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Effects of carotenoid and vitamin E supplementation on oxidative stress and plumage coloration in house finches (*Haemorhous mexicanus*)

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ABSTRACT

There has been much recent interest from both applied and basic scientists in the broad series of benefits that animals reap from acquiring high concentrations of dietary antioxidants, such as carotenoids and vitamins (e.g., vitamin E, or tocopherol). Most attention has been paid to separate effects of these compounds on, for example, coloration, health state, development, and vision, but because of possible interactions between these lipid-soluble molecules, we are in need of more studies that co-manipulate these substances and examine their possible synergistic impacts on animal physiology and phenotype. We capitalized on a model avian system (the house finch, *Haemorhous mexicanus*), where extensive information is available on the fitness roles of carotenoids, to test how variation in carotenoid and/or vitamin E concentrations in the diet impacts body accumulation of these compounds, factors related to oxidative damage (e.g., breast muscle and plasma oxidative-stress susceptibility, plasma nitric-oxide levels), and plumage color development. As in a previous study of ours on carotenoids and health in finches, we employed a 2×2 factorial experimental design on birds in both molting and non-molting conditions, to understand how seasonal shifts in carotenoid use (i.e., pigment incorporation into plumage) might alter the accumulation and roles of carotenoids and vitamins. As expected, lutein supplementation increased the level of circulating carotenoids in both experiments and the color of newly molted plumage. By contrast, vitamin E provisioning did not significantly affect plasma carotenoid levels or plumage coloration in either experiment. Interestingly, carotenoid provisioning decreased circulating vitamin E levels during molt, which suggests either molecular competition between carotenoids and tocopherol at the absorption/transport stages or that vitamin E serves as an antioxidant to offset harmful actions that carotenoids may have at very high concentrations. Finally, in both experiments, we found a reduction in breast-muscle oxidative damage for tocopherol-supplemented birds, which constitutes the first demonstration of a protective effect of vitamin E against oxidative stress in wild birds. Taken together, these findings provide an interesting contrast with our earlier work on season-specific physiological benefits of carotenoids in finches and point to complex associations between indicators of antioxidant and oxidative state in wild-caught animals.

1. Introduction

Minimizing oxidative damage to cells and tissues is a major physiological challenge to organisms. Animals employ various antioxidant mechanisms to offset oxidative stressors (Surai, 2002), including both endogenous (e.g., hormone-initiated cellular processes, enzymatic) and exogenous sources (e.g., dietary vitamins, carotenoids) of detoxification (McGraw et al., 2011). Though there has been extensive work on oxidative balance under controlled laboratory conditions, relatively little is known about the costs and benefits of antioxidants in free-ranging organisms (Cohen et al., 2008; Nussey et al., 2009; McGraw et al., 2010).

Among the various antioxidant systems to study, carotenoids have received particular attention in the last two decades, largely because these pigments also conspicuously color the integument of animals (e.g., bird feathers, fish scales; McGraw, 2006). Carotenoid colors are used widely as condition-dependent signals of quality (e.g., nutrition, health; Hill, 2006), and it naturally follows that carotenoid pigmentation may specifically reflect an individual's antioxidant status (von Schantz et al., 1999). However, there are few direct studies on this topic, and those that have been conducted have yielded ambiguous results and even spawned new hypotheses. Though one of the first tests found that circulating carotenoid levels and antioxidant defenses were significantly positively correlated in zebra finches (*Taeniopygia guttata*) (Alonso-Alvarez et al., 2004), Costantini and Moller (2008) showed in a

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meta-analysis that there is poor overall evidence that carotenoids serve as important antioxidants in birds. Detrimental effects of carotenoids (e.g., on muscle deterioration) have even been reported in wild-caught animals fed large quantities of carotenoids (e.g., in American goldfinches, *Spinus tristis*; Huggins et al., 2010). Some have even argued that carotenoid-based colors might better reveal the antioxidant capacities of others compounds, such as vitamins A, C and E, which can protect carotenoids from oxidation and make them available for coloration (Hartley and Kennedy, 2004); there are now several studies of birds and fish that support this notion (Bertrand et al., 2006; Perez et al., 2008; Pike et al., 2007; but see Karu et al., 2008).

A limitation of this line of work on carotenoids, antioxidants, and coloration, however, has been the emphasis on single antioxidant types when evaluating a link to oxidative stress, coloration, or other fitness proxies. In reality, different antioxidant systems likely work interactively to shape oxidative balance and other physiological and morphological characters (like coloration) of organisms (McGraw et al., 2011). In fact, to our knowledge, no study has assessed the effect of simultaneous manipulation of carotenoids and non-carotenoid antioxidants on oxidative stress, health, and color expression in animals.

Therefore, we performed a laboratory experiment on wild-caught colorful birds (house finches, *Haemorhous mexicanus*) to investigate how supplementation with dietary carotenoids and/or vitamin E (tocopherol) affected circulating levels of these substances, oxidative damage in tissue, levels of plasma nitric oxide (reaction of nitric oxide with superoxide produces a highly reactive intermediate molecule, peroxynitrite, which is one of the most important initiators of free radical damage, Sild and Horak, 2009), and the expression of sexually attractive, carotenoid-based coloration (Hill, 2002). In addition to the extensive general information on the condition-dependence of male carotenoid coloration in this species (Hill, 2002), we recently found that carotenoid enrichment enhances immunity, but only during the molt phase, in house finches (McGraw et al., 2011); no study has yet examined links between carotenoids and oxidative stress markers in this species. We chose to co-examine the role of vitamin E because of its similar lipid-solubility to carotenoids and because of its positive effects on carotenoid coloration in another bird species (yellow-legged gull, *Larus michahellis*; Perez et al., 2008). We predicted that carotenoid and vitamin E supplementation would decrease nitric oxide levels, increase coloration, and reduce the levels of oxidative damage markers. We chose to assess nitric oxide levels in our experiments because Selvaraj and Klasing (2006) reported that lutein suppresses inducible nitric oxide synthase and nitric oxide production when supplied to chicken macrophages in vitro. Moreover, we focused on oxidative damage markers in breast muscle tissue because Huggins et al. (2010) recently showed that carotenoids at high concentration increased creatine kinase (indicator of skeletal muscle breakdown) and affected flight performance. Finally, we also predicted that simultaneous intake of both antioxidant supplements would be doubly beneficial, given the recycling nature of vitamin E as an antioxidant and thus protection of carotenoids from oxidative damage (Perez et al., 2008). As in our prior study of carotenoids and immunity in house finches (McGraw et al., 2011), we ran this experiment two times, in April (during breeding) and September (during molt), to assess whether or not there are special seasonally restricted antioxidant benefits of carotenoid and/or vitamin E supplementation.

2. Materials and methods

2.1. Study animals

In January and April 2011, we captured 32 and 42 male house finches (*H. mexicanus*), respectively, using baited basket traps (Hill, 2002) from various sites in Phoenix, Arizona, USA. We housed birds individually in small wire cages (0.6 × 0.4 × 0.3 m) in an environmental chamber on the ASU campus, at a constant temperature of 20 °C and a photoperiod that mimicked natural conditions. Birds were fed an ad libitum diet of black oil sunflower seeds and tap water.

2.2. Experimental procedure

2.2.1. Experiment 1

From 24 May–23 June 2011, 32 males caught in January were randomly assigned to one of four treatment groups: carotenoid-supplemented, tocopherol-supplemented, carotenoid- and tocopherol-supplemented, or control (N = 8 for each group). Supplements were mixed into the drinking water; we used the same dose of lutein (10 μg/mL of purified beadlets from DSM Inc., Parsippany, NJ, USA) as in our previous study (McGraw et al., 2011). We chose a mixed-tocopherol (d-alpha, d-beta, d-gamma and d-delta tocopherols; DSM Inc., Parsippany, NJ, USA) dose of 360 μg/mL because our preliminary analyses of vitamin E in sunflower seed indicated that house finches were ingesting 1.08 mg of tocopherol each day by eating 3 g/day of sunflower seed. Thus, as house finches drink 3 mL of water/day on average, we doubled their daily dose by having them consume an additional 1.08 mg per day in water.

At the beginning and end of the experiment, we drew 150 μL of whole blood through the alar vein into heparinized capillary tubes. Plasma was then centrifuged (10,000 *g* for 3 min) and frozen at −80 °C for later analysis of carotenoids, tocopherol, nitric oxide and oxidative damage markers. Every week, we weighed each bird to the nearest 0.1 g with a digital scale. Finally, at the end of the experiment, we euthanized all birds by rapid decapitation to collect breast muscle tissue samples for analyses of oxidative damage markers (see more below).

2.2.2. Experiment 2

From 2–30 September 2011 (which is during the molt period of these birds), 42 males captured in April were randomly assigned to one of four treatment groups: carotenoid-supplemented (N = 10), tocopherol-supplemented (N = 11), carotenoid- and tocopherol-supplemented (N = 10), or control (N = 11). We followed the same treatment-administration and sampling procedures that we used in Experiment 1, except that we measured the keel length instead of tarsus length as an index of body size. Every other week, we weighed each bird to the nearest 0.1 g with a digital scale. We also digitally photographed each bird at the beginning and end of the experiment to measure plumage coloration (Oh and Badyaev, 2006; Giraudeau et al., 2012). Because house finch plumage does not reflect significantly in the UV (Keyser and Hill, 1999; McGraw and Hill, 2000), techniques that rely on visible-light are sufficient to capture variation in bird-visible and carotenoid-relevant coloration (Butler et al., 2011). Using a Canon PowerShot SD1200S (Lake Success, NY, USA), we took two separate photographs of the breast of each bird against a neutral gray-board, using identical distance from camera to object, shutter, exposure, and flash settings for each photograph and including a color/size standard in each photo to control for any slight variations in object illumination. Digital images (JPEG, 3648 × 2736 pixels) were imported into Adobe Photoshop (San Jose, CA, USA) to determine plumage hue of the breast; we used the lasso marquee to select the carotenoid-pigmented plumage regions and the color picker function to obtain hue values. Hue values for the two pictures of each bird were averaged for statistical analyses (repeatability = 0.99, calculated using the method of Lessells and Boag (1987)).

2.3. Measurements of carotenoids, tocopherol, oxidative damage markers, and nitric oxide levels

Methods for plasma carotenoid extractions and high-performance liquid chromatography analyses follow those described in Toomey and McGraw (2010). We recovered four tocopherol types, all of which had λ_{max} = 299 nm: Δ-tocopherol (t_R = 4.6 min), γ-tocopherol

(t_R = 5.0 min), α-tocopherol (t_R = 5.7 min), and an unknown (t_R = 6.6 min). Because levels of individual carotenoid and tocopherol types are tightly positively intercorrelated, we use total carotenoid and total tocopherol concentrations in statistical analyses (McGraw et al., 2011). Oxidative damage was measured in breast muscle and plasma using a miniaturized thiobarbituric acid reactive substances (TBARS) assay modified from a commercially available kit (Oxi-Tek TBARS assay kit, ZeptoMetrix Corp., Buffalo, NY, USA). The TBARS method quantifies oxidative damage by measuring levels of lipid peroxidation, a major biomarker of oxidative damage in animal tissues (Oakes and Van Der Kraak, 2003; Almroth et al., 2005; Isaksson et al., 2009). Specifically, this assay involves the reaction of malondialdehyde (MDA), a naturally occurring product of lipid peroxidation, with thiobarbituric acid (TBA) under conditions of high temperature and acidity to generate an adduct that can be measured by spectrophotometry. Briefly, 20 μL homogenized tissue was mixed with 20 μL 8.1% sodium dodecyl sulfate (SDS) and 500 μL TBA buffer reagent. The TBA buffer reagent was prepared by mixing 50 mg TBA with 10 mL acetic acid and 10 mL 50 mM NaOH. Samples were then vortexed and incubated at 95 °C in capped tubes for 60 min. Thereafter, the sample was placed on ice for 10 min and centrifuged at 5000 *g* for 15 min. After centrifugation, the supernatant was removed and absorbance measured at 532 nm (Bio-Tek μQuant microplate spectrophotometer, Winooski, Vermont, USA). Sample concentrations were calculated by interpolation from a standard curve of MDA in concentration from 0–100 nmol·mL^{-1}and are expressed in nmol·mL^{-1} of MDA equivalents. Higher values correspond to greater oxidative damage. Subtle differences are difficult to detect using this assay since it can be confounded by the TBARS generated during the assay (Halliwell and Gutteridge, 2007). However, this method has been commonly used to assess how anthropogenic activities may affect oxidative damage in wild animals (Hoffman, 2002; Berglund et al., 2007).

We quantified circulating levels of nitric oxide (NO) at the start and end of the experiment by following the protocol described in Sild and Horak (2009), but amended for larger (15 μL) plasma volumes. Specifically, we added 15 μL plasma to 40 μL of 75 mmol ZnSO4 and 50 μL of 55 mmol NaOH, centrifuged the samples for 10 min at 16,000 *g*, and then combined 80 μL of supernatant with 80 μL of glycine buffer (pH = 9.7). Next, we added two activated cadmium granules and agitated the samples at maximum force (Vortex Genie 2; Scientific Industries, Inc.; NY, USA) for 15 min. We then combined 120 μL supernatant with 120 μL Griess reagent (G4410 Sigma-Aldrich, St. Louis, MO, USA), agitated the samples at maximum force for 15 min, and transferred 200 μL of each sample to a 96 well plate. We then read the absorbance of each well at λ = 540 nm using an iMark Microplate Reader (Bio-Rad Laboratories, UK), and converted absorbance values to NO concentration based on a standard curve (R^2 = 0.98).

2.4. Statistics

We used repeated-measures analyses of variance (rmANOVAs) to test for effects of diet treatments (tocopherol and carotenoids), time point, and the interactions between these variables. When variables were not normally distributed, we used non-parametric Kruskal–Wallis tests to examine effects of diet treatments on the change between the start and the end of the experiment (levels of plasma carotenoids and plasma oxidative stress markers during the second experiment). We used one-way ANOVAs to examine potential differences between groups at the start of the experiments and to test the effect of dietary treatments on breast muscle oxidative damage levels. When variables were non-normally distributed at the start of the experiment, we used non-parametric Kruskal–Wallis to test for potential differences between groups. Scheffé post-hoc tests were used to test for group differences (i.e., among the four diets). Data were analyzed using STATISTICA 6.0 software (Statsoft, Tulsa, OK, USA).

3. Results

3.1. Experiment 1

At the beginning of the experiment, birds in the four experimental groups did not differ in body mass ($F_{3,28}$ = 0.17, P = 0.90), tarsus length ($F_{3,28}$ = 1.06, P = 0.40), or plasma levels of nitric oxide ($F_{3,28}$ = 0.53, P = 0.66), oxidative damage ($F_{3,28}$ = 2.08, P = 0.13), tocopherol (X^2 = 3.0, P = 0.39) and carotenoids ($F_{3,28}$ = 0.7, P = 0.50). Repeated-measures analysis of variance revealed significant effects of time and the time * carotenoid treatment interaction on body mass during this experiment (Table 1; Fig. 1). Body mass increased during the experiment, but this increase was slower at the beginning of the experiment for carotenoid-supplemented birds than for non-supplemented individuals.

Plasma carotenoid levels were significantly affected by carotenoid treatment (Fig. 2), but not by tocopherol treatment or by the interaction between the two treatments (Table 1). At the end of the experiment birds that received supplemental lutein and both lutein and tocopherol circulated higher levels of carotenoids than did control and tocopherol-supplemented birds ($H_{3,32}$ = 24.10, P < 10^{-4}; P < 0.02 for the post-hoc comparisons of the differences between lutein and lutein + tocopherol groups compared to control and tocopherol groups). This increase in circulating carotenoids was unexpectedly high in carotenoid-supplemented birds, which circulated levels 15 or 20 times higher than non-carotenoid-supplemented birds.

The change in circulating levels of vitamin E during the experiment tended to be influenced by the carotenoid treatment, but not by the tocopherol treatment or the interaction between the two dietary treatments (Fig. 3, Table 1). We also found that levels of oxidative damage markers in breast muscle were significantly affected by the tocopherol treatment (Fig. 4). Post-hoc tests revealed that birds that received only tocopherol (post-hoc test: P = 0.037) and tocopherol + carotenoid (post-hoc test: P = 0.0066; Fig. 4) had significantly lower levels of breast-muscle oxidative damage markers at the end of the experiment than birds who received the carotenoid treatment. However, at the end of the experiment none of the groups had levels of breast oxidative damage markers significantly different than the control group (all P > 0.3). We did not find any carotenoid treatment, tocopherol treatment, time, or interaction effects on plasma oxidative damage markers (Fig. 5) or plasma nitric oxide levels (Fig. 6) (all P > 0.01).

3.2. Experiment 2

At the start of the experiment, treatment groups did not differ in body mass ($F_{3,36}$ = 0.42, P = 0.74), keel length ($F_{3,36}$ = 0.35, P = 0.79), plasma carotenoid concentration ($F_{3,36}$ = 0.42, P = 0.74), plasma vitamin E levels ($F_{3,36}$ = 0.21, P = 0.89), plasma oxidative damage levels ($H_{3,36}$ = 2.16, P = 0.54) or breast hue ($F_{3,36}$ = 0.71, P = 0.55). However, plasma nitric oxide levels were significantly different between groups at the beginning of the experiment ($F_{3,36}$ = 4.80, P = 0.007), and we found significant effects of time (a decrease in nitric oxide levels during the experiment) and of tocopherol supplementation on this variable (Fig. 5). This tocopherol effect was mainly explained by the difference in nitric oxide levels at the start of the experiment, since nitric oxide levels at the end of the experiment did not differ among groups (carotenoid: $F_{1,36}$ = 0.03, P = 0.86; tocopherol: $F_{1,36}$ = 2.12, P = 0.15; carotenoid * tocopherol: $F_{1,36}$ = 0.02, P =0.88). Body mass did not change according to diet treatment or over time during the experiment (Fig. 1); in addition, no significant interactions between dietary treatments and time were found (Table 1).

As we found in the first experiment, change in plasma carotenoid levels during the experiment differed significantly by diet treatment (Fig. 2), such that both groups that were supplemented with lutein increased significantly in circulating carotenoid levels during the study (post-hoc tests, P < 0.05). Again, the increase in circulating carotenoids

Table 1
Results of statistical tests examining the effects of carotenoid and tocopherol supplementation on various physiological and morphological variables in house finches during two experiments (Experiment 1 = breeding experiment; Experiment 2 = molting experiment). Values in bold are statistically significant ($P < 0.05$).

	VARIABLES	FACTORS	F	P
Experiment 1	*Body mass*	Carotenoid treatment	0.0001	0.99
		Tocopherol treatment	0.017	0.90
		Carotenoid * tocopherol treatment	0.18	0.68
		Time	**15.27**	**10^{-7}**
		Time * carotenoid treatment	**3.13**	**0.03**
		Time * tocopherol treatment	1.18	0.32
		Time * carotenoid treatment * tocopherol treatment	2.01	0.11
	Plasma carotenoid levels	Carotenoid treatment	**35.80**	**10^{-5}**
		Tocopherol treatment	0.76	0.39
		Carotenoid * tocopherol treatment	1.05	0.31
		Time	**29.39**	**10^{-5}**
		Time * carotenoid treatment	**36.01**	**10^{-6}**
		Time * tocopherol treatment	0.84	0.37
		Time * carotenoid treatment * tocopherol treatment	0.66	0.42
	Change in plasma vitamin E levels	Carotenoid treatment	2.75	0.11
		Tocopherol treatment	0.71	0.41
		Carotenoid * tocopherol treatment	0.32	0.58
	Oxidative damage in breast muscle	Carotenoid treatment	0.43	0.52
		Tocopherol treatment	**14.96**	**0.0006**
		Carotenoid * tocopherol treatment	2.45	0.13
	Oxidative damage in plasma	Carotenoid treatment	0.32	0.58
		Tocopherol treatment	0.69	0.41
		Carotenoid * tocopherol treatment	1.66	0.21
		Time	3.22	0.08
		Time * carotenoid treatment	1.38	0.25
		Time * tocopherol treatment	2.01	0.17
		Time * carotenoid treatment * tocopherol treatment	0.0007	0.99
	Nitric oxide levels	Carotenoid treatment	0.44	0.51
		Tocopherol treatment	0.10	0.75
		Carotenoid * tocopherol treatment	0.002	0.96
		Time	2.01	0.17
		Time * carotenoid treatment	0.02	0.88
		Time * tocopherol treatment	1.8	0.19
		Time * carotenoid treatment * tocopherol treatment	1.33	0.26
Experiment 2	*Body mass*	Carotenoid treatment	0.37	0.54
		Tocopherol treatment	0.054	0.82
		Carotenoid * tocopherol treatment	0.75	0.39
		Time	2.60	0.08
		Time * carotenoid treatment	1.15	0.32
		Time * tocopherol treatment	1.05	0.35
		Time * carotenoid treatment * tocopherol treatment	0.54	0.58
	Change in plasma carotenoid levels	Carotenoid treatment	**85.24**	**10^{-6}**
		Tocopherol treatment	1.89	0.18
		Carotenoid * tocopherol treatment	**4.19**	**0.05**
	Change in plasma vitamin E levels	Carotenoid treatment	**7.01**	**0.01**
		Tocopherol treatment	**11.17**	**0.002**
		Carotenoid * tocopherol treatment	0.57	0.45
	Oxidative damage in breast muscle	Carotenoid treatment	1.94	0.17
		Tocopherol treatment	**4.16**	**0.05**
		Carotenoid * tocopherol treatment	1.57	0.22
	Change in plasma oxidative damage	Carotenoid treatment	0.49	0.49
		Tocopherol treatment	0.08	0.78
		Carotenoid * tocopherol treatment	0.0009	0.98
	Nitric oxide levels	Carotenoid treatment	0.01	0.92
		Tocopherol treatment	**9.47**	**0.004**
		Carotenoid * tocopherol treatment	1.91	0.18
		Time	**4.99**	**0.03**
		Time * carotenoid treatment	0.14	0.71
		Time * tocopherol treatment	1.33	0.26
		Time * carotenoid treatment * tocopherol treatment	2.93	0.10
	Plumage hue	Carotenoid treatment	**14.25**	**0.0006**
		Tocopherol treatment	0.05	0.83
		Carotenoid * tocopherol treatment	1.09	0.30
		Time	**22.96**	**0.0002**
		Time * carotenoid treatment	**4.71**	**0.037**
		Time * tocopherol treatment	0.11	0.74
		Time * carotenoid treatment * tocopherol treatment	0.38	0.54

was unexpectedly high (30–40 times higher) in carotenoid-supplemented compared to carotenoid-unsupplemented birds.

Carotenoid and tocopherol treatments significantly affected plasma vitamin E levels in this experiment (Fig. 3). Dietary lutein treatment reduced the concentration of vitamin E in circulation, whereas the dietary tocopherol treatment increased tocopherol levels in circulation (Table 1). Levels of oxidative damage markers in breast muscle tissue were affected by the tocopherol treatment as well (Table 1); birds

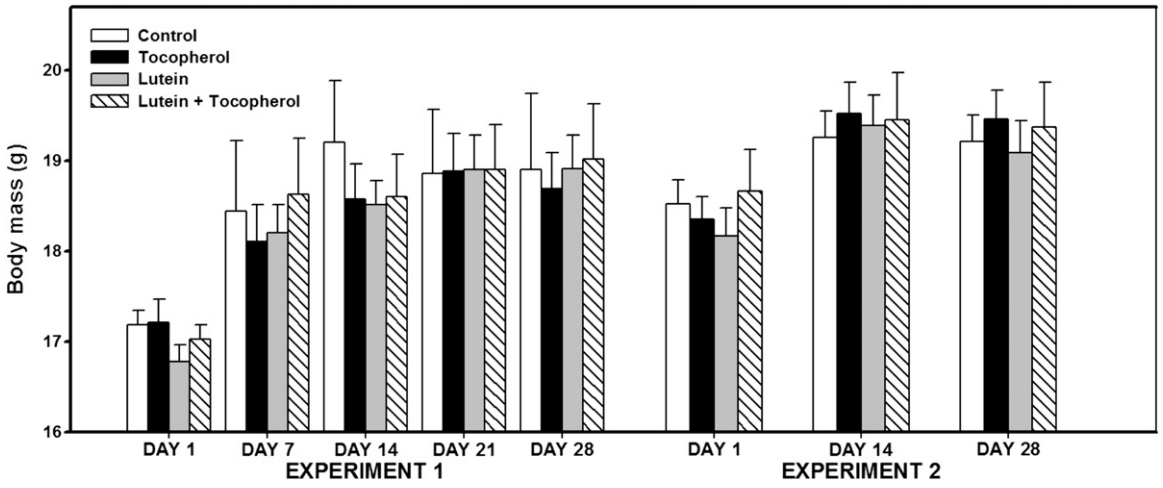

Fig. 1. Body mass (mean + SE) of captive male house finches supplemented or not with dietary carotenoids and/or tocopherols during the breeding (Experiment 1) and molt (Experiment 2) experiments.

supplemented with tocopherol (tocopherol and tocopherol + lutein birds) showed lower levels of oxidative damage markers than lutein-supplemented birds (post-hoc tests, $P < 0.03$, Fig. 4), but not with the control group (post-hoc tests, $P > 0.1$, Fig. 4).

We did not find any effect of our treatments on the change in the levels of plasma oxidative damage markers during the experiment ($H_{3,39} = 1.05$, $P = 0.78$; Fig. 5). Finally, diet treatment significantly influenced plumage color expression during the experiment. Birds supplemented with carotenoids had a more colorful plumage (hue values around 25° for a yellow plumage) than non-supplemented birds and birds supplemented with tocopherol alone (hue values around 16° for a gray plumage) at the end of the experiment (Fig. 7).

4. Discussion

Here we examined possible independent and interactive effects of lutein and tocopherol supplementations on plasma accumulation of these nutrients, oxidative damage markers in breast muscle and plasma, and plumage color development in male house finches — a popular study subject for the control and function of carotenoid coloration in animals (Hill, 2002). We did this in two separate experiments – one run during the breeding season and the other during the molt period – to address the potential for seasonal variation in such physiological effects (McGraw et al., 2011).

