Research

Complexity in relationships between antioxidants and individual life-history parameters in a seabird and a songbird

Alan A. Cohen, Robert A. Mauck, Nathaniel T. Wheelwright, Charles E. Huntington and Kevin J. McGraw

A. A. Cohen (aacohen1.bus@gmail.com), Dept of Biology, Univ. of Missouri-St. Louis, 8001 Natural Bridge Road, St. Louis, MO 63121–4499, USA. Present address: Centre for Global Health Research, St Michael's Hospital, 70 Richmond St East Suite 202A, Toronto, ON, Canada. – R. A. Mauck, Biology Dept, Kenyon College, Gambier, OH 43022, USA. – N. T. Wheelwright and C. E. Huntington, Dept of Biology, Bowdoin College, Brunswick, ME 04011, USA. – K. J. McGraw, School of Life Sciences, Arizona State Univ., Tempe, AZ 85287, USA.

Selection on physiological traits is thought to mediate the evolution of individual life-history parameters like reproduction, longevity, and the tradeoffs between them, but almost nothing is known about the relationships between physiological and life-history parameters in the wild. Antioxidants are strong candidates to correlate with life histories because they play a critical role in preventing free radical damage to macromolecules, and many types are involved in sexual signaling and embryo provisioning. Here for the first time we present data on associations between serum antioxidant measures (antioxidant capacity and concentrations of uric acid, vitamin E and carotenoids) and indices of reproductive rate and age in two bird species. After controlling for age, 36% of the variation in hatching rate in Leach's storm-petrels $Oceanodroma$ $leucorhoa$ was accounted for by a negative association with antioxidant capacity. Age was negatively associated with uric acid levels. Savannah sparrows $Passerculus$ $sandwichensis$ showed no association between antioxidant capacity and fledging rate, but serum β-carotene levels were weakly positively associated with fledging rate. Because antioxidant levels are known to vary markedly within individuals over time, detection of associations between long-term measures of reproduction and instantaneous antioxidant levels suggests strong (though not necessarily causal) relationships. Relationships between antioxidants and life histories appeared to differ in sparrows and storm-petrels though, likely due to variation in diet, ecology, and life-history evolution in these distantly related species.

Evolutionary ecologists have long had an interest in understanding the tradeoffs between reproduction and longevity, i.e. current versus future reproduction (Williams 1957, Stearns 1992). Although some studies have investigated the role of hormones in regulating behavior that affects these tradeoffs, very little is known about more direct physiological causes and consequences of the tradeoffs (Ketterson et al. 1996, Ricklefs and Wikelski 2002). Why does reproductive effort incur physiological costs, and would understanding these mechanisms help explain why some species appear to be more likely than others to increase reproductive effort in response to need or opportunity? Such physiological costs are a critical assumption of the theoretical framework of evolutionary ecology and life-history tradeoffs, but are still understood only as broad and largely unsubstantiated hypotheses.

One such hypothesis is that oxidative damage – a by-product of aerobic metabolism – accrues in proportion to stress and/or metabolic exertion, likely including reproductive effort, eventually causing or contributing to the aging process (Beckman and Ames 1998, Barja 2004). In recent years oxidative balance has emerged as an important sub-discipline of physiological ecology, with a number of studies examining both antioxidant defenses and oxidative damage, primarily in birds (Costantini and Dell'Omo 2006, Hõrak et al. 2006, Alonso-Alvarez et al. 2007, Cohen et al. 2008b, Isaksson and Andersson 2008). For example, artificially increased brood size in zebra finches $Taeniopygia$ $guttata$ was associated with increased oxidative stress in parents (Alonso-Alvarez et al. 2004, Wiersma et al. 2004). However, to date there are no studies establishing relationships within species between oxidative balance and individual life-history parameters such as reproductive rate and age.

Across bird species, higher reproduction and lower survival are often associated with higher antioxidant levels, potentially reflecting tradeoffs (Cohen et al. 2008b). However, within species there might be countervailing effects of individual quality – where high reproduction is associated with general health (Ardia 2005) – and of tradeoffs, where high current reproduction is associated with physiological damage (Wiersma et al. 2004, Knowles et al. 2009). Our goal here is to assess the relationship in the field between antioxidant levels and age and reproductive rate in two distantly related bird species as a first step

toward understanding whether and how oxidative balance may help mediate life-history tradeoffs.

Physiological systems are complex, and antioxidants are an imperfect measure of oxidative balance. Much of the important variation may be due to regulation of the rate of free radical production, although it appears that antioxidants may respond to need, such that high levels indicate greater oxidative stress (Lopez-Torres et al. 1993, Brand 2000). Antioxidant systems also vary greatly across tissues and across species. In many tissues, and primarily in mitochondria, antioxidant enzymes are the key defense against oxidative damage (Barja 2004). In circulating systems (measured in this study), micromolecular antioxidants such as uric acid, vitamins C and E, and carotenoids play a more important role (Miller et al. 1993). Antioxidant enzymes, which have a specialized function, are likely regulated to adjust antioxidant protection specifically, whereas micromolecular antioxidant levels depend on dietary intake and on their other physiological roles such as signaling. Additionally, the diverse roles of micromolecular antioxidants mean that different trends might be seen for different types of antioxidants. In particular, carotenoids are important in sexual signaling and immune function, making it possible that carotenoids would correlate positively with reproductive rate even while other types of antioxidants might correlate negatively, particularly if reproductive rates reflect quality and high antioxidant levels reflect high oxidative stress (Bortolotti et al. 2003, McGraw 2006). Across species, variation in diet and in other aspects of physiology mean that antioxidants may vary for reasons unrelated to life-history tradeoffs (Tella et al. 2004).

We assessed the relationship between circulating antioxidant concentrations and age and reproductive rate in two species that have been studied at the same site for many years: Leach's storm-petrel *Oceanodroma leucorhoa*, a long-lived seabird, and Savannah sparrow *Passerculus sandwichensis*, a short-lived migratory passerine. From blood samples, we determined antioxidant capacity and levels of three classes of antioxidant: uric acid, vitamin E and carotenoids. We use a measure of reproduction – average annual number of offspring from first breeding attempt through year of sampling – that allows us to compare living individuals of different ages. Theoretical considerations could lead to several conflicting predictions for associations between antioxidant levels and individual life-history parameters; given the absence of previous studies on the subject we treat this as an exploratory analysis. We show that there are associations between some antioxidants and individual life-history parameters in both species, but that the specifics of the associations differ markedly between the species.

Methods

Study species

Leach's storm-petrels have been studied for 53 years at the Bowdoin Scientific Station on Kent Island, New Brunswick, Canada (44°35′N, 66°46′W), and the biology of the species is well-characterized (Huntington et al. 1996). The species is long-lived – maximum recorded lifespan is 36 years and annual adult return rates are around 89%. Diet consists of plankton, nekton, copepods, squid and small fish. Females lay a single egg in June, and both parents alternate incubation bouts of 1–7 days in burrows. Parents return to burrows at night after feeding for several days. Mean mass on first day of incubation is 51 g, with an average daily weight loss of 4–7%. The incubation period is usually 40–44 days. Many individuals in the population are banded with US Fish and Wildlife Service bands and have a known reproductive history, and at time of sampling there were marked individuals in the population that had been breeding as long as 28 years.

Savannah sparrows on Kent Island have been studied from 1963–1966 and from 1986–2006 (Wheelwright et al. 2006, Wheelwright and Rising 2008). The maximum lifespan is about eight years, annual return rates for adults are about 50%, and adult mass averages 20 g for males and 17.5 g for females. During breeding, diet consists mostly of spiders and adult and larval insects, with less fruit and seeds than at other times of year. Males defend territories in open grasslands and females lay 1–4 clutches of 3–5 eggs, depending on nest predation rates. Polygyny is common, with 15–43% of males mated to more than one female, depending on site and year. About 45% of young are sired through extra-pair fertilizations (Wheelