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ARTICLE

Circulating carotenoid levels are negatively associated with previous reproductive success in Florida Scrub-Jays (*Aphelocoma coerulescens*)

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Abstract: The relationship between individual fitness and antioxidants and oxidative stress has come under increasing scrutiny of late. In particular, associations between oxidative balance indicators and reproductive success in the wild have been inconsistent in the limited prior work on this topic. Studies spanning multiple seasons and antioxidant types are particularly lacking. Here, we examined associations between reproductive success over two breeding seasons and several metrics of circulating antioxidants (antioxidant capacity, uric acid, carotenoids, and vitamin E, measured in the intervening nonbreeding season) in Florida Scrub-Jays (*Aphelocoma coerulescens* (Bosc, 1795)). We found that carotenoid levels in the nonbreeding season were negatively associated with reproductive success in the preceding breeding season but unassociated with that in the subsequent breeding season. This correlation may be driven by the cost of reproduction (i.e., carotenoid depletion while breeding) or some other unmeasured and intercorrelated variable such as diet. Antioxidant capacity, uric acid, and vitamin E were not associated with reproductive success. These data are consistent with an emerging theme in physiological ecology: that antioxidants and oxidative stress are but one part of a suite of integrative physiological systems that interact and trade-off in complex ways, making full understanding of their ecological roles challenging.

Key words: antioxidant, fitness, reproduction, uric acid, vitamin E, Florida Scrub-Jays, *Aphelocoma coerulescens*.

Résumé : Le lien entre la valeur adaptative individuelle et les antioxydants et le stress oxydatif suscite un intérêt croissant. Un manque de cohérence est notamment observé en ce qui concerne les associations entre les indicateurs d'équilibre oxydatif et le succès de reproduction en milieu naturel relevées dans les quelques études antérieures sur le sujet. Les études s'étalant sur plusieurs saisons et qui s'intéressent à plusieurs types d'antioxydants sont particulièrement rares. Nous avons examiné les associations entre le succès de reproduction sur deux saisons de reproduction et plusieurs paramètres relatifs aux antioxydants circulants (capacité antioxydante, acide urique, caroténoïdes et vitamine E mesurés durant la période entre les saisons de reproduction) chez des geais à gorge blanche (*Aphelocoma coerulescens* (Bosc, 1795)). Nous avons observé une association négative entre les concentrations de caroténoïdes durant la période de non-reproduction et le succès de reproduction durant la saison de reproduction antérieure, mais aucune association de ces concentrations avec le succès de reproduction pour la saison de reproduction subséquente. Cette corrélation pourrait découler du coût de reproduction (c.-à-d. la réduction des teneurs en caroténoïdes durant la reproduction) ou d'une autre variable non mesurée et intercorrélée, comme le régime alimentaire. Aucune association n'a été notée entre le succès de reproduction et la capacité antioxydante, l'acide urique ou la vitamine E. Ces données sont compatibles avec une notion émergente en écologie physiologique voulant que les antioxydants et le stress oxydatif ne constituent qu'un volet d'un ensemble de systèmes physiologiques intégrés qui interagissent de manière complexe, rendant ainsi difficile une compréhension exhaustive de leurs rôles écologiques. [Traduit par la Rédaction]

Mots-clés : antioxydant, valeur adaptative, reproduction, acide urique, vitamine E, geai à gorge blanche, *Aphelocoma coerulescens*.

Introduction

Physiological ecologists have a long-standing interest in understanding how physiology relates to fitness and life-history traits (Ricklefs and Wikelski 2002). Studies of oxidative balance are growing in importance, based on the hypothesis that efficient regulation of oxidative damage is an important aspect of fitness that varies across individuals or species (Monaghan et al. 2009; Nussey et al. 2009). Oxidative stress is the process through which free radicals and other metabolic and immune derivatives cause oxidative damage, notably to DNA, proteins, and lipids (Davies 2000; von Schantz et al. 1999). Antioxidants can counteract free radicals and oxidizing agents, thus representing a defense mechanism against oxidative stress. Although there is considerable evidence in captive and domesticated organisms that oxidative balance (i.e., the relative levels of oxidative molecules and antioxidants) is associated with survival and longevity (Hulbert et al. 2007), fewer studies have been conducted on wild adult organisms or in relation to reproductive success, with decidedly mixed results (reviewed in Monaghan et al. 2009; Selman et al. 2012).

Birds have emerged as a model for such questions. Experimentally increased reproductive effort resulted in oxidative stress in both captive Zebra Finches (*Taeniopygia guttata* (Vieillot, 1817)) and wild Great Tits (*Parus major* L., 1758) (Christe et al. 2012; Wiersma et al. 2004). However, in Adélie Penguins (*Pygoscelis adeliae*

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(Hombron and Jacquinot, 1841)), experimentally increased reproductive effort resulted in higher antioxidant levels but unchanged levels of oxidative damage (Beaulieu et al. 2011). In Alpine Swifts (*Apus melba* (L., 1758)) oxidative stress resistance was positively associated with both survival and reproductive success (Bize et al. 2008). Measures of reproductive success and subsequent circulating antioxidant levels were correlated in two wild bird species—Leach's Storm-Petrels (*Oceanodroma leucorhoa* (Vieillot, 1818)) and Savannah Sparrows (*Passerculus sandwichensis* (Gmelin, 1789))—but the species differed both in which antioxidants were associated with reproduction and in the direction of the association (Cohen et al. 2009*a*). In two mammal species, reproductive effort was unassociated with oxidative damage to lipids (Bergeron et al. 2011; Nussey et al. 2009).

It is intuitive to expect that high antioxidant levels would be associated with better health and fitness; however, several studies have shown circulating antioxidant levels to be higher in individuals that are stressed or in species with accelerated life histories (e.g., Cohen et al. 2008; Costantini and Verhulst 2009; Hõrak et al. 2006). Thus, it is far from clear what to expect or how to interpret the results from existing avian studies, especially considering that circulating antioxidants only represent one component in the organism's defense system against oxidative stress. If high antioxidant levels indicate greater stress (i.e., the body's response to an environmental or physiological stressor), then we might expect negative associations. Conversely, if antioxidants are indicators of health, then we might expect positive relationships. A more likely scenario is that physiological needs and strategies differ across species and possibly within species (e.g., depending upon life-history stage), so that high and low levels may indicate different things depending on the antioxidant, the species, and perhaps other factors. For example, carotenoid levels may be positively associated with health and fitness in species with high carotenoid content in both diet and plumage, whereas such an association may be unlikely in species with plumage that does not rely on carotenoids for pigmentation, as evidenced by lower levels of circulating carotenoids in species that have less red pigmentation (Simons et al. 2012). This suggests that physiological roles of carotenoids are evolving in response to their dietary availability. Not only are circulating carotenoid levels different among species, they differ by orders of magnitude and often have nonoverlapping distributions (Cohen and McGraw 2009). Additionally, timing of sampling is critical, given known seasonal fluctuations in both availability and utilization of carotenoids (Negro et al. 1998; Safran et al. 2010).

Florida Scrub-Jays (*Aphelocoma coerulescens* (Bosc, 1795); henceforth, scrub-jays) are an excellent species in which to study associations between antioxidants and reproductive success. They are nonmigratory and maintain territories year-round, making it possible to study individuals or populations across generations (Woolfenden and Fitzpatrick 1996). Long-term studies make it possible to incorporate individual histories, experimental manipulations, and other physiological markers to address a wide range of questions (e.g., Schoech et al. 2007; Schoech et al. 2008). Because scrub-jays do not use carotenoids in their plumage and have only two types in circulation (lutein and zeaxanthin, both at very low levels; Heiss et al. 2011), they are a good species in which to test the generality of carotenoid–fitness implications. Although the importance of carotenoids' role as antioxidants in birds has been questioned (Costantini and Møller 2008), some evidence points towards their association with oxidative stress (Pérez-Rodríguez 2009). We have also shown associations between circulating vitamin E levels and clutch initiation (Heiss et al. 2011); the latter measure has been shown to be positively correlated with breeding success in this species (Schoech et al. 2008).

Here, we compare scrub-jay reproductive success in two successive breeding seasons to determine links between reproductive success and antioxidant levels during the intervening nonbreeding season. This sampling design allows us to distinguish between hypotheses related to cost of reproduction and individual quality (e.g., Mauck et al. 2004). (Individual quality is a complex concept; for our purposes here it can be crudely considered to be positive correlations among fitness correlates.) If antioxidant levels decrease as a cost of reproduction, antioxidants in the nonbreeding season should correlate negatively with breeding success in the preceding breeding season, but birds with higher nonbreeding carotenoid levels would be expected to have better breeding success in the succeeding breeding season. This should result in a negative correlation in breeding success between 2004 and 2005. If antioxidant levels reflect individual quality, then they should correlate positively with reproduction in both years, and the 2 years should correlate positively. Reproductive success should also correlate positively with reproductive effort in this case. In addition, we investigate whether age or individual body condition is associated with reproductive output, carotenoid levels, or both.

Materials and methods

Study species and food supplementation

We studied free-living scrub-jays at Archbold Biological Station, Highland County, Florida (27°10′N, 81°21′W; elevation 38–68 m) as previously described (see Schoech 1996; Schoech and Bowman 2003). Scrub-jays are cooperative breeders that defend large all-purpose territories throughout the year. Approximately half of these territories are occupied by breeding pairs with nonbreeding helpers that are usually the offspring of one or both of the breeders; most helpers assist in territory defense and provisioning of nestlings and fledglings (Schoech et al. 1996). The other half of the territories are occupied by a breeding pair only (DeGange et al. 1989). As a part of our ongoing research with this population, all birds were uniquely marked with both color bands and a U.S. Geological Survey band and we tracked the fate of nearly every egg laid during a bird's lifetime. The sex of all birds was determined through sex-specific behaviors (e.g., vocalizations) or genetic sexing, and their status (breeder or nonbreeding helper) was determined by intensive monitoring of all scrub-jays in the population.

Scrub-jays breed between March and June, with considerable interyear variation in the onset of breeding (Schoech et al. 1996, 2007). Clutch size ranges from 1 to 5 eggs, with a mean of 3 eggs (Woolfenden and Fitzpatrick 1996). Nests were found for each breeding pair during the building or laying stages. If the date on which the first egg laid was unknown for a given nest, we counted back from the date of first hatch (incubation lasts for ~18 days). After location, nests were monitored daily to determine the date of first clutch initiation and subsequent nesting attempts.

Study birds were also part of a large-scale field experiment tangential to this study (Schoech et al. 2007). The 68 territories that made up the study tract in 2004–2005 were divided into three treatment groups. Group 1 (N = 30) was a control group that received no supplemental food. Group 2 (N = 16) was supplemented ad libitum with dried dog food (25%–27% crude protein, 14% fat) beginning in the third week of January 2005 through to the date the first egg for a given group was laid (see Reynolds et al. 2003; Schoech et al. 2004; Schoech and Bowman 2003), and group 3 (N = 22) was supplemented ad libitum with the same dried dog food starting in mid-October 2004 and ending with the date of first clutch initiation. Supplemental food was provided in feeders placed near the center of each territory, and use of the feeders by members of the focal group was confirmed through observation. In some instances, it was impossible to prevent individuals from nonsupplemented territories from taking supplemented food. Although territories were heavily defended from intrusion by other jays (as noted by Woolfenden and Fitzpatrick 1996), there were extensive invasions and disruptions of long-term supplemented territories. Although the treatment groups were not of primary interest here, they have the potential to confound measures of interest in the current study. Therefore, we statistically controlled for food supplementation by including the groups as a variable in all analyses (Heiss et al. 2011 and data not shown). Treatment group did not affect the conclusions and analyses presented here.

