Notes and Comments

Do Dietary Antioxidants Alleviate the Cost of Immune Activation? An Experiment with Greenfinches

Peeter Hõrak,[1,*] Lauri Saks,[1,†] Mihkel Zilmer,[2,‡] Ulvi Karu,[1,§] and Kersti Zilmer[2,‖]

1. Institute of Zoology and Hydrobiology, Tartu University, Vanemuise 46, 51014 Tartu, Estonia;
2. Department of Biochemistry, Tartu University, Ravila 19, 50411 Tartu, Estonia

Submitted November 27, 2006; Accepted May 9, 2007; Electronically published August 17, 2007

Online enhancements: appendixes.

ABSTRACT: Reactive oxygen and nitrogen species produced by metabolism and immune defenses can cause extensive damage to biomolecules. To counteract this damage, organisms rely on exogenous and endogenous antioxidants, although their relative importance in maintaining redox balance is unclear. We supplemented captive greenfinches with dietary antioxidants—carotenoids and vitamin E—and injected them with an inflammatory agent, phytohemagglutinin. Compared to controls, immune-challenged birds circulated more lipid peroxidation products but also increased total plasma antioxidativity. Carotenoid (but not vitamin E) supplementation generally reduced lipid peroxidation, but this did not compensate for the effects of immune activation. Levels of an endogenous antioxidant—uric acid—strongly contributed to plasma antioxidativity. We found no evidence that dietary antioxidants are immunostimulatory. These results demonstrate the antioxidant function of carotenoids in birds and show that simultaneous assessment of oxidative stress-driven damage, antioxidant barrier, and individual antioxidants is critical for explaining the potential costs of immune system activation.

Keywords: immune challenge, malondialdehyde (MDA), phytohemagglutinin, plasma carotenoids, total antioxidant capacity, uric acid.

* Corresponding author; e-mail horak@ut.ee.

† E-mail: slauris@ut.ee.

‡ E-mail: zilmer@ut.ee.

§ E-mail: ulvika@ut.ee.

‖ E-mail: kerstiz@ut.ee.

Am. Nat. 2007. Vol. 170, pp. 625–635. 0003-0147/2007/17004-42240$15.00.
DOI: 10.1086/521232

Cellular metabolism generates reactive oxygen and nitrogen species (RONS) that can damage lipids, proteins, and nucleic acids (Halliwell and Gutteridge 1999). To counteract harmful effects of excess RONS, organisms rely on a complex antioxidant network that includes endogenously produced enzymes and low-molecular-weight compounds and exogenous, food-derived antioxidants. The situation where RONS overwhelm the antioxidant defenses is defined as oxidative stress (OxS; Halliwell and Gutteridge 1999). Understanding how organisms adjust their antioxidant defenses to cope with OxS is important for several major areas of evolutionary ecology. It is believed that OxS represents the main proximate mechanism driving the aging process (Beckman and Ames 1998), and diversity of antioxidant defense systems in animals may explain evolutionary patterns of metabolic rates (reviewed by Perez-Campo et al. [1998]). Also, because RONS generation is accelerated by a high work load, OxS may increase with reproductive effort (Alonso-Alvarez et al. 2004*b*; Wiersma et al. 2004). The concept of OxS is also relevant to host-parasite coevolutionary research because production of RONS in the course of an immune response is an important source of immunopathology, which contributes to the costs of immunity (von Schantz et al. 1999; Slettstoesser and Schuff-Werner 2002). Finally, many types of animal pigments (McGraw 2005), particularly carotenoid-based ones (Lozano 1994), possess antioxidative properties and thus have the potential to honestly reveal the ability of their bearers to cope with OxS (von Schantz et al. 1999). Recently, however, the antioxidant role of carotenoids has been debated by Hartley and Kennedy (2004), who proposed that carotenoid-based traits might instead signal the availability of other nonpigmentary antioxidants that protect carotenoids from oxidation and thereby make them available for sexual displays.

Despite the prominence of the topic, attempts by animal ecologists to assess the importance of OxS and antioxidant defenses in vertebrate models have progressed slowly. Since the publication of a seminal paper by von Schantz et al. in 1999, only a handful of studies have managed to address

these issues. The few successful examples in birds include demonstrations that embryos are protected from oxidative damage by maternally derived carotenoids (Blount et al. 2002*a*, 2002*b*; McGraw et al. 2005*a*) and that reduced antioxidant protection is associated with increased reproductive effort (Alonso-Alvarez et al. 2004*b*; Wiersma et al. 2004; Bertrand et al. 2006*a*). On the other hand, attempts to demonstrate the antioxidant function of carotenoids in the postembryonic stage have been unsuccessful (Alonso-Alvarez et al. 2004*a*; Isaksson et al. 2005; Costantini et al. 2006; Hõrak et al. 2006; Tummeleht et al. 2006; Cohen et al. 2007). Studies aimed at assessing whether immune system activation leads to oxidative damage have yielded contradictory results. Bertrand et al. (2006*b*) showed that immune challenge of zebra finches (*Taeniopygia guttata*) with bacterial lipopolysaccharide (LPS) reduced the antioxidant barrier of blood. In the same model system, Alonso-Alvarez et al. (2004*a*) did not detect the effect of immune challenge on the blood antioxidant barrier, although immune activation diverted carotenoids from plasma. Contrary to this, Costantini and Dell'Omo (2006) found that in the nestlings of Eurasian kestrels (*Falco tinnunculus*), immune challenge with phytohemagglutinin (PHA) resulted in increased circulating levels of carotenoids, in addition to increasing the levels of reactive oxygen metabolites and decreasing the serum antioxidant barrier. Hõrak et al. (2006) did not detect any effects of repeated humoral immune challenge with sheep red blood cells on the plasma antioxidant barrier or carotenoid levels in captive greenfinches (*Carduelis chloris*).

