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Serum antioxidant levels in wild birds vary in relation to diet, season, life history strategy, and species

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Abstract Micronutrient antioxidants are thought to be generally important for health in many animals, but factors determining levels in individuals and species are not well understood. Diet and season are obvious environmental variables that might predict the degree to which species can accumulate such nutrients. We analyzed antioxidant levels [Trolox-equivalent antioxidant capacity (TEAC), uric acid (UA), vitamin E, and four carotenoids] in 95 bird species and compared these to species-level data on diet from the literature. Using compositional principal components analysis, we identified two main axes of diet variation: invertebrate consumption and seed-to-fruit ratio. We then examined associations between diet axes and antioxidant measures, with and without control for life-history variation and phylogeny. We also analyzed a subset of 13 species for which we had data on seasonality of antioxidant levels and diet, assessing the variance in antioxidant levels explained by seasonality, diet, and species. Unsurprisingly, there were strong associations between antioxidant levels and diet. TEAC and UA concentration were consistently positively associated with invertebrate consumption and seed-to-fruit ratio, and carotenoid concentrations (e.g. zeaxanthin and β-carotene) were negatively associated with invertebrate consumption. However, vitamin E was not associated with diet as measured here. Importantly, there is much variation in antioxidants that is not explained by diet, and we are able to identify diet-independent effects of species, season/breeding stage, and life history on antioxidant levels. Circulating antioxidant concentrations within and across species can therefore be viewed as a function of multiple factors, including but not limited to diet, and antioxidant metabolism appears to differ across species and seasons irrespective of diet.

Keywords Carotenoid · Fruit · Insect · Seed · Uric acid · Vitamin E

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Introduction

Antioxidants sit at the intersection of several branches of physiological ecology: oxidative balance, immunological functioning, nutrition, and sexual selection. Recent studies have examined antioxidant levels in the context of animal ecology and evolution, including impacts on development, relationship to immunocompetence, response to stress, and associations with life history strategy (e.g., Alonso-Alvarez et al. 2007; Cohen et al. 2008b; Costantini et al. 2007a;

Costantini and Dell'Omo 2006; Hõrak et al. 2007). One class of antioxidants, carotenoids, has been examined in detail for its roles in sexual selection, neonatal nutrition, and immune-system activation (e.g., Blount et al. 2003; McGraw and Ardia 2003; Surai et al. 2001). Recently, circulating carotenoid levels have been shown to be largely independent of overall antioxidant capacity and oxidative stress (Costantini et al. 2007b; Costantini and Møller 2008; Isaksson and Andersson 2008). Most of the antioxidants in these studies are obtained or modified from dietary sources, and while the relationship between diet and circulating carotenoid levels has been studied (e.g., McGraw et al. 2003; Tella et al. 2004), there has been no systematic examination of the role of diet in determining more general antioxidant levels in animals, especially across species.

Enzymatic antioxidants are important for protection within cells (Pérez-Campo et al. 1994), and are not expected to respond as directly to diet, since they are manufactured within cells like other proteins. However, micromolecular antioxidants such as uric acid (UA), vitamin E, and carotenoids are largely dependent on dietary intake. Vitamin E and carotenoids cannot be synthesized in vivo (though the latter can be modified to other carotenoid types; McGraw et al. 2006). Uric acid is the main form of nitrogen excretion in birds, and thus responds to protein intake and catabolism (Tsahar et al. 2006). However, circulating levels of these antioxidants should also depend on a number of other proximate factors, including uptake from the gut, consumption (i.e., free radical production/oxidative stress), localization in specific tissues, and excretion (e.g., McGraw et al. 2006; Wyss et al. 2001). Quantifying the effect of diet on antioxidants is thus important for understanding the relative role of other factors, and also for understanding how facultative adjustment of diet and poor nutritional conditions may play a role in mediating or exacerbating oxidative stress.

Nearly all that is presently known about the relationship between diet and antioxidants in wild animals comes from research on carotenoid pigments in birds (McGraw 2006). Applied research on domesticated animals like chickens and trout, where dietary carotenoid provisioning is key for maintaining the color of skin and egg yolk as well as the health of animals (Hill 2006), has been extended to free-ranging species recently, and the role of diet in maintaining carotenoid status is well-established in animals like house finches (Hill et al. 2002) and guppies (Grether et al. 1999). There have also been several comparative studies linking carotenoid accumulation and diet in birds (Mahler et al. 2003; Olson and Owens 2005; Tella et al. 2004), but in no study was any other form of antioxidant considered, despite the fact that several other antioxidants have been mentioned in the context of carotenoid accumulation and signaling (e.g. melatonin, Bertrand et al. 2006; vitamin E, Hartley and Kennedy 2004). With the exceptions of the carotenoid studies cited above, population- and species-level studies of the relationship between diet and circulating antioxidants have been confined largely to humans. In an epidemiological study, ~10% and 25% of the variance in plasma β-carotene and vitamin E levels, respectively, was explained by diet, but the percentage for β-carotene was much lower in smokers (Stryker et al. 1988). This is consistent with dietary intake being but one among many factors that can influence circulating antioxidant levels. Additionally, in taxa as diverse as fish, birds, and humans, increased dietary intake of one type of antioxidant often affects levels of others, potentially in both directions (e.g., Cohen et al. 2007; Huang et al. 2005; Kiron et al. 2004). For example, in chickens, vitamin E supplementation increases UA levels whereas lutein supplementation decreases them; in humans, vitamin C supplementation decreases UA levels.

Here, we measured antioxidant levels in 95 bird species and reviewed the literature to gather species-level data on relative proportions of fruit, seeds, and invertebrates in the diet. Antioxidants measured include "TEAC" (Trolox-equivalent antioxidant capacity—a common assay for circulating antioxidant capacity), UA, vitamin E, and four types of carotenoids, including both carotenes and xanthophylls. Because antioxidant levels in this data set are positively associated with faster pace of life [i.e., larger clutch, higher basal metabolic rate (BMR), smaller body size, lower survivorship, and faster development, Cohen et al. 2008b], and because diet is likely also associated with life history strategy, we analyze the data with and without control for seven life history variables and accounting for phylogenetic relationships. This allows us to assess life-history-independent antioxidant-diet relationships, and also confirms that our previous result was not attributable to failure to control for diet. We also take a subset of species for which we gathered antioxidant data in both June and July in Michigan, a time period during which most species are finishing nest-attendance, and use season-specific diet data on these species to look at the effects of season and diet on antioxidants within species.

We expected levels of all antioxidants studied here to be significantly associated with diet. Because TEAC is known to be tightly associated with UA, and because UA is a by-product of protein metabolism (Cohen et al. 2007; Tsahar et al. 2006), these measures were expected to be highest when invertebrate consumption was high and lowest when fruit consumption was high. Carotenoid content of items varies substantially within our categories of diet, but in general is highest in fruit and lowest in seeds, and we expected circulating levels to reflect this (Olson and Owens 2005). Vitamin E, in contrast, is lowest in fruits and highest in invertebrates, but with substantial heterogeneity in levels in invertebrates relative to those in fruits and seeds (Barker

et al. 1998; McLaughlin and Weihrauch 1979). Because physiology as well as dietary intake can contribute to circulating antioxidant levels, life history strategy and diet were expected to remain significant predictors of antioxidant concentrations after controlling for each other, and diet was expected to explain only a portion of the variance in antioxidant levels across species. Dietary shifts toward more insects during the breeding season were expected to explain most of the seasonal variation in circulating antioxidant levels.

Methods

Collection of avian serum and measurement of antioxidant levels

A total of 745 individuals from 95 bird species (Table S1) were caught during the day, and blood samples were taken from a wing vein using non-heparinized microcapillary tubes. Samples were centrifuged in a Zip-spin portable centrifuge, and serum was removed and kept on ice for up to 6 h until it could be frozen at −80°C. Ninety-two of our study species (97%) were small forest and edge species, mostly passerines (Order: Passeriformes), caught in mist nets in Panama and Michigan (Table S1). Netting was conducted at several locations in and around Gamboa, Panama, in March 2004 and March 2005, and at Kellogg Biological Station near Kalamazoo, Michigan, from 27 May–8 June and 9–18 July 2004, and 7–25 July 2005. The additional three species include savannah sparrows (*Passerculus sandwichensis*) and tree swallows (*Tachycineta bicolor*) sampled on Kent Island, New Brunswick, Canada (44°35′ N, 66°46′ W) from 18 to 25 June 2005, and house sparrows (*Passer domesticus*) caught in Princeton, New Jersey from 1 to 5 September 2005. In addition, one blue jay (*Cyanocitta cristata*), two northern cardinals (*Cardinalis cardinalis*), two eastern towhees (*Pipilo erythrophthalmus*), and 11 gray catbirds (*Dumetella carolinensis*) were caught in Princeton with the house sparrows, though these species are represented in greater numbers in the sampling from Michigan and Panama. Two species, the house wren (*Troglodytes aedon*) and yellow warbler (*Dendroica petechia*), had temperate and tropical subspecies that were considered separately in the analysis (*T. a. aedon* and *T. a. musculus*; *D. p. aestiva* and *D. p. erithachorides*, respectively).

Levels of individual carotenoid types and vitamin E were quantified by HPLC. TEAC and UA were quantified by spectrophotometric assays. TEAC quantifies circulating antioxidant capacity of micromolecular antioxidants: antioxidant activity of proteins and enzymes are not incorporated into the assay (Cohen et al. 2007). Details of all assays are provided in the Supplementary material "Methods supplement (S1)".

Diet classification

Data on diet type were obtained from published primary literature (see Supplementary material "Methods supplement S1" for details), and thus are not directly a measure of what was consumed by the individuals we sampled. In fact, we expect that regional, temporal, and stochastic differences mean that any given individual may have had a diet substantially different from what was indicated in the literature. Nevertheless, there are clear differences in species diets, and we have no reason to expect any systematic error in classifying generalized types of foods consumed by a species. Many species have diets that change over the course of the year, and when possible we subdivided our sample by month. For example, gray catbirds were caught in March (Panama), June and July (Michigan), and September (New Jersey), and we thus assigned separate diet types for these four months/sites as closely as available data allowed.