Capture protocol

Scrub-jays were trapped in continuously monitored Potter traps baited with peanuts (Schoech 1996) at Archbold Biological Station and the nearby suburbs between January and February 2005. Some birds were recaptured, and some were not breeders; after excluding these, we had data for 79 individuals. Males constituted 54% of the sample and ages ranged from 1 to 13 years. Following venipuncture of the brachial vein with a 25-gauge needle, blood samples were collected in microhematocrit tubes. All blood samples were taken within 120 s of capture to avoid effects of stress on antioxidant levels (Cohen et al. 2007). Morphometric data (body mass, head breadth, and the distance from the nares to bill tip, as well as the lengths of the wing cord, head-plus-bill, culmen, and tail) were taken immediately after blood samples were obtained. Samples were immediately placed on ice and kept cool (0–5 °C) and, upon return to the laboratory (usually within 1–3 h), were centrifuged to remove the plasma portion, which was then frozen and stored at −80 °C until analysis.

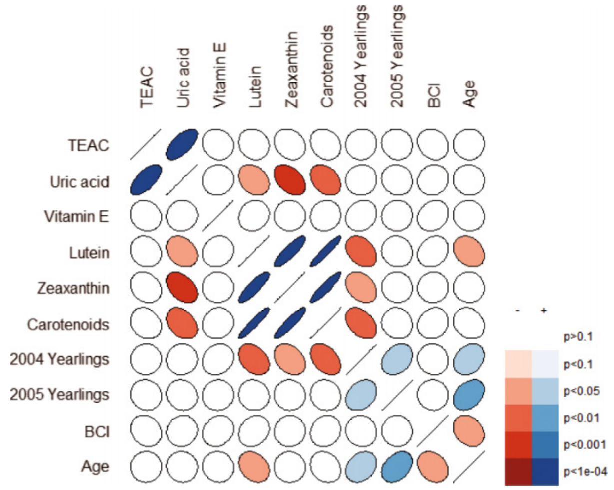
Antioxidant analysis

Trolox-equivalent antioxidant capacity (TEAC) of 1–5 μL serum was measured following Cohen et al. (2007). The assay uses a chromogenic free radical, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS; Sigma), activated by H_2O_2. As the free radical is activated, the solution shows a linear increase in absorbance; any micromolecular antioxidants in the sample delay the start of this increase by quenching the free radical as it is activated. When the antioxidants are exhausted, absorbance increase begins. Antioxidant capacity is thus quantified by measuring the delay in start of increase relative to Trolox (Aldrich), a water-soluble vitamin E analogue used as the standard. This assay gives a functional measure of antioxidant capacity; it reveals how effective the sample is at quenching free radicals. It does not measure antioxidant contributions of proteins or enzymes, and says nothing about antioxidant activity in tissues. Primary antioxidants contributing to the assay include uric acid, vitamins E and C, carotenoids, phenolics, and bilirubin (Miller et al. 1993). All measurements were conducted on a VersaMax spectrophotometer (Molecular Devices, Sunnyvale, California, USA). Interassay coefficient of variation (CV) for individual samples was 7%.

Uric acid concentration was quantified with a spectrophotometric kit based on uricase and a chromogen (Sterling Diagnostics, Sterling Heights, Michigan, USA). The samples can be run alongside the antioxidant samples in the same microplate. Interassay CV for samples was 5%.

Lipids were extracted by sequentially adding 100 μL ethanol and 100 μL *tert*-butyl methyl ether to 5–10 μL plasma, vortexing, and centrifuging for 15 s at 10 000 rev/min. We transferred the supernatant to a fresh tube, evaporated the solvent to dryness under a stream of nitrogen, and redissolved the extract in 200 μL of 42:42:16 (*v*/*v*/*v*) methanol–acetonitrile–dichloromethane. Carotenoids and vitamin E were subsequently analyzed using high-performance liquid chromatography (HPLC), following previously published methods (McGraw and Parker 2006), with the following slight modifications. Pigment extracts were injected into a Waters Alliance 2695 HPLC system (Waters Corporation, Milford, Massachusetts, USA) fitted with a Waters YMC Carotenoid 5.0 μm column (4.6 mm × 250 mm) 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: first, isocratic elution with 42:42:16 (*v*/*v*/*v*) methanol–acetonitrile–dichloromethane for 11 min, followed by a linear gradient up to 42:23:35 (*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 40 min. Data were collected from 250 to 600 nm using a Waters 2996 photodiode array detector. We identified molecules by comparing their respective retention times and absorbance maxima (λ_{max}) to those of pure standards. Concentrations were quantified by comparison to standard curves.

Fig. 1. Correlations between antioxidant levels, reproductive success, body condition (BCI), and age of Florida Scrub-Jays (*Aphelocoma coerulescens*). Narrowness of ellipses represent $|r|$, with a line for $|r| = 1$ and a circle for $|r| = 0$. Right-tilted and blue (on the Web; grey in print) ellipses represent positive correlations, whereas left-tilted and red (on the Web; grey in print) ellipses represent negative correlations. Darkness of shading indicates level of significance. There were significantly more correlations that reached the α threshold of 0.05 than randomly expected (χ^2 test for proportions, $p < 0.001$).

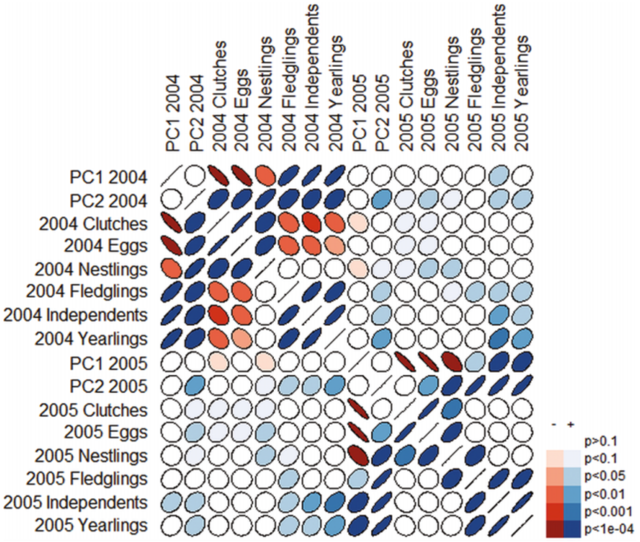


Data analysis

All statistical analyses were performed using R version 2.10.0 for Windows. Data were checked for normality using Q-Q plots and the Shapiro–Wilk test. All antioxidant measures (concentrations of plasma uric acid, vitamin E, lutein, and zeaxanthin) were non-normally distributed and therefore were log- or square-root-transformed to meet the assumptions of normality; transformed values were used in all subsequent analyses. Antioxidant covariation was initially analyzed with a correlation matrix (Fig. 1); lutein and zeaxanthin levels were tightly correlated ($r = 0.85$, $p < 0.0001$) and we made a simplified, normally distributed combined metric for our statistical analyses, "Carotenoids", by taking the log of the sum of their standard normal transformations (subtract mean, divide by standard deviation). Vitamin E levels were not associated with other antioxidant measures ($|r| < 0.15$, $p > 0.2$ for all), whereas uric acid was weakly negatively associated with carotenoids (Fig. 1).

