Carotenoid Coloration in Greenfinches Is Individually Consistent Irrespective of Foraging Ability

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Accepted 4/19/2007; Electronically Published 9/5/2007

ABSTRACT

Carotenoid-based plumage coloration of birds has been hypothesized to honestly reflect individual quality, either because carotenoids are difficult to acquire via food or because of a trade-off in allocation of carotenoids between maintenance and signaling functions. We tested whether differential foraging ability is a necessary precondition for maintaining individual differences in carotenoid-based plumage coloration in male greenfinches (*Carduelis chloris*). Wild-caught birds were brought into captivity, where half of them were supplemented with carotenoids while the other half was maintained on a carotenoid-poor diet. Color of the yellow parts of tail feathers, grown under natural conditions, was compared with that of the replacement feathers, grown in captivity. Carotenoid supplementation increased feather chroma (saturation). Color of wild-grown feathers significantly correlated with the color of lab-grown feathers. This result demonstrates the existence of a significant component of variation in carotenoid coloration, which reflects physiological qualities or genetic differences among individuals independent of foraging ability. Among both experimental groups, plasma carotenoid concentration during feather growth strongly correlated with chroma of the feathers grown in captivity. This indicates that carotenoid-based plumage coloration can reveal circulating carotenoid levels over a very wide range of concentrations, suggesting the ample signaling potential of such a mechanism.

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Physiological and Biochemical Zoology 80(6):663–670. 2007. © 2007 by The University of Chicago. All rights reserved. 1522-2152/2007/8006-0168$15.00
DOI: 10.1086/521084

Introduction

Many animals, particularly birds and fish, rely extensively on red, yellow, and orange carotenoid pigments for coloration of signal traits used in mate attraction and other types of social communication (reviewed in Lozano 1994; Olson and Owens 1998; Møller et al. 2000). Because animals cannot synthesize these pigments, they must be obtained through dietary sources (Goodwin 1950), which led researchers to propose that honesty of carotenoid-based signals is based on their limited availability for signalers (Endler 1980; Kodric-Brown 1985; Hill 1992). Under this hypothesis, individual differences in expression of color mainly result from differential foraging ability. This idea is especially appealing in the context of mate choice of bird species where a male's food-providing ability is a likely target of female choice. The hypothesis of foraging ability was contrasted by Hudon (1994), who proposed that carotenoids are probably not generally limiting in the environment and that the expression of color reflects individual physiological condition rather than foraging ability. Further challenges for the foraging-ability hypotheses emerged after Lozano (1994) emphasized important physiological maintenance functions of carotenoids, such as immunoenhancement, immunomodulation, detoxification, and free-radical scavenging. Under this view, carotenoid-based ornaments enable individuals to signal their past and/or current health state: carotenoids can be allocated to signaling only if and when they are not needed for maintenance purposes at the same time. This debate about the information content and function of carotenoid-based signals continues (Hill 1994, 1999; Olson and Owens 1998; Lozano 2001), and there is still no consensus about which particular individual qualities carotenoid displays most clearly reflect (e.g., Hill et al. 2002; Hartley and Kennedy 2004; McGraw et al. 2005).

Despite their different assumptions concerning carotenoid availability, the "foraging hypothesis" and the "health hypothesis" are difficult to tease apart (e.g., Linville and Breitwisch 1997). For instance, the fact that nutritional condition or infection status directly affects carotenoid ornaments does not necessarily mean that they do not affect foraging ability (e.g., Thompson et al. 1997; Hill 2002, 2006). Similarly, successful alteration of ornament expression by manipulation of dietary carotenoid access does not necessarily imply that the ornament expression is independent of other factors affecting physiological state (e.g., Hill et al. 2002; McGraw et al. 2005). On the other hand, there is no reason to expect that all differences in health state between individuals can be entirely ascribed to

factors determining foraging ability. This holds specifically for genetically determined parasite resistance. For instance, Hõrak et al. (2006*a*) showed that greenfinches maintained individually under uniform feeding conditions differed remarkably in resistance to standardized coccidian infection. In free-living American kestrels (*Falco spaverius*), carotenoid-dependent skin coloration predicted future (but not current) resistance to hematozoan parasites, and similar relationships have also been described in other bird species (Dawson and Bortolotti 2006). Ornament expression is assumed to reflect parasite resistance in models of sexual selection (Hamilton and Zuk 1982; Andersson 1994); thus, the question of whether an individual's health state is related to ornamentation directly or via indirect pathways is worthy of further experimental investigation.