Diet is more complex than can be incorporated well into a quantitative analysis, and literature data are often incomplete, so we simplified diet into three types of food: invertebrates, fruit, and seeds. These three food types account for most items ingested across our species and are likely to capture much of the substantive variation in intake of protein, carbohydrates, and micronutrients. We used the literature to estimate to the nearest one-sixth the relative proportions of these three components (invertebrates, fruit, and seeds) in the diet of each species in each month sampled. For example, "consumes mostly insects with some fruit" would be interpreted as two-thirds insectivorous and one-third frugivorous. In most cases more quantitative estimates were available, but we nonetheless rounded to the nearest one-sixth because of the difficulty inferring from literature accounts what the individuals we sampled were consuming. We assigned scores between zero and three reflecting these estimates, constraining the sum to three and using levels of 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0. A fully insectivorous species would thus have respective scores of 3, 0, 0, whereas a fully omnivorous species would have scores of 1, 1, 1. A species estimated to consume two-thirds insects, one-sixth fruit, and one-sixth seeds would have scores of 2, 0.5, 0.5.

As studied here, diet is a compositional variable: all scores are constrained to have a fixed sum, resulting in one less degree of freedom than normal. Compositional variables cannot be analyzed by standard statistics because they are not independent. For example, the fact that a species consumes two-thirds insects constrains its seed consumption to be less than or equal to one-third. Special transformations are needed to account for this non-independence

(Aitchison 1982). We used the "rcomp" function in the "compositions" package in R (v.2.6.0) to run a compositional principal components analysis (PCA). The two principal component axes by definition account for 100% of the variation in the three compositional diet variables (Aitchison 1983).

However, compositional analyses do not deal well with 0 or 100% values for the variables; because many of our species had specialized diets, and because we estimated rather than measured diet, we added into the diet estimates a small random correction factor from a uniform distribution between 0.00 and 0.33% of total diet, constraining it so that all components of diet are greater than zero and sum to exactly 100%. For example, the Acadian flycatcher (*Empidonax virescens*), an insectivorous species, had diet scores of 2.9840, 0.0074, 0.0086 instead of 3, 0, 0. We ran the analyses with other sizes of deviation and found only negligible differences in results (data not shown); some deviation is necessary so that the program runs properly, but the deviations used here are small enough that they do not bias our results. Moreover, birds classified as specialists likely consume small quantities of other foods, so introducing some deviation is probably more accurate. The deviations are random, but for the sake of consistency we used a single set of deviations for all analyses rather than generate new deviations for each analysis.

Statistical analyses

Full data set

We used nine antioxidant variables in our analyses. Because TEAC and UA correlate tightly, we also used the residual of the regression of UA on TEAC, i.e., residual or non-UA antioxidant capacity (Res). This is important for distinguishing TEAC from factors such as protein intake and nitrogen excretion that are unrelated to antioxidants but affect UA (Cohen et al. 2007). In addition, we had direct measurements of concentrations of vitamin E and four individual carotenoids: lutein, zeaxanthin, β-cryptoxanthin, and β-carotene. Several other carotenoids were present at low levels in only a few species and are ignored here. We do not use total carotenoid levels because in this data set total concentration was basically equivalent to levels of lutein, which was the most abundant carotenoid and present in all species. Instead, we use a carotenoid factor generated by factor analysis that represents overall variation in the four main carotenoid types and in carotenoid diversity (Cohen and McGraw 2009). This factor represents 36% of the total variation of the individual carotenoid measures, and each measure has a loading between 0.69 and 0.79 on the factor.

We began by looking at simple correlations between the diet axes and the antioxidant variables using species means by month (SAS proc corr). We then controlled for several life history variables: body mass, clutch size, incubation period, nestling period, survival rate, basal metabolic rate, and tropical vs temperate climate zone. These data were collected for a previous analysis of antioxidant-life history relationships (Cohen et al. 2008b); here we used the set as a simple summary of life history variation. General linear models (SAS proc glm) were used to control for these variables simultaneously. We also present total antioxidant variation explained by life histories after controlling for diet and antioxidant variation explained co-linearly by the two diet variables we identify. Lastly, we used linear models to quantify the antioxidant variance explained by the two diet axes with and without control for each of the life history variables.

In order to control for phylogeny, we used a phylogenetic regression module available for SAS (Grafen 1989, 2006). There has been considerable debate about when phylogenetic correction is important and which method to use (e.g., Freckleton et al. 2002), but the lack of strong phylogenetic effect we see here suggests that phylogenetic control is not a crucial issue for this study (see Results, Table 1). The phylogeny used was the same as for Cohen et al. (2008a); methods of construction can be found there, and the phylogeny is available in supplementary material as shown in Newick format (S1). To address the non-independence of species diets in different months (e.g., gray catbirds in June vs July), we ran the analyses (including those without phylogenetic control) three ways: (1) including all data, and, for species with multiple months of data, categorizing the months as sub-taxa of the species separated by the minimum possible branch length (0.1); (2) including only June data for species sampled in other months too; and (3) including only July data for species sampled in other months too. The differences in the results are negligible, and only the last analysis is presented here, with discussion of the few differences. Phylogenetic regression does not generate r^2-values, so we use it only to confirm standard linear models, not to calculate percent of variance explained.

