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No simple measures for antioxidant status in birds: complexity in inter- and intraspecific correlations among circulating antioxidant types

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Summary

1. Recently, ecologists have shown interest in examining antioxidant protection in wild animals; carotenoids in particular have received attention as antioxidants that play an important role in mediating health, life-history trade-offs and sexual selection. However, we know almost nothing about the relationships among levels of different antioxidants in wild animals or whether variation in antioxidants can be viewed as a single coherent system.

2. Here we use a data set of 903 individuals from 99 bird species to examine covariation among concentrations of three types of antioxidants (uric acid, vitamin E and four carotenoids), and their relationship to a summary measure, Trolox-equivalent antioxidant capacity (TEAC), both inter- and intraspecifically in 30 species.

3. Three axes were necessary to adequately describe variation in nine antioxidant measures, and these axes corresponded to the basic categories of antioxidant measured: uric acid, vitamin E and carotenoids. There was substantial heterogeneity in the correlations across species.

4. TEAC covaried strongly with uric acid levels, both interspecifically and in 23 of the 30 species. Concentrations of different carotenoids covaried both inter- and intraspecifically, but there was also substantial variance explained by each carotenoid independent of the others. Vitamin E concentration did not robustly correlate with any other antioxidant parameters.

5. Overall, we show that although antioxidants tend to covary with those of similar type, these correlations demonstrate evolutionary lability and/or ecological heterogeneity. Even the most general associations are absent in some species, suggesting that conceptualization of a single antioxidant system is oversimplified and that ecological studies attempting to examine antioxidant function should use multiple measures.

Key-words: avian, carotenoid, comparative, correlation, TEAC, uric acid, vitamin E

Introduction

A number of recent studies have examined the role of antioxidant protection in avian ecology and physiology (e.g. Hõrak *et al.* 2006; Tummeleht *et al.* 2006; Alonso-Alvarez *et al.* 2007; Isaksson *et al.* 2007). Antioxidants are valuable both for protection against free radical damage – considered to be an important mechanism underlying aging – and for proper functioning of the immune system. However, results have not always been straightforward. Depending on species and ecology, higher antioxidant capacity can be indicative of animals in good or poor condition (Costantini & Dell'Omo 2006; Costantini, Cardinale & Carere 2007), and can rise or fall in response to stress (Cohen, Klasing & Ricklefs 2007). At the same time, one type of antioxidant – carotenoids – has repeatedly been shown to be important for good health and for sexual signalling in a number of species (e.g. Blount *et al.* 2003; McGraw & Ardia 2003), though it is not clear that these benefits come from the role of carotenoids as antioxidants. Supplementation of the common carotenoid lutein in chickens (*Gallus domesticus*) decreased overall antioxidant capacity, though vitamin E supplementation increased antioxidant capacity (Cohen *et al.* 2007). In contrast, in zebra finches (*Taeniopygia guttata*), carotenoid supplementation increased

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overall antioxidant protection (Alonso-Alvarez *et al*. 2004). Intermediate but not high levels of vitamin E supplementation improved body condition in nestling barn swallows (*Hirundo rustica*) (de Ayala, Martinelli & Saino 2006).

This range of conflicting results is particularly difficult to interpret given our poor understanding of the interactions among components of antioxidant systems and of complexity in physiological systems more generally. The growing use of markers of overall antioxidant capacity implies a conception in the literature of antioxidants as a unified system that can be summarized by a single variable. If antioxidant function is modulated as a coherent whole via regulation within individuals or selection across individuals or species, we should expect strong correlations among different types of antioxidants at those respective levels, either positive (suggesting synergism) or negative (suggesting compensatory effects). However, it is not clear that overall antioxidant function in circulating systems is more than the sum of a number of largely independent processes affecting the levels of individual antioxidant types, potentially neither regulated nor subject to selection with regard to antioxidant function (Hartley & Kennedy 2004). Even to the extent that each antioxidant type is regulated for its antioxidant function, the functions may be type-specific enough that there is relatively little signal in overall levels. For example, although carotenoid levels are quite low relative to overall antioxidant levels, they are considered to play important free radical scavenging roles in membranes (Young & Lowe 2001). Carotenoids are now considered not to contribute much too overall antioxidant status (Isaksson *et al*. 2007; Costantini & Møller 2008).

For these reasons, the question of how to measure antioxidants is related to a fundamental understanding of the physiological roles of different antioxidant types and the degree of their integration into a single coherent system. These questions can be addressed for at least three different ecological levels: across species, reflecting regulation over evolutionary time; within species across individuals, reflecting covariation based on genetics, diet or condition; and within individuals over time. We have observed strong covariation among antioxidants within individuals in response to capture stress, and describe this relationship elsewhere (Cohen 2007). Here, we focus on inter- and intraspecific variation.

Antioxidant systems involve both enzymes and micromolecules and vary across tissues. Enzymatic antioxidants are particularly important in mitochondria at the site of most free radical production (e.g. Van Remmen *et al*. 2003). Here we focus on circulating micromolecular systems, known to affect both immune function and oxidative damage (Konjufca *et al*. 2004; McGraw & Ardia 2004; Niki 2004). Most recent work on antioxidants in an ecological context has focused on micromolecular antioxidants in birds. There is clear evidence that for some antioxidants dietary availability is a critical factor. Vitamin E and carotenoids cannot be produced endogenously, though some types of carotenoids can be modified to others, depending on the presence of the appropriate enzymes (Surai 2002). Others, such as vitamin C and uric acid, can be produced endogenously at least in some birds, and additionally uric acid is the main by-product of amino acid metabolism in birds, and its levels thus may reflect protein intake or regulation of nitrogen excretion as much as antioxidant function (Wright 1995). In many bird species, uric acid is also by far the most abundant of the circulating micromolecular antioxidants (as opposed to the enzymatic antioxidants, which function primarily in mitochondria), and uric acid levels have been shown to correlate well with the Trolox-equivalent antioxidant capacity measure (TEAC, Cohen *et al*. 2007; Hõrak *et al*. 2007). Whether or not there are particularly strong correlations within groups of antioxidants such as lipid- vs. water-soluble molecules or those with exogenous vs. endogenous sources will clarify the functional relevance of these classes.

