Carotenoid Plasma Concentration, Immune Profile, and Plumage Ornamentation of Male Barn Swallows (*Hirundo rustica*)

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ABSTRACT: Carotenoids exert immunomodulating, immunostimulating, and antioxidant actions in mammals and are major determinants of coloration in animals. Honest advertisement models of sexual selection propose that male ornaments, including coloration, are reliable indicators of male quality. Because of their simultaneous effects on male coloration and immunity, carotenoids might mediate the hypothesized relationship between the expression of epigamic coloration and parasitism in vertebrates. We analyzed the relationship between immune profile and concentration of lutein, the most abundant carotenoid in the plasma of male barn swallows (*Hirundo rustica*). Consistent with our predictions, lutein plasma concentration was negatively correlated with gamma-globulin plasma levels and concentration of selected leukocyte types in peripheral blood, suggesting that, to exert immune function, carotenoids are taken up from plasma, thus becoming unavailable for epigamic signaling. The coloration of red feathers of the throat of adult males was positively related to plasma concentration of lutein, but not with immunologic variables, consistent with the idea that more brightly colored males do not pay a larger immunological cost for their coloration compared with less brightly colored males. Length of male tail ornaments, which is currently under directional sexual selection, was positively correlated with lutein plasma levels. In species where carotenoids limit immune function, demands for pigments for sexual signaling might compete with those for immunity, thus generating a mechanism that enforces honesty on the signal.

Keywords: immunity, immunoglobulins, leukocytes, sexual selection, signaling.

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Many secondary sexual characters are extravagant traits with carotenoid-based coloration (e.g., Andersson 1994; Gray 1996). Female vertebrates have been shown to prefer males with a larger amount of bright carotenoid-based coloration (e.g., Endler 1983; Burley and Coopersmith 1987; Hill 1990, 1991, 1992, 1994; Milinski and Bakker 1990). The predominance of carotenoid-based sexual signals in vertebrates has led to speculation about the functional basis for the evolution of such signals. A relatively neglected possibility is that carotenoids are directly involved in host-parasite interactions and therefore are of functional importance for choosy females. Genetic models of parasite-mediated sexual selection (Hamilton and Zuk 1982) predict that condition-dependent male ornaments honestly reveal genetically based individual resistance to parasites via an effect of parasitism on condition. The mechanism(s) enforcing honesty on sexual signals, however, are unclear (Folstad and Karter 1992; Andersson 1994; Møller et al. 1998a). The possibility that carotenoids might mediate a mechanism of honest expression of male ornaments has been discussed by Zuk et al. (1990), Lozano (1994), and Skarstein and Folstad (1996).

Carotenoids are a large family of natural pigments synthesized mainly in plants and algae (Fox 1976, 1979; Brush 1978), and carotenoids found in animal tissues are therefore exclusively acquired through the diet. In vertebrates, carotenoids can be stored in skin, flanks, eyes, other organs such as the liver, and plasma (Goodwin 1984; Parker 1996). Together with melanins and porphyrins, carotenoids determine the chemical coloration of plumage as well as other tissues in birds, fish, reptiles, and mammals (e.g., Fox et al. 1967; Brush and Johnson 1976; Goodwin 1984; Hudon and Brush 1990; Hill et al. 1994; Stradi et al. 1995; Gray 1996).

The expression of carotenoid-based coloration is affected by parasitism. A negative correlation between parasitism and carotenoid coloration has been identified in

fish and birds (Milinski and Bakker 1990; Zuk et al. 1990; Houde and Torio 1992; Thompson et al. 1997; Hill and Brawner 1998; but see, e.g., Folstad et al. 1994; Skarstein and Folstad 1996). Several studies have shown that in chickens, carotenoid skin coloration and plasma concentration are negatively related to infection by pathogens (e.g., Bletner et al. 1966; Kowalski and Reid 1970; Yvore and Mainguy 1972; Ruff et al. 1974).