The species used in the analysis vary substantially in sample size, and in such situations it is generally appropriate to weight by the square-root of sample size. However, in this data set, sampling was non-random with respect to species characteristics—over-represented species were much more likely to be common species, omnivorous, temperate, and so forth, all of which are likely to bias the results if a weighting is used. Thus, when there is a discrepancy in the results with and without weights, it is not clear which result should be used. We have explored this issue in some detail elsewhere (Cohen and McGraw 2009); here, we present only the unweighted analyses, but make note of cases where there is a discrepancy.

Michigan June–July data set

We had 13 species that were captured in both June and July in Michigan. The beginning of June is at the peak of breeding, whereas by the middle of July most nests of most species have fledged (see Results, Table S2); this transition is generally accompanied by diets lower in insects and higher in fruit (see Results, Table S3). For these reasons, we analyzed these 13 species separately using the more accurate estimates of diet in these months (to the nearest percent rather than the nearest one-sixth). We excluded other species that did not have specific literature data available on June and July diet, unless these species were specialized enough for us not to expect a change in the proportions of invertebrates, fruit, and seeds in the diet. For example, the warblers we studied are known to be basically fully insectivorous year-round, so we included them in the June–July analysis to improve our ability to distinguish seasonal effects from diet effects. We ran the compositional PCA again for this data set to account for the revised diet estimates and the different species set.

We tested whether June- and July-caught birds were representative of different breeding stages, peak (most birds laying/incubating/feeding nestlings) versus tail end (most birds finished). Antioxidant levels likely change even between laying and incubation (Negro et al. 1998); our analyses were not intended to clarify all such finer scale effects, but rather to look at the relative contribution of broad-scale seasonal variation to overall variation in antioxidant levels. Nesting status was not known for individuals sampled, but we had data on seasonality of nesting at our study site in our study years for 10 of the 13 species. We recorded presence or absence of eggs and nestlings in nests by week starting at the beginning of June and continuing for 8 weeks. For each nest, we calculated the last week in which an egg or nestling was present, and then averaged this by species for egg and nestling periods separately, counting weeks from the beginning of June.

To assess general changes in antioxidant levels between June and July, we used a mixed effects model of antioxidant levels with species as a random effect and month as a fixed effect. We compared this to similar models containing year and species × month interaction. Next, we set up linear models of antioxidant levels as a function of diet, species, month, and year. Inclusion of year in the model was necessary based on *t*-tests comparing antioxidants levels in July 2004 and July 2005 (data not shown). We did not have any individuals captured in June 2005, so we could not include year × month interactions, and the model thus assumes there are none. Given our hypotheses and the results from the overall analyses, we expected that diet would explain much of the variance in antioxidants; what was of interest here was the shared variance, so we performed variance components analysis (Sokal and Rohlf 1969). For example, once we know how diet differs in different species in different months, we can partition the antioxidant variance into that explained by diet after controlling for all other factors, that explained by the shared contribution of diet and species, that explained by the shared contribution of diet and month, etc. These are not all mutually exclusive partitions, but each can be informative. If there is significant shared diet-month variance, for example, we can infer that some of the antioxidant variance is explained by how diet changes across months.

We ran multiple models including different sets of predictor variables in order to be able to show what we felt were the primary partitions of interest: (1) total variance explained by each diet variable without control, (2) shared variance between the two diet variables, (3) shared variance of each diet variable with species, (4) shared variance of each diet variable with month, (5) unique (i.e. independent) variance of each diet variable after controlling species, month, and year together, and (6) unique variance of each variable in a full model: antioxidant as a function of both diet variables, species, month, and year. Shared variance was calculated from Type I sums of squares, Type III sums of squares, and total sums of squares using the appropriate model. For example, shared diet-month variance of TEAC was calculated from the model TEAC = Diet + Month by subtracting Type III sum of squares for Diet from Type I sum of squares for Diet. This was then divided by the total sum of squares to get the portion of the variance explained by Diet that disappears when we control for Month—the shared variance. Phylogeny was not incorporated into the variance components analysis because higher-level variance is accounted for when we quantify the species-level variance component; species-level variance should thus be interpreted with respect to all effects at the species level or higher, not with respect to effects at the level of species within genus.

Results

Principal components analysis of diet

For the full diet data set, the first PCA axis explains 61% of the variance and has loadings for invertebrates, fruit, and seed of 0.82, −0.38, and −0.44, respectively. The second axis explains 39% of the variance and has loadings of 0.0, −0.72, and 0.69, respectively. In other words, the first axis explains invertebrate consumption and the second axis explains the relative balance of fruits and seeds, with higher scores indicating more seed in the diet. The validity of

these axes was confirmed by examining correlations of the axes with the raw variables and with factors generated with a non-compositional factor analysis (SAS proc corr and proc factor; data not shown). For ease of reference, we refer to these axes as "Inverts" and "Seed/fruit." For the June–July Michigan data set, the first axis explained 56% of the variance and had loadings of invertebrates at 0.82, fruit at −0.41, and seed at −0.41. The second axis explained 44% of the variance and had respective loadings of 0.0, −0.71, and 0.71, respectively; in other words, both axes can be interpreted essentially identically to those from the full data set.