Reproductive success was measured in both 2004 and 2005, the seasons before and after the blood sampling. Multiple measures of success were collected and analyzed, including laying date and numbers of clutches, eggs, nestlings, fledglings, independent young, and yearlings produced, as well as several composite indices based on these measures. Our conclusions did not depend on how we measured reproductive success (data not shown). For brevity and clarity, we present primarily yearling-based analyses here, as the number of fledged yearlings is the closest measure

Fig. 2. Correlations among various reproductive measures of Florida Scrub-Jays (*Aphelocoma coerulescens*) for 2004 and 2005. PC1 and PC2 are the first and second axes from a principal components (PC) analysis of the reproductive measures conducted separately for each year. Narrowness of ellipses represent $|r|$, with a line for $|r| = 1$ and a circle for $|r| = 0$. Right-tilted and blue (on the Web; grey in print) ellipses represent positive correlations, whereas left-tilted and red (on the Web; grey in print) ellipses represent negative correlations. Darkness of shading indicates level of significance. There were significantly more correlations that reached the α threshold of 0.05 than randomly expected (χ^2 test for proportions, $p < 0.001$).



that we have to an ultimate measure of success. Associations among reproductive measures are shown in Fig. 2.

To include a measure of body condition, we calculated a body condition index (BCI; see Green 2001) by regression of mass against the first principal component of wing-cord, tarsus, tail, and head-plus-bill lengths (eigenvalue = 1.5, variance explained = 56%, 0.42 < all loadings < 0.54; regression $r^2 = 0.34$, $p < 0.0001$). The residuals from this regression were saved as the BCI.

Correlation matrices were used to assess the potential for confounding covariates; detailed analyses were then run using general linear models of reproductive success as a function of levels of each antioxidant and various combinations of covariates (sex, BCI, and treatment group). As they are not statistically justified (Harrell 2001), we did not use stepwise procedures. Instead, we checked all apparent significant results to see if the pattern was robust when including different combinations of covariates, as well as to see if the pattern was robust when using alternative metrics such as carotenoids versus lutein. We accounted for multiple testing by using global tests for false discovery rates by table or figure. We used χ^2 tests for proportions to calculate whether the number of significant results ($p < 0.05$) in each table or figure was greater than expected by chance.

Results

Basic statistics for variables used are presented in Table 1. Reproductive success was positively correlated between the 2 years, as measured by the number of independents and yearlings ($0.0001 < p < 0.05$ for all; Fig. 2). In 2004, there were strong negative associations between numbers of eggs and clutches and number of yearlings and independents, indicating that nest failure was a big driver of overall success and supporting a distinction between reproductive effort (clutches, eggs) and reproductive success (yearlings, independents). In 2005, a better year for reproduction (Schoech et al. 2007), there was less contrast between early and late breeding measures. The correlation structure in Fig. 2 shows that use of yearlings as a proxy for overall reproductive success is justified in both years.

Table 1. Basic statistics on measured variables from Florida Scrub-Jays (*Aphelocoma coerulescens*).

Variable	*n*	Mean ± SD
TEAC	78	1.37±0.48
Uric acid (mg/dL)	79	24.56±10.0
Vitamin E (absolute units)	59	4363±4303
Lutein (μg/dL)	59	0.91±0.88
Zeaxanthin (μg/dL)	59	0.80±1.2
Carotenoids	59	1.71±2.0
BCI	73	0.00±3.9
Age	79	5.52±3.0
2004		
Yearlings	70	0.59±0.84
Independents	70	0.90±1.1
Fledglings	70	1.47±1.4
Nestlings	70	3.26±1.6
Eggs	70	5.83±3.6
Clutches	70	1.77±1.02
2005		
Yearlings	74	0.73±0.98
Independents	74	0.96±1.1
Fledglings	74	1.72±1.2
Nestlings	74	2.95±1.9
Eggs	74	5.18±3.1
Clutches	74	1.65±0.82

Note: BCI, body condition index; TEAC, Trolox-equivalent antioxidant capacity.

Figure 1 shows that all carotenoid measures were negatively correlated with 2004 reproductive success but unassociated with 2005 reproductive success. TEAC, uric acid levels, and vitamin E levels were not correlated with yearling production in either year. BCI was also uncorrelated with reproductive success and with antioxidant levels. Antioxidant levels were generally not correlated with the covariates considered (age, sex, and BCI; $p > 0.2$ except lutein with age: $r = -0.28$, $p = 0.02$).

Regression models were used to assess possible mediation of observed correlations by covariates (Table 2). These models confirm a consistent negative association between circulating carotenoid levels and 2004 reproductive success. In no model did the carotenoid effect disappear completely, and in only one did it fail to reach significance for any carotenoid measure (model IV). Note that even in this case the effect size is largely unchanged and the trend is consistent with other models. However, these models also confirm the absence of effects for TEAC and uric acid in 2004 and for all antioxidants in 2005. Note the consistent effect sizes across models for carotenoids and 2004 reproduction. Overall, the models in Table 2 show that the relationships between antioxidant levels and reproductive success—whether strong or absent—do not depend on covariates such as sex, age, body condition, or treatment group.