If there is a trade-off between expression of carotenoid-based male coloration and immunocompetence, we should expect a negative relationship between allocation of carotenoids to visual signals and immunocompetence, because parasitized individuals will allocate more carotenoids to immune function (Shykoff and Widmer 1996). Indeed, carotenoids have been associated with a multitude of functions related to immunity. They are reported to enhance T- and B-lymphocyte proliferative responses, stimulate effector T-cell function, enhance macrophage and cytotoxic T-cell capacities, increase the population of specific lymphocyte subpopulations, and stimulate the production of various cytokines and interleukins in mammals (see Bendich 1989; Chew 1993; van Poppel et al. 1993, and references therein). We assume here that they have similar effects in birds. For example, astaxanthin and lutein are known to enhance in vitro and in vivo antibody production to T-dependent antigens in mice (e.g., Jyonouchi et al. 1994 and references therein). Carotenoids are well known for their activity as oxygen-radical scavengers, acting as free radical traps and efficient quenchers of singlet oxygen, thus decreasing immunosuppressive peroxides (Bendich 1989). Hence, it might be speculated that allocation of carotenoids by parasitized individuals to the metabolic pathway of immunomodulation or antioxidant activity reduces the amount of carotenoids stored in the signal.

Our main aim in this study was to analyze covariation in concentration of plasma carotenoids, immune profile, and condition of male barn swallows (*Hirundo rustica*). We predicted that individuals with the largest activation of the immune system, that is, with large concentrations of gamma-globulins and leukocytes in peripheral blood, should have the smallest concentration of plasma carotenoids. The reason for this prediction is that healthy individuals should have relatively low circulating titers of gamma-globulins and leukocytes. Because males with long ornamental tails—a character under directional sexual selection—have been shown to have lower levels of parasite infestation (e.g., Møller 1994), a positive correlation between carotenoid plasma levels and size of tail ornaments was also expected. Finally, we predicted a positive relationship between the size of tail ornaments and carotenoid-based coloration of feathers if long-tailed males were in better condition than short-tailed males.

The barn swallow is a socially monogamous passerine that feeds on the wing on insect prey species (see Møller 1994) that have been reported to contain many carotenoid types (see Goodwin 1984, and references therein), including lutein, which is the predominant carotenoid in swallow plasma (see "Material and Methods"). Both male and female plumage consists of a metallic blue-black dorsal side, and white-to-rufous breast and belly feathers. Red-to-chestnut coloration of forehead, chin, and throat patch is mainly caused by melanin, although small amounts of lutein have also been found in these feathers (R. Stradi, unpublished data). Molt mainly occurs during winter, in Africa. Several experiments have shown that females prefer males with relatively long outermost tail feathers both as mates and extrapair fathers of their offspring (Møller 1994; see also Møller et al. 1998*c* for a recent review).

Material and Methods

This study was done in two colonies located southeast of Milano (northern Italy) during spring 1996 and included 41 mated males. Individuals were captured at sunrise in mist nets. At first capture we measured length of the left and right outermost tail feathers, length of the innermost tail feathers, left and right wing chord, length of bill, keel, and right tarsus (all in mm), and body mass (g). Tail and wing lengths were then expressed as the mean value of the left and right character. Body condition was expressed as body mass$^{1/3}$ divided by keel length. We also measured the level of infestation by two species of ectoparasitic mallophaga (*Machaerilaemus malleus*, *Myrsidea rustica*) (see Møller 1994). From each individual we took a blood sample in capillary tubes after puncture of the ulnar vein. Blood was stored in a cool bag in the field and then in a refrigerated room (4°C). Blood samples were then centrifuged for 10 min at 11,500 rpm. Plasma was subsequently stored at −30°C.

Assay of Lutein Concentration in Plasma

We extracted carotenoids from plasma with 4 mL of CH_3CN and sonication of the extract for 15 min. The extract was then filtered by a disposable syringe filter (0.2 μm) and evaporated. HPLC/UV-VIS analysis of extracted carotenoids was performed on a Gymcotek A110 instrument equipped with an isocratic Gymcotek pump, by using two sequential Lichrocart purospher RP-18 columns (250 × 4-mm i.d.) maintained at 40°C by a column block heater (model 7970 Hichrom Ltd., Reading). The mobile phase was CH_3CN/CH_3OH (70/30), and the flow rate was 0.8 mL/min. Samples were injected by using a Rheodyne 7125 valve equipped with a 20-μL loop. Data were acquired between 230 and 600 nm by using a Diode Array Detector

(HP 1050 series) with peak areas being integrated at λ 450 nm. Lutein concentration was estimated by an external standard method. As a result of technical limitations, for the plasma lutein concentration assay the 41 males included in this study had to be randomly assigned to two different groups containing 20 and 21 individuals, respectively, which were therefore analyzed in two different assays. In a separate assay we measured the within-assay repeatability of lutein plasma concentration by splitting in two parts the blood samples collected from 10 individuals and analyzing the two parts separately. Within-assay lutein concentration measures were found to be highly repeatable ($R = 0.94$, $F = 33.43$, $n = 10$ birds, $P < .0001$; Falconer and Mackay 1996). Hence, within-assay measurements of lutein concentration appeared to be highly reliable. We have no firm explanation for the difference in mean lutein concentration observed for the males in the two assays (see "Results"). It should be emphasized, however, that all the laboratory analyses have been done by the same experienced chemist, with exactly the same equipment and analytical protocol, and samples were assigned to either assay randomly. However, we statistically controlled for the difference between the two assays by entering assay as a factor in the analyses involving lutein concentration.

Leukocyte Counts and Concentrations

Leukocytes and red blood cells were counted by an experienced person after blood smears had been air dried and stained by using the May-Grunwald-Giemsa staining method. Blood smears were scanned at ×630 magnification by using standard routines. In each microscopic field we counted red blood cells and leukocytes classified as lymphocytes, monocytes, eosinophils, heterophils, and basophils. In each smear we counted 95–160 leukocytes and the corresponding red blood cells. This allowed us to calculate the number of leukocytes of the different types per 10,000 red blood cells (absolute counts) and an index of concentration of leukocyte types, calculated as the product of absolute counts and hematocrit.

Protein and Gamma-Globulin Assay

Protein assay was done by spectrophotometric analysis of diluted small amounts of plasma by using the Bio-Rad protein microassay procedure. Gamma-globulins were assayed by densitometric analysis after electrophoretic separation of plasma proteins on agarose gels (Paragon SPE Kit, Beckman Instruments) (Saino et al. 1997). The relative concentration of gamma-globulins was expressed as the percentage of the densitometric profile occupied by the gamma-globulin region. The absolute concentration was expressed as the product of plasma protein concentration

and the concentration of gamma-globulins relative to total plasma proteins.

Colorimetry

Analysis of plumage color was performed on two feathers, plucked from a standard position in the center of the throat, by using a spectroradiometer (Ocean Optics Europe). Color was measured in an area of the visible surface of the feather ~1 mm^2 wide and situated at 1 mm from the distal end of the feather. The illuminant was a halogen light source (DH 2000). The light was transferred to the feather through a quartz optic fiber (Ocean Optics) and reached the feather at 90°. The sampling optic was placed at 45° to the surface of the sample and was connected to a spectrometer (S2000) by a second quartz fiber-optic cable. Data from the spectrometer were converted into digital information by DAQ Card 700 and passed into a computer with appropriate software (Spectrawin 3.1). The measurements were relative and referred to a standard white reference tile (WS-2) and to the dark. For each feather we obtained a measure of transmittance for each 1-nm interval in the interval 400–700 nm, and values were averaged between the two feathers before being transformed into absorbance values ($= -\log_{10}$[transmittance value]). Because lutein absorbance peaks at 450 nm (Stradi et al. 1995), absorbance as a result of this carotenoid was calculated from mean transmittance data in the interval 445–455 nm with the same equation. Absorbance data were used for subsequent analyses (see "Results").

We tested the consistency of measurement of absorbance in the lutein band in a number of different tests by means of repeatability analyses (Falconer and Mackay 1996). First, we measured three consecutive times on the same date the absorbance of a single feather and found a high repeatability ($R = 0.92$, $F = 36.89$, $n = 96$ birds, $P < .0001$). Second, we measured the absorbance of the same feather on two different days and found a significant repeatability ($R = 0.56$, $F = 3.56$, $n = 18$ birds, $P = .0053$). Third, we measured two feathers from the central part of the badge and found a significant repeatability ($R = 0.88$, $F = 15.60$, $n = 114$ birds, $P < .0001$). Finally, we measured a feather from the central, the right, and the left part of the badge and found a significant repeatability ($R = 0.67$, $F = 7.13$, $n = 48$ birds, $P < .0001$). Thus all measurements were reliable.

Results

Lutein Plasma Concentration and Male Condition

Analyses of lutein plasma concentration of males on the basis of the two assays gave qualitatively similar results.

Table 1: ANCOVA of lutein plasma concentration and immune system variables, protein plasma concentration, and body condition

	F	P	Slope
Absolute leukocyte count:			
Basophils	1.79	NS	−.84
Heterophils	9.98	.003	−3.16
Eosinophils	7.24	.01	−2.97
Lymphocytes	1.02	NS	−7.10
Proteins:			
Gamma-globulins/plasma proteins			
(%)	13.44	.001	−3.51
Concentration of gamma-globulins	5.21	.03	−2.42
Protein plasma concentration	.56	NS	1.55
Body condition	.41	NS	−.001

Note: Body condition is expressed as body mass$^{1/3}$ divided by the keel length. See "Material and Methods" for measurement units. Carotenoid assay was entered as a factor. df = 1, 38 in all analyses.

Mean lutein plasma concentration was significantly different between the two groups to which the 41 males had to be randomly assigned for lutein assay (first group: 3.02 μg/mL [SE = 0.18], $n = 20$; second assay: 2.38 μg/mL [SE = 0.20], $n = 21$; $t = 2.40$, df = 39, $P = .02$). Individuals considered in the two assays did not differ in any feature of their morphology, immune profile, or age (t values always associated with $P > .05$), thus suggesting that males in the first and second group were sampled from the same statistical population. To control for the effect of assay, we included assay as a factor in all analyses involving lutein plasma concentrations. ANCOVA of lutein plasma concentration in which assay (first or second) was entered as a classification factor did not show any significant effect of calendar date or age ($P > .20$ in both cases).

Absolute counts of heterophils and eosinophils in peripheral blood were significantly negatively correlated with lutein concentration (table 1; equation of the lines fitted to heterophil counts: males in the first assay: count = 17.961 [SE = 6.02] − 4.130 [SE = 1.926] × lutein concentration, $F = 4.60$, $R^2 = 0.20$, $n = 20$ males, $P = .046$; second assay: count = 10.65 [SE = 2.224] − 2.415 [SE = 0.877] × lutein concentration, $F = 7.59$, $R^2 = 0.29$, $n = 21$, $P = .013$; equation of the lines fitted to eosinophil counts: first assay: count = 16.37 [SE = 5.136] − 2.807 [SE = 1.645] × lutein concentration, $F = 2.91$, $R^2 = 0.14$, $n = 20$, $P = .11$; second assay: count = 14.89 [SE = 3.881] − 3.097 [SE = 1.529] × lutein concentration, $F = 4.10$, $R^2 = 0.18$, $n = 21$, $P = .057$), whereas nonsignificant negative relationships were found for lymphocytes and basophils, respectively (table 1). Qualitatively similar results were obtained when concentrations of leukocyte types, rather than absolute counts, were considered (results not shown). Gamma-globulin level relative to plasma proteins was significantly negatively correlated with lutein concentration (table 1; equations of the lines fitted to gamma-globulin level: males in the first assay: gamma-globulin level = 32.06 [SE = 3.463] − 3.52 [SE = 1.108] × lutein concentration, $F = 10.07$, $R^2 = 0.36$, $n = 20$ individuals, $P = .005$; second assay: gamma-globulin level = 31.475 [SE = 3.889] − 3.5 [SE = 1.532] × lutein concentration, $F = 5.22$, $R^2 = 0.22$, $n = 21$, $P = .03$), and a qualitatively similar relationship was found between absolute gamma-globulin concentration and lutein concentration (table 1). However, lutein concentration was weakly and nonsignificantly correlated with plasma protein concentration (table 1). These significant relationships persisted also when we controlled for the effect of age for immunological variables (heterophil absolute counts and concentrations) that varied with age.

Lutein Plasma Concentration and Male Morphology

An ANCOVA with group of males as factor showed a significant positive association between mean length of outermost tail feathers and lutein plasma concentration (tail length was missing for one individual; $F = 7.86$, df = 1, 37, $P = .008$, coefficient = 0.050; fig. 1). However, no significant correlation between any of the other morphological characters measured and lutein plasma con-
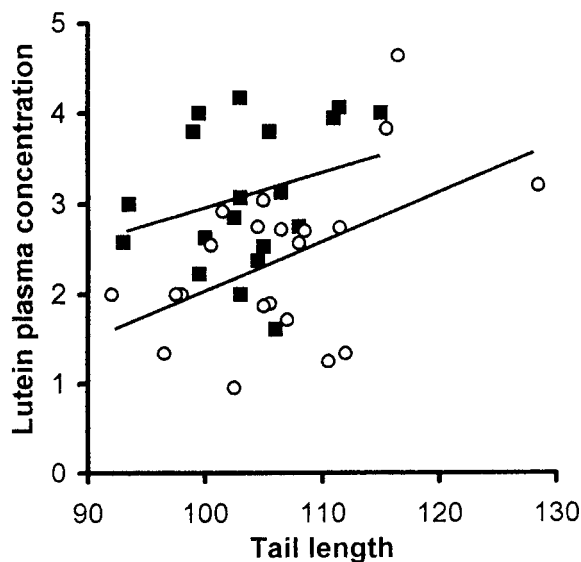

Figure 1: Lutein plasma concentration (μg/mL) during the breeding season in relation to tail length (mm) of male barn swallows. The regression lines for the two different assays have the following equations: first assay: lutein concentration = −1.292 [SE = 3.341] + 0.042 [SE = 0.032] × tail length, $F = 1.72$, $R^2 = 0.09$, $n = 19$ individuals, $P = .21$; second assay: lutein concentration = −3.368 [SE = 2.376] + 0.054 [SE = 0.022] × tail length, $F = 5.89$, $R^2 = 0.24$, $n = 21$. $P = .025$.

centration was found (*F* values always associated with *P* > .05).

Absorbance of light by the red throat feathers in the spectrum around the wavelength of lutein (445–455 nm), which is proportional to the amount of lutein included in the feathers, significantly positively covaried with concentration of lutein in the plasma of the males (fig. 2; $F = 16.17$, df = 1, 33, $P < .001$, coefficient = 0.053). In addition, absorbance in the lutein light absorption band was significantly positively correlated with male tail length ($r = 0.53$, df = 34, $P < .001$), although it was not significantly correlated with the size of other morphological characters (*r* values always associated with *P* > .05). Absorbance of light by the red throat feathers in the total spectrum significantly positively covaried with concentration of lutein in the plasma of the males ($F = 13.93$, df = 1, 33, $P = .001$, coefficient = 0.037). This finding demonstrates that individuals with high lutein plasma concentrations had overall greater absorbance.

Feather Color and Immunological Variables

Absolute count and concentration of the different leukocyte types, respectively, were not significantly correlated with absorbance in the lutein band (absolute counts: *r* values from −0.01 to −0.32; concentrations: *r* values from −0.004 to −0.31; $n = 36$, $P > .05$ for all correlations). Absorbance in the lutein band was significantly negatively correlated with relative concentration of gamma-globulins but not with absolute concentration (relative concentration: $r = -0.38$, $n = 36$, $P = .025$; absolute concentration: $r = -0.21$, $n = 36$, NS). However, no significant correlations existed between absorbance in the lutein band and immunological variables while partialling out the effect of lutein plasma concentration.

No significant correlations were found between intensity of infestation by two species of ectoparasites and feather coloration or, respectively, lutein plasma concentration (results not shown).

Discussion

Carotenoid concentration in the plasma of male barn swallows was negatively correlated with measures of immunological condition. It seems to be established that both eosinophils and heterophils have a major function in immune response to parasite infestation in vertebrates (e.g., Tizard 1991; Pastoret et al. 1998). Gamma-globulins are the source of antibodies involved in immune response to parasitic infestations and protozoan, bacterial, and viral infections (DeVaney and Augustine 1988; Russell and Ezeifka 1995; Tizard 1991; Pastoret et al. 1998).

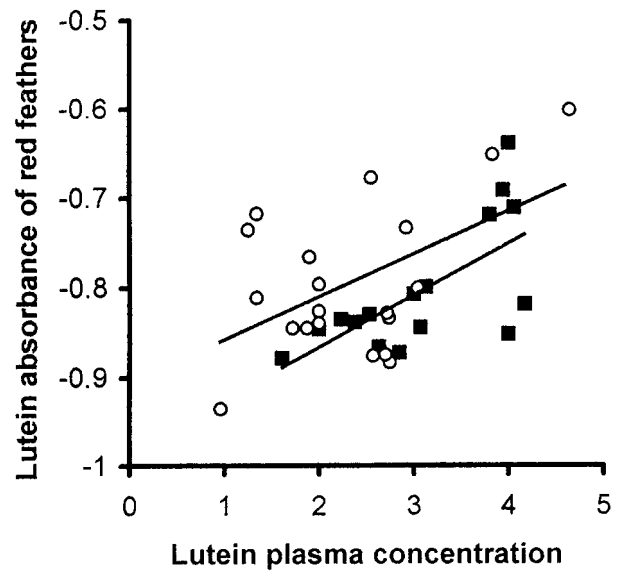High levels of eosinophil, heterophil, and gamma-glob-

Figure 2: Absorbance of light around the wavelength of lutein (445–455 nm) for red throat feathers of male barn swallows in relation to lutein plasma concentration (μg/mL) during the breeding season. The regression lines for the two different assays have the following equations: first assay: absorbance = −0.986 [SE = 0.055] + 0.059 [SE = 0.017] × lutein concentration, $F = 11.77$, $R^2 = 0.46$, $n = 16$ individuals, $P = .004$; second assay: absorbance = −0.907 [SE = 0.048] + 0.048 [SE = 0.019] × lutein concentration, $F = 6.36$, $R^2 = 0.26$, $n = 20$, $P = .021$.

ulin concentration in peripheral blood are likely to reflect infection by pathogens and parasitism (Tizard 1991; Pastoret et al. 1998). The negative correlation of lutein plasma concentration with variables reflecting the activation of the immune system is consistent with our predictions. If turnover or metabolism of carotenoids is increased in infected individuals, perhaps because of use for their immuno-modulating or immunostimulating activities, infection should result in a reduction of plasma carotenoids. Indeed, experimental infection of chickens by coccidia resulted in decreased concentration of carotenoids in peripheral blood as well as visible tissues (e.g., Yvore and Mainguy 1972; Ruff et al. 1974), consistent with the idea of recruitment of carotenoids from storage tissues for immune function.