The diversity of outcomes of those experiments can probably be partly ascribed to different study species, antigens, and experimental conditions. However, it is also possible that the difficulties in establishing the costs of immune activation relate to assessment methods of antioxidant protection and interpretations of results. For instance, the antioxidant barriers measured in the abovementioned studies are based on the capacity of the whole blood (or plasma or serum) to resist controlled free-radical attack ex vivo. These measurements, which are based on the cumulative action of all the antioxidants in the sample, may reflect the dynamics of protective mechanisms aimed at maintaining redox balance. This means that an increased antioxidant capacity of blood may not be a sign of optimal health condition if it reflects a compensatory response to increased oxidative stress. Similarly, a decrease in the antioxidant barrier may not necessarily signal deteriorated condition if it occurs in response to decreased production of reactive species (e.g., Prior and Cao 1999). Such problems of interpretation, for instance, frequently occur in sports medicine, where exercise-induced inflammatory responses elicit compensatory increases in the antioxidant barrier (e.g., Vider et al. 2001), so that often, the only evidence that oxidative stress has occurred may be the upregulation of antioxidant defense systems (Halliwell and Gutteridge 1999). Because of such complications, assessments of antioxidant protection should preferably also include estimates of oxidative stress-induced damage, such as lipid or protein peroxidation products (e.g., Prior and Cao 1999), which have been measured in only a few ecological studies (Blount et al. 2002*a*, 2002*b*; McGraw et al. 2005*a*).

The aims of this study are to ascertain whether activation of the immune system exacts oxidative costs in a passerine bird and to examine to what extent exogenous dietary antioxidants mitigate potential damage from RONS. For this purpose, captive greenfinches were first supplemented with combinations of lutein and vitamin E, two exogenous antioxidants that can be acquired in the diet, and subsequently either injected with saline or immune-challenged with a plant lectin PHA. Specifically, we examined four hypotheses. First, we hypothesized that activation of the immune system will lead to oxidative damage in tissue, as measured by increased lipid peroxidation, and a decline in plasma carotenoid levels and total antioxidant protection. Alternatively, an increase in plasma carotenoids and/or antioxidant protection would indicate that immune challenge promotes mobilization of antioxidant defenses. Second, we predicted that exogenous supplementation of carotenoids and vitamin E would alleviate the oxidative costs of immune system activation by reducing lipid peroxidation and increasing antioxidative protection. Third, we hypothesized that birds receiving antioxidant supplementation would mount stronger immune responses than birds receiving no supplementation, as documented in previous studies. Finally, we tested for correlations between measures of lipid peroxidation, total antioxidant protection, carotenoids, and an endogenous antioxidant, uric acid, to examine the relationships that promote redox balance and alleviation of oxidative stress. We predicted that exogenous and endogenous levels of antioxidants would be positively correlated with each other and negatively correlated with oxidative stress–induced damage after immune challenge.

Methods

Greenfinches weigh about 30 g and are sexually dichromatic, gregarious, granivorous passerines native to the western Palearctic region. Carotenoid-based plumage coloration in males is sexually selected (Eley 1991) and affected negatively by intestinal, viral, and hematozoan infections (reviewed by Hõrak et al. [2004]). Male greenfinches ($n = 94$) were caught in mist nets in the Sõrve Bird Observatory on the island of Saaremaa (57°55′N; 22°03′E) on January 22, 2006 (day 1). Birds were trans-

ported to Tartu and housed indoors (two identical rooms) in individual cages (27 cm × 51 cm × 55 cm) with sand bedding. Average temperature in the aviary during the experiment was 16.0° ± 1.6°C (SD), and average humidity was 51.5% ± 2.4% (SD). The birds were supplied ad lib. with sunflower seeds and filtered tap water. Birds were held on the natural day-length cycle. All blood samples were collected before the lights were turned on, in order to obtain the values of biochemical parameters characteristic of the state of overnight fast. Before each blood sampling, body mass of birds was recorded with a precision of 0.1 g. Blood sampling (by two persons) started 2.5 h before sunrise and lasted up to 4 h; "night" was artificially extended on blood-sampling days. Birds were brought from the aviary rooms into the bleeding room one at time, and maximum effort was made to avoid waking up the rest of birds (by keeping silent and using flashlights with small focus). Order of bleeding did not affect systematically any of the studied parameters (app. B in the online edition of the *American Naturalist*). Sampling of a single bird lasted about 4–5 min. Birds were released into their natural habitat on April 1 (day 72). The study was conducted under a license from the Estonian Ministry of the Environment.

Experimental Time Line

After transportation to Tartu, birds were allowed a 17-day acclimatization period (days 3–19). Birds were divided into six treatment groups (15–16 birds in each). These groups were set to have similar average body mass at capture and age composition (eight or nine first-year and seven older birds in each group). On the morning of day 20, preexperimental blood samples were collected, and subjects in each treatment group (except controls) started to receive different antioxidant supplements, as indicated in figure 1.

