraspecific comparisons showed different trends, independent of whether the species had any external carotenoid-dependent coloration. Two of the three species showed significant but weak correlations, which do not allow for the detection of the fine individual variations in plasma carotenoids obtained by spectrophotometry (Bortolotti et al. 1996; Negro et al. 1998). In fact, color scores of both white-crowned sparrows and American kestrels mainly ranged between 3 and 5, while in some instances actual concentrations

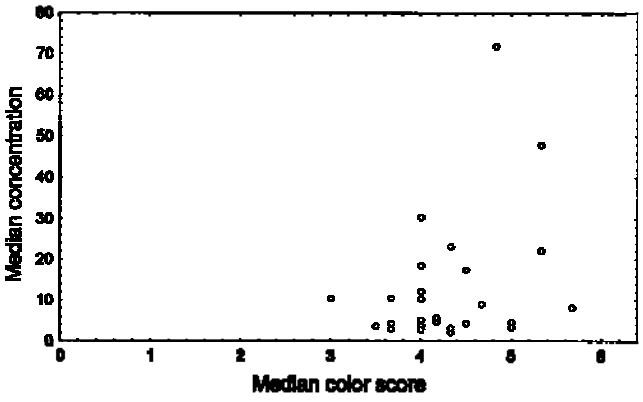

Figure 1. Relationship between plasma color scores and total carotenoid concentration (mg/mL) obtained by spectrophotometry. Dots correspond to median values of different species (n Å 26).

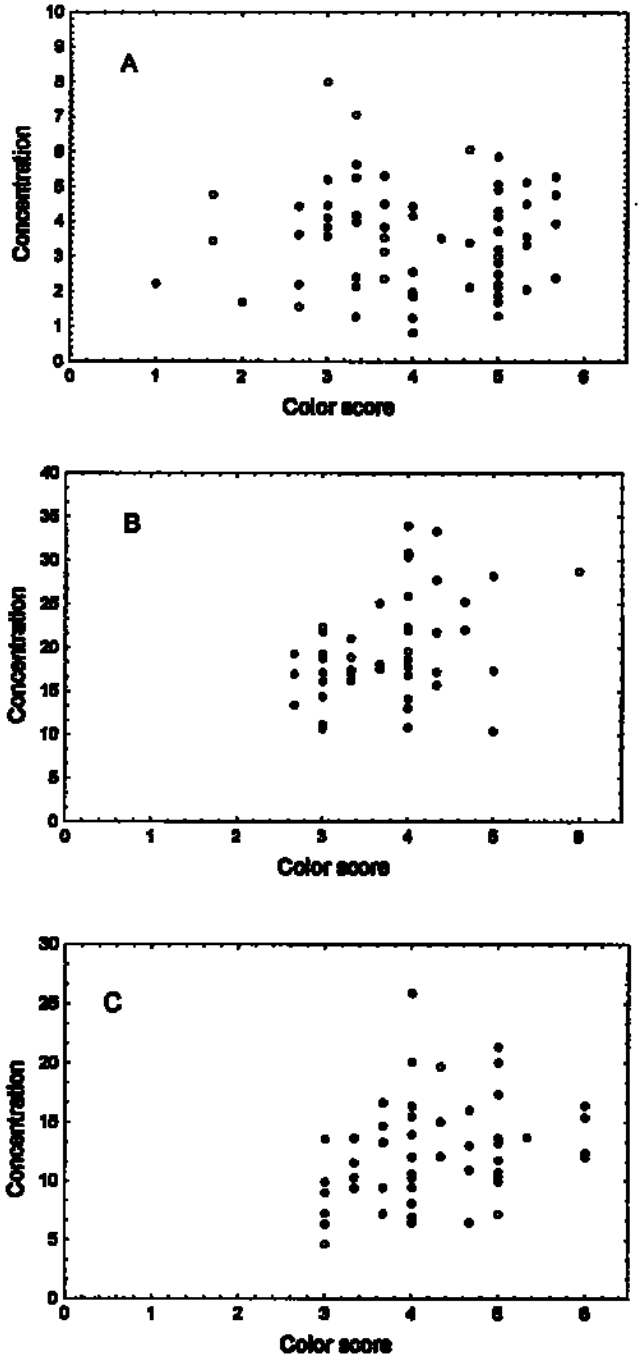

Figure 2. Relationship between plasma color scores and total carotenoid concentration (mg/mL) obtained by spectrophotometry, in (*A*) common ground doves (*n* Å 67), (*B*) white-crowned sparrows (*n* Å 50), and (*C*) American kestrels (*n* Å 62).