However, the correlational nature of this study does not allow inferences about the causative effects behind the relationships between immune profile and lutein plasma levels, and alternative interpretations should therefore be considered. Carotenoids may simply be acquired through the diet in proportion to food intake but have no specific effect on immune function, resulting in better-fed males being free from parasites and also having relatively large concentrations of carotenoids in their plasma. This hypothesis is not particularly likely because carotenoids have

been shown to exert a specific effect on several components of immunity in most studies (see introduction), and we found no indication of a positive relationship between carotenoid plasma concentration and individual body condition. Foraging experience, as influenced by age, could also affect carotenoid plasma concentration. If relatively old individuals are, on average, healthier than young birds as a result of selection against individuals in poor condition, then the correlations observed might be determined by the effect of age. This is also unlikely because we could show no effect of age on lutein concentration. Similarly, if carotenoid availability in insect prey declines during the season and arrival date is negatively correlated with health status, then a negative correlation between lutein levels and immunological variables could arise because of a seasonal effect. This hypothesis is also contradicted by the lack of significant correlation between blood sampling date and lutein levels.

We found positive correlations between feather coloration and lutein concentration, respectively, and tail length, which is a secondary sexual character subject to a directional mate preference (Møller 1994; Møller et al. 1998a). These correlations are unlikely to result from long-tailed males being better able to select lutein-rich food. Indeed, it is known from correlational and experimental studies that a long tail imposes a predation and a flight cost to its bearer (Møller 1994; see Møller et al. 1998c). Alternatively, long-tailed males may have larger lutein plasma concentration when smaller amounts are taken up for their specific activity, because such individuals are relatively free from disease. If this is the case, our results are compatible with the Hamilton and Zuk (1982) hypothesis positing that long-tailed males are healthy, and that a female preference for these males might have the function of acquiring good genes for parasite resistance.

Feather coloration in the lutein absorption band developed in the African winter quarters was found to be positively correlated with plasma concentration of lutein during the breeding season. These relationships indicate a consistency in carotenoid content during the annual cycle and suggest that there are marked differences among individuals in their availability and/or utilization of carotenoids. If relative carotenoid concentrations are consistent among males during the annual cycle, and if males have to sacrifice immune function to allocate more carotenoids to feather coloration, we should predict a negative correlation between feather absorbance in the lutein band and immunological variables. However, this turned out not to be the case in this study, because no correlation between immunological variables and feather color was observed after controlling for lutein plasma concentration. This finding is consistent with handicap models of parasite-mediated sexual selection (Hamilton and Zuk 1982), because more brightly colored males apparently did not pay more in terms of immunological condition compared with less colored males.

Previous studies of the barn swallow have identified multiple secondary sexual characters that all are positively correlated and independently influence female choice. These characters include length and symmetry of outermost tail feathers, size of the white tail spots of tail feathers, song rate, and color of red throat feathers (review in Møller 1994; Møller et al. 1998b; Kose and Møller 1999). In particular, the coloration of the red throat feathers is related to male mating status in a pairwise comparison of mated and unmated males first captured on the same date (which is a good approximation of arrival date), with mated males being more brightly colored than unmated males (P. Ninni, N. Saino, and A. P. Møller, unpublished data). Given the relatively strong positive correlations between the different sexual signals, we can assume that they all reflect aspects of the same underlying phenotypic quality. This observation supports the suggestion that red feather coloration will be indirectly affected by sexual selection through correlated responses to selection on other traits, and perhaps even directly through selection on color per se.

Our results suggest a simple mechanism ensuring that male coloration honestly signals health. Individuals with no or few parasites will allocate larger amounts of carotenoids to plumage coloration than will parasitized males, without paying the costs of reduced immune function. Carotenoid-based feather coloration will therefore signal to choosy females the amount of carotenoid that could be allocated to plumage, at the time of feather development, after concomitant demands of carotenoids or their metabolites for immune function have been met (Skarstein and Folstad 1996). Larger allocation of carotenoids to plumage would therefore indicate better health and allow females to choose males with no or few parasites.

In conclusion, the results we presented in this study might serve as a basis for elucidating the mechanisms enforcing honesty on carotenoid-based sexual signals because they suggest a simple pathway for the causal relationship between parasitism and male color as mediated by carotenoid effects on both ornamentation and immunity.

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