Full data set

TEAC and UA were both strongly positively associated with seed-to-fruit ratio and invertebrate consumption (Table 1; Fig. 1). Although the July-only data set presented in Table 1 shows the effects to be consistent across models, the June-only and full data set analyses show no association for seed-to-fruit ratio after controlling for life history. The TEAC-UA residual was not associated with diet, suggesting that the TEAC effect is attributable to the large portion of its variance explained by UA (Table 1). This is further supported by the larger effect sizes for UA relative to TEAC. Vitamin E showed no significant associations with diet in the unweighted analyses (Table 1), but was significantly positively associated with invertebrate consumption when weights were added ($r^2 = 0.19$, $P = 0.0001$). The effect was not maintained after control for life history variables ($P = 0.4$). Carotenoids differed in their associations with diet (Table 1). β-cryptoxanthin showed the least association: the apparent relationship with seed-to-fruit ratio after controlling for life histories and phylogeny was not evident in the other models (data not shown). Lutein consistently showed a weak negative relationship with invertebrate consumption after controlling for life histories (Table 1). Zeaxanthin, β-carotene, and the carotenoid factor showed consistent negative associations with invertebrate consumption regardless of control for phylogeny or life histories (Table 1). The effect was strongest for zeaxanthin and sometimes only marginally significant for the carotenoid factor. There were no robust changes in these results after controlling for phylogeny; both sets of results are presented because the r-values from the uncontrolled analyses are more interpretable than the F-values from the phylogenetic regressions. For all antioxidant variables, a large portion of the variance explained by the life history variables was independent of the diet variables (Table 1). Antioxidant variance explained by diet after controlling for individual life history variables is shown in supplementary material (Fig. S1).

June–July data set

Analysis of nesting data confirms that the June–July division of our data is consistent with the winding down of the breeding season in most species (Table S2). For nine of the ten species for which we had data, the mean end of the nestling period was between the time of our last June sample and our first July sample; cedar waxwings (*Bombycilla cedrorum*) finished later. Some individuals sampled may have been at a different stage than what the species averages suggest, but for at least nine species it is reasonable to analyze June and July samples discretely as representative of different breeding stages as well as different diets.

According to the literature surveyed, 10 of the 13 species (all except the two warblers and the house finch, *Carpodacus mexicanus*) had different diets in June and July, with more invertebrates consumed in June (Table S3). In mixed models with species as a random effect, TEAC, UA, and Res were higher in June and zeaxanthin and the carotenoid factor were higher in July ($P < 0.001$ for all, various models); vitamin E, lutein, β-cryptoxanthin, and β-carotene showed less clear trends across months and the results depended heavily on which co-variates were included in the models (year, diet, and species × month interaction; data not shown).

Variance partitioning in linear random-effects models shows that dietary shifts are associated with changes in antioxidants. This can be seen in the "Shared Variance with Month" columns of Table 2: shifts in invertebrate consumption between June and July are associated with changes between those months in all antioxidant measures except Res; shifts in seed-to-fruit ratio are associated with changes in TEAC and UA but not other antioxidants, consistent with the results from the full data set. The percent of the variance explained is much higher for "Shared variance with species" (Table 2), suggesting that species differences in diet are more important in determining antioxidant levels than changes in diet within species over time. Note, though, that the shared variance with species does not control for phylogeny; this portion of the variance is thus a blanket category covering the phylogenetic inertia of diet, antioxidants, and likely other covariates such as physiology and ecology.

The shared variance between diet and species or month was generally a significant portion of the carotenoid variance, but, with the exception of zeaxanthin, diet did not uniquely explain carotenoid variance after controlling simultaneously for species, month, and year. TEAC and UA, on the other hand, had significant variance explained by nearly every partition of predictor variables tested, with the exception of unique year variance. Unique year variance was significantly associated only with variance of lutein and the carotenoid factor, but was retained in the

Table 1 Associations of diet with antioxidants across the full data set

		Inverts				Seed:Fruit				Diet-independent LH[a]	Diet combined[b]
		No LH		LH Control		No LH		LH Control			
		No Phy	Phy	No Phy	Phy	No Phy	Phy	No Phy	Phy		
TEAC	r^2/F	**0.19**	**16.2**	**0.09**	**5.5**	**0.22**	**17.6**	**0.06**	1.36	0.23	**0.41**
	P	**<0.0001**	**0.0001**	**0.009**	**0.03**	**<0.0001**	**<0.0001**	**0.04**	0.26		**<0.0001**
UA (mg/dl)	r^2/F	**0.31**	**31.1**	**0.14**	**12.7**	**0.24**	**24.4**	**0.06**	**5.38**	0.22	**0.56**
	P	**<0.0001**	**<0.0001**	**0.0006**	**0.003**	**<0.0001**	**<0.0001**	**0.03**	**0.03**		**<0.0001**
Res	r^2/F	0	−0.38	−0.01	−0.54	0.02	1.35	0.06	0.34	0.23	0.03
	P	0.55	0.54	0.56	0.47	0.16	0.25	0.17	0.57		0.32
VitE (absorbance units)	r^2/F	0.04	1.74	0	0.06	−0.01	−0.88	−0.06	−1.52	0.25	0.05
	P	0.11	0.19	0.76	0.82	0.42	0.35	0.19	0.24		0.19
Lut (mg/l)	r^2/F	−0.02	−2.55	−0.13	**−7.59**	0	−0.23	−0.07	−3.09	0.46	0.02
	P	0.26	0.12	0.06	**0.02**	0.64	0.64	0.15	0.1		0.48
Zea (mg/l)	r^2/F	**−0.11**	**−8.23**	**−0.18**	**−7.04**	−0.01	−0.58	−0.06	−1.77	0.47	**0.11**
	P	**0.005**	**0.006**	**0.02**	**0.02**	0.46	0.45	0.19	0.21		**0.02**
Bcrypt (mg/l)	r^2/F	0	0.09	0	−0.23	0	0.02	−0.11	**−6.25**	0.39	0
	P	0.98	0.77	0.99	0.64	0.82	0.9	0.11	**0.03**		0.97
Bcar (mg/l)	r^2/F	**−0.08**	**−7.69**	**−0.09**	**−5.22**	0	0.06	−0.07	−2.61	0.59	**0.08**
	P	**0.01**	**0.008**	**0.04**	**0.04**	0.83	0.81	0.08	0.13		**0.05**
CarotFac	r^2/F	**−0.07**	**−5.19**	−0.1	−3.49	0	−0.04	−0.11	−4.03	0.54	0.07
	P	**0.02**	**0.03**	0.08	0.08	0.81	0.84	0.06	0.07		0.08

TEAC Trolox-equivalent antioxidant capacity, *UA* uric acid, *Res* TEAC-UA residual; *VitE* vitamin E, *Lut* lutein, *Zea* zeaxanthin, *Bcrypt* β-cryptoxanthin, *Bcar* β-carotene, *CarotFac* carotenoid factor. Diet variables (*Inverts*; *Seeds/Fruits*) are compositional principal component analysis (PCA) axes. *LH* life histories, a set of seven life history variables. *Phy* phylogenetic regression. The data set excludes June-caught birds of species caught in July too, but results are generally consistent if July-caught birds are excluded instead, or if June and July are modeled as subspecies in the phylogenetic regression (see text for differences). "Phy" columns have *F*-values, "No Phy" columns have r^2 values taken from linear models of antioxidant as a function of diet, with or without life history variables in the model, but these values are negative when the *r*-value was negative to show direction of the association

Bold indicates $P < 0.05$

[a] Percent of antioxidant variance explained by all life history variables after controlling for the diet variables. Significance depends on the set of LH variables and cannot be meaningfully assessed

[b] Percent of antioxidant variance explained by both diet variables together (colinearity)

model because many antioxidant measures in July differed between the two years (data not shown).

Vitamin E was only weakly associated with diet measures, and not at all after controlling for species and month; given the known associations between diet and month and species, there is little evidence that diet as we measured it is linked to vitamin E levels. As in the full data set, there was no evidence for an association between diet and the TEAC-UA residual, suggesting that TEAC associations with diet are fully attributable to effects of diet on UA.

Discussion

Results from our study are consistent with the hypothesis that diet type affects levels of some circulating antioxidants in birds. This was true across species and within species across months, and for both UA and carotenoids. It was notably not true for vitamin E, and the effect of TEAC on diet appears completely attributable to the effect of UA. Many of the associations we found are not surprising. Uric acid, the main form of nitrogen excretion in birds, is a by-product of protein catabolism and is expected to be higher in birds that consume lots of protein (Tsahar et al. 2006). Accordingly, we found positive associations with both invertebrate consumption and the seed-to-fruit ratio. Likewise, carotenoids were hypothesized to be higher in species that consume many fruits compared to those that consume many invertebrates, and this is the relationship we found.

However, this appears to be an oversimplification. We did not find evidence that carotenoid levels are higher in birds with a low seed-to-fruit ratio as expected, and the highest carotenoid levels detected were in Yellow Warblers (*Dendroica petechia*), which are basically fully insectivorous. Many invertebrates do contain carotenoids, and not all fruits and seeds are highly concentrated with carotenoids

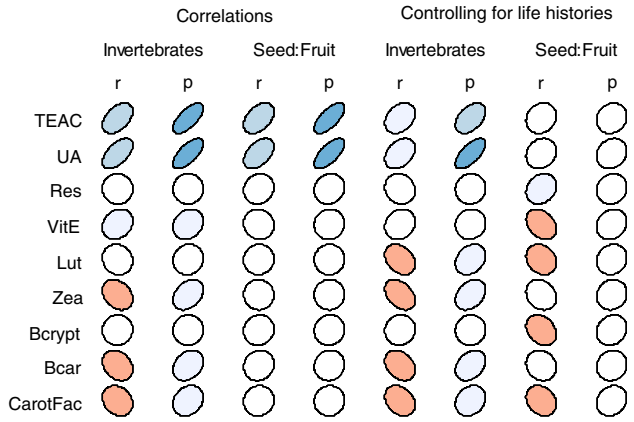

Fig. 1 Associations between antioxidant levels (*rows*) and diet axes (*columns*; Invertebrates and seed-to-fruit ratio) with and without control for seven life history variables. *r*-value columns show ellipses that are darker and narrower for stronger correlations, and are right-slanted for positive correlations and left-slanted for negative correlations. *P*-value columns are based on transformations ($\log(p)/-6$) of the *P*-values such that shaded ellipses have $P < 0.05$; degree of shading and narrowness indicates level of significance

(Olson and Owens 2005). Many of the American robins and gray catbirds that we caught were actively feeding on berry bushes and had feces that were clear, liquidy, and full of berry seeds. These same birds had low carotenoid levels, as did their species in general. Of course, species that rely more heavily on carotenoid pigmentation for sexual displays may have evolved more efficient uptake mechanisms for absorbing carotenoids in the diet (McGraw 2005). Rigorous analysis of serum carotenoid levels in relation to integument levels is beyond the scope of this paper, but casual examination of our results (Table S1) supports the findings of Tella et al. (2004), i.e., that species with substantial carotenoid pigmentation in the integument (e.g., feathers, bill, legs) have higher circulating levels, and that phylogeny is also important.

Although we found effects of diet on circulating antioxidant levels, we also found substantial independent effects of season, species, and life-histories. In some cases, we were able to pinpoint more specific relationships: for example, seasonal changes in diet correspond to seasonal changes in antioxidants. A relationship between diet and circulating levels of diet-derived antioxidants is unsurprising; a more substantive conclusion is that diet is but one of many factors that interact to determine antioxidant levels. For example, the antioxidant variance explained uniquely by month in the June–July analysis can be interpreted as diet-independent changes in physiology between breeding and nonbreeding stages, potentially mediated by shifts in hormone levels, oxidative stress, or disease status (e.g., Wingfield and Farner 1993). Because our diet measures are imperfect and are based on species- rather than individual-level data,

Table 2 Partitioning of variance in antioxidant levels between diet, species, and season in 13 Michigan species

	Correlations		Shared diet variance	Shared variance with species		Shared variance with month		Control for species month year		Full model				
	Inverts	Seed:Fruit		Inverts	Seed:Fruit	Inverts	Seed:Fruit	Inverts	Seed:Fruit	Overall model	Inverts	Seed:Fruit	Species	Month	Year
TEAC	0.46*	0.24*	0.19*	0.33*	0.18*	0.15*	0.07[a]	0.02***	0.03**	0.62*	0.00	0.02***	0.06***	0.03**	0.00
UA (mg/dl)	0.47*	0.37*	0.25*	0.37*	0.28*	0.12*	0.07*	0.04*	0.06*	0.65*	0.01***	0.03*	0.05***	0.00	0.00
Res	0.00	0.01	−0.01	−0.01	0.01	0.00	−0.01	0.01	0.00	0.16***	0.01	0.00	0.06	0.08**	0.00
VitE (absorbance units)	0.11**	0.00	0.00	0.11[a]	0.00	0.03[a]	0.00	0.01	0.00	0.36**	0.00	0.00	0.23***	0.03***	0.00
Lut (mg/l)	0.22*	0.48*	0.20[a]	0.22*	0.48*	−0.05[a]	0.00	0.00	0.00	0.81*	0.00	0.00	0.30*	0.01***	0.02**
Zea (mg/l)	0.00	0.05**	−0.01	−0.18*	−0.13[a]	−0.04[a]	0.00	0.04***	0.07**	0.63*	0.01	0.04***	0.37*	0.00	0.00
Bcrypt (mg/l)	−0.10*	0.11*	0.07[a]	−0.10*	0.11*	−0.07[a]	0.00	0.00	0.00	0.68*	0.00	0.00	0.44*	0.00	0.01
Bcar (mg/l)	0.13*	0.48*	0.13*	0.12*	0.48*	−0.10[a]	0.00	0.00	0.01	0.75*	0.00	0.01	0.21*	0.00	0.01
CarotFac	0.07*	0.28*	0.07*	0.04[a]	0.26*	−0.10a	−0.01	0.00	0.00	0.81*	0.00	0.00	0.42*	0.00	0.02***

Variance (r^2) explained by various model components, calculated from sums of squares in general linear models. Antioxidant (row) is modeled as a function of various combinations of diet, month, species, and year. Columns numbers are calculated (when necessary) based on the differences between variance explained in different models

* $P < 0.0001$, ** $0.0001 < P < 0.005$, *** $0.005 < P < 0.05$

Negative values in "shared variance" columns indicate that more antioxidant variance was explained by diet when the other factor (species or month) was included in the model and implies an interaction

[a] *P*-values in "shared variance" columns were not precisely estimable, but $0.0001 < P < 0.05$. Negative values in "shared variance" columns indicate that more antioxidant variance was explained by diet when the other factor (species or month) was included in the model and implies an interaction

it is possible that diet is more tightly associated with antioxidant levels than indicated by these analyses; this will be particularly true if the relevant variation in diet was not captured by our coarse categorization. Nonetheless, in the full data set, life history variables still explained a substantial portion of antioxidant variance after controlling for diet. Likewise, in the June–July data set, species and month remained significantly associated with most antioxidant measures after controlling for all other sources of variation. Circulating antioxidant levels are a function not only of dietary intake but also of storage (in tissues), usage, and excretion. Our data support the idea that usage and/or excretion patterns depend on both species-specific physiology and on seasonal changes in physiology.

We expected a tighter association between diet and dietary antioxidants than between diet and endogenously synthesized antioxidants. However, the reverse was true—UA was more strongly associated with diet than either vitamin E or carotenoid concentrations. One potential explanation is that our divisions of diet might not reflect micronutrient levels as well as they do protein content, which affects UA. However, for both carotenoids and vitamin E, there are substantial differences in levels between invertebrates, seeds, and fruit as well as within these classes (Barker et al. 1998; McLaughlin and Weihrauch 1979; Olson and Owens 2005). If our diet classification is too crude with respect to micronutrient content, our estimates of the relationship between diet and UA should be more accurate than between diet and the micronutrients; the latter should be interpreted as minimum possible effects in that case. However, it is also possible that vitamin E and carotenoid levels depend more strongly on patterns of metabolism (McGraw 2005) than UA levels do. This would be consistent with UA functioning primarily in nitrogen excretion, not as an antioxidant. Vitamin E in general has shown few associations with anything that we have measured, including life histories across species, reproductive success, age, and body size within species, and now diet within and across species (Cohen 2007; Cohen et al. 2008b); potentially this is due to recycling of vitamin E and short-term variation not easily detectable in our large-scale analyses.

Carotenoids showed a diversity of patterns, especially among different pigment types. Many researchers who performed comparative studies of plasma carotenoids in birds have used a single measure for carotenoids (total concentration, e.g., Tella et al. 2004), and we have shown elsewhere that the carotenoid factor used here is a reasonable summary of much of the variation in carotenoids in this group of passerine birds that we sampled (Cohen and McGraw 2009); however, with respect to the diet variables we measured, it is clear that the important variation in carotenoid concentrations is not the shared variation but the unique variation. Lutein and β-cryptoxanthin, though correlated with diet in the June–July analysis, did not have any variance independently explained in either analysis; the correlations appear to be completely due to diet covariance with species. Zeaxanthin, on the other hand, had substantial unique variance explained by diet in both analyses, though in the full data set the association was only with invertebrate consumption, not seed-to-fruit ratio. β-carotene was associated only with invertebrate consumption in the full data set. Given the diversity of associations among the carotenoids, it is clear that a simplified set of all antioxidant variables would not have accurately conveyed the patterns observed here. We did not even find a clear breakdown between the two major types of carotenoids, carotenes and xanthophylls, despite generally strong inter-correlations among carotenoid types in our sample (Cohen and McGraw 2009).

The xanthophylls lutein and zeaxanthin are the most abundant avian carotenoids and typically co-occur in avian foods and plasma (McGraw et al. 2001, 2003, 2006), but here we found that they associated differently with diet. This suggests that selection can shape differential availability or use of these pigments, either as colorants or as antioxidants/immunostimulants. Zeaxanthin is generally the rarer of the two pigments and may play stronger physiological (e.g. in the immune system) or morphological (e.g. as a pigment) roles than lutein, as it has been shown to do experimentally in zebra finches (*Taeniopygia guttata*), American goldfinches (*Carduelis tristis*; McGraw et al. 2005), and chickens (*Gallus domesticus*; Wang et al. 2007). β-cryptoxanthin and β-carotene are much less common in the diets of birds, especially those in our sample (largely inland songbirds). Given the comparative rarity of β-carotene, along with zeaxanthin, it is perhaps not surprising that these two pigments had similar associations with diet, even if they are thought of as members of different subclasses of carotenoids (xanthophylls vs carotenes). β-carotene is also a vital pro-vitamin A carotenoid, becoming hepatically or duodenally converted, so residual amounts in plasma may indicate physiological value beyond vitamin synthesis and still have a dietary basis. Very little is known about β-cryptoxanthin availability in wild birds except in a few species such as house finches, where its important dietary role for coloration has been advocated (Hill 2000; McGraw et al. 2006). The fact that it showed similar patterns with lutein, however, may also be a function of their physiological link, as lutein may be converted into β-cryptoxanthin in some animals (Deviche et al. 2008; Khachik et al. 2007; McGraw et al. 2002). Overall, given the complex integration of processes that a single measurement of plasma carotenoids captures (e.g. dietary intake, physiological use, withdrawal from tissue stores), it is a key confirmation that diet can explain significant plasma carotenoid variance, though by no means would we expect it to explain all. Future studies

should add measures of oxidative stress exposure/loads and other functional uses of carotenoids (e.g., coloration, deposition in internal tissues) to better disentangle the relative effects of these different factors on plasma carotenoid circulation.

Ultimately we would like to understand the co-evolution of diet and antioxidant physiology in birds. How does a change in diet affect the long-term evolution of oxidative-stress and antioxidant physiology in a species? Conversely, how does a change in oxidant physiology affect antioxidant demand, and can this result in a long-term shift in diet? Because of the reciprocal causality involved here, this study cannot answer such questions—experimental approaches and/or detailed studies of natural experiments will be necessary. However, we are able to establish a framework here for understanding the relative contributions of diet, season, and other factors to differences in circulating antioxidant levels in wild birds. Although diet and species each independently explained substantial variation in antioxidants, there was also a large portion of the variance for which diet and species effects were not easily disentangled. This is exactly the portion in which we expect reciprocal causality to be operating. Over evolutionary time, diet likely adjusts to the physiological needs of each species (e.g., for increased immune response to a new pathogen with minimal collateral damage), while at the same time the physiological strategies are adjusting to make use of what is present in the diet.

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