corresponding to a single chart unit varied fourfold (6–25.5 mg/mL; see Fig. 2). Charts with a larger number of color chips would facilitate a finer distinction of colors, but repeatability between observers would surely decrease.

Excluding highly hemolyzed samples, color charts still provided poor estimations of plasma carotenoid concentration. This could be due to partial hemolysis or to other substances such as bilirubin and plasma proteins, which are known to give yellow and orange colorations to the plasma (see, e.g., Linch et al. 1972). These problems make difficult the study of subtle patterns of sex, age, and season variations in plasma carotenoids through color charts and may also pose problems in interspecific studies. Our intraspecific analyses suggest that all species are not equally affected by hemolysis, which could lead to the absence of an interspecific relationship between visual estimations and actual carotenoid concentrations, even if we worked with a variability in plasma carotenoids higher than those found in populations of birds with large carotenoid displays (Bortolotti et al. 1996) and when considering larger numbers of species and sample sizes (J. L. Tella et al., unpublished data). In fact, the effects of hemolysis on other serum chemistry measurements are known to vary between species (see Andreasen et al. [1996] and references therein).

In conclusion, color charts may be used in some species, but only after testing its validity. In any case, the accuracy of the method would increase if undesirable plasma substances were removed with an organic solvent such as acetone. On the other hand, spectrophotometry is a straightforward and inexpensive laboratory method. For total carotenoid determination in plasma, a visible light spectrophotometer, or colorimeter, is needed, and this is commonly found in most laboratories. With adequate standards, results obtained with different spectrophotometers are comparable, even using different solvents and machines of variable resolution (see, e.g., Wellburn 1994). Color-chart matching, however, is influenced by a wide array of biases related to individual human vision (Endler 1990). More elaborate methods such as HPLC (see, e.g., Stradi et al. 1995) allow the precise identification and quantification of different types of carotenoids, but this may be unnecessary when the researcher focuses on total plasma carotenoids.

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Appendix

Species and Number of Sampled Birds Grouped by Order and Family

Superscript letters denote carotenoid-dependent coloration in [a]plumage, and [b]exposed integuments.

Order Ciconiforms. Family Threskiornithidae: *Plegadis chihi,* 2. Family Cathartidae: *Cathartes aura,* 13.

Order Charadriiforms. Family Charadriidae: *Charadrius semipalmatus,*[b] 2; *Calidris mauri,* 11.

Order Falconiforms. Family Accipitridae: *Parabuteo unicinctus,*[b] 2. Family Falconidae: *Falco sparverius,*[b] 68.

Order Galliforms. Family Phasianidae: *Callipepla californica,* 6.

Order Columbiforms. Family Columbidae: *Zenaida macroura,* 1; *Zenaida asiatica,* 2; *Columbina passerina,* 141.

Order Piciforms. Family Picidae: *Melanerpes uropygialis,*[a] 4; *Colaptes auratus,*[a] 1; *Picoides scalaris,* 1.

Order Passeriforms. Family Tyrannidae: *Pyrocephalus rubinus,*[a] 1. Family Troglodytidae: *Campylorhynchus brunneicapillus,* 12. Family Mimidae: *Toxostoma cinereum,* 2; *Mimus polyglottos,* 1. Family Emberizidae: *Pheucticus ludovicianus,*[a] 1; *Cardinalis cardinalis,*[a] 3; *Cardinalis sinuatus,*[a] 5; *Pipilo chlorurus,*[a] 4; *Pipilo fuscus,* 1; *Chondestes grammacus,* 14; *Zonotrichia leucophrys,* 50. Family Fringillidae: *Carpodacus mexicanus,*[a] 4. Family Passeridae: *Passer domesticus,* 6.

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