Indirectly, the relative importance of the foraging and health hypotheses can be assessed by standardizing the foraging conditions in captive birds (Hill 1992). Assuming that carotenoid-based plumage coloration reflects especially long-term physiological aspects of individual quality (e.g., Thompson et al. 1997; Saks et al. 2003*b*), one may expect that individual differences in naturally obtained plumage color will retain the same rank order when ornamental feathers are removed and regrown under standardized conditions. This scenario thus implies that individuals that are able to grow relatively conspicuous carotenoid-colored feathers in one situation are also capable of growing such ornaments later in their life. Alternatively, if natural variation in carotenoid plumage coloration is exclusively caused by differential foraging ability of individuals, one should not detect a significant correlation between the color rankings of wild-grown feathers and those of birds reared under standardized feeding conditions. Tests of these predictions on two American passerine species have yielded mixed results (Hill 1992; McGraw and Hill 2001); thus, more research on the mechanisms underlying variation in carotenoid plumage coloration is warranted.

We tested the predictions of the foraging hypothesis and the health hypothesis with captive greenfinches. Greenfinches are medium sized (ca. 28 g), sexually dichromatic, gregarious, seed-eating passerines native to the western Palearctic region. The expression of carotenoid-based plumage coloration in male greenfinches is a sexually selected trait (Eley 1991). Chroma (saturation) of the yellow parts of tail feathers directly reflects the carotenoid content of those feathers, which primarily contain canary xanthophylls A and B (Saks et al. 2003*a*). To manipulate feather coloration, we provided half of the birds in our experiment with supplementary carotenoids in their drinking water. To study the individual consistency of plumage coloration, we removed one tail feather from all the birds before carotenoid supplementation and compared its color to that of the replacement feather grown during the experiment. In addition, we examined the extent to which the amount of circulating carotenoids during feather growth is reflected in the feather color and assessed the relationship between the natural coccidian infection intensity and feather color. Coccidians from the genus *Isospora* (Protozoa, Apicomplexa) directly inhibit the uptake of essential dietary components, including carotenoids, in the intestinal tract and consequently depress carotenoid-based pigmentation. Pathogenicity of isosporan coccidians (which can ultimately lead to the host's death) has been well documented (reviewed in Hõrak et al. 2004; Hill 2006). In the context of the vivid interest of animal ecologists in carotenoid-based ornaments as potential signals of phenotypic quality, such parasites should be especially suitable for the detection of mechanisms ensuring the honesty of signals. Plasma carotenoid levels are important health state indicators because of sensitivity to parasitism and potential immunoenhancing properties (Lozano 1994; Møller et al. 2000; Blount et al. 2003; McGraw and Ardia 2003).

Methods

Fifty-six male greenfinches were caught in mist nets in the Sõrve Bird Observatory on the island of Saaremaa (57°55′N, 22°03′E) on January 25 (day 0) and 26, 2005. Birds were transported to the lab and housed in individual indoor cages (27 cm × 51 cm × 55 cm) with sand bedding. The birds were supplied ad lib. with sunflower seeds (which are poor in carotenoids) and filtered tap water. During the study, birds were held on the natural day-length cycle. To minimize disturbance, lights were turned off during any visits to the aviary, and most procedures were done in the dark (with minimal light) before illumination or after the lights were turned off. Average temperature in the aviary during the experiment was 14.6° ± 1.2°C (SD), and average humidity was 55.5% ± 7.6% (SD). The experiment complied with the current laws of the Estonian Republic.