Discussion

This study shows an association between antioxidant levels and reproductive success in a free-living animal species, specifically, a negative association between reproductive success and subsequent plasma carotenoid levels. However, only carotenoids were associated with reproductive success, whereas TEAC, uric acid, and vitamin E were not. Moreover, carotenoid levels measured between the two breeding seasons were only associated with the previous breeding season but not the subsequent one. In contrast, we recently found a positive correlation between levels of some

Table 2. Regression coefficients of the effect of various antioxidants on yearling production by Florida Scrub-Jays (*Aphelocoma coerulescens*) under various models.

	Model			
	I	II	III	IV
2004				
TEAC	0.20	0.19	−0.06	0.01
Uric acid	0.15	0.15	−0.02	−0.35
Vitamin E	0.24	0.24	0.32*	0.24
Lutein	**−0.70‡**	**−0.70†**	**−0.69†**	**−0.70†**
Zeaxanthin	**−0.46†**	**−0.46†**	**−0.48†**	−0.42*
Carotenoids	**−0.31‡**	**−0.31‡**	**−0.31†**	**−0.35†**
2005				
TEAC	−0.42	−0.41	−0.27	−0.62
Uric acid	−0.42	−0.42	−0.35	−0.66
Vitamin E	0.05	0.05	0.09	0.03
Lutein	−0.19	−0.22	−0.14	0.44
Zeaxanthin	−0.06	−0.07	−0.09	0.45
Carotenoids	−0.08	−0.09	−0.07	0.17

Note: Model I: no control; model II: control for sex; model III: control for sex and body condition; model IV: control for sex, body condition, and treatment group. TEAC, Trolox-equivalent antioxidant capacity. There were significantly more associations that reached the α threshold of 0.05 than randomly expected (χ^2 test for proportions, $p < 0.001$). *, $0.05 \leq p < 0.2$; †, $0.01 \leq p < 0.05$; ‡, $p < 0.01$, with values in boldface type indicating any $p < 0.05$.

antioxidants and clutch initiation date (i.e., individuals with higher levels tended to delay laying) in the subsequent breeding season in female scrub-jays (Heiss et al. 2011). Associations between antioxidants and reproductive success have been found in other species as well; however, differences in both the antioxidant measured and the direction of the relationship makes interpretation difficult. In Adélie Penguins, increased reproductive effort resulted in increased antioxidant capacity (as measured by the OXY-ADSORBENT assay; Beaulieu et al. 2011). Alpine Swift resistance to oxidative stress (as measured by the KRL assay, which integrates antioxidant levels and other information) was associated with larger clutch size and better egg hatchability (Bize et al. 2008). In Leach's Storm-Petrels, TEAC and mean annual reproductive success were negatively correlated, and in Savannah Sparrows, β-carotene levels were positively related to reproductive success (Cohen et al. 2009*a*). Thus, no two species have yet shown consistent patterns; to our knowledge, the present study is the first to show a negative association between carotenoid levels and previous reproductive success.

At first glance, the negative correlation between 2004 reproductive output and carotenoid levels the following winter appears to suggest a cost of reproduction. Birds with higher reproductive output in 2004 may have suffered oxidative stress as a result of increased reproductive effort, and their low carotenoid levels in early 2005 may thus reflect poorer health, as suggested by the results of Wiersma et al. (2004). Such cost-of-reproduction hypotheses are plausible, but not all our results are confirmatory. For 2004, we found a negative association between reproductive effort (number of clutches, eggs, or nestlings) and reproductive success (number of independents or yearlings; Fig. 2). Furthermore, if there were trade-offs, we would expect a negative correlation between reproductive output in 2004 and 2005, and a positive correlation between carotenoid levels and 2005 reproduction. We see neither of these, and in fact see a positive association between reproductive successes in the 2 years. This suggests that reproductive success as we measure it may be indicative of individual quality, the effects of breeding experience, differential territory quality, or a combination of these factors.

However, there is also no evidence that high carotenoid levels are an indicator of individual quality or health in scrub-jays. BCI was not associated with carotenoid levels. Out of 78 species examined in a recent study, the scrub-jays had the sixth-lowest mean carotenoid levels, 1.8 μg/dL (cross species mean of 17.0 and range of 0.7–81.1; Cohen and McGraw 2009). This implies that carotenoids may be less important physiologically in scrub-jays than in some other species, and even the individuals with the lowest levels may have sufficient carotenoids for their needs. Accordingly, there are many plausible but unsubstantiated explanations for the associations that we observed. Carotenoids may be unrelated to health but correlated with an extraneous variable related specifically to 2004 reproductive success, such as the presence of a nest predator in territories having a certain vegetation composition. Previously successful breeders may favor a diet low in carotenoids but beneficial in some other way. Given our inability to exclude the numerous such hypotheses, we do not believe it is productive to speculate on all the potential explanations without more evidence.

Even if there is a direct causal relationship between reproductive success and subsequent carotenoid levels, such as a cost of reproduction, the implications of this are not clear for the role of oxidative stress in life-history trade-offs. Costantini and Møller (2008) argue that carotenoids are not important components of antioxidant defense in birds based on their molar levels, and this is supported by a number of detailed studies (e.g., Isaksson and Andersson 2008; Isaksson et al. 2007). However, a recent meta-analysis found a weak but significant positive association between carotenoid levels and antioxidant capacity (Simons et al. 2012). Comparative data suggest that these relationships vary across species (Cohen and McGraw 2009). All of these studies are based on blood samples and may miss important relationships occurring in other tissues; additionally, a focus on antioxidants alone ignores other aspects of oxidative stress such as damage levels and repair mechanisms (Selman et al. 2012). Even within blood, a focus on molar levels may obscure important antioxidant roles of carotenoids within membranes that do not depend on their levels relative to overall antioxidant capacity. Carotenoids have other physiological roles as well (Hartley and Kennedy 2004), and it would be a mistake to assume that the relationships detected here necessarily reflect oxidative stress, especially in the absence of confirmatory data from other antioxidant measures. As argued above, the physiological role and importance of carotenoids likely evolves in response to dietary intake and other factors, making it particularly difficult to draw clear conclusions in any given study.

Taking in and maintaining sufficient carotenoids during breeding can be a challenge. For example, Safran et al. (2010) found that years in which Barn Swallows (*Hirundo rustica* L., 1758) sustained elevated carotenoid levels throughout the breeding season were characterized by increased reproductive output. In females of many species, egg laying both requires substantial amounts of carotenoids and depletes physiological reserves (Negro et al. 1998; Bortolotti et al. 2003; Blount 2004). The study by Safran et al. (2010) highlights the need to acquire multiple, independent assessments of antioxidant levels or oxidative state across time, given that free-radical quenching can occur on the scale of seconds and antioxidant supply from food or tissues can be elevated or markedly reduced on the order of minutes to hours. Despite such fine-scale variation, here we are able to demonstrate that longer-term associations are also present. Whether these directly reflect carotenoid use and storage is less clear—it is not known how long it might take to recover carotenoid levels in scrub-jays.

Carotenoids aside, we found no other associations between reproduction and antioxidant metrics, including plasma antioxidant capacity, vitamin E concentration, and uric acid concentration. This was despite sufficient sample sizes and measurements of antioxidants that were repeatable and have been found to be biologically relevant in other studies (Cohen et al. 2008, 2009*a*; 2009*b*). We did not measure antioxidants during reproduction, so we had a reduced likelihood of detecting a significant association between these two variables from the out-set. In addition, because we found limited links between these antioxidant measures and other potential indicators of quality (e.g., body condition, age), it is possible that this suite of antioxidants is neither limiting nor differentially valuable to fitness in our population of scrub-jays. The multidimensional nature of antioxidant variation here is consistent with a complex picture of antioxidant function in which not all measures are correlated, where measures sometimes reflect important aspects of function but are also sometimes incidental, and where the information content of a given measure varies across species, conditions, and other factors. Such transient and species-specific effects would also explain the dependence of lay date on somewhat complex interactions between vitamin E, sex, and supplementation treatment in these scrub-jays (Heiss et al. 2011), as well as the differing results across species (Selman et al. 2012).

It is reasonable to question whether the effects we detect are due to multiple testing issues. We are effectively measuring levels of four antioxidants relative to two breeding seasons, so false discovery rates should be considered. Even with a Bonferroni correction, most of our significant results would remain significant or nearly so. However, approaches like Bonferroni corrections are problematic for many reasons (Moran 2003; Perneger 1998). In this particular context, they are not appropriate because we report and are interested in the negative results for the other antioxidants and breeding season, and because we have multiple tests using correlated measures and thus with correlated results. Other approaches to false discovery rate were considered (Pike 2011), but methods based on q-values raise concerns under the assumption of variable dependency (Kim and van de Wiel 2008). We have addressed the false discovery rate concern by showing that (*i*) the results are robust whether looking at simple correlations or regression models with various covariates included or removed; (*ii*) the relative strengths of the associations vary across the models as expected for a real association; (*iii*) the results can be replicated with other measures of reproductive success (data not shown); and (*iii*) we detect significantly more significant results than expected by chance, based on the tablewise proportion of expected false positives. False discovery rates with a normal α = 0.05 threshold are still only 1 in 20, and we believe the combined validation analyses bring us to at least this level of certainty, though precise quantification is impossible.

The results of this study integrate well into an emerging picture of oxidative physiology as a complex dynamic system (Cohen et al. 2012; Costantini et al. 2011). There may be no straightforward relationship between antioxidant levels and fitness, but oxidative balance may nonetheless be important for fitness in ways that are difficult to measure with a limited sampling scheme such as ours. Given the potential confounding effects of diet, physiological history of individuals, etc., drawing firm conclusions is problematic. Some species may have a tighter relationship between oxidative balance and fitness than others. There appears to be an equally complex relationship between oxidative balance and other variables such as immune function and environmental quality (Costantini and Møller 2009; Isaksson et al. 2005). This complexity is problematic for both experimental studies, which manipulate only a small number of variables, and for observational studies such as this, which can measure many factors but cannot pinpoint their causal relationships. Future studies of oxidative balance will require greater detail than we are able to provide here, such as more physiological measures and an understanding of how they covary over time within individuals. Experimental approaches might be possible, but risk detecting effects that depend strongly on experimental conditions. Measures of cumulative damage and repeated measurements in individuals over varying time scales may help clarify when oxidative balance is a key driver in fitness processes and when it is a spurious covariate (Selman et al. 2012).

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References

Beaulieu, M., Reichert, S., Le Maho, Y., Ancel, A., and Criscuolo, F. 2011. Oxidative status and telomere length in a long-lived bird facing a costly reproductive event. Funct. Ecol. **25**(3): 577–585. doi:10.1111/j.1365-2435.2010.01825.x.

Bergeron, P., Careau, V., Humphries, M.M., Réale, D., Speakman, J.R., and Garant, D. 2011. The energetic and oxidative costs of reproduction in a free-ranging rodent. Funct. Ecol. **25**(5): 1063–1071. doi:10.1111/j.1365-2435.2011.01868.x.

Bize, P., Devevey, G., Monaghan, P., Doligez, B., and Christe, P. 2008. Fecundity and survival in relation to resistance to oxidative stress in a free-living bird. Ecology, **89**(9): 2584–2593. doi:10.1890/07-1135.1. PMID:18831179.

Blount, J.D. 2004. Carotenoids and life-history evolution in animals. Arch. Biochem. Biophys. **430**(1): 10–15. doi:10.1016/j.abb.2004.03.039. PMID:15325906.

Bortolotti, G.R., Negro, J.J., Surai, P.F., and Prieto, P. 2003. Carotenoids in eggs and plasma of red-legged partridges: effects of diet and reproductive output. Physiol. Biochem. Zool. **76**(3): 367–374. doi:10.1086/375432. PMID:12905123.

Christe, P., Glaizot, O., Strepparava, N., Devevey, G., and Fumagalli, L. 2012. Twofold cost of reproduction: an increase in parental effort leads to higher malarial parasitaemia and to a decrease in resistance to oxidative stress. Proc. R. Soc. Lond. B Biol. Sci. **279**(1731): 1142–1149. doi:10.1098/rspb.2011.1546.