Birds were divided into the following treatment groups: (1) Carotenoid birds received 12 μg/mL carotenoid solution on alternate days, for a total of 13 days of antioxidant supplementation. (2) High–vitamin E birds received 500 μg/mL tocopherol solution on alternate days (13 days). (3) Low–vitamin E birds received 250 μg/mL tocopherol solution on alternate days (13 days). (4) Carotenoid + high–vitamin E birds received 12 μg/mL carotenoid solution and 500 μg/mL tocopherol solution on alternate days, a total of 26 days of supplementation. (5) Carotenoid + low–vitamin E birds received 12 μg/mL carotenoid solution and 250 μg/mL tocopherol solution on alternate days (26 days). (6) Control birds received just filtered tap water. Carotenoid supplementation consisted of lutein and zeaxanthin (20 : 1, w/w), prepared from OroGlo liquid solution of 11 g/kg xanthophyll activity (Kemin AgriFoods Europe, Herentals, Belgium). Vitamin E supplementation consisted of a mixture of 20 mg/mL d-α-tocopherol, 12 mg/mL d-γ-tocopherol, 4 mg/mL d-β-tocopherol + d-δ-tocopherol, and 2 mg/mL total tocotrienols, prepared from water-soluble vitamin E solution Aqua-E (Yasoo Health, Johnson City, TN). Those solutions were freshly prepared each evening using filtered (Brita Classic; BRITA, Taunusstein, Germany) tap water and were provided in 50-mL doses in opaque dispensers in order to avoid oxidation of carotenoids and vitamin E. Carotenoid dose was determined on the basis of previous work (Hõrak et al. 2006). The high dose of vitamin E was set to approximately double the content of tocopherols (mainly α-tocopherol) contained in sunflower seeds (Yoshida et al. 2002); the low dose of vitamin E was 50% of the high dose.

On day 34 (14 days after antioxidant treatments started), a second blood sample was collected (*mid exp.* in fig. 2). Subsequently, all the birds were subjected to 4-day anticoccidian treatment with Intracox Oral (Interchemie, Castenray, The Netherlands). The birds received 2 mL/L of the solution containing 25 mg/L toltrazuril in their drinking water. Antioxidant supplementation was temporarily interrupted for that period. On day 47, all the birds were orally infected with ~2,000 coccidian (*Isospora*) oocysts, a mixed stock originating from multiple hosts. The prepatent period of *Isospora* is 6–7 days (Mehlhorn et al. 1986; U. Karu, unpublished data). Because we blood-sampled the birds 4 days after experimental infection, we could not determine whether the resistance to novel coccidian strains affected their condition. However, we could test whether the efficiency of anticoccidian treatment affected the studied variables. For this purpose, we compared the hematological parameters and PHA responses of birds whose parasites were more susceptible to anticoccidian treatment (i.e., with zero prevalence of coccidians at the postexperimental blood sampling) with those of birds who had naturally relapsed infections by that time.

On the evening of day 48 (between 1630 and 1840 hours), eight or nine birds from each treatment group were injected intradermally in the wing web with 0.2 mg of PHA in 0.04 mL of sterile isotonic saline. At the same time, the rest of the birds (seven or eight individuals per group) were injected with saline. Wing web thickness was measured before injection, and the swelling response was measured 24 h later. Average increase in wing web thickness was 0.73 ± 0.31 mm ($n = 50$) for immune-challenged and 0.07 ± 0.11 mm ($n = 41$) for saline-injected birds ($t = 13.0$, $P < .00001$). Cutaneous hypersensitivity reaction resulting from PHA injection reflects the combined responses of T cells, cytokines, and inflammatory cells (Martin et al. 2006). We followed the simplified pro-

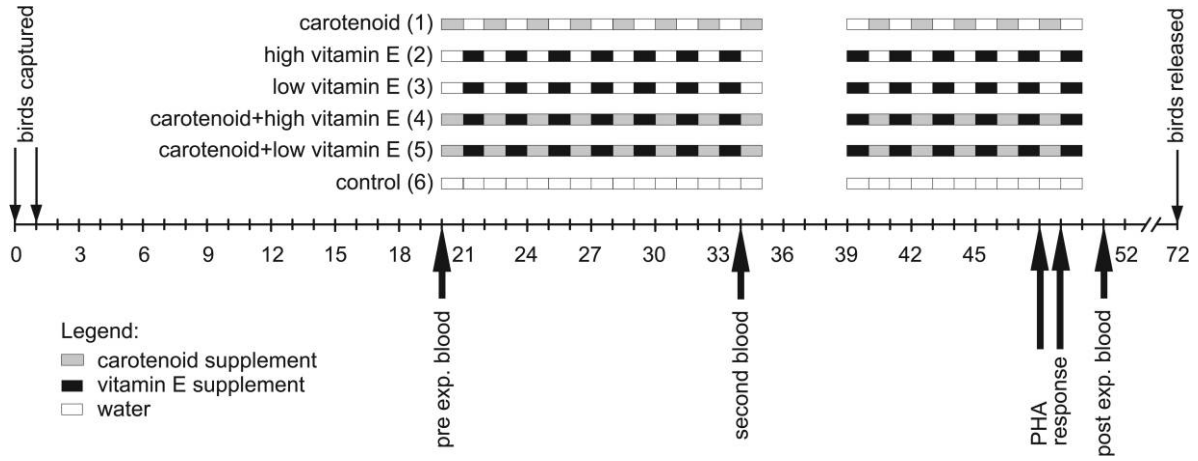

Figure 1: Course of the experiment. Day 0 = January 20. The break in antioxidant supplementation (days 35–39) occurred because of anticoccidian treatment of all birds.

tocol (Smits et al. 1999) as described in detail by Saks et al. (2003).