Here, we studied circulating antioxidants in more than 900 individuals from 99 wild bird species. For almost all individuals, we quantified TEAC, uric acid and non-uric acid (residual) antioxidant capacity. For 428 individuals we also measured vitamin E and carotenoid levels. Four different types of carotenoids, including both carotenes and xanthophylls, were found in enough species to be analyzed individually, in addition to total carotenoid concentration and number of carotenoid types present. At least *in vitro*, carotenes generally are more effective antioxidants than xanthophylls due to the absence of a hydroxyl group on the β-ring (Miller *et al*. 1996).

We examined correlations among these antioxidant measures at the interspecific level and at the intraspecific level for 30 different species for which we had sufficient sample size ($n \geq 5$) and confirmed these analyses with multi-level models assessing the heterogeneity of correlations across species and the partitioning of variance between the individual and species levels. We were interested in understanding the correlation structure among different antioxidant types, its variation across species, and how well a summary measure such as TEAC would capture this variation. A correlational study such as this is not intended to shed light on causal links between these antioxidants (e.g. whether they are co-regulated, associated because of diet, etc.; see Discussion), but is an important starting point from which experiments on specific antioxidants can be designed and interpreted. For example, an experiment showing that immune stimulation causes an increase in antioxidant capacity and a decrease in carotenoid levels has different implications depending on whether antioxidant capacity and carotenoids are known to consistently covary on a broader scale. Heterogeneity in the correlations across different species would suggest that the associations, rather than being strictly physiologically constrained, either evolve themselves or are determined by the evolution of other traits such as diet and habitat. Most importantly, all these patterns can serve as a proxy for understanding complexity in physiological systems more generally.

Methods

COLLECTION OF AVIAN SERUM

Nine hundred and three individuals of 99 bird species (see Appendix S1 in Supporting Information for full list) were captured for this study.

Blood samples were taken within 5 min of capture (to avoid effects of stress) through standard wing venipuncture and collected into un-heparinized microcapillary tubes. Samples were centrifuged in a Zip-spin portable centrifuge and serum was removed and kept on ice for 1–6 h. until it could be frozen at –80 °C.

Ninety-two of our study species were small forest and edge birds, mostly passerine, caught in mist nets in Panama and Michigan. Birds from these species were netted at several locations in and around Gamboa, Panama, in March 2004 and March 2005, and at Kellogg Biological Station near Kalamazoo, Michigan, in June and July 2004, and July 2005. The additional seven species include Leach's storm-petrels (*Oceanodroma leucorhoa*), savannah sparrows (*Passerculus sandwichensis*) and tree swallows (*Tachycineta bicolor*) sampled on Kent Island, New Brunswick, Canada (44°35′ N, 66°46′ W) between 18 and 25 June 2005; Florida scrub-jays (*Aphelocoma coerulescens*) caught at Archbold Biological Station, Lake Placid, FL throughout 2005, but mostly in January and February; and house sparrows caught in Princeton, NJ from 1 to 5 September 2005. Waved albatross (*Diomedea irrorata*; caught 8–10 May 2002) and nazca booby (*Sula granti*; caught 16–23 August 2003) samples were provided by Dave Anderson and Victor Apanius from their studies in the Galápagos Islands, and were sampled following our protocol of taking serum immediately upon capture, centrifuging and freezing. In addition, one blue jay (*Cyanocitta cristata*), two northern cardinals (*Cardinalis cardinalis*), two eastern towhees (*Pipilo erythrophthalmus*) and 11 gray catbirds were caught in Princeton with the house sparrows, though these species are represented in greater number in the sampling from Michigan.

Most species were sampled during the breeding season. For species in Panama, breeding season is generally more diffuse than in temperate species, and March is generally before peak breeding. For species in Michigan, breeding generally tails off by the end of July. Only nazca boobies were sampled entirely outside the breeding season, though most Florida scrub-jays and birds caught in Princeton, NJ were not breeding. Breeding and non-breeding Florida scrub-jays did not significantly differ in levels of any antioxidants (data not shown).

We measured TEAC and uric acid using spectrophotometric methods, and vitamin E and carotenoid levels using High Performance Liquid Chromatography (McGraw & Parker 2006; Cohen *et al*. 2007), though not all measures were available for all individuals. TEAC reflects levels of circulating micromolecular antioxidants including uric acid, vitamin C, vitamin E and carotenoids, but does not reflect levels of enzymatic antioxidants or other macromolecules with antioxidant properties. See the Methodological Appendix S4 in Supporting Information for details.

DATA ANALYSIS

TEAC and concentrations of uric acid, vitamin E and all individual carotenoids were log-transformed for normality. TEAC–uric acid residuals were calculated following Cohen *et al*. (2007). This residual indicates non-uric acid antioxidant capacity. Four carotenoid types were present in enough species to be considered individually in our analyses: lutein, zeaxanthin, β-cryptoxanthin and β-carotene. Additionally, canthaxanthin was used in the intraspecific analysis of northern cardinals, canary xanthophylls in the analysis of cedar waxwings (*Bombycilla cedrorum*) and α-cryptoxanthin in the analysis of savannah sparrows and tree swallows. Total carotenoid concentrations were calculated, but are not presented here because lutein accounts for most of the variation and thus there is no additional information gained by including this variable. Carotenoid number is the number of types of carotenoids detected in an individual or species.

We tested the dimensionality of relationships among the nine antioxidant variables by first using a principal components analysis (PCA, R v2·5·0, princomp function). We then used a factor analysis (proc factor, SAS, v9·1, SAS Institute, Carey, NC) to confirm these results and subsequently to generate individual-specific scores for a carotenoid factor (CarFac: factor loadings: lutein = 0·76; zeaxanthin = 0·69; β-cryptoxanthin = 0·77; β-carotene = 0·79; carotenoid number = 0·95). Antioxidant measurements for all individuals are provided in the Appendix S2 in Supporting Information.

We calculated Pearson correlation coefficients (SAS, proc corr) at the interspecific level with species average values. Statistically, such correlations should be weighted by sample size, but because sample size is not random with respect to species characteristics (e.g. species with large sample sizes tended to be temperate and omnivorous) we present results from both weighted and unweighted analyses. For each variable, portions of the variance at the individual and species levels and partial correlation coefficients were calculated using a nested ANOVA (SAS, proc nested). Differences among species in each antioxidant variable were tested using a general linear model with random effects (SAS, proc glm, random statement). Pearson correlations were also used for intraspecific analyses on 30 species, but sample sizes were too small for robust PCA or factor analyses. Phylogenetic independent contrasts were run on interspecific analyses as described in the Methodological Appendix S4.

We assessed whether correlations among levels of antioxidants were heterogeneous across species using multilevel random effects models. We chose a subset of six correlations to focus on as representative of the larger set of 45: TEAC–UA, lutein–zeaxanthin, TEAC–vitamin E, TEAC–carotenoid factor, vitamin E–carotenoid factor and UA–zeaxanthin. Because multilevel models use a regression framework with an independent and a dependent variable whereas correlations assess a symmetrical relationship, we transformed all antioxidant variables into standard normal random variables by subtracting the species-specific mean and dividing by the species-specific standard deviation. Regression intercepts thus become zero and slopes become equal to the correlation coefficient, obviating questions about how to assign dependent and independent variables. An example of the models used is as follows:

$$TEAC_{sn} = (\rho_1 + \rho_{2s}) * UA_{sn} + \varepsilon_s \quad \text{eqn 1}$$

$$UA_{sn} = (\rho_1 + \rho_{2s}) * TEAC_{sn} + \varepsilon_s \quad \text{eqn 2}$$

$$\rho_{2s} \sim \text{normal}\ (0, \sigma^2) \quad \text{eqn 3}$$

$$\varepsilon_s \sim \text{normal}\ (0, 1 - (\rho_1 + \rho_{2s})^2) \quad \text{eqn 4}$$

where $TEAC_{sn}$ and UA_{sn} are standard normal TEAC and UA, respectively, ρ_1 is the average correlation across species, ρ_{2s} is the species-specific deviation from ρ_1 for species s, ε_s is the species-specific random error, and σ^2 is the variance of the species-specific deviations from ρ_1. Equations (1) and (2) are equivalent – both yield identical estimates of ρ_1, each ρ_{2s}, and σ^2. Larger σ^2 indicate greater heterogeneity across species.

Before running the models, we culled the data set separately for each correlation to include only species with three or more individuals without missing data for either antioxidant in the correlation. Data preparation was done in R v. 2·6·0; models were run using Monte Carlo Markov Chain simulations and Gibbs sampling in WinBUGS v. 1·4·3. For each correlation, the model ran 210 000 iterations and discarded the first 10 000 (burn-in). We tabulated 95% credible intervals based on the posterior probability distributions for the parameters

ρ_1, each ρ_{2s}, and σ^2. For readers who are not familiar with multilevel models and Bayesian modelling approaches, we have provided an additional introduction and our WinBUGS and R code in Appendix S4 in Supporting Information.

There are many factors at both the individual and species levels that are potentially related to antioxidant levels, including individual quality, breeding status, life-history strategy and diet. We explore all of these factors in other publications (Cohen 2007; Cohen, Hau & Wikelski 2008a; Cohen *et al.* 2008b, AAC, unpublished manuscripts), and cannot control simultaneously for all of them here. However, in this study such control would actually limit our ability to characterize the nature of the variation within antioxidant systems (as opposed to pinpoint its causes). In fact, adequate characterization of the variation is a prerequisite for robust analyses of factors that may determine the variation. This study is thus limited more by the factors that were controlled for in our sampling than by the factors that were not: we are unable to make generalizations to wintering birds or to unsampled taxa about the variation we detect. We explore sex differences within 16 species in Appendix S5 in Supporting Information.

Some of our analyses use multiple non-independent variables to assess similar questions – for example, as TEAC and uric acid are mechanistically dependent and highly correlated, if TEAC correlates with lutein, it is likely that uric acid will correlate with lutein as well. When multiple tests are performed, it is common to use a correction method such as a sequential Bonferroni adjustment; however, there is considerable debate as to whether this is generally appropriate (Perneger 1998; Moran 2003). In particular, when the tests are not fully independent, there is no widely accepted methodology for accounting for multiple testing issues. We believe, in this early stage of our observational research, that it is best to present raw *P*-values and acknowledge that multiple tests were performed in our interpretation of them rather than rely on an arbitrary standard such as $\alpha = 0{\cdot}05$. Most importantly, our conclusions do not hinge on the significance of individual tests but rather on the broad patterns seen across multiple tests, or from Bayesian estimates of heterogeneity in parameters. Multiple testing and false discovery rates are often considered irrelevant in a Bayesian framework such as this (Jeffreys & Berger 1992). In particular, as we are only trying to assess 'significance' (in this context, a conclusion that a parameter is substantially different from zero) for a small subset of the parameters estimated in the models, multiple testing is of little concern. As will be seen, some patterns are clear and consistent, some are clearly absent, and others are ambiguous and noted as such.

Results

INTERSPECIFIC LEVEL

Summary statistics for all antioxidant measures at the species and individual level are presented in Appendices S1 and S2, respectively. For the nine antioxidant variables, the first three PCA axes had eigenvalues > 1·0 and explained 75% of the variation (37%, 25% and 14%, respectively). A biplot of the first two axes shows that slight rotations of these axes would yield a carotenoid axis, an axis explaining TEAC, UA, Res and an axis explaining vitamin E levels (Fig. 1). Closely related species tend to group closely on the biplot, implying an effect of phylogeny and/or ecology. For example, all three woodcreepers (4, 17 and 62 on the plot) have low carotenoid levels but moderately high TEAC–UA values. In some cases,

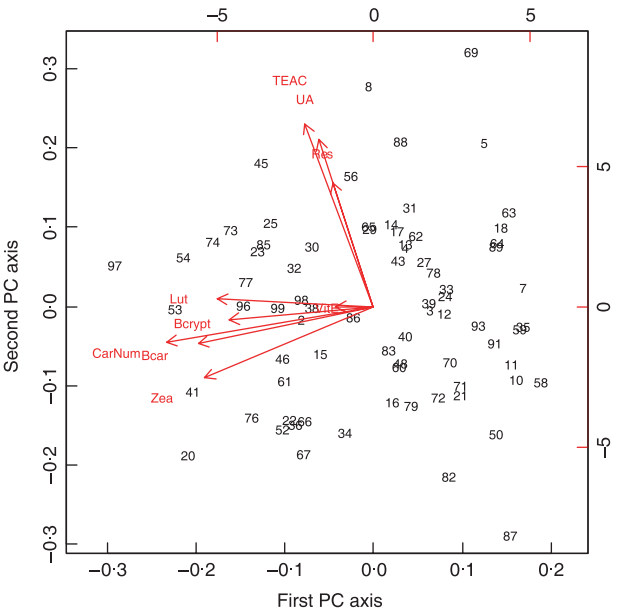

Fig. 1. Loadings of antioxidant variables and scores of species on a biplot of the first two principal component (PC) axes. Numbers represent species and vectors represent antioxidant variables. The bottom and left axes scale the factor loadings of the antioxidant variables to λ; the top and right axes scale the PC scores of the species to λ. 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; CarNum, number of carotenoid types. Note that related species tend to group together, either in bi-variate space or along a single axis in that space. Species number codes are shown in Appendix S1.

related species group along one axis but not another – for example, the antbirds (7, 59 and 89 on the plot) all have very low carotenoid levels but varying TEAC–UA levels. Ignoring such phylogenetic groupings, species are randomly distributed in this 'antioxidant space', without certain combinations of TEAC and carotenoid levels either over- or under-represented. When we compared the actual distribution to a null model randomly assigning species to points within the distribution range, 33 out of 100 random models were less clumped and 67 were more clumped.

The clear identification of three orthogonal axes of variation corresponding to the three biochemical types of antioxidant we measured provides strong justification for use of three summary measures to represent these axes. Varimax rotation in a three-factor factor analysis confirms the identity of these axes (Table 1). TEAC, UA and Res are not statistically independent measures: TEAC is a combination of the other two. Thus, TEAC alone is a good summary of the second axis, on which it loads at 0·98. Likewise, the third axis is roughly equivalent to vitamin E (although β-cryptoxanthin does load somewhat onto this axis); accordingly, we simplify presentation and discussion by using vitamin E as a proxy for this axis. We identified the single axis best describing the five carotenoid variables in a second factor analysis (Table 1), and used an individual-level version of this analysis to generate individual-specific summary scores for carotenoid level (see Methods).

Table 1. Factor loadings for interspecific variation in antioxidant levels. Factor analyses were performed on species means of 99 species with varimax rotation. All loadings > 0·30 are in bold

	Full factor analysis				Carotenoid-only factor	
	Factor 1	Factor 2	Factor 3	Final communality	Factor 1	Final communality
Percent variance explained	35%	25%	15%	75%	36%	36%
TEAC	0·06	**0·98**	0·05	0·97		
Uric acid	0·0008	**0·89**	0·12	0·8		
TEAC–UA residual	0·04	**0·66**	–0·08	0·45		
Vitamin E	–0·03	0·03	**0·86**	0·74		
Lutein	**0·77**	0·23	–0·28	0·72	**0·69**	0·48
Zeaxanthin	**0·8**	–0·16	0·28	0·74	**0·82**	0·68
β-cryptoxanthin	**0·54**	0·09	**0·56**	0·62	**0·65**	0·43
β-carotene	**0·87**	0·02	0·11	0·77	**0·83**	0·69
Carotenoid number	**0·92**	0·06	0·29	0·93	**0·96**	0·92

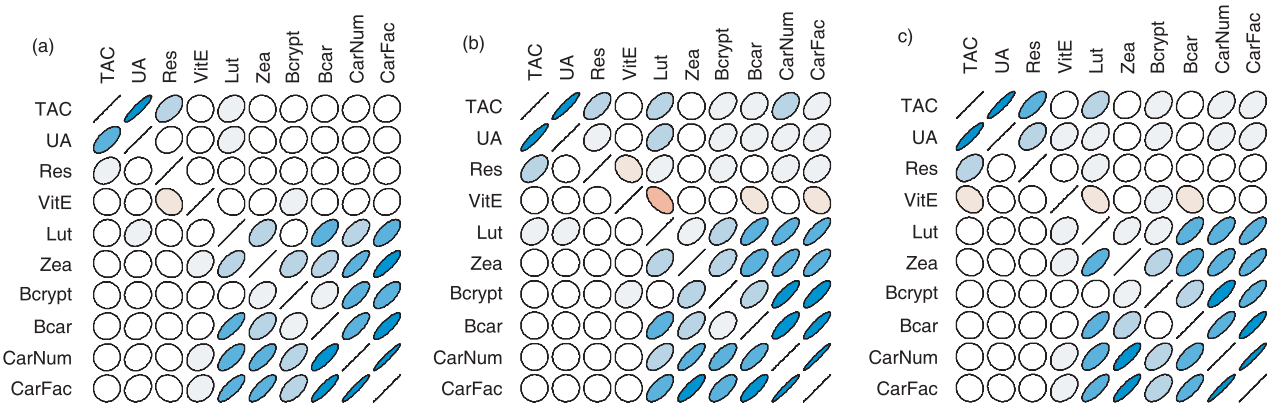

Fig. 2. Correlations among antioxidant variables (a) across species, with and without phylogenetic control (PICs; below and above diagonal, respectively); (b) across species, weighted and unweighted for sample size (above and below diagonal, respectively); (c) across species, separated for large- and small-sample size species ($n > 5$, $n \leq 5$; above and below diagonal, respectively). Direction of the correlation is indicated by direction of the ellipse and colour (blue: positive; red: negative). Strength of the correlation (r-value) is indicated by shading and narrowness. 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; CarNum, number of carotenoid types.

However, there is substantial residual carotenoid variation unexplained by this axis, indicating unique effects of the individual carotenoid types.

In a simple correlation matrix (unweighted for sample size and without phylogenetic control), we found strong positive correlations within the three antioxidant groups (TEAC–UA variables, vitamin E and carotenoids) but few correlations among them (Fig. 2). Controlling for phylogeny slightly weakened some of the associations, but did not produce any major changes in the overall patterns (Fig. 2a). Of the 27 correlations between vitamin E, carotenoids and TEAC–UA variables, only two are significant without phylogenetic control and five with phylogenetic control (Table S1 in Supporting Information). The significance levels do not fall below $P = 0{\cdot}03$, so there is relatively little suggestion of overall associations, and many of the correlations may be spurious. Only one of these is present both with and without phylogenetic control: the correlation between lutein and uric acid ($r = 0{\cdot}25$, $P = 0{\cdot}03$). A nested ANOVA shows that for all antioxidant variables, 29%–78% of the variation is at the species level, confirming strong effects at both inter- and intraspecific levels (Table S2 in Supporting Information).

Exploration of a statistical issue – weighting for sample size – led us to discover that interspecific associations appear to depend on the set of species included. When we weighted correlations by sample size per species, we detected many more positive correlations between TEAC–UA and carotenoid variables, and some negative correlations between vitamin E and carotenoids (Fig. 2b). Because this difference could be due to species characteristics correlated with sample size (diet, tropical vs. temperate, or common vs. rare, to name a few), we ran unweighted analyses separately on the 33 species with $n > 5$ and the 66 with $n \leq 5$ (Fig. 2c). There were no significant correlations between TEAC–UA variables and carotenoids in the small sample size group, but there were stronger correlations in the large sample size group than in the full unweighted analysis. This was not sensitive to varying the sample size cut-off point, and there were no such differences

Table 2. Heterogeneity of antioxidant correlations across species. Parameters were estimated using 200 000 iterations of a Monte Carlo Markov Chain model after a 10 000 iteration burn-in. Only species with at least three individuals with both measurements were included in each analysis

Correlation	Parameter	Mean	SD	CV	2·5% CI	97·5% CI
TEAC–UA						
(42 species, 767 individuals)	ρ_1	0·72	0·04	0·06	0·65	0·80
	σ	0·25	0·04	0·16	0·18	0·35
Lut–Zea						
(22 species, 357 individuals)	ρ_1	0·61	0·06	0·10	0·48	0·72
	σ	0·23	0·05	0·22	0·14	0·35
TEAC–VitE						
(22 species, 355 individuals)	ρ_1	0·22	0·08	0·36	0·07	0·37
	σ	0·22	0·08	0·36	0·08	0·40
TEAC–CarFac						
(22 species, 355 individuals)	ρ_1	–0·02	0·07	3·50	–0·17	0·12
	σ	0·17	0·08	0·47	0·04	0·34
VitE–CarFac						
(22 species, 357 individuals)	ρ_1	0·16	0·09	0·56	–0·02	0·33
	σ	0·28	0·10	0·36	0·06	0·48
UA–Zea						
(22 species, 357 individuals)	ρ_1	–0·07	0·08	1·14	–0·22	0·09
	σ	0·22	0·08	0·36	0·07	0·38

CI, Bayesian credible interval; ρ_1, overall mean correlation across species; σ, standard deviation of species-specific deviations from ρ_1; TEAC, Trolox-equivalent antioxidant capacity; UA, uric acid; Lut, lutein: Zea, zeaxanthin; VitE, vitamin E; CarFac, carotenoid factor.

between tropical and temperate species (data not shown). The lack of correlations in the small sample size group cannot easily be attributed to the greater error in estimating means for these species because: (i) there were nearly twice as many species in this group, and (ii) the correlations within the TEAC–UA and carotenoid groups were just as pronounced as for the species with large sample size. We also ran the phylogenetic independent contrasts on several different trees and found that tree topology and inclusion of different sets of species had a large effect on which relationships between TEAC–UA variables and carotenoids were significant (data not shown). Thus, relationships between TEAC–UA variables and carotenoids are present at the interspecific level in certain sets of species but not others; though we are currently unable to specify which characteristics of species sets predict these relationships.

HETEROGENEITY OF ANTIOXIDANT CORRELATIONS ACROSS SPECIES

A random effects model showed significant differences among species in all antioxidant measures ($P < 0{\cdot}0001$ for all). We then chose six correlations to explore in more detail across species. The TEAC–UA correlation is important because it shows how general and strong the effect of uric acid is in determining overall antioxidative status of serum, and also serves as an example of two variables from within the TEAC–UA set. The correlation between lutein and zeaxanthin was chosen as an example of correlations among carotenoid variables because all species had individuals with both of these types. Uric acid–zeaxanthin is a representative correlation between two variables from different axes of variation. The remaining three correlations are between the variables best representing the main axes of variation: TEAC, vitamin E and the carotenoid factor. Based on the interspecific findings, we expected that correlations would be stronger and more common within these axes than across them.

Correlations among some antioxidant types varied across species (Table 2, Fig. 3). The TEAC–UA and lutein–zeaxanthin associations both showed strong overall correlations across species ($\rho_1 = 0{\cdot}72$, $0{\cdot}61$, respectively) and substantial heterogeneity among species ($\sigma = 0{\cdot}25$, $0{\cdot}23$, respectively). Because σ is constrained to be non-negative, its credible interval will never span zero; heterogeneity of correlations among species is thus indicated by narrow credibility intervals for σ relative to the magnitude of σ and by large σ relative to ρ_1 (Table 2). The TEAC–vitamin E correlation was weak overall ($\rho_1 = 0{\cdot}22$), and there was no evidence the other three correlations differed from zero (TEAC–carotenoid factor $\rho_1 = -0{\cdot}02$, vitamin E carotenoid factor $\rho_1 = 0{\cdot}16$, and UA–zeaxanthin $\rho_1 = -0{\cdot}07$). The wide credible intervals for σ for all these four correlations indicate insufficient power in our data set to infer whether or not there are substantial differences among species; however, except for the vitamin E–carotenoid factor correlation, it is unlikely that any species has a strong correlation ($|\rho_1 + \rho_{2s}| < 0{\cdot}5$ for all s).

INTRASPECIFIC LEVEL

We summarize the main findings from the 45 correlations calculated for 20 species by presenting five key correlations for each species (Fig. S1; Table S3 in Supporting Information). Standard errors of the species-specific correlation estimates are too large to test for phylogenetic signal, but Supporting

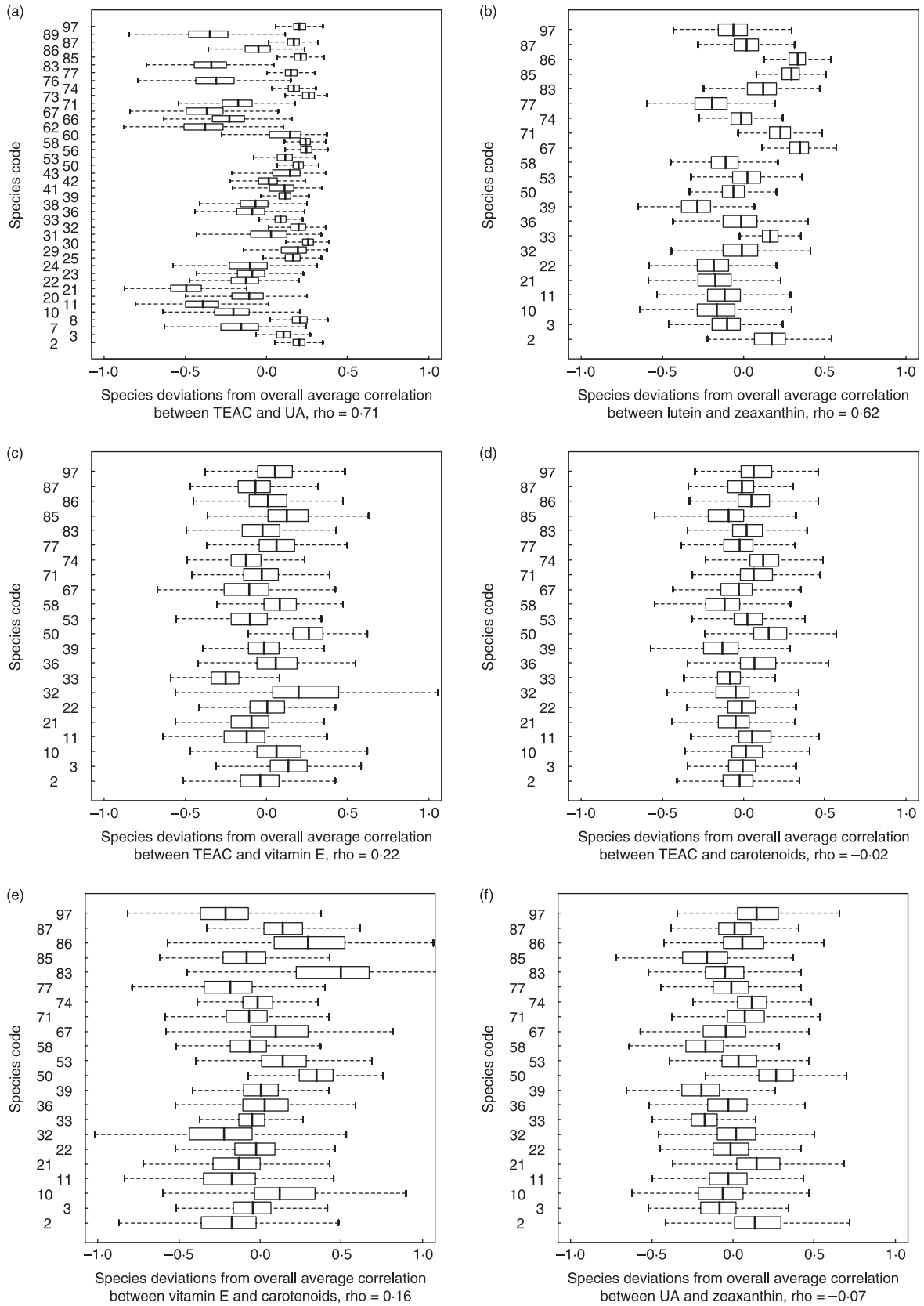

Fig. 3. Estimated Bayes values and 50% and 95% credible intervals for species-specific deviations (ρ_{2s}) from the overall average correlation coefficients (ρ_1) for six antioxidant correlations. (a) TEAC–uric acid; (b) Lutein–zeaxanthin; (c) TEAC–vitamin E; (d) TEAC–carotenoid factor; (e) Vitamin E–carotenoid factor; (f) Uric acid–zeaxanthin. Species codes are listed in Appendix S1. Boxes indicate 50% CIs and whiskers indicate 95% CIs. Overall mean correlation across species is indicated below each plot.

Fig. S1 shows that even closely related species often show markedly different associations among antioxidants. In the case of the TEAC–uric acid association, heterogeneity across species appears tightly associated with habitat (tropical vs. temperate vs. seabird, Fig. S2 in Supporting Information). Some of the species-specific associations between TEAC–UA variables and carotenoids variables are shown in Table S4 in Supporting Information, and all correlations among the full set of antioxidant variables in all species with sufficient sample size are shown in Appendix S3 in Supporting Information. Sex differences in 16 species are explored in Appendix S5 in Supporting Information.

Discussion

MULTIVARIATE NATURE OF ANTIOXIDANT SYSTEMS

Analysis of covariation among antioxidant types showed clearly that univariate measures are not sufficient to characterize patterns of variation in circulating antioxidants in birds at either the inter- or intraspecific level. In this study at least three largely orthogonal axes were necessary to adequately describe the data, and these axes corresponded well with biochemical categories of antioxidant: uric acid, carotenoids and vitamin E. More axes would likely have been necessary if we had included other measures such as vitamin C and intracellular enzyme levels. The summary measure of antioxidant capacity used here, TEAC, was tightly associated with uric acid but largely unassociated with vitamin E or carotenoids, consistent with the high molar levels of uric acid relative to other types.

One implication of this is that the characterization of 'antioxidant systems' may be inaccurate, at least at the levels of analysis used here. There is evidence that different antioxidants are regulated in concert within individuals over time (Cohen 2007), but the forces that determine levels at the inter- and intraspecific levels do not appear to result in a single coherent pattern of variation across antioxidant types. As discussed below, this may be attributable to specificity of function among antioxidants and to alternative physiological strategies for controlling oxidative damage. It also may indicate that antioxidant function is incidental to other functions for at least some of these molecules (Costantini & Møller 2008). In terms of antioxidant measurement in an ecological context, studies should include multiple measures of antioxidants. Studies of antioxidants in relation to oxidative stress should likely also include measures of reactive oxygen species or oxidative damage such as thiobarbituric acid reactive substances (TBARS), reactive oxygen metabolites (ROM) or H_2O_2 levels (Esterbauer 1996; Brunet-Rossinni 2004; Costantini & Dell'Omo 2006). Alternatively, studies might focus on one specific class of antioxidants such as carotenoids but avoid all pretense of measuring overall antioxidant status. Enzymatic antioxidant activity is important but hard to measure in ecological studies; future studies might explore circulating levels of mineral co-factors such as selenium, zinc and manganese as a proxy, though confounding by diet and sequestration variability may prove insuperable. Summary measures such as TEAC should be used in concert with other measures and should be interpreted with caution.

CAROTENOID LEVELS DO NOT CONTRIBUTE MEANINGFULLY TO TEAC

Based on our data, carotenoids are generally present at much lower levels in the bloodstream than uric acid, but if carotenoids are important general antioxidants then they should have been associated with overall TEAC, at least in species with high carotenoid levels. In addition, there could have been TEAC–carotenoid associations due to coherent regulation of antioxidant systems or to co-fluctuations due to shared physiological determinants or shared dietary sources. Roles for carotenoids are well established both in colour communication and in egg yolk for enhanced offspring development (e.g. Surai *et al*. 2001; McGraw *et al*. 2003a). It would have been convenient if we could clearly assert that this was due to general enhancement of antioxidant protection, but it appears that this is not the case. Not only are there few consistent correlations between TEAC and carotenoids, but the species that show correlations often lack carotenoid-pigmented plumage (Table S4 in Supporting Information).

Other studies have also shown a lack of association between overall antioxidant measures and carotenoid levels (e.g. Isaksson *et al*. 2007), and a meta-analysis confirms this trend across studies (Costantini & Møller 2008). If carotenoids benefit health through a simple increase in overall antioxidant capacity, then it is hard to explain why carotenoids have consistently been shown to affect health status in birds, given that they do not covary strongly with TEAC and that high TEAC itself has sometimes been associated with poor health status (Cohen 2007; Hõrak *et al*. 2007). Much recent evidence points to a more complex explanation. Carotenoids may benefit health via gene regulatory and cellular communication functions, especially in tissue regeneration and the immune response, and through membrane stabilization rather than via a direct role as antioxidants (Young & Lowe 2001; Hartley & Kennedy 2004). However, the antioxidant function of carotenoids is highly specific and not necessarily interchangeably carried out by other antioxidants (Surai 2002), so it is equally possible that carotenoids are essential as antioxidants but only within certain tissues or membranes or in certain species. In this case, the minor contribution of carotenoids to overall measures of circulating antioxidant capacity may be a poor reflection of their importance as antioxidants.

IMPLICATIONS FOR PHYSIOLOGY OF SPECIFIC ANTIOXIDANTS

Almost all correlations between carotenoid variables were significant at both the inter- and intraspecific levels, except between lutein and β-cryptoxanthin, for which most analyses at both levels showed little or no relationship. Such consistent intercorrelations among carotenoid concentrations likely reflect attributes of both diet and carotenoid physiology. First, carotenoid-replete foods tend to be rich in several

different carotenoids (Goodwin 1980, 1984; also see studies of fruit and vegetable diets in humans, e.g. Rock *et al*. 1992; McEligot *et al*. 1999). Lutein and zeaxanthin, for example, are sister molecules, differing only in structure by the placement of a single C = C bond, and rarely are found without one another in plant or animal matter (but see McGraw *et al*. 2003b for a special case in bird feathers). Also, animals are considered to use a generalized mechanism for carotenoid uptake from food, via passive diffusion with other dietary lipids across the intestinal mucosa (Erdman, Bierer & Gugger 1993; Furr & Clark 1997). Hence, species are not generally expected to show high specificity for accumulating more of one type of carotenoid than another. There is experimental evidence in some situations and species that certain carotenoids out-compete others during uptake (van den Berg 1999) or are more valuable for antioxidant function (Mortensen & Skibsted 1997) or colour acquisition (McGraw *et al*. 2004; McGraw, Nolan & Crino 2006), but data to date suggest that most free-living/unmanipulated birds tend to accumulate more of all types of carotenoids when many are present in foods (e.g. McGraw *et al*. 2003a; McGraw & Gregory 2004).

Carotenoid intercorrelations tended to be weaker when they involved the non-polar carotenoids – for example, carotenes and cryptoxanthins. These types of carotenoids are far less common in the insects and plant foods consumed by the majority of the species in our sample (Goodwin 1980) and in fact were undetectable in many species. The lowest total carotenoid levels observed in this study are similar to human levels (*c.* 0·9 mg L^{-1}, Walston *et al*. 2005), but the highest levels are two orders of magnitude higher. In humans, however, both lycopene and β-carotene are more abundant than the combination of lutein and zeaxanthin, whereas not a single bird here had more β-carotene than lutein, and only 2 out of 428 had more β-carotene than zeaxanthin. Lycopene was not detected in these birds.

A number of recent nutritional ecology studies have used a measure of total carotenoids rather than breaking them down by type (e.g. Alonso-Alvarez *et al*. 2004; Tella *et al*. 2004; Costantini *et al*. 2006). Our data give a mixed review to this approach. It is simpler and more straightforward to have one variable than several, and clearly carotenoids are not always completely independent from each other. Also, understanding the roles of the different carotenoid types in an ecological or life-history context can be a challenge. However, we have shown that some carotenoid types do behave differently than others, consistent with a number of previous studies (McGraw *et al*. 2004, 2006). In our data set, total carotenoid concentration is essentially a measure of lutein in most species and was thus excluded from analysis, but lutein levels do not necessarily reflect levels of other carotenoids. β-carotene, for example, appears to correlate with reproductive success in savannah sparrows, even though lutein may not (AAC, MS submitted). The best composite measure, a factor such as we used here, can only be calculated if the individual types are measured. Any composite measure is likely to obscure some patterns of interest, even though it may be an inevitable and not wholly inaccurate alternative for many studies.

Vitamin E was not consistently associated with other antioxidants in this study, though several species did show some associations. Little is known about the nutritional ecology of vitamin E in wild avian species. In general, vitamin E is considered particularly important for protection of membranes and lipids against oxidative damage (Surai 2002; Niki 2004). It also has a number of regulatory roles in addition to its antioxidant function, but there is some evidence that it can be pro-oxidant in certain biochemical environments (Neuzil & Stocker 1994; Surai 2002). Birds rely heavily on fatty acids for metabolism during flight (McWilliams *et al*. 2004), suggesting that lipid-protective antioxidants such as vitamin E could be particularly important in birds relative to mammals. We do not see any strong evidence for this – for example, we might have expected a negative correlation with carotenoids if the two were interchangeable in protecting lipids; or alternatively a positive correlation if birds with higher metabolic rates needed higher levels of both. Vitamin E does not systematically correlate with either basal metabolic rate or mass-adjusted basal metabolic rate in the species in this study (Cohen *et al*. 2008b).

DETERMINANTS OF ANTIOXIDANT LEVELS AND CORRELATIONS OVER EVOLUTIONARY TIME

There was substantial heterogeneity in correlations across species. The strongest association, between TEAC and uric acid, was weak or absent in most tropical species, was generally strong in temperate species, and was extremely strong in seabirds. This cannot be attributed to the low levels of uric acid in tropical species because seabirds had even lower levels. The most consistent association, between lutein and zeaxanthin, was positive though not necessarily significant in all species tested, but there was significant heterogeneity in the strength of the correlation. Rarely was there any apparent pattern with regard to which species showed which correlations. Such heterogeneity suggests evolutionary and/or ecological determinants not only of antioxidant levels, but also of antioxidant associations.

Carotenoids that may be essential for survival or that increase reproductive success in one species were often below detection levels in many others in our sample, implying considerable dietary or environmental constraints on their availability and subsequent evolutionary lability of their roles. Once a nutrient is present in a species' diet, it is possible that the species could evolve more varied physiological uses for it and could conceivably evolve a need for it. Species that make greater use of carotenoids in plumage displays should exhibit greater physiological reliance on them and get greater health benefits – immunological or cell signalling, for example – from increased levels in the diet. It is even possible that certain carotenoids could have negative health effects in species not accustomed to consuming them. There are clearly health benefits from some carotenoids in some species, as demonstrated experimentally (e.g. McGraw & Ardia 2003) and in accord with their role in sexual displays and provisioning in egg yolk (e.g. Saino *et al*. 2003).

In a larger physiological context, our results agree with a series of recent studies demonstrating the complexity of physiological systems across evolutionary time. For example, some of our work has shown a similar complexity in other aspects of antioxidant systems, including the stress response, relationships with corticosterone and metabolic rate, relationships with individual quality, and relationships with life-history variation (Cohen 2007; Cohen *et al*. 2008a,b). Hõrak *et al*. (2006) have shown similar complexity in relationships among carotenoids, TEAC and immune response in greenfinches. Hormone systems may be equally complex (Wingfield *et al*. 1998; Gill, Alfson & Hau 2007), and the evidence for such patterns among immune variables is now quite strong (Adamo 2004). Even within ducks, for example, it is difficult to find immunological patterns that are consistent at both the individual and species level (Matson *et al*. 2006). Immune variables do not seem to partition easily into intuitive units for analysis, but there are real associations that are meaningful when viewed in the appropriate context.

Given these broader patterns, the results here are consistent with levels of antioxidants evolving in response to a large number of factors, including diet, physiological need (ultimately relating to life-history strategy) and abundance of other antioxidants. (When we say that antioxidant levels 'evolve', we include evolution of indirect mechanisms such as changes in diet, habitat and metabolic activity as well as evolution of direct physiological regulation.) Functions of and relationships among antioxidants likely vary among species to some extent, reflecting evolutionary lability. For example, a short-term shortage of a given antioxidant in the diet of a species might lead to roles of other, more abundant antioxidants evolving to compensate and potentially even to evolutionary or behavioural alterations of diet to accord with the new optimal levels of various micronutrients. In other situations, levels of a given antioxidant may not be particularly important with regard to health, and may fluctuate for other reasons. Such a large number of sometimes conflicting selective pressures, and such a variety of potential solutions to physiological problems, should lead to exactly the sort of patterns we observe: correlations that can be generalized but rarely are universal, and which are often hard to predict based on ecology and life histories. Even when general patterns are absent, however, some potential associations may be categorically excluded – none of our species showed a negative correlation between TEAC and uric acid, for example. The gradual accumulation of information on the conditions relating to variation in strength of antioxidant associations should lead to improved understanding of these systems, even if predictive power is limited.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Species mean antioxidant levels

Appendix S2. Individual antioxidant levels

Appendix S3. Intraspecific correlations among antioxidants

Appendix S4. Methodological details

Appendix S5. Sex differences

Fig. S1. Intraspecific correlations in 20 species.

Fig. S2. Intraspecific correlations between TEAC and UA.

Table S1. Species-level antioxidant correlations with and without phylogenetic contrasts

Table S2. Individual- and species-level variance and partial correlation coefficients

Table S3. Correlations among main antioxidant variables within species

Table S4. Associations between TEAC–UA variables and carotenoids within various species

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