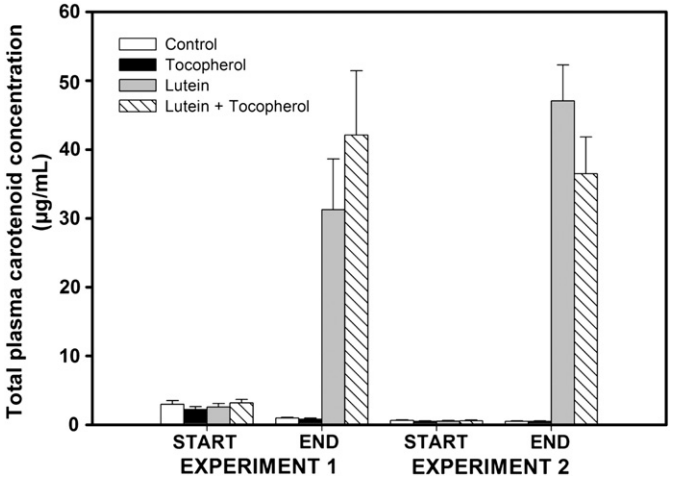

Fig. 2. Circulating carotenoid levels (mean + SE) of male finches supplemented or not with dietary carotenoids and/or tocopherols at the start and the end of the breeding (Experiment 1) and molt (Experiment 2) experiments.

Our carotenoid manipulation was effective in significantly elevating circulating carotenoid levels in both experiments. In fact, unexpectedly, plasma carotenoid levels were extremely high in lutein-supplemented birds, often attaining total concentrations >40 μg/mL, which is fairly rare in nature for house finches from our study population at any time of year (Toomey and McGraw, 2010; Toomey et al., 2010) and was considerably higher than in our prior experiment where we used the same dose (males circulated an average of 25 μg/mL; McGraw et al., 2011). This high increase of circulating carotenoids in carotenoid-supplemented birds was associated with negative effects, such as a reduction in circulating vitamin E compared to birds who did not receive carotenoid supplementation during the molt experiment (only a tendency with a similar trend during the breeding experiment). This result may be explained by a process of molecular competition between carotenoids and vitamin E during the absorption or transport of these dietary compounds. For example, carotenoids and vitamin E are lipid-soluble substances (Schiedt, 1998) that are circulated through the body by lipoproteins (Parker, 1996; Surai, 2002). Thus, lutein saturation of lipoproteins in carotenoid-supplemented birds may have inhibited tocopherol uptake by these molecules, especially during the molt period when the demand for carotenoid pigments is high for coloring the new plumage. Such differential uptake is possible, as McGraw et al. (2004) previously showed competition between xanthophyll carotenoid types when one (zeaxanthin) was in higher dietary concentration (than lutein) in American goldfinches. An alternative hypothesis to explain the decrease in circulating vitamin E that we observed in carotenoid-supplemented birds is that vitamin E was used as an antioxidant to offset harmful actions that carotenoid may have at high concentrations. Huggins et al. (2010) showed, for example, that high intake of carotenoid pigments in American goldfinches led to an increase in creatine kinase, an indicator of skeletal muscle breakdown, and a reduction in vertical flight performance. At high concentrations, carotenoids can lose their antioxidant activity and have pro-oxidant properties through single-electron oxidations or reductions (Palozza et al., 1995; Palozza, 1998; Martin et al., 1999; Russel, 1999; Hartley and Kennedy, 2004). Our results showing that (1) birds that received lutein showed the highest levels of breast oxidative damage markers in both experiments and (2) birds that received lutein + tocopherol showed significantly lower levels of breast oxidative damage markers than birds that received only lutein are in accordance with this latter hypothesis. Unfortunately, in these avian systems, we know very little at present at the

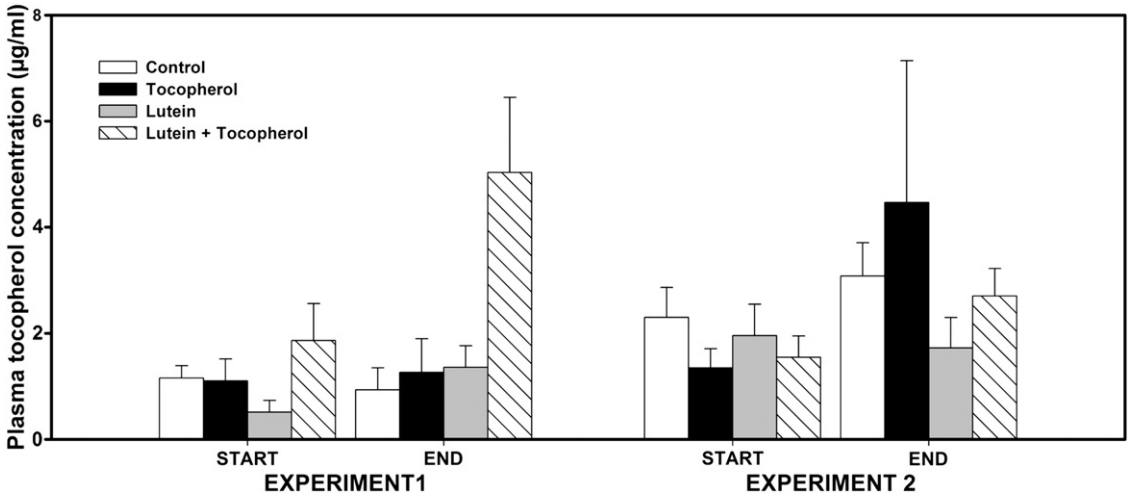

Fig. 3. Plasma vitamin E levels (mean + SE) of male house finches supplemented or not with dietary carotenoids and/or tocopherols at the start and the end of the breeding (Experiment 1) and molt (Experiment 2) experiments.

cellular and biochemical levels about the factors that influence the pro- and anti-oxidant behavior of carotenoids and how birds can limit the detrimental effects of carotenoids using others antioxidant compounds. Future experiments examining interactions among carotenoids and other lipid-soluble nutrients like tocopherol should carefully consider both the physiological mechanisms underlying these interactions as well as their consequences for their emergent effects on phenotype and fitness.