On the morning of day 51 (3 days after immune challenge), a third blood sample (time point *post exp.* in fig. 2) was collected. In all cases, 100–300 μL of blood was drawn from brachial or tarsal veins. After centrifugation, plasma was stored at −75°C until analyzed. During the few hours between blood collection and centrifugation, samples were maintained in refrigerator at 4°C. Lipid peroxidation products were determined only from the third blood sample (postexperimental); the rest of analyses were conducted for all three sampling points.

Chemical Analyses

Antioxidants. Concentrations of carotenoids were determined spectrophotometrically using acetone-resistant microtiter plates, as described by Hõrak et al. (2006). In this process, 150 μL of acetone was added to 15 μL of plasma and centrifuged for 10 min at 16,800 *g*. Absorbance of supernatant was measured at 449 nm. Concentration of uric acid was determined from 5-μL plasma samples by enzymatic colorimetric test with lipid clearing factor (uric acid liquicolor, HUMAN, Wiesbaden, Germany).

Total Antioxidant Potential. The capacity of fluids to inhibit redox reaction induced by free radicals was used to assess the total antioxidant potential (AOP) of plasma. Reduced AOP levels may reflect hosts' inability to deal with increased free-radical load. Increased AOP levels may represent a compensatory enhancement of antioxidant defenses (e.g., Prior and Cao 1999). The AOP was estimated using the BIOXYTECH AOP-490 assay (Oxis Research, Portland, OR), which is based on the reduction of Cu^{++} to Cu^{+} by the combined action of all antioxidants present in a sample. A chromogenic reagent, bathocuproine (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline), selectively forms a 2 : 1 complex with Cu^{+} that has a maximum absorbance at 490 nm. The color change of plasma incubated with reagent containing Cu^{++} and chromogen for 3 min at room temperature is measured. A standard of known uric acid concentration was used to create the calibration curve, so that the results are quantified in millimole-per-milliliter uric acid equivalents. The assay was adapted for small (5-μL) plasma samples. Previous research (Hõrak et al. 2006; Tummeleht et al. 2006) has

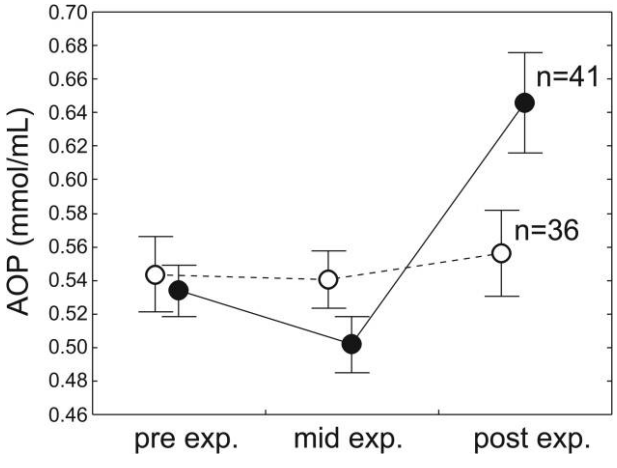

Figure 2: Dynamics of plasma antioxidant potential (AOP) among immune-challenged (*filled circles*) and control (*open circles*) birds. Error bars represent SE.

shown that this method is precise and that the measurements of antioxidativity obtained by the AOP kit strongly and linearly correlate with estimates obtained by the trolox equivalent antioxidant capacity assay (Randox TAS kit, Randox, Crumlin, United Kingdom). All spectrophotometric analyses (except lipid peroxidation) were performed with Tecan microplate reader (Sunrise, Tecan Austria, Grödig/Salzburg, Austria).

Lipid Peroxidation. Lipid peroxidation (LPO) is a well-established mechanism of cellular injury used to indicate OxS in cells and tissues. Lipid peroxides are unstable and decompose to form a complex series of compounds, including reactive carbonyl compounds. Polyunsaturated fatty acid peroxides generate malondialdehyde (MDA) and 4-hydroxyalkenals (HAEs) upon decomposition. The LPO-586 assay (Oxis Research) is based on the reaction of a chromogenic agent, N-methyl-2-phenylindole, with MDA and HAEs at 45°C. One molecule of MDA or HAE reacts with two molecules of reagent to yield a stable chromophore with maximal absorbance at 586 nm.

After separation of plasma, 1 μL of 0.125 M butylated hydroxytoluene (BHT) solution in acetonitrile was added to a 100-μL plasma sample in order to preserve it from oxidation. The assay was performed according to the kit instructions, which were adapted for 50-μL plasma samples by reducing the amounts of all required chemicals four times. Absorbance was measured against sample blank containing 50 μL plasma and a mixture of acetonitrile and diluent. The standard curve was prepared from the acetal (tetramethoxypropane [TMOP]; stock solution provided with the kit), which is hydrolyzed during the acid incubation step at 45°C, generating MDA. The results are expressed as nanomoles of MDA per milliliter of plasma.