Cohen, A.A., and McGraw, K.J. 2009. No simple measures for antioxidant status in birds: complexity in inter- and intraspecific correlations among circulating antioxidant types. Funct. Ecol. **23**(2): 310–320. doi:10.1111/j.1365-2435.2008.01504.x.

Cohen, A.A., Klasing, K.C., and Ricklefs, R.E. 2007. Measuring circulating antioxidants in wild birds. Comp. Biochem. Physiol. Part B Biochem. Mol. Biol. **147**(1): 110–121. doi:10.1016/j.cbpb.2006.12.015.

Cohen, A.A., McGraw, K.J., Wiersma, P., Williams, J.B., Robinson, W.D., Robinson, T.R., Brawn, J.D., and Ricklefs, R.E. 2008. Interspecific associations between circulating antioxidant levels and life history variation in birds. Am. Nat. **172**(2): 178–193. doi:10.1086/589456. PMID:18624664.

Cohen, A.A., Mauck, R.A., Wheelwright, N.T., Huntington, C.E., and McGraw, K.J. 2009*a*. Complexity in relationships between antioxidants and individual life-history parameters in a seabird and a songbird. Oikos, **118**(12): 1854–1861. doi:10.1111/j.1600-0706.2009.17785.x.

Cohen, A.A., McGraw, K.J., and Robinson, W.D. 2009*b*. Serum antioxidant levels in wild birds vary in relation to diet, season, life history strategy, and species. Oecologia, **161**(4): 673–683. doi:10.1007/s00442-009-1423-9. PMID:19669166.

Cohen, A.A., Martin, L.B., Wingfield, J.C., McWilliams, S.R., and Dunne, J.A. 2012. Physiological regulatory networks: ecological roles and evolutionary constraints. Trends Ecol. Evol. **27**(8): 428–435. doi:10.1016/j.tree.2012.04.008.

Costantini, D., and Møller, A.P. 2008. Carotenoids are minor antioxidants for birds. Funct. Ecol. **22**(2): 367–370. doi:10.1111/j.1365-2435.2007.01366.x.

Costantini, D., and Møller, A.P. 2009. Does immune response cause oxidative stress in birds? A meta-analysis. Comp. Biochem. Physiol. A Mol. Integr. Physiol. **153**(3): 339–344. doi:10.1016/j.cbpa.2009.03.010.

Costantini, D., and Verhulst, S. 2009. Does high antioxidant capacity indicate low oxidative stress? Funct. Ecol. **23**(3): 506–509. doi:10.1111/j.1365-2435.2009.01546.x.

Costantini, D., Monaghan, P., and Metcalfe, N.B. 2011. Biochemical integration of blood redox state in captive zebra finches (*Taeniopygia guttata*). J. Exp. Biol. **214**(7): 1148–1152. doi:10.1242/jeb.053496. PMID:21389200.

Davies, K.J. 2000. Oxidative stress, antioxidant defenses, and damage removal, repair, and replacement systems. IUBMB Life, **50**(4–5): 279–289. doi:10.1080/713803728. PMID:11327322.

DeGange, A.R., Fitzpatrick, J.W., Layne, J.N., and Woolfenden, G.E. 1989. Acorn harvesting by Florida Scrub Jays. Ecology, **70**(2): 348–356. doi:10.2307/1937539.

Green, A.J. 2001. Mass/length residuals: measures of body condition or generators of spurious results? Ecology, **82**(5): 1473–1483. doi:10.1890/0012-9658(2001)082[1473:MLRMOB]2.0.CO;2.

Harrell, F.E., Jr. 2001. Regression modeling strategies. Springer-Verlag, New York.

Hartley, R.C., and Kennedy, M.W. 2004. Are carotenoids a red herring in sexual display? Trends Ecol. Evol. **19**(7): 353–354. doi:10.1016/j.tree.2004.04.002.

Heiss, R.S., Cohen, A.A., Bowman, R., Boughton, R.K., Bridge, E., McGraw, K.J., and Schoech, S.J. 2011. Circulating carotenoid concentrations are positively correlated with later clutch initiation in Florida Scrub-Jays (*Aphelocoma coerulescens*). J. Exp. Zool. A Ecol. Genet. Physiol. **315A**(2): 101–110. doi:10.1002/jez.654.

Hõrak, P., Zilmer, M., Saks, L., Ots, I., Karu, U., and Zilmer, K. 2006. Antioxidant protection, carotenoids and the costs of immune challenge in greenfinches. J. Exp. Biol. **209**: 4329–4338. doi:10.1242/jeb.02502. PMID:17050848.

Hulbert, A.J., Pamplona, R., Buffenstein, R., and Buttemer, W.A. 2007. Life and death: metabolic rate, membrane composition, and life span of animals. Physiol. Rev. **87**: 1175–1213. doi:10.1152/physrev.00047.2006. PMID:17928583.

Isaksson, C., and Andersson, S. 2008. Oxidative stress does not influence carotenoid mobilization and plumage pigmentation. Proc. R. Soc. Lond. B Biol. Sci. **275**(1632): 309–314. doi:10.1098/rspb.2007.1474.

Isaksson, C., Ornborg, J., Stephensen, E., and Andersson, S. 2005. Plasma glutathione and carotenoid coloration as potential biomarkers of environmental stress in Great Tits. EcoHealth, **2**(2): 138–146. doi:10.1007/s10393-005-3869-5.

Isaksson, C., McLaughlin, P., Monaghan, P., and Andersson, S. 2007. Carotenoid pigmentation does not reflect total non-enzymatic antioxidant activity. Funct. Ecol. **21**(6): 1123–1129. doi:10.1111/j.1365-2435.2007.01317.x.

Kim, K.I., and van de Wiel, M.A. 2008. Effects of dependence in high-dimensional multiple testing problems. BMC Bioinformatics, **9**: 114. doi:10.1186/1471-2105-9-114. PMID:18298808.

Mauck, R.A., Huntington, C.E., and Grubb, T.C. 2004. Age-specific reproductive success: evidence for the selection hypothesis. Evolution, **58**(4): 880–885. doi:10.1111/j.0014-3820.2004.tb00419.x. PMID:15154562.

McGraw, K.J., and Parker, R.S. 2006. A novel lipoprotein-mediated mechanism controlling sexual attractiveness in a colorful songbird. Physiol. Behav. **87**(1): 103–108. doi:10.1016/j.physbeh.2005.09.001.

Miller, N.J., Rice-Evans, C., Davies, M.J., Gopinathan, V., and Milner, A. 1993. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. Clin. Sci. **84**(4): 407–412. PMID:8482045.

Monaghan, P., Metcalfe, N.B., and Torres, R. 2009. Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. Ecol. Lett. **12**(1): 75–92. doi:10.1111/j.1461-0248.2008.01258.x. PMID:19016828.

Moran, M.D. 2003. Arguments for rejecting the sequential Bonferroni in ecological studies. Oikos, **100**(2): 403–405. doi:10.1034/j.1600-0706.2003.12010.x.

Negro, J.J., Bortolotti, G.R., Tella, J.L., Fernie, K.J., and Bird, D.M. 1998. Regulation of integumentary colour and plasma carotenoids in American Kestrels consistent with sexual selection theory. Funct. Ecol. **12**(2): 307–312. doi:10.1046/j.1365-2435.1998.00176.x.

Nussey, D.H., Pemberton, J.M., Pilkington, J.G., and Blount, J.D. 2009. Life history correlates of oxidative damage in a free-living mammal population. Funct. Ecol. **23**(4): 809–817. doi:10.1111/j.1365-2435.2009.01555.x.

Pérez-Rodríguez, L. 2009. Carotenoids in evolutionary ecology: re-evaluating the antioxidant role. BioEssays, **31**(10): 1116–1126. doi:10.1002/bies.200900070. PMID:19705366.

Perneger, T.V. 1998. What's wrong with Bonferroni adjustments. Br. Med. J. **316**: 1236–1238. doi:10.1136/bmj.316.7139.1236.

Pike, N. 2011. Using false discovery rates for multiple comparisons in ecology and evolution. Methods Ecol. Evol. **2**: 278–282. doi:10.1111/j.2041-210X.2010.00061.x.

Reynolds, S.J., Schoech, S., and Bowman, R. 2003. Diet quality during pre-laying and nestling periods influences growth and survival of Florida scrub-jay (*Aphelocoma coerulescens*) chicks. J. Zool. (Lond.), **261**: 217–226. doi:10.1017/S0952836903004023.

Ricklefs, R.E., and Wikelski, M. 2002. The physiology/life-history nexus. Trends Ecol. Evol. **17**(10): 462–468. doi:10.1016/S0169-5347(02)02578-8.

Safran, R.J., McGraw, K.J., Wilkins, M.R., Hubbard, J.K., and Marling, J. 2010. Positive carotenoid balance correlates with greater reproductive performance in a wild bird. PLOS One, **5**: e9420. doi:10.1371/journal.pone.0009420. PMID:20195540.

Schoech, S.J. 1996. The effect of supplemental food on body condition and the timing of reproduction in a cooperative breeder, the Florida Scrub-Jay. Condor, **98**: 234–244. doi:10.2307/1369141.

Schoech, S.J., and Bowman, R. 2003. Does differential access to protein influence differences in timing of breeding in Florida Scrub-Jays (*Aphelocoma coerulescens*) in suburban and wildland habitats? Auk, **120**: 1114–1127. doi:10.2307/4090282.

Schoech, S.J., Mumme, R.L., and Wingfield, J.C. 1996. Prolactin and helping behaviour in the cooperatively breeding Florida scrub-jay, *Aphelocoma coerulescens*. Anim. Behav. **52**(3): 445–456. doi:10.1006/anbe.1996.0189.

Schoech, S.J., Bowman, R., and Reynolds, S.J. 2004. Food supplementation and possible mechanisms underlying early breeding in the Florida Scrub-Jay (*Aphelocoma coerulescens*). Horm. Behav. **46**: 565–573. doi:10.1016/j.yhbeh.2004.06.005. PMID:15555498.

Schoech, S.J., Bowman, R., Bridge, E.S., and Boughton, R.K. 2007. Baseline and acute levels of corticosterone in Florida Scrub-Jays (*Aphelocoma coerulescens*): effects of food supplementation, suburban habitat, and year. Gen. Comp. Endocrinol. **154**: 150–160. doi:10.1016/j.ygcen.2007.05.027. PMID:17624348.

Schoech, S.J., Bridge, E.S., Boughton, R.K., Reynolds, S.J., Atwell, J.W., and Bowman, R. 2008. Food supplementation: a tool to increase reproductive output? A case study in the threatened Florida Scrub-Jay. Biol. Conserv. **141**: 162–173. doi:10.1016/j.biocon.2007.09.009.

Selman, C., Blount, J.D., Nussey, D.H., and Speakman, J.R. 2012. Oxidative damage, ageing, and life-history evolution: where now? Trends Ecol. Evol. **27**(10): 570–577. doi:10.1016/j.tree.2012.06.006.

Simons, M.J.P., Cohen, A.A., and Verhulst, S. 2012. What does carotenoid-dependent coloration tell? Plasma carotenoid level signals immunocompetence and oxidative stress in birds—a meta-analysis. PlosONE, **7**(8): e43088. doi:10.1371/journal.pone.0043088.

vonSchantz, T., Bensch, S., Grahn, M., Hasselquist, D., and Wittzell, H. 1999. Good genes, oxidative stress and condition-dependent sexual signals. Proc. R. Soc. Lond. B Biol. Sci. **266**(1414): 1–12. doi:10.1098/rspb.1999.0597.

Wiersma, P., Selman, C., Speakman, J.R., and Verhulst, S. 2004. Birds sacrifice oxidative protection for reproduction. Proc. R. Soc. Lond. B Biol. Sci. **271**(Suppl.): S360–S363. doi:10.1098/rsbl.2004.0171.

Woolfenden, G.E., and Fitzpatrick, J.W. 1996. Florida Scrub-jay (*Aphelocoma coerulescens*). *In* The birds of North America, No. 228. *Edited by* A.F. Poole and F.B. Gill. The Academy of Natural Sciences, Philadelphia, Pa., and The American Ornithologists' Union, Washington, D.C.