When we assessed effects of our dietary antioxidant manipulations on levels of oxidative damage, we found that tocopherol supplementation decreased levels of oxidative damage in breast muscle (though not in plasma) compared to birds that received lutein during both experiments. These results show for the first time in wild birds the protective effect of tocopherol against oxidative damage. Consistent with our results, Larcombe et al. (2008) previously showed that adult budgerigars (*Melopsittacus undulatus*) fed an antioxidant-rich diet (including tocopherol) suffered less DNA damage and had lower levels of MDA (malondialdehyde, a by-product of lipid peroxidation) than birds fed a low-antioxidant diet. Similarly, studies in humans and rats showed that vitamin E supplementation reduces oxidative stress induced by exercise (Simon-Schnass and Pabst, 1988; Meydani et al., 1993) and pesticide exposure (Giray et al., 2001; John et al., 2001), respectively. As avian metabolic demands (and associated free radical generation) for flight are very high (Lindström et al., 1999), and because vitamin E is a very common constituent of avian diets (Surai, 2002), it is possible that our results on the physiological benefits of vitamin E may extend widely to many other bird species.

Our result showing a reduction in the levels of muscle oxidative damage markers for birds that received tocopherol during the first experiment may appear surprising, given that we did not significantly elevate plasma vitamin E concentration in tocopherol-supplemented birds (though we did demonstrate this in Experiment 2). However, these results are similar to others obtained in previous avian studies. Biard et al. (2006) failed to find an effect of increased dietary antioxidants on plasma antioxidant levels in nestlings great tits (*Parus major*) and blue tits (*Parus caeruleus*), but, similar to our study, did find effects on others parameters (e.g., color in great tits and body condition in both species). It is possible that our tocopherol-supplemented birds had greater vitamin E levels in other tissues/organs (e.g., liver; Surai, 2002; Karadas et al., 2005), though not in blood plasma (Larcombe et al., 2008).

We did not find effects of carotenoid provisioning on any measure of oxidative damage or on plasma nitric oxide levels. These results are consistent with the recent findings suggesting that carotenoids have weak antioxidant properties in birds. For example, Costantini et al.

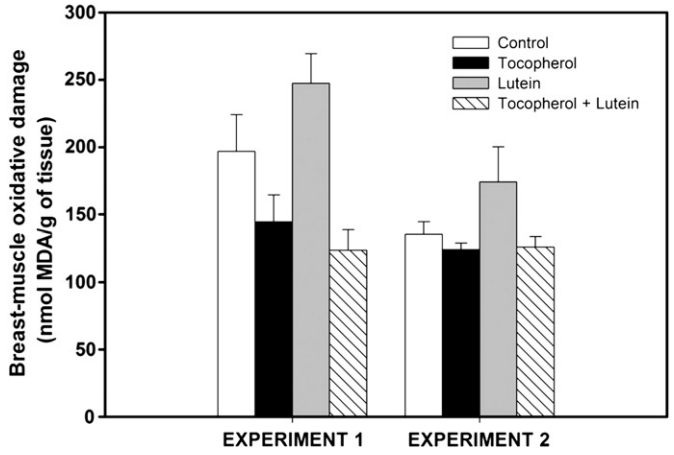

Fig. 4. Oxidative damage levels (mean + SE) in breast muscle of male finches supplemented or not with dietary carotenoids and/or tocopherols at the end of the breeding (Experiment 1) and molt (Experiment 2) experiments.

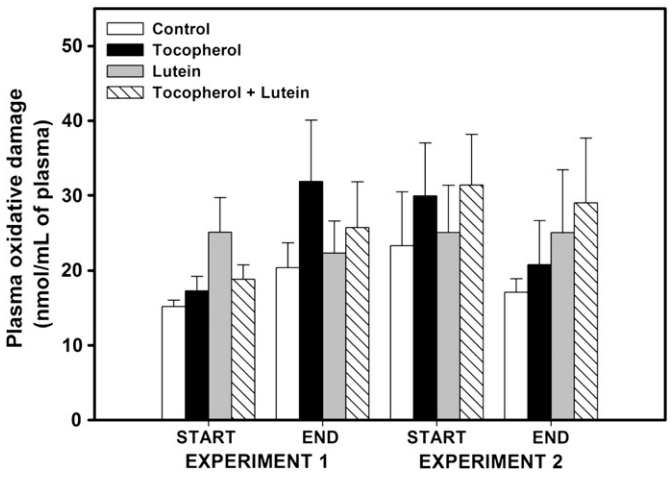

Fig. 5. Levels of oxidative damage (mean + SE) in plasma of male finches supplemented or not with dietary carotenoids and/or tocopherols at the start and the end of the breeding (Experiment 1) and molt (Experiment 2) experiments.