Statistics

Effects of experimental treatments on the dynamics of AOP, uric acid, and body mass were analyzed by repeated-measures ANOVAs. Carotenoid and vitamin E treatments and PHA versus saline injections were considered as factors with two, three, and two levels, respectively. The blood-sampling event was a within-individual repeated measure termed "time" (with three levels: preexperiment, mid-experiment, and postexperiment; fig. 1). We started examination of full models with interaction terms between all factors and time, dropping nonsignificant effects one at a time. In the final models, only significant main effects and interactions were retained. The effects of treatments were assumed to be revealed by significant time × treatment interaction terms. Assumptions for this analysis (normality, homogeneity of variances, and sphericity) were met for these variables (for AOP and uric acid, this required ln transformation). Because carotenoid concentrations were not normally and homogenously distributed, we could not apply repeated-measures ANOVA for testing the treatment effects. Therefore, individual changes within treatment groups were tested with Friedman's ANOVA and between-treatment differences with *U*-tests (table 1). The LPO products were measured only once during the experiment (after PHA challenge). Treatment effects on LPO and swelling response to PHA were tested in ANOVA, starting with full models of all factors and interactions. The PHA response was normally distributed, and distribution of LPO values became normal after ln transformation. Assumption of homogeneity of variances was met for both analyses. All initial and final models retained in the analyses are presented in appendix A in the online edition of the *American Naturalist.* Relationships between individual antioxidants and LPO, AOP, and swelling response were assessed on the basis of Spearman rank correlations. Because vitamin E supplementation did not affect any of the studied parameters (app. A), data were pooled over experimental groups 1, 4, and 5 (carotenoid supplemented) and over groups 2, 3, and 6 (unsupplemented with carotenoids) for the final analyses of the effects of immune challenge and carotenoid supplementation. Sample sizes differ between some analyses because of our inability to

Table 1: Average plasma carotenoid concentrations (μg/mL) in different treatment groups and *P* values and *Z* statistics for between-group comparisons (Mann-Whitney *U*-tests)

	No carotenoid supplementation						Carotenoid supplementation							
	PHA		Saline		*P*		PHA		Saline		*P*		Difference[a]	
Time	Mean ± SD	*n*	Mean ± SD	*n*	diff.	*Z*	Mean ± SD	*n*	Mean ± SD	*n*	diff.	*Z*	*P* diff.	*Z*
Preexp.	8.5 ± 8.7	24	8.1 ± 6.6	20	.777	−.28	10.2 ± 7.6	24	7.3 ± 6.0	20	.199	1.29	.310	1.02
Mid-exp.	3.4 ± 4.8	25	3.3 ± 3.2	19	.619	−.50	17.8 ± 8.3	21	15.4 ± 11.3	19	.140	1.48	<.00001	6.60
Postexp.	1.6 ± 2.6	24	2.7 ± 3.3	18	.322	−.99	10.6 ± 5.7	22	9.7 ± 9.9	18	.289	1.06	<.00001	5.83

Note: "Preexp.," "Mid-exp.," and "Postexp." stand for the preexperimental, mid-experimental, and postexperimental blood samples, respectively. Individual carotenoid levels changed during the experiment both among unsupplemented ($\chi^2 = 43.45$, $P < .00001$, $n = 40$) and carotenoid-supplemented birds ($\chi^2 = 34.37$, $P < .00001$, $n = 38$; Friedman ANOVA).

[a] Difference between unsupplemented and carotenoid-supplemented groups.

collect sufficient amount of blood from all the birds. Average trait values ± SD are presented. Age (first year vs. older) did not affect any of the studied parameters (app. B).

Results

Three days after injection, immune-challenged birds circulated 19% higher levels of lipid peroxidation (LPO) end products in their plasma than saline-injected birds (PHA-injected: 1.51 ± 0.71 nmol MDA/mL plasma, $n = 41$; saline-injected: 1.23 ± 0.68 nmol MDA/mL, $n = 33$; $t = 2$, $P = .049$; untransformed means ± SD, *t*-test on ln-transformed values). Plasma antioxidant potential (AOP) of immune-challenged birds increased significantly after PHA injection compared to that of nonchallenged birds, whose AOP remained stable during the study period (fig. 2; $F = 7.7$, df = 2, 150, $P = .0007$ for time × immune challenge interaction term in repeated-measures ANOVA with main effects of immune challenge [$F = 0.4$, df = 1, 75, $P = .552$] and time [$F = 9.4$, df = 2, 150, $P = .0001$]). Immune challenge did not affect the dynamics of plasma carotenoid levels (table 1). Immune challenge did not affect the dynamics of plasma uric acid levels ($F = 2.6$, df = 2, 112, $P = .076$ for time × immune challenge interaction term in repeated-measures ANOVA with main effects of immune challenge [$F = 0.2$, df = 1, 56, $P = .688$] and time [$F = 6.6$, df = 2, 112, $P = .001$]). Vitamin E treatment did not affect LPO ($F = 0.4$, df = 2, 62, $P = .690$) or dynamics of AOP ($F = 0.3$, df = 4, 130, $P = .864$), plasma carotenoids at the end of the experiment ($H = 0.05$, $P = .977$, $n = 85$; Kruskal-Wallis ANOVA), or uric acid ($F = 1.1$, df = 4, 92, $P = .378$); see appendix A.