On the fourth day of captivity, the right outermost tail feather was plucked from all the birds for color measurement of naturally grown feathers. In the morning of day 15, the birds were divided into two equal (28 birds) treatment groups with similar average body mass at capture and age composition (10 first-year and 18 older birds in each group). Birds in the carotenoid supplementation group received a 10-μg/mL water solution of lutein and zeaxanthin (20 : 1, w/w), prepared from OroGlo liquid solution of 11-g/kg xanthophyll activity (Kemin Agri-Foods Europe, Herentals, Belgium). All solutions were freshly prepared each morning using filtered (Brita Classic, Brita, Taunusstein, Germany) tap water at 4°C and were provided in 30-mL doses in opaque dispensers in order to avoid oxidation of carotenoids. Unsupplemented birds received filtered tap water. On day 21 (7 d after the start of carotenoid supplementation), a blood sample of 100 μL was collected from tarsal or brachial veins for determination of the carotenoid concentrations during the regrowth of the tail feathers. (Yellow parts of these feathers are formed approximately between the fifteenth and twenty-second days of feather growth; L. Saks, unpublished data.) Plasma was stored at −75°C until analyzed. On the forty-eighth day of the experiment, when the replacement feathers

were fully grown, carotenoid supplementation was terminated, lab-grown feathers were plucked, and the birds were released into their natural habitat.

In the course of the study, birds were monitored for their individual levels of coccidian infection by fecal examination as described in Hõrak et al. (2006*a*, 2006*b*). Infection intensities were determined on days 4, 6, 8, 10, 12, 14, 16, 18, 20–22, 24–34, 36, 37, 39, 41, 44, 47, and 48 of the experiment. On days 24–30, all the birds received sulfonamide coccidiostatic treatment (sulfathiazole sodium pentahydrate, 2 g/L water) in their drinking water in order to equalize their infection status. The treatment, however, was not fully efficient, because by the end of treatment (day 25), 27% (15/56) of the birds were still shedding oocysts. The effect of treatment almost completely vanished by day 34, when 95% of birds (53/56) had started to shed oocysts again. For the analyses, individual infection intensities were averaged over the period between the fourth and twenty-fourth days of experiment (i.e., 12 measurements over 21 d), which coincided with the period of growth of replacement feathers (Hõrak et al. 2004). Average coccidian infection intensity over that period correlated with the average intensity at the end of experiment (from day 34 to day 48): $r_s = 0.70$, $P < 0.00001$, $N = 56$.

The most prevalent carotenoids in the plasma of greenfinches are lutein and its structural isomer, zeaxanthin (McGraw 2004). Concentration of carotenoids was determined spectrophotometrically (e.g., Tella et al. 1998; Bortolotti et al. 2000; Peters et al. 2004) using a microplate reader (Tecan Sunrise; Tecan Austria, Grödig/Salzburg, Austria) and acetone-resistant microtiter plates. One hundred fifty microliters of acetone was added to 15 μL of plasma and centrifuged for 10 min at 1,500 *g*. Absorbance of supernatant was measured at 449 nm, corresponding to the maximum absorbance of lutein in acetone (Zsila et al. 2001). Calibration curves were prepared using lutein (Sigma X 6250) as a standard. Repeatability (Lessells and Boag 1987) of carotenoid measurements between different microtiter plates was 0.95 ($F_{15,20} = 46.0$, $P < 0.0001$).

Feather color was measured in an approximately 1-mm^2 area of the visible carotenoid-pigmented surface of the feather using a spectrophotometer (Ocean Optics USB2000, with Ocean Optics DH2000 lamp). The measurements were taken from the standard position from the dorsal side of the feather on both vanes, about 5 mm inward (away, toward the body from the apex of the feather) from the end of the black-colored tip area of the feather (Fig. 1 in Saks et al. 2003*a*). Measurements were taken twice from both inner and outer vane of the feather, and the resulting four measurements were averaged to obtain total feather coloration estimates. Light was transferred to the feather through a quartz optic fiber (Ocean Optics), reaching the feather at 90°. The sampling optic was placed at 45° to the surface of the sample and connected to a spectrophotometer by a quartz fiber-optic cable. Data from the spectrophotometer were digitized and passed into a computer with appropriate software (OOIBase). The measurements were relative and referred to a standard white reference tile (WS-2) and to the dark. Each measurement provided a measure of reflectance for each 1-nm interval in the range of 400–700 nm. To estimate color, we calculated values of chroma according to Endler (1990). Chroma is a measure of the "purity" or "saturation" of a color and was calculated as follows:

$$\text{chroma} = \sqrt{(R - G)^2 + (Y - B)^2},$$

where $B = (Q_b/Q_t)$, $G = (Q_g/Q_t)$, $Y = (Q_y/Q_t)$, $R = (Q_r/Q_t)$; Q_r denotes the summed reflectance in the red area of the reflectance spectrum (between 625 and 700 nm), Q_g is summed reflectance in green area of the spectrum (between 475 and 550 nm), Q_y is summed reflectance in the yellow area of the spectrum (between 550 and 625 nm), Q_b is summed reflectance in the blue area of the spectrum (between 400 and 475 nm), and Q_t (brightness) is summed reflectance between 400 and 700 nm. The repeatabilities (Lessells and Boag 1987) for measurements of chroma were high, ranging from 0.91 to 0.98 ($F =$ 32–122, $P < 0.00001$ in all cases, $N = 50$–56). First-year and older birds did not differ in their plumage chroma before the experiment ($t_{54} = 0.56$, $P = 0.574$).

Values of infection intensity were ln transformed to obtain normality. The *P* values are for two-tailed tests. Sample sizes differ between some analyses for technical reasons. Mean trait values ± SD are presented.

Results

Color of the replacement feathers grown in captivity depended on both carotenoid supplementation and the original feather color (Table 1; Fig. 1). Correlation between the colors of wild-grown and lab-grown feathers was not different among carotenoid-supplemented and unsupplemented birds, as indicated by the nonsignificant interaction term in Table 1. Lab-grown feathers were paler (i.e., less saturated) than original ones in both the control (13.7% difference: wild-grown chroma 0.321 ± 0.012 vs. lab-grown chroma 0.277 ± 0.012; paired *t*-test: $t = 15.0$, $P < 0.0001$, $N = 24$) and the supplemented group (6.8% difference: wild-grown chroma 0.324 ± 0.014 vs. lab-grown chroma 0.302 ± 0.014; paired *t*-test: $t = 7.60$, $P <$ 0.0001, $N = 25$). Chroma of lab-grown feathers was 8.3% higher among carotenoid-supplemented birds than among controls ($t = 6.86$, $P < 0.0001$, $N = 49$).

Coccidian infection intensity did not predict the color of replacement feathers in the model accounting for the original feather color and the effect of carotenoid supplementation. However, when analyzed separately in both treatment groups, a negative correlation between infection intensity and chroma emerged among unsupplemented birds (Fig. 2). This relationship was not significant among carotenoid-supplemented birds (Fig. 2) or in the whole sample; neither did we detect a sig-

Table 1: Effect of carotenoid supplementation, coccidian infection intensity, and natural plumage color on chroma of lab-grown feathers

Factor	F_{df}	P
Carotenoid supplementation	$49.5_{1, 46}$	<.00001
Wild-grown chroma	$9.3_{1, 46}$	.004
Carotenoid supplementation × wild-grown chroma	$.7_{1, 45}$	.396
Infection intensity	$1.0_{1, 45}$	.312
Carotenoid supplementation × infection intensity	$2.4_{1, 44}$	.126

Note. Only significant factors were maintained in the final model; factors and interaction terms after the first two were entered into the final model one at time, with the exception of carotenoid supplementation × infection intensity interaction term, which was entered into the model with all main effects.

nificant carotenoid supplementation × infection interaction term ($F_{1,45} = 1.97$, $P = 0.167$) in a model with main effects of infection intensity ($F_{1,45} = 0.88$, $P = 0.353$) and carotenoid supplementation ($F_{1,45} = 0.07$, $P = 0.786$). For both groups, plasma carotenoid levels that were determined during feather growth straightforwardly predicted the color of replacement feathers (Fig. 3).

Discussion

Our experiment clearly showed (1) that dietary manipulation of carotenoid availability affects plumage coloration and (2) that despite the uniform foraging conditions of captive birds, natural plumage coloration still significantly predicts that of feathers grown in captivity. The first result is not surprising and compares favorably with that of many carotenoid supplementation experiments performed in captivity (see Hill 2006 for a review).