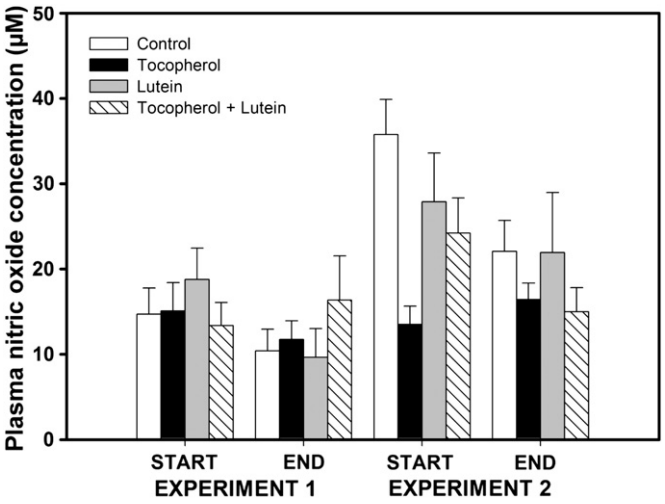

Fig. 6. Plasma nitric oxide concentration (mean + SE) of males supplemented or not with dietary carotenoids and/or tocopherols at the start and the end of the breeding (Experiment 1) and molt (Experiment 2) experiments.

(2007) found no effect of xanthophyll (lutein + zeaxanthin) supplementation on serum levels of oxidative damage or antioxidant capacity in nestling Eurasian kestrels (*Falco tinnunculus*). Moreover, Costantini and Moller (2008) showed in a meta-analysis that there is no significant relationship between carotenoids and antioxidant protection in avian studies published to date. These results are intriguing in light of our prior work showing that carotenoid supplementation boosts immunity in house finches (McGraw et al., 2011; also see McGraw and Ardia, 2003, McGraw and Klasing, 2006, Sepp et al., 2011 for evidence in other species). Taken together, it appears that the immunoenhancing action of carotenoids in this species does not occur via their antioxidant potency, but perhaps via gene-regulatory or other cellular- or receptor-mediated processes (Park et al., 1999). Given that there has been interest among evolutionary biologists in carotenoids, immunity, and antioxidants in colorful animals for nearly two decades (Lozano, 1994), it is due time that we begin to isolate the fine-scale biochemical and physiological mechanisms by which carotenoids bolster immunity.

In accordance with abundant previous work in this and other avian species (reviewed in McGraw, 2006), carotenoid provisioning significantly increased the color of newly grown ornamental feathers. However, contrary to our prediction, tocopherol supplementation did not enhance the expression of carotenoid-based coloration. It was previously demonstrated that supplementation with non-pigmentary antioxidants increases carotenoid-based ornamental coloration in zebra finches (Bertrand et al., 2006), yellow-legged gulls (Perez et al., 2008), and sticklebacks (*Gasterosteus aculeatus*; Pike et al., 2007). On the other hand, vitamin E supplementation did not enhance carotenoid-based plumage coloration of male greenfinches (*Carduelis chloris* L., Karu et al., 2008) or nestling blue tits (*Cyanistes caeruleus*, Larcombe et al., 2010). Overall, our results are not consistent with the hypothesis of Hartley and Kennedy (2004), which stipulates that carotenoid-based coloration may reflect the availability of uncolored antioxidants such as vitamins E or C. One intriguing pattern and interpretation that emerges from our findings, when combined with the literature reviewed above, is that carotenoid coloration of plumage is less sensitive to vitamin E than carotenoid-based bare parts (e.g., beaks, legs, skin). However, it is possible that the supplemental amount of dietary tocopherol given in our experiment was not sufficient or long enough to have an effect on plumage coloration. Moreover, the plumage color developed by our control and tocopherol-supplemented groups was very pale, given that they only received very carotenoid-depleted sunflower seed diets (McGraw et al., 2001). Thus, follow-up studies are needed, where tocopherol is enriched physiologically under carotenoid-replete conditions, to better ascertain effects of vitamin E on plumage color development in house finches.

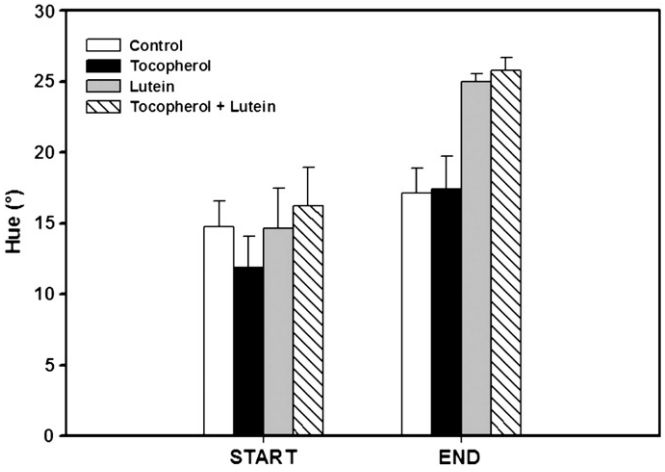

Fig. 7. Hue (mean + SE) of newly grown feathers of male house finches supplemented or not with dietary carotenoids and/or tocopherols at the beginning and end of the molt experiment (Experiment 2).

In conclusion, we have uncovered intriguing independent and interactive effects of two lipid-soluble dietary compounds – tocopherol and carotenoids – on physiology and morphology in a wild species of songbird. Vitamin E circulation declined when animals were fed a high concentration of the xanthophyll carotenoid lutein, suggesting either a process of competition between these two compounds or that tocopherol served as an antioxidant to offset harmful actions that carotenoids may have at high concentrations. In addition, we demonstrate for the first time in wild birds a protective role for tocopherol against tissue oxidative damage. We hope that these results on a complex physiological system spark additional interest in the simultaneous study of multiple antioxidants and oxidative stress markers in organisms (Costantini, 2008).

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