Carotenoid supplementation significantly increased plasma carotenoid levels, while among unsupplemented birds, plasma carotenoids declined throughout the study (table 1). Carotenoid supplementation significantly reduced LPO (MDA levels in supplemented birds: 1.21 ± 0.62 nmol/mL, $n = 39$; in unsupplemented birds: 1.59 ± 0.75 nmol/mL, $n = 37$; app. A). The effect of carotenoid supplementation on LPO remained significant in ANOVA models adjusting for the effect of immune challenge on LPO ($F = 7.3$, df = 1, 71, $P = .008$). However, carotenoid treatment did not significantly alleviate the effect of immune challenge on LPO, as indicated by the nonsignificant interaction term between immune challenge and carotenoid supplementation (fig. 3; $F = 0.11$, df = 1, 70, $P = .735$) in a model with main effects of immune challenge ($F = 3.4$, df = 1, 70, $P = .068$) and carotenoid treatment ($F = 7.0$, df = 1, 70, $P = .010$). As shown above, carotenoid supplementation did not affect the dynamics of AOP in the model adjusting for the effects of immune challenge on AOP dynamics. Neither did we find any evidence that carotenoid supplementation had modulated the effect of immune challenge on the dynamics of AOP ($F = 1.8$, df = 2, 130, $P = .166$ for a three-way interaction between time × immune challenge × carotenoid supplementation terms in repeated-measures ANOVA). Carotenoid supplementation did not affect the dynamics of uric acid ($F = 1.2$, df = 2, 92, $P = .307$). Experimental treatments did not affect body mass dynamics (app. A); neither did body mass correlate significantly with any of the measured biochemical parameters or swelling response to PHA.

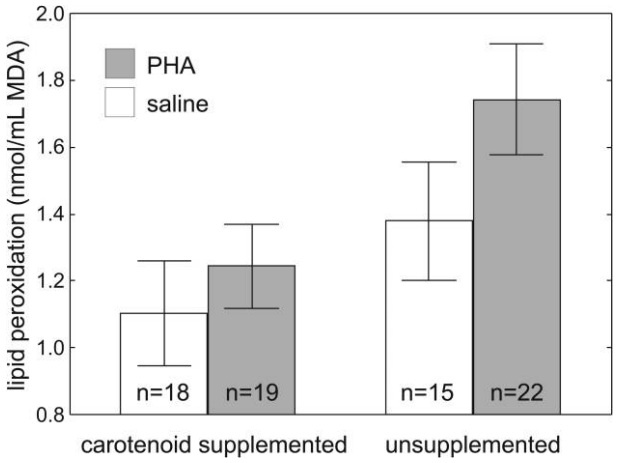

Figure 3: Effect of carotenoid supplementation and immune challenge on lipid peroxidation, measured as plasma malondialdehyde (MDA) concentration. Error bars represent SE. See "Results" for ANOVA statistics.

Antioxidant supplementation did not affect the magnitude of the swelling response to PHA injection ($F = 0$, df = 1, 44, $P = .980$ for carotenoids; $F = 0.3$, df = 2, 44, $P = .70$ for vitamin E; and $F = 0.7$, df = 2, 44, $P = .50$ for carotenoid × vitamin E interaction). Magnitude of swelling response to PHA did not correlate with concentrations of lipid peroxidation products, carotenoids, or uric acid measured 3 days after injection ($r_s = 0$–0.1, $P = .7$–$.9$, $n = 32$–46).

At the end of the experiment, the antioxidant barrier increased with increasing plasma uric acid levels (AOP vs. uric acid: $r_s = 0.81$, $P < .0001$, $n = 65$; fig. 4) and LPO (AOP vs. LPO: $r_s = 0.42$, $P = .0003$, $n = 72$; fig. 4). Lipid peroxidation also correlated positively with plasma uric acid levels (uric acid vs. LPO: $r_s = 0.37$, $P = .005$, $n = 55$; fig. 4). Plasma carotenoid levels were not related to antioxidant barrier, LPO, or uric acid levels ($r_s = -0.2$ to 0.2, $P = .06$–$.4$, $n = 64$–82). Relapse of coccidian infection after medication was not related to any of the physiological parameters measured at the postexperiment blood sampling or to swelling response to PHA (app. B).

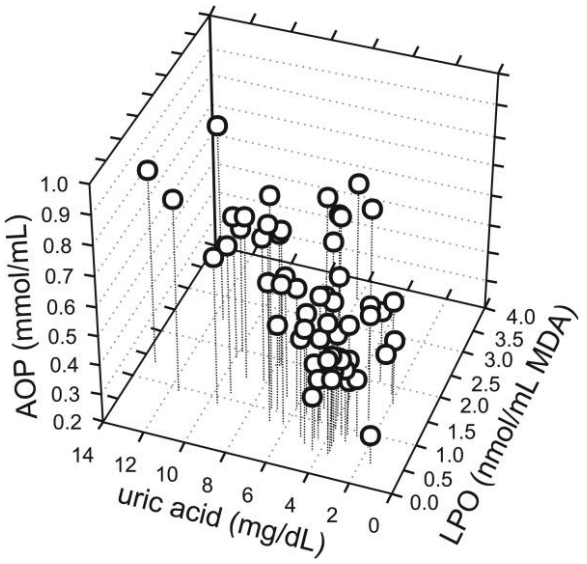

Figure 4: Relationships between lipid peroxidation (*LPO* measured as plasma malondialdehyde [MDA] concentration), plasma uric acid concentration, and antioxidant potential. See "Results" for the correlation statistics.

Discussion

This study clearly showed that activation of the immune system in greenfinches increased lipid peroxidation and elevated plasma antioxidant potential. Carotenoid- (but not vitamin E-) supplemented birds circulated lower levels of lipid peroxidation products, but the effect of supplementation was not strong enough to reduce lipid peroxidation caused by immune challenge. Levels of an endogenous antioxidant—uric acid—strongly and positively correlated with total plasma antioxidant potential. Finally, supplementation with dietary carotenoids or vitamin E failed to stimulate immune function in greenfinches.

Lipid peroxidation involves a chain reaction whereby free radicals remove electrons from the lipids of membranes surrounding cells and organelles such as mitochondria, lysosomes, and peroxisomes (Halliwell and Gutteridge 1999). It most often affects polyunsaturated fatty acids (PUFAs), which are responsible for maintenance of physiologically important membrane properties, including fluidity and permeability. Peroxidation products of PUFAs, such as MDA and alkenals, are also mutagenic and cytotoxic and can damage membrane proteins (Halliwell and Gutteridge 1999). To our knowledge, this study presents the first direct evidence in a wild animal species that immune system activation by a nonparasitic foreign antigen leads to lipid peroxidation. Notably, a similar study in kestrel nestlings found that PHA challenge increased the serum concentrations of reactive oxygen metabolites that are able to propagate the chain reactions that may eventually lead to lipid peroxidation (Costantini and Dell'Omo 2006).

Phytohemagglutinin (PHA) is a plant lectin derived from the red kidney bean (*Phaseolus vulgaris*) where it is believed to serve as a defense against herbivory. Injection with PHA induces a complicated immunological cascade beginning with proliferation of (mainly T) lymphocytes and secretion of proinflammatory cytokines that recruit and activate effector cells and phagocytes such as basophils, heterophils, and macrophages (Martin et al. 2006). Eventually, infiltration and additional cytokine secretion by these phagocytes is believed to induce the local swelling response (Martin et al. 2006). Besides the local leukocyte infiltration, PHA injection in birds can also elevate the concentration of circulating heterophils in the peripheral blood for as long as 1 week after challenge (Hõrak et al. 2000), which points to the induction of systemic inflammation. Heterophils involved in this process represent the first line of immune defense in terms of ingestion and destruction of potential pathogens. During particle ingestion, they produce large amounts of RONS, which, in addition to the pathogens, can damage the phagocytes themselves and other exposed cells (Splettstoesser and Schuff-Werner 2002). We consider such inflammation-induced damage as the most parsimonious explanation for the PHA-induced lipid peroxidation observed in our experiment. Such an interpretation would be consistent with evidence of occurrence of lipid peroxidation in inflammations associated with infectious and degenerative diseases (Romero et al. 1998) or exhaustive physical exercise (e.g., Vider et al. 2001). Further research in this area would benefit from application of experimental immune suppression treatments. For instance, Alonso-Alvarez et al. (2007) found that experimental reduction of testosterone levels in zebra finches enhanced the resistance of erythrocytes to controlled free-radical attack and also increased the magnitude of swelling response to PHA.

In addition to increased lipid peroxidation in response to immune challenge, the other physiological processes accompanying this effect are of considerable interest. Approximately 60 h after immune challenge, the plasma antioxidant barrier of challenged birds increased 25% compared to the values measured 14 days before immunization. Among the saline-injected birds, the increase was only 4%. This result contrasts with the previous finding in kestrel nestlings (Costantini and Dell'Omo 2006), where immunostimulation with PHA caused a significant decrease of the serum antioxidant barrier 24 h after injection. A similar decrease in the whole-blood antioxidant barrier (measured as erythrocyte resistance to controlled free-radical attack) was observed in zebra finches 24 h after injection of bacterial lipopolysaccharide (LPS; Bertrand et

al. 2006*b*). On the other hand, Cohen et al. (2007) found no effect of LPS injection on plasma antioxidativity in chickens. Our results are more similar to those observed in exercise-induced inflammation, where plasma antioxidativity and/or antioxidant enzyme levels increased in parallel with lipid peroxidation (e.g., Vider et al. 2001; Tauler et al. 2006). These results exemplify the compensatory activation of endogenous antioxidant machinery in response to free-radical attack imposed by immune system activation.

Our result of a strong correlation between plasma antioxidativity and uric acid levels compares favorably with recent findings of Cohen et al. (2007), who demonstrated positive relationships between these variables in 92 wild bird species. The antioxidative properties of uric acid and its high concentrations in bird plasma have been suggested as one of the reasons for greater longevity of birds than mammals of similar size (Klandorf et al. 2001). The positive correlation between uric acid and lipid peroxidation (fig. 4) might indicate production of this antioxidant in response to increased lipid peroxidation. For instance, plasma uric acid has been shown to increase in parallel with lipid peroxidation in broiler chickens during chronic corticosterone exposure (Lin et al. 2004). It should be noted, however, that uric acid could not be considered a single major antioxidant contributing to the increased antioxidant barrier in response to immune challenge in our study, because the treatment effect on its temporal dynamics was not significant. Evidence in humans suggest that proteins and micromolecular antioxidants, such as ascorbate, polyphenols, and glutathione, can also importantly contribute to plasma antioxidativity (e.g., Erel 2004).

Despite the considerable variation in plasma carotenoid levels induced by dietary manipulation (table 1), plasma carotenoids did not correlate with plasma antioxidativity. This result is consistent with previous findings in captive greenfinches (Hõrak et al. 2006), breeding great tits (Tummeleht et al. 2006), and kestrel nestlings (Costantini and Dell'Omo 2006). On the other hand, in this study, carotenoid supplementation reduced lipid peroxidation by 24%. Although this effect was not strong enough to compensate for the increased LPO due to immune challenge, it indicates that the antioxidant function of carotenoids in avian models cannot be totally discounted, as suggested by Hartley and Kennedy (2004). Our results thus demonstrate the necessity of composite assessment of AOP and indices of oxidative stress-induced damage, because the antioxidative effect of carotenoid supplementation could be demonstrated only by measuring LPO products. We would have reached a different conclusion about the antioxidant function of carotenoids on the basis of AOP data alone.

The antioxidant function of carotenoids in birds has been well established in protection of embryos and hatchlings (Surai 2002; McGraw et al. 2005*a*). It remains to be shown whether these antioxidant properties are also of any ecological relevance in adult birds. In this context, it is important to note that the carotenoid manipulation in our study was relatively moderate. At the time of LPO measurement, supplemented birds circulated only about 10 μg/mL and unsupplemented birds about 2 μg/mL of carotenoids in their plasma. Indirect evidence suggests that the amounts circulated during molt in the wild may range up to at least 30 μg/mL (Karu et al. 2007). Thus, it appears feasible that plasma carotenoid levels (which are directly reflected in plumage coloration) may indeed signal the birds' ability to resist oxidative stress, especially if the energetic costs imposed by molt contribute to LPO. This contention is supported by evidence that nutritional limitation during molt (independent of carotenoid availability) reduces carotenoid ornament expression (Hill 2000; McGraw et al. 2005*b*). To prove that carotenoids exert any important physiological effect in birds as antioxidants, measurements of LPO, carotenoids, and other sources of antioxidant protection must be associated with components of fitness, preferably in the field. This study shows that measuring the antioxidant barrier alone is not sufficient to demonstrate the antioxidant function of carotenoids (see also Alonso-Alvarez et al. 2004*a*; Costantini et al. 2006).

Lipid peroxidation was not affected by vitamin E supplementation, although vitamin E is considered the main lipophilic antioxidant involved in membrane defense (Halliwell and Gutteridge 1999). Neither did carotenoid or vitamin E supplementation (separately or in combination) affect the strength of the swelling response to PHA. Immunostimulatory effects of vitamin E have been repeatedly demonstrated in poultry (Surai 2002). Possibly, the effects of vitamin E supplementation in our study could be masked by the high levels of this antioxidant in dietary sunflower seeds (Yoshida et al. 2002). To our knowledge, the only previous experiment of vitamin E supplementation in wild birds has been performed on nestling barn swallows (*Hirundo rustica*); no effect on PHA response was found (de Ayala et al. 2006).

Interest of animal ecologists in the immunostimulatory properties of carotenoids has emerged since publication of Lozano (1994). Since then, the associations between immune function and carotenoids have been implicated in at least eight avian species (reviewed by McGraw et al. [2006]); however, results demonstrating a lack of association have also started to accumulate (Navara and Hill 2003; McGraw and Ardia 2005; Hõrak et al. 2006; McGraw and Klasing 2006; McGraw et al. 2006). One reason for these inconsistencies may relate to the ways ecologists assess and interpret immunocompetence. For instance, the

PHA-induced skin swelling, which is arguably the most popular immune assay in the wild birds, results from the activation of both adaptive and innate components of the immune system (Martin et al. 2006), all of which might be differentially affected by different carotenoids. Furthermore, indices of immunocompetence obtained by measuring responses to novel antigens are notoriously difficult to interpret in the context of real parasite resistance (Adamo 2004). This also holds for the PHA-induced skin swelling that has been enhanced because of carotenoid supplementation in some but not in other studies (reviewed by McGraw et al. [2006]). In our greenfinch model, for instance, the strongest swelling response to PHA was produced by the individuals most susceptible to infection with novel coccidian strains (Saks et al. 2006). Such outcomes are perhaps expected, given that different compartments of immune system, orchestrated by secretion of cytokines by Th1 and Th2 lymphocytes, are in a cross-regulatory balance (reviewed by Graham et al. [2005]). This means that humoral Th2 responses may exert anti-inflammatory action by downregulating Th1 cell–mediated immunity and vice versa. Recently, it has been proposed that the immune system's constrained ability to achieve the optimal balance between those different arms of defense serves as a major evolutionary reason why natural selection has not eliminated immunopathology, which is implicated in the etiology of most diseases (Graham et al. 2005). Realizing that immune function is not a distinct and easily quantifiable entity is also necessary for understanding the costs of expression of signals based on carotenoids and other antioxidant pigments.

In conclusion, this study assessed for the first time a measure of oxidative stress–driven damage—lipid peroxidation—in a wild bird species in postembryonic stage. We found that this damage was induced by inflammatory immune challenge and reduced by carotenoid supplementation, although this reduction was not sufficient to compensate for the effect of immune system activation. These results demonstrate the importance of antioxidant function of carotenoids in birds. In addition, we found that immune challenge induced an increase in plasma total antioxidant capacity. This capacity was strongly correlated with the plasma levels of an endogenous antioxidant, uric acid, which is a simple end product of nitrogen metabolism. These results indicate that caution is required in interpreting the potential costs of immune system activation on the basis of assessment of blood antioxidant barrier alone. Identifying the mechanisms by which organisms cope with oxidative stress is necessary for understanding the major processes in important domains of evolutionary ecology. This study shows that these mechanisms cannot be properly elucidated without simultaneous assessment of oxidative damages, antioxidant barriers, and individual antioxidants.

Acknowledgments

We thank the Sõrve Bird Observatory for providing facilities and M. Martinson for the help in bird trapping. S. Van Dyck (Kemin Agrifoods Europe) kindly donated OroGlo carotenoid supplement. I. Ots helped us with the experiment and analyses, and E. Sonn and H. Tamm aided in bird maintenance. G. Lozano and two anonymous reviewers provided thorough and constructive criticism on the manuscript. The study was financed by Estonian Science Foundation grant 6222 to P.H.

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Associate Editor: Ellen D. Ketterson
Editor: Monica A. Geber

Male greenfinch (photograph by Arne Ader; http://www.loodusemees.ee).