Our second main finding is more interesting because, to our knowledge, this is the second demonstration of individual consistency of carotenoid-based plumage coloration under uniform foraging conditions. We do not think that the correlation between the chromas of wild-grown and lab-grown feathers could be ascribed to a carryover effect of foraging in the wild, because in such a case we would have observed a stronger correlation among unsupplemented birds than among carotenoid-supplemented ones. This, however, was not the case (Fig. 1; Table 1). In a similar experiment performed by McGraw and Hill (2001), the winter plumage color of male American goldfinches (*Carduelis tristis*) also strongly predicted the extent to which they expressed breeding plumage pigmentation after completing their molt in captivity. In contrast, Hill (1992) did not detect any significant correlation between natural and lab-grown plumage coloration in male house finches (*Carpodacus mexicanus*). It should be noted, however, that the birds in Hill's study were fed fully oxidized 4-keto-carotenoids not present in the birds' normal diet and present only in small amounts in the normal species' plumage in the wild. An additional reason why the results obtained in greenfinches and goldfinches differ from those obtained in house finches may stem from differing biochemical pathways of carotenoid metabolism. In contrast to the hypervariable red-to-yellow plumage coloration of house finches, which contains a suite of 12 carotenoid pigments (Inouye et al. 2001), the plumage of greenfinches and goldfinches varies more subtly in yellow color and contains only two main yellow carotenoids (canary xanthophylls A and B), which are derived from circulating lutein and zeaxanthin by a simple dehydrogenation process (Saks et al. 2003*a*; McGraw et al. 2005).

The foraging-ability hypothesis has gained indirect support in several studies. For instance, Hill et al. (2002) found a correlation between carotenoid levels in gut contents during molt and plumage coloration in house finches. Similarly, nestling great tits (*Parus major*) that were more frequently fed with

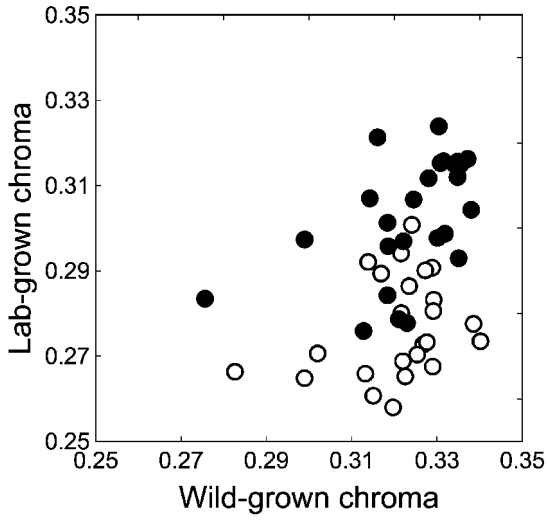

Figure 1. Relationship between the colors of wild-grown and replacement feathers grown in captivity. Filled circles are for carotenoid-supplemented birds; open circles are for unsupplemented birds. See Table 1 for statistics. The relationship between the colors of wild-grown and lab-grown feathers remains significant ($F_{1,44} = 5.94$, $P = 0.020$) after removal of the two individuals with lowest values of wild-grown chroma.

