The Auk 123(4):1161–1171, 2006

CAROTENOIDS, IMMUNITY, AND INTEGUMENTARY COLORATION IN RED JUNGLEFOWL (*GALLUS GALLUS*)

Kevin J. McGraw[1] and Kirk C. Klasing

Department of Animal Science, University of California at Davis, One Shields Avenue, Davis, California 95616, USA

Abstract.—There is widespread interest in the roles that carotenoids play as yolk and shank pigments, antioxidants, and immune-system regulators in chickens, but nothing is known of such functions in the wild ancestors of chickens—the Red Junglefowl (*Gallus gallus*). We manipulated carotenoid access in the diet of captive male and female Red Junglefowl to investigate its effects on the coloration of the red comb and buff-brown legs and beak as well as on several indices of immunocompetence. Comb, leg, and beak did not differ in coloration between control and carotenoid-supplemented groups; in fact, biochemical analyses showed that, unlike in chickens, leg and beak tissue contained no carotenoids. Carotenoids showed variable effects on immunological performance, boosting the potency of whole blood in males to kill bacterial colonies, while inhibiting the ability of macrophages to phagocytize bacterial cells and having no significant effect on the accumulation of haptoglobin—an acute-phase protein whose production was induced by a simulated infectious challenge with lipopolysaccharide. These results bring into question interpretations of the evolutionary significance of carotenoid-based and sexually dichromatic shank coloration in domestic chickens, which was apparently derived through artificial selection, and suggest that carotenoids can exert different, mechanism-specific actions on the many lines of immune defense in birds. *Received 1 July 2005, accepted 4 January 2006.*

Key words: carotenoid pigmentation, chickens, diet, *Gallus gallus*, immunocompetence, Red Junglefowl, sexual selection.

Carotenoides, Inmunidad y Coloración Integumentaria en *Gallus gallus*

Resumen.—Existe un amplio interés en el papel que tienen los carotenoides como pigmentos de la yema y parte inferior de las patas, así como también en antioxidantes y reguladores del sistema inmunológico en gallinas, pero nada se sabe sobre estas funciones en el ancestro silvestre de las gallinas, *Gallus gallus.* Manipulamos el acceso a carotenoides en la dieta de machos y hembras en cautiverio de *G. gallus* para investigar los efectos sobre la coloración roja de la cresta y coloración café de las patas y el pico, como así también en varios índices de inmuno-competencia. La cresta, las patas y el pico no difirieron en coloración entre el grupo control y el suplementado con carotenoides. De hecho, los análisis bioquímicos mostraron que, contrariamente a lo observado en las gallinas, los tejidos de las patas y el pico no contienen carotenoides. Los carotenoides mostraron efectos variados sobre el desempeño inmunológico. En los machos, impulsaron el potencial del tejido sanguíneo para matar colonias bacterianas, mientras que inhibieron la habilidad de los macrófagos de fagocitar células bacterianas y no tuvieron un efecto significativo sobre la acumulación de haptoglobina—una proteína de fase aguda cuya producción

[1]Present address: School of Life Sciences, Arizona State University, Tempe, Arizona 85287, USA. E-mail: kevin.mcgraw@asu.edu

fue inducida por una infección simulada con lipo-polisacáridos. Estos resultados cuestionan las interpretaciones del significado evolutivo de la coloración con carotenoides y de la coloración di-cromática sexual de la parte inferior de las patas en gallinas domesticas, la cual aparentemente derivó a partir de selección artificial y sugiere que los carotenoides pueden ejercer acciones mecánicas específicas y diferentes sobre las variadas líneas de defensa inmunológica en aves.

There has been considerable interest during the past decade in the health-revealing function of carotenoid-based colors in birds (Olson and Owens 1998, Møller et al. 2000). Diet-derived carotenoids serve important antioxidant and immunoregulatory roles in many animals (reviewed in Chew and Park 2004), and their incorporation into avian feathers and bare parts for sexually attractive coloration (Hill 1999) raises the possibility that individuals advertise their health status with their colors (Lozano 1994). Several empirical studies conducted within the past three years have tested this hypothesis, and most have found support for the idea that carotenoids boost immune performance in colorful birds (Fenoglio et al. 2002, Blount et al. 2003, McGraw and Ardia 2003, Alonso-Alvarez et al. 2004, Peters et al. 2004). However, there are a few notable cases in which carotenoids have not boosted health (e.g., Navara and Hill 2003, McGraw and Ardia 2005), and these exceptions call for additional studies in more species to better understand when and how carotenoids provide immune benefits to birds.

The first avian studies showing immunomodulatory effects of carotenoids were performed with domestic chickens (*Gallus gallus domesticus*; hereafter "chickens"; reviewed in Møller et al. 2000). High dietary supplies of carotenoids in chickens enhance antibody production (McWhinney et al. 1989) and the phagocytic activity of neutrophils (Mazurkiewicz et al. 1990) and discourage tumor growth (Nishino et al. 1992). Moreover, chickens display carotenoid-based coloration in their beak and legs (shanks; Koutsos et al. 2003a, b), and this color is responsive to health state (Tyczkowski et al. 1991). There are obvious commercial reasons why studies of carotenoids, immunity, and pigmentation in chickens are useful, but they may be of limited value for understanding the evolutionary significance of carotenoids in a sexual-signaling context. Better models would be the wild ancestors of chickens—Red Junglefowl (*Gallus gallus*; hereafter "junglefowl") from Asia. The enlarged, fleshy red combs of male junglefowl have been well studied as a sociosexual signal of androgen titers and health state (Ligon et al. 1990, Zuk et al. 1995, Zuk and Johnsen 2000, Parker and Ligon 2003), but to date nothing is known of the role that carotenoids play as immunomodulators or as integumentary colorants in junglefowl.

We experimentally investigated the relationship between carotenoids, immunity, and coloration in a captive group of junglefowl, derived from the same stock used recently to sequence the chicken genome (the UCD001 line; International Chicken Genome Sequencing Consortium 2004). We supplemented the base diet of a treatment group of individually housed male and female junglefowl with carotenoids for six weeks, and after that time compared their integumentary (beak, comb, and leg) coloration and three measures of innate immunity—bactericidal efficiency, bacterial phagocytosis, and acute-phase-protein production in response to a simulated bacterial challenge—with those of a control group fed only the base diet. We focused on indices of innate immunity because recent results in chickens indicate that this branch of the immune system is especially influenced by carotenoids, compared with adaptive immunity (Selvaraj et al. 2006). We also performed biochemical analyses to determine the types and amounts of carotenoids present in the red comb and buff-brown beak and legs of all birds after the study.

Methods

Housing and feeding animals.—Birds were housed in March and April 2004 in individual poultry pens at the Hopkins Avian Facility on the campus of the University of California, Davis. Birds were provided water and commercial chicken feed (Layena 6501, Purina Mills, St. Louis, Missouri; following Koutsos et al. 2003b), which contains some carotenoids (~8 mg per kilogram of feed; K. J. McGraw and K. C. Klasing unpubl. data), for *ad libitum* consumption. We randomly divided 14 male

and 14 female junglefowl into treatment and control groups of 7 birds of each sex. To the base Layena diet of the treatment group, we added 38 mg xanthophyll carotenoids (93% lutein, 7% zeaxanthin) per kilogram of pellet diet, in the form of dry Oro-Glo (Kemin, Des Moines, Iowa). We selected this dietary level and time course because in chickens it is sufficient to elevate circulating and integumentary carotenoid levels (Koutsos et al. 2003a, b) and is well below the upper limit of the physiological range (~100 mg kg^{-1}; Marusich and Bauernfeind 1981, McGraw 2005a).

Survey of innate immunity.—We chose three assays of innate immunity that encompass several constitutive and inducible components of this branch of the immune system. On 21 April, after the six-week carotenoid-supplementation period, we drew 300 μL blood from each bird via the jugular vein and examined the bactericidal activity of the whole blood and the capacity of cellular components to phagocytize bacteria. These assays require fresh whole blood, so the samples were transported back to the lab for use within 30 min of collection. The first assay tests the efficiency of whole blood in killing *Escherichia coli* bacteria (E^{power} microorganisms ATCC no. 8739, 10^7, MicroBioLogics, St. Cloud, Minnesota), as described previously by Tieleman et al. (2005). Briefly, ~200 *E. coli* were added to whole blood and medium (CO_2 independent medium + 4 mM glutamine + 5% heat-inactivated fetal calf serum), to give a final dilution of 1:10, and incubated at 41°C for 15 min. After incubation, we transferred 75-μL aliquots of each sample to two 4% tryptic soy agar plates, dispersed the solution homogeneously across the plate with a sterile glass spreader, and incubated the plate overnight at 37°C. We returned to count the number of bacterial colonies per plate and determined average (for the two plates per bird) killing efficiency in comparison with control plates prepared with only medium and *E. coli*. Killing efficiency was highly repeatable for our duplicate samples ($R_i = 0.77$, $F = 5.2$, df = 27 and 28, $P < 0.001$; see Lessells and Boag 1987), so we used averages in our statistical analyses.

The second assay run on this post-treatment blood sample tested the ability of macrophages to phagocytize introduced microorganisms. Here, standard amounts of fluorescently labeled *Staphylococcus aureus* (Wood strain without protein A; Alexa Fluor 488 conjugate, Molecular Probes, Eugene, Oregon) were incubated with blood, and fluorescent microscopy was used to count the frequency with which macrophages phagocytized these particles. *Staphylococcus aureus* were first suspended in tissue-culture-grade PBS with 2 mM sodium azide at a dilution of 20 mg mL^{-1} and stored at 4°C in the dark before use. One hundred milligrams of blood was diluted 1:20 in the aforementioned medium + 1% 100× penicillin-streptomycin (GIBCO, Invitrogen, Carlsbad, California), and 66 μL of the blood–medium mix was added to 250 μL of *S. aureus* (diluted to a 0.67% solution in sterile CO_2 independent medium) in the wells of a 96-well plate. The plate was covered with foil and incubated (in the absence of CO_2) for 15 min at 41°C, after which it was placed on ice for 5 min to stop phagocytosis. Wells were washed gently twice with 300 μL refrigerated, sterile CO_2 independent medium, and then with 300 μL methanol on ice for 5 min. *Staphylococcus aureus* were then visualized at 60× with fluorescence microscopy at 505/513 nm (excitation/absorption) in a dark room. We counted the first 100 individual macrophages that had adhered to the well and determined the percentage of cells that phagocytized a fluorescent bacterium. Assays were performed in duplicate, and repeatability was moderately high ($R_i = 0.49$, $F = 2.9$, df = 27 and 28, $P = 0.004$), so we used averages in our statistical analyses.

Our final immune assay involved the simulation of an infectious challenge in live junglefowl, through the administration of lipopolysaccharide (LPS), an inflammatory component of the cell-wall of gram-negative bacteria that induces an acute-phase response (Koutsos et al. 2003a). Among the many immunological changes associated with the inflammatory response, levels of the acute-phase protein haptoglobin increase to remove heme released by damaged cells (which can be cytotoxic when it intercalates into cell membranes; Tolosano and Altruda 2002) and render iron reserves unavailable to invading, nutrient-seeking pathogens. Following the protocol used by Koutsos et al. (2003a) for chickens, we collected 300 μL whole blood via the jugular vein on 23 April (to determine prechallenge levels of haptoglobin) and then injected 0.5 mL of a 1-mg mL^{-1} saline solution of LPS (Sigma-Aldrich, St. Louis, Missouri) subcutaneously. We returned 24 h later to collect 0.5 mL blood via

cardiac puncture for postchallenge haptoglobin titers, as well as to collect comb, beak, and leg tissue from freshly euthanized birds for pigment analysis (see below). Haptoglobin levels were assayed in duplicate from plasma using a commercially available kit (PHASE haptoglobin colorimetric assay, Tri-Delta Diagnostics, Morris Plains, New Jersey) and an ELISA plate reader. Repeatability of the haptoglobin assay was moderately high ($R_i = 0.43$, $F = 2.5$, df = 27 and 28, $P = 0.01$), and averages (mg mL^{-1}) were used in statistical analyses.

Measuring plasma carotenoids.—On 8 March, the day before we began experimental feeding, we drew 100 μL blood from the wing vein of each bird to determine pretreatment plasma-carotenoid levels. We again obtained a blood sample for carotenoid analysis after the six-week feeding period (21 April) and after injecting lipopolysaccharide (24 April), to ensure that our diet treatment had its intended effect as well as to test whether this *in vivo* simulated infectious challenge decreased circulating carotenoid levels. Plasma was stored at –80°C until carotenoids were extracted with organic solvents (see McGraw and Ardia 2003). Extracts were then dissolved in 200 μL methanol, and we injected 50 μL of each sample into a Waters Alliance 2695 high-performance liquid chromatographic (HPLC) system (Waters, Milford, Massachusetts) fitted with a Develosil RPAqueous RP-30 column (250 × 4.6 mm; Nomura Chemical, Aichi, Japan) and a built-in column heater set at 30°C. We used a three-step gradient solvent system to analyze both xanthophylls and carotenes in a single run, at a constant flow rate of 1.2 mL min^{-1}: first, isocratic elution with 50:46:4 (v/v/v) methanol: acetonitrile:dichloromethane for 11 min, followed by a linear gradient up to 50:15:35 (v/v/v) methanol:acetonitrile:dichloromethane through 21 min, held isocratically at this condition until 30 min, and finishing with a return to the initial isocratic condition from 30 to 48 min. Data were collected from 250–600 nm using a Waters 2996 photodiode array detector. We identified pigments by comparing their respective retention times and absorbance maxima with those of authentic reference carotenoids run as external standards. Lutein and zeaxanthin were the only carotenoids present in all plasma samples (λ_{max} = 447 and 453 nm, respectively; retention times = 7.6 and 8.1 min, respectively). Total carotenoid concentration was determined using EMPOWER, version 5.0 (Waters, Milton, Massachusetts), by adding lutein and zeaxanthin peak areas at lambda-max and fitting these to an external standard curve.

Scoring integumentary colors.—If the red comb or buff legs and beak of junglefowl were colored by carotenoids, we expected that carotenoid supplementation would deepen the color of these integumentary tissues. Moreover, because the size of the combs in males is health-dependent, our treatment may have increased comb size in treatment birds compared with controls. After our six-week feeding regime, we scored these integumentary features using digital photography. Leg coloration was scored both before (on 8 March) and after (on 21 April) our experiment to account for potential pre-experimental differences between groups and to examine color change with respect to diet treatment; beaks and combs were photographed only after the experiment. Birds were photographed in lateral profile in a standard region of the housing room under natural light using the autofocus function of a PowerShot S30 digital camera (Canon USA, Lake Success, New York). Because there may have been slight lighting and distance-to-image differences among photographs, we included in each a color and size standard. Images were imported into PHOTOSHOP CS, version 8.0 (Adobe, San Jose, California), at a resolution of 2,048 × 1,536 pixels. First, we adjusted all photographs so that the color and size of the standard matched predetermined (with a ruler and spectrophotometer) actual values. The body region of interest was then selected with the lasso tool, and we used RGB values from the histogram palette to calculate hue, saturation, and brightness scores (using the Color Picker function); number of pixels occupied by the comb was also determined with the histogram palette, as our measure of comb size. We took two photographs of the comb, beak, and legs of each bird, and all of our measurements of ornamentation were significantly repeatable (in most cases, >0.8; Table 1). We used the average of the hue, saturation, brightness, and comb-size values from each bird in our statistical analyses.

Analyzing tissues for carotenoids.—To determine the carotenoid content of integumentary tissues, we excised ~50 mg of beak tissue, ~25 mg of leg scales, and ~250 mg of comb from all freshly euthanized animals at the end of the

TABLE 1. Repeatabilities of scoring beak color, leg color, comb color, and comb size in Red Junglefowl from two photographs per body region. Hue is measured in degrees around a 360-degree color wheel, with red arbitrarily set at 0°. Saturation and brightness are both measured as percentages, with saturation capturing spectral purity and brightness capturing amount of light reflected.

Measure	Pre- or post-treatment?	R_i	F [a]	P
Leg hue	Pre-treatment	0.84	11.6	<0.0001
Leg saturation	Pre-treatment	0.99	76.5	<0.0001
Leg brightness	Pre-treatment	0.90	18.9	<0.0001
Leg hue	Post-treatment	0.61	4.1	0.0006
Leg saturation	Post-treatment	0.93	28.1	<0.0001
Leg brightness	Post-treatment	0.89	16.8	<0.0001
Beak hue	Post-treatment	0.51	3.1	0.0060
Beak saturation	Post-treatment	0.85	12.3	<0.0001
Beak brightness	Post-treatment	0.85	12.6	<0.0001
Comb hue	Post-treatment	0.84	11.6	<0.0001
Comb saturation	Post-treatment	0.87	14.1	<0.0001
Comb brightness	Post-treatment	0.87	14.7	<0.0001
Comb size	Post-treatment	1.00	316.8	<0.0001

[a] df = 27 and 28.

study (24 April). These were frozen at –80°C prior to analysis one year later. We extracted lipids from tissues by adding 2 mL methanol in a zirconia jar and grinding the tissue with a zirconia grinding ball using a Retsch MM 200 mixer mill (Retsch, Newtown, Pennsylvania) for 15–30 min (until fully ground) at 30 Hz. The solution was then transferred to a 5-mL glass tube and centrifuged at 2,500 RPM for 3 min, after which we removed the supernatant and evaporated the solvent to dryness under a stream of nitrogen. We resuspended the extract in 200 μL methanol and injected 50 μL into the HPLC for analysis, as described above.

Statistical analyses.—We used two-way analyses of variance (ANOVA) to examine the effects of treatment (carotenoid supplementation), sex, and the treatment-by-sex interaction on plasma-carotenoid status, integumentary coloration, and immunocompetence. We log-transformed any non-normally distributed variables to meet assumptions of parametric statistics. Again, for all comparisons in the study, $n = 7$ for each treatment group.

RESULTS

Effect of carotenoid supplementation on plasma-carotenoid status.—Before the experiment, carotenoid concentration of plasma did not differ between dietary treatment groups (F = 0.98, df = 1 and 24, P = 0.33; Fig. 1). There was a sex difference in plasma-carotenoid concentration at the start of the study (F = 13.9, df = 1 and 24, P = 0.001; F = 1.7, df = 1 and 24, P = 0.21 for the treatment*sex interaction), with females (mean ± SE: 2.03 ± 0.22 μg mL^{-1}) circulating 65% more than males (1.23 ± 0.06 μg mL^{-1}). After carotenoid supplementation, however, there was a significant diet-treatment effect on plasma-carotenoid concentration (F = 5.6, df = 1 and 24, P = 0.03;

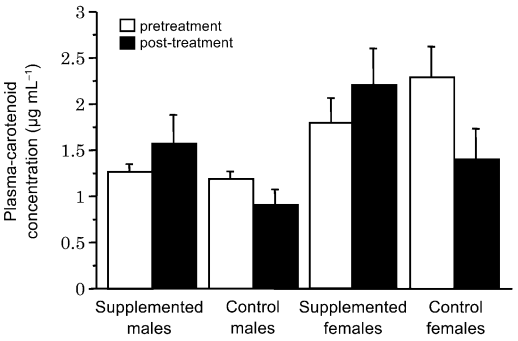

FIG. 1. Response of plasma carotenoids to a carotenoid feeding experiment in captive Red Junglefowl. Control males and females were fed a base pelleted chicken diet, and supplemented birds were given a dose of powdered carotenoids that were sprinkled among the food pellets. Bars show mean + SE.

Fig. 1). Plasma carotenoid concentration was significantly higher (72% and 58%, respectively) in supplemented males (1.55 ± 0.32 μg mL^{-1}) compared with control males (0.90 ± 0.17 μg mL^{-1}) and in supplemented females (2.21 ± 0.39 μg mL^{-1}) compared with control females (1.40 ± 0.33 μg mL^{-1}). The effects of sex ($F = 3.5$, df = 1 and 24, $P = 0.07$) and the treatment*sex interaction ($F = 0.06$, df = 1 and 24, $P = 0.81$) were not significant. The same patterns were true for the pre- to post-treatment change in plasma carotenoids (treatment: $F = 6.1$, df = 1 and 24, $P = 0.02$; sex: $F = 1.6$, df = 1 and 24, $P = 0.22$; treatment*sex: $F = 0.38$, df = 1 and 24, $P = 0.54$).

Effect of carotenoids on immunocompetence.—We found no significant effect of sex ($F = 0.04$, df = 1 and 24, $P = 0.84$) or diet treatment ($F = 0.26$, df = 1 and 24, $P = 0.61$) on bactericidal efficiency, but there was a significant treatment*sex interaction ($F = 6.8$, df = 1 and 24, $P = 0.02$; Fig. 2). *Post hoc* pairwise tests revealed that there was no significant treatment effect in females (Fisher's protected least-significant-difference test [PLSD], $P = 0.17$), but that whole blood from carotenoid-supplemented males killed more *E. coli* colonies than blood from control males (Fisher's PLSD test, $P = 0.04$). There was a significant effect of diet treatment on macrophage phagocytosis ($F = 5.7$, df = 1 and 24, $P = 0.03$), but in the direction opposite to that of the bactericidal-activity assay (Fig. 2); macrophages from treatment birds phagocytized fewer fluorescent bacterial particles than did those from control junglefowl (effect of sex: $F = 0.21$, df = 1 and 24, $P = 0.65$; treatment*sex interaction: $F = 1.16$, df = 1 and 24, $P = 0.29$). Lastly, there was no effect of treatment ($F = 2.0$, df = 1 and 24, $P = 0.17$) , sex ($F = 1.1$, df = 1 and 24, $P = 0.30$), or treatment*sex interaction ($F = 0.53$, df = 1 and 24, $P = 0.47$) on haptoglobin production in response to an LPS challenge (Fig. 2). Interestingly, however, when we compared plasma-carotenoid levels before and after the LPS challenge, carotenoid concentration declined significantly (by an average of 28%; paired *t*-test, $t = 4.3$, $P = 0.0002$; Fig. 3). There were no dietary treatment ($F = 0.34$, df = 1 and 24, $P = 0.56$), sex ($F = 3.7$, df = 1 and 24, $P = 0.07$), or dietary treatment*sex interaction ($F = 1.08$, df = 1 and 24, $P = 0.31$) effects on the LPS-induced change in plasma carotenoid levels.

Effect of carotenoids on integumentary coloration.—Leg hue, saturation, and brightness did not differ between treatment groups before the experiment, nor were there sex differences in these scores or a treatment*sex interaction (Table 2). After carotenoid supplementation, we found no significant effects of diet treatment on any color measure for any integumentary tissue (leg, beak, comb), nor was there an effect on comb size or on the pre- to post-treatment change in leg hue, saturation, or brightness (Table 2). The only significant effects we found were sex differences in post-treatment leg

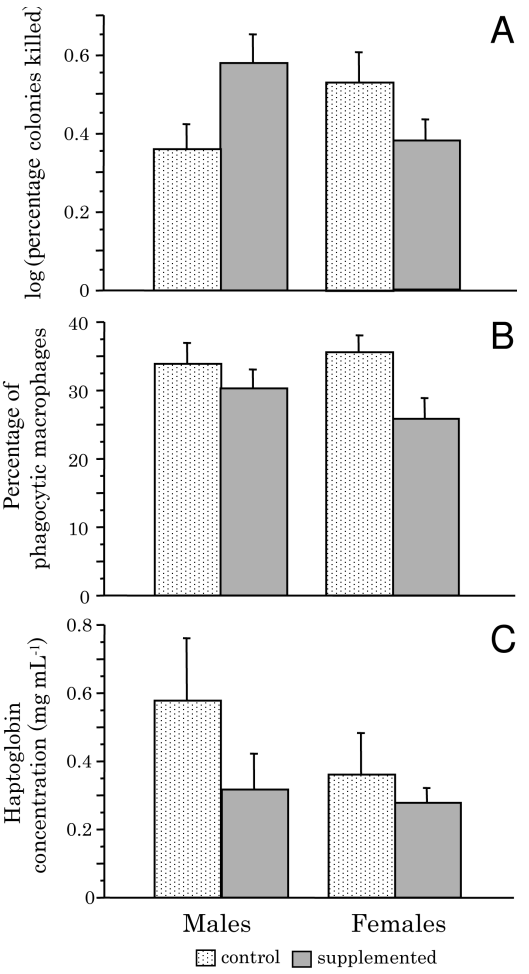

Fig. 2. Effect of carotenoid supplementation on three measures of immunocompetence in male and female Red Junglefowl: (A) *in vitro* bactericidal efficiency, (B) *in vitro* bacterial phagocytosis, and (C) *in vivo* production of acute-phase proteins (haptoglobin) in response to a simulated infectious challenge (lipopolysaccharide). Bars show mean + SE.

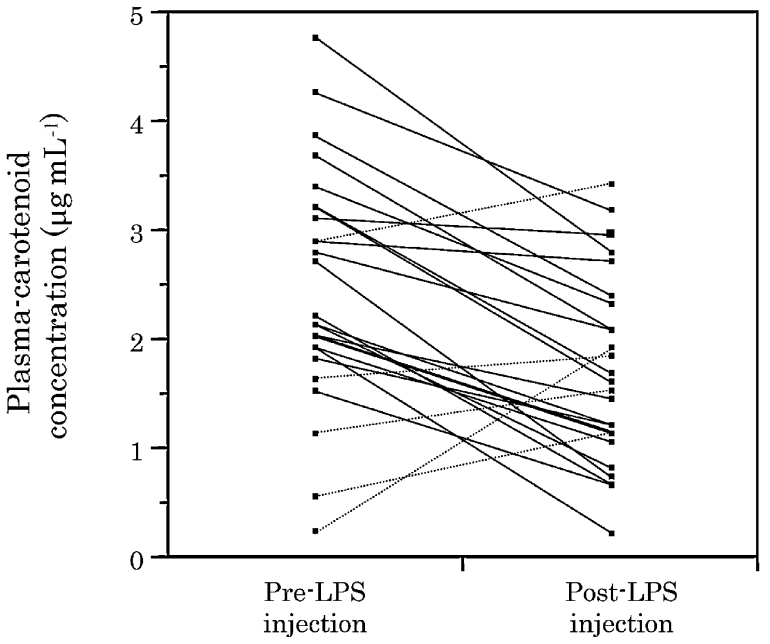

FIG. 3. Effect of LPS-induced acute-phase response on plasma carotenoid levels in Red Junglefowl. Data points and lines are for individual birds and show the changes in carotenoid levels that each bird experienced as a result of the LPS challenge. Dashed lines indicate the few ($n = 5$) birds whose plasma carotenoid levels increased in response to the treatment, whereas solid lines signify birds whose carotenoid levels diminished after the immune challenge.

TABLE 2. Results from two-way ANOVAs comparing the effects of dietary-carotenoid treatment and sex on measures of integumentary coloration in Red Junglefowl. Statistically significant effects are indicated with asterisks. Recall that $n = 7$ for all four treatment groups in all comparisons. See Table 1 for more information about color measures.

	Treatment		Sex		Treatment*sex	
Color measure	F [a]	P	F [a]	P	F [a]	P
Pretreatment leg hue	1.74	0.20	0.41	0.5300	0.02	0.90
Pretreatment leg saturation	0.84	0.37	3.05	0.0900	0.04	0.84
Pretreatment leg brightness	1.67	0.21	0.24	0.6300	0.33	0.57
Post-treatment leg hue	0.02	0.90	1.04	0.3200	0.89	0.35
Post-treatment leg saturation	0.03	0.86	7.02	0.0100*	1.37	0.25
Post-treatment leg brightness	3.65	0.07	8.00	0.0100*	0.78	0.39
Change in leg hue	1.36	0.26	0.00	0.9800	0.17	0.68
Change in leg saturation	0.81	0.38	0.18	0.6700	0.48	0.50
Change in leg brightness	0.09	0.76	4.13	0.0500	0.83	0.37
Post-treatment beak hue	0.13	0.72	2.81	0.1100	0.83	0.37
Post-treatment beak saturation	1.55	0.23	0.26	0.6100	0.91	0.35
Post-treatment beak brightness	1.35	0.26	0.02	0.9000	0.00	0.97
Post-treatment comb hue	0.91	0.35	32.10	<0.0001*	0.37	0.55
Post-treatment comb saturation	2.25	0.15	29.80	<0.0001*	0.02	0.88
Post-treatment comb brightness	0.55	0.47	1.92	0.1800	0.19	0.67
Post-treatment comb size	2.12	0.16	747.00	<0.0001*	2.94	0.00

[a] df = 1 and 24.

saturation and brightness, with males having more saturated and brighter legs than females, and in comb hue, saturation, and size, with males having redder, more saturated, and larger combs than females (Table 2).

After the experiment was over, we proceeded to use HPLC to analyze the carotenoid content of beak, skin, and comb tissue from all control and supplemented birds. In combs, we detected trace amounts of the yellow dietary carotenoid lutein (0.36 ± 0.16 μg carotenoid per gram of comb), which we presume came from blood in the excised tissue. No red carotenoids were detected. No carotenoids were detected in beak or leg tissue from any animal. The minimum detection limit of our analyses was 0.01 μg per gram of tissue.

Discussion

We experimentally investigated the relationship between carotenoids, innate immunity, and coloration in male and female junglefowl. Our aims were to determine (1) whether carotenoids can modulate immunity in the wild ancestor of chickens, (2) whether distinct immune responses may be differentially sensitive to carotenoids, and (3) whether colorful red and yellow-brown integumentary tissues in junglefowl are carotenoid-containing, responsive to carotenoid supplementation and, thus, perhaps reflective of an individual's immunocompetence. We found that carotenoids boosted the bactericidal efficiency of blood in male junglefowl. The bactericidal activity of whole blood is attributable to both humoral and cellular factors (Keusch et al. 1975). Complement, natural antibodies, and a variety of other pathogen-recognition proteins are important humoral components of this activity in whole blood, whereas phagocytosis by macrophages, heterophils, and thrombocytes are primary cellular components. A likely immunoprotective mechanism for carotenoids in this system is antioxidant action that protects these proteins or immune cells from oxidative damage and, thus, permits them to kill bacteria (Alonso-Alvarez et al. 2004). Many cells also have receptors for proteins such as complement, so carotenoids may also enhance immune-cell signaling cascades by binding to such receptors and regulating gene expression (e.g., Nikawa et al. 1995, Sharoni et al. 2002).

Although we demonstrated that carotenoids increased bactericidal activity of whole blood in male junglefowl, the same was not true of females. The lack of effect in females may result from females having higher carotenoid levels than males and, thus, being able to allocate sufficient carotenoids to meet the demands of this immune response regardless of treatment group. Alternatively, the sexes may prioritize different lines of immune defense in relation to the different ecological, endocrinological, and parasitological pressures they face (Zuk and McKean 1996). Thus, male junglefowl may have prioritized constitutive innate immunity more than females in our study and thus devoted more carotenoids to such a defense (see Koutsos et al. [2003a] for evidence of tissue prioritization of carotenoids in immune-challenged chickens). Compared with the wealth of information on sex differences in adaptive immunity (as revealed by traditional tests using phytohaemagglutinin [PHA] or lymphocyte counts; reviewed in Zuk and McKean 1996), next to nothing is known of sex differences in innate immunity (e.g., natural antibodies, phagocytes, acute-phase proteins) in birds.

In our *in vivo* immune assay, control junglefowl performed equally well in response to a simulated infectious challenge with lipopolysaccharide as carotenoid-supplemented birds. This, combined with recent studies, supports the notion that carotenoids do not provide benefits for all immune defenses in birds. Studies of Zebra Finches (*Taeniopygia guttata*; McGraw and Ardia 2005) and chickens (Haq et al. 1995, 1996; Okotie-Eboh et al. 1997) similarly report some significant and some nonsignificant effects of carotenoids on various immune parameters (also see Navara and Hill [2003] for no effect of carotenoids on three immune parameters in male American Goldfinches [*Carduelis tristis*]). Altogether, these results underscore the need to better understand how carotenoids can affect different measures of immunity. Selecting standard, reliable, and biologically relevant and interpretable techniques for probing molecular and cellular mechanisms of immunity will be invaluable for identifying how and why carotenoids are only occasionally immunomodulatory in birds and other animals.

In our third immune test, carotenoid-replete birds had macrophages that phagocytized significantly fewer bacterial particles than control individuals. To our knowledge, this is the first

study to document negative effects of carotenoid enrichment on immunity in birds. Carotenoids are known to act as toxic pro-oxidants, rather than as antioxidants, under certain circumstances, such as at unusually high concentrations (reviewed in Lowe et al. 2003). Preliminary tests in captive American Goldfinches suggest that these birds may experience liver damage when provisioned with pharmacological doses of dietary xanthophylls (Huggins et al. 2005). Although we have several reasons to believe that we did not deliver globally toxic levels of carotenoids to supplemented junglefowl (e.g., levels in blood were lower than levels in nearly all other avian species studied to date [McGraw 2005a] and 3× lower than the physiological range for chickens [Marusich and Bauernfeind 1981]), at this time we cannot speak to the specific antioxidant or pro-oxidant sensitivities of different immune responses. Thus, these other studies and our results suggest the potential for carotenoid toxicity in avian systems and underscore the need to know the physiologically relevant doses of dietary carotenoids for various parts of the immune system in each study species.

Carotenoid supplementation had no effect on color expression of the beak, legs, and comb of junglefowl, but this turned out to be quite unsurprising, because no carotenoids were detected in these colorful tissues. Decades of dietary and physiological studies on beak and shank coloration demonstrate its carotenoid basis in domestic chickens (Marusich and Bauernfeind 1981), so the absence of carotenoids in their wild ancestors indicates that chickens acquired carotenoid-derived integumentary coloration through a process of artificial selection. To our knowledge, no studies have been performed on the functional significance of carotenoid coloration in chickens, but clearly any future work on this topic should bear in the mind the history of domestication behind the expression of this trait. Junglefowl instead use melanins to color their legs and beak (K. J. McGraw and K. C. Klasing pers. obs.), and the sex difference in leg coloration that we have newly uncovered here suggests that there may have been historical sexual-selection pressures for melanin-based sexual dichromatism that were capitalized on and transferred (via artificial selection) to a carotenoid-dependent trait.

The absence of red carotenoids in combs also further supports the idea that we cannot always judge a pigment by its color (McGraw et al. 2004). Many have assumed red, orange, and yellow traits to be carotenoid-based in birds, without solid biochemical evidence (reviewed in McGraw 2005b). Our HPLC analyses of comb extracts instead revealed the presence of hemoglobin (with an absorbance maximum of 400 nm). Previous studies show that the size of blood-filled combs signals health state in male junglefowl (Zuk and Johnsen 2000), but our experiment demonstrates that it is not through a carotenoid-facilitated mechanism. Instead, perhaps it is the antioxidant potential of heme (reviewed in McGraw 2005c), like that of carotenoids, that reinforces the health-signaling role of junglefowl combs and other blood-based signals (e.g., nestling mouth flushes).

Acknowledgments

All procedures reported here were approved by the Institutional Animal Care and Use Committee at the University of California, Davis (UC-Davis) (protocol no. 6476). We thank G. Hu, J. Pisenti, J. Bennett, and C. Uytingco for assistance with animal care and blood collection, J. Bennett for help with running the immune assays, O. Crino for assistance with HPLC data collection, and P. Nolan, A. Dufty, Jr., and two anonymous referees for helpful comments on the manuscript. This research was funded by the U.S. Department of Agriculture (grant to K.C.K.) and by the Department of Animal Science at the UC-Davis. K.J.M. was supported by the School of Life Sciences and College of Liberal Arts and Sciences at Arizona State University during data analysis and manuscript preparation.

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Associate Editor: A. M. Dufty, Jr.