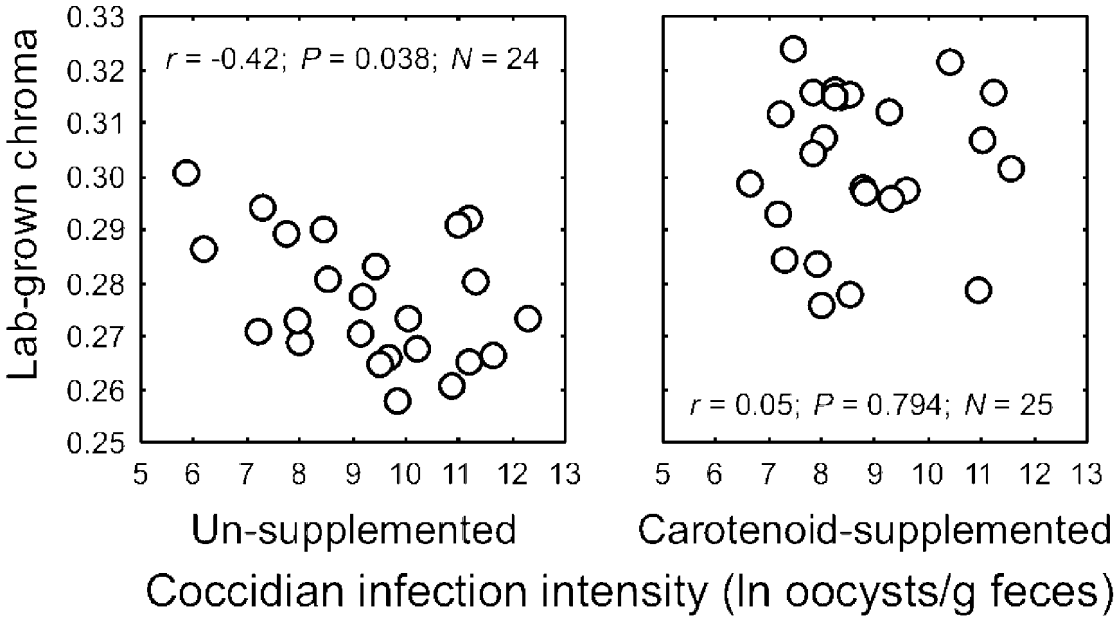

Figure 2. Relationship between coccidian infection intensity during feather growth and color of replacement feathers grown in captivity.

carotenoid-rich lepidopteran larvae (Slagsvold and Lifjeld 1985) or had higher larval densities in their territories (Eeva et al. 1998) developed yellower plumage coloration. In siskins (*Carduelis spinus*), expression of carotenoid ornaments correlated with behavioral indexes of foraging skills (Senar and Escobar 2002). Still other studies have demonstrated habitat and geographic (see Hill 2002) or interannual (Linville and Breitwisch 1997) correlations between carotenoid-rich food availability and variation of carotenoid ornaments at population level. On the other hand, several experiments have also convincingly shown that expression of carotenoid ornaments depends on parasitic infections (reviewed in Hill 2002, 2006; Hõrak et al. 2004), immune system activation (e.g., Blount et al. 2003; Faivre et al. 2003; McGraw and Ardia 2003), or individual nutritional state (Hill 2000; McGraw et al. 2005). Other studies have also shown that birds kept on a uniform diet do not converge on the same plasma carotenoid levels (e.g., Bortolotti et al. 1996; Negro et al. 2001).

Therefore, it seems that a multitude of exogenous and intrinsic factors usually interact to form carotenoid ornaments in birds, and given the strong support for both explanations, carotenoid-based color may more typically be a product of both limitations (Hill 2006). Our study, in line with the experiment on goldfinches (McGraw and Hill 2001), demonstrated a significant component of variation in carotenoid coloration that reflects physiological qualities of individuals independent of access to food. It is perhaps not incidental that such pattern was revealed in feather ornaments. Feathers possess the potential to be a particularly honest signal of condition because molt usually occurs several months before using feathers in sexual display. Thus, to be useful for mate choice, plumage coloration has to signal especially long-term aspects of individual quality (e.g., Thompson et al. 1997; Saks et al. 2003*b*), which probably covers many more diverse intrinsic properties than just foraging

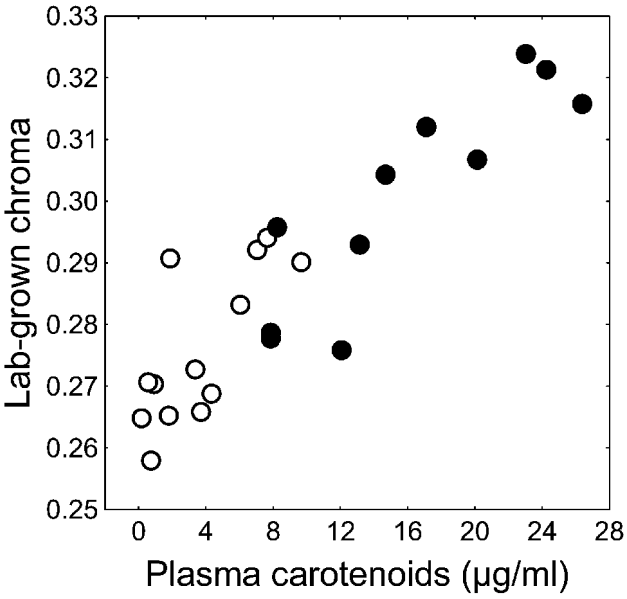

Figure 3. Relationship between plasma carotenoid concentration during feather growth and chroma of the yellow parts of tail feathers grown in captivity. For carotenoid-supplemented birds (*filled circles*): $r = 0.88$, $P = 0.0004$, $N = 11$. For unsupplemented birds (*open circles*): $r = 0.74$, $P = 0.004$, $N = 13$.

skills. We are not aware of the factors responsible for the individual consistency of plumage coloration in our study. First, it is possible that this pattern was caused by differential ability of individuals to store or remobilize carotenoids. Second, it is well known that infections by coccidian intestinal parasites reduce the absorption of carotenoids in birds, as has been experimentally demonstrated in greenfinches (Hõrak et al. 2004). In the current study, preexperimental infection intensities correlated negatively with plasma carotenoid levels, while carotenoid treatment did not affect the intensity of coccidiosis (Hõrak et al. 2006*b*). Unsupplemented birds with higher natural infection intensities grew replacement feathers with lower values of chroma (Fig. 2). This correlation, however, did not hold for the carotenoid-supplemented birds, and the interaction between carotenoid treatment and infection intensity was not significant. Thus, admitting the possible contribution of coccidian parasitism to individual variation in plumage coloration, we cannot consider it a single major factor responsible for maintaining that variation. It is equally possible that other microbial infections could have affected carotenoid absorption, transportation, or deposition patterns. Third, at least theoretically, we also cannot exclude the nonfunctional explanation that individual consistency of plumage coloration in greenfinches could be caused by genetic differences between individuals that are unrelated to "health" or physiological condition.

Another notable result of our study is that the color differences between the wild-grown and lab-grown feathers among both carotenoid-depleted and carotenoid-supplemented birds remained moderate (13.7% and 6.8%, respectively), as compared to the results of our previous study (Hõrak et al. 2004), where the lab-grown feathers of greenfinches had, on average, a 48.5% lower value of chroma than wild-grown feathers. We think that this difference is due to the time spent in captivity before feather removal. In the current experiment, birds started to grow their replacement feathers 3–4 d after capture, while in the previous study, they had spent 91–109 d in captivity before feather removal. Explanations for the greater reduction in color production with time in captivity involve increasing parasite loads, social stress, or missing nutrients. We acknowledge a limitation of this study, that regrowth of ornamental feathers was induced in January instead of the period of molt, which occurs from late July to early November (Cramp and Perrins 1994). Paler coloration of lab-grown feathers could thus, at least partly, result from the seasonal downregulation of physiological mechanisms for uptake and deposition of carotenoids. On the other hand, this experiment design enabled us to demonstrate that individual differences in carotenoid deposition into plumage persist even in a situation where the competition for carotenoids within the bodies of these birds is reduced. (By contrast with previous studies, where the birds performed a complete molt in captivity, our greenfinches were replacing just a single tail feather.)

Finally, we would like to draw attention to the relationship between plasma carotenoid levels and feather chroma. As can be seen in Figure 3, this relationship was very similar for the carotenoid-limited and carotenoid-supplemented birds, which supports the view that the processes of carotenoid transformation and deposition in greenfinches are indeed rather robust. Ecologically, this means that carotenoid-based plumage coloration in greenfinches signals circulating carotenoid levels over a very wide range of concentrations, which once more highlights the potential for honest signaling by such a mechanism. Biochemically, this result compares favorably with those of McGraw et al. (2005), who showed in a food restriction experiment in American goldfinches that the relative decline in pigment concentration found in the feathers matched that in the blood. However, greenfinches and American goldfinches use similar biochemical pathways to color their plumage, so it remains to be shown whether similar relationships between plasma carotenoid levels and plumage coloration also exist in another species with more complicated mechanisms of plumage color determination.

Acknowledgments

We thank the Sõrve Bird Observatory for providing facilities and Mati Martinson for the help in bird trapping. Indrek Ots helped us in many ways and performed plasma carotenoid analyses. Stefaan Van Dyck (Kemin Agrifoods Europe) kindly donated OroGlo carotenoid supplement. George Lozano and two anonymous referees provided constructive criticism on the manuscript. The study was financed by Estonian Science Foundation grant 6222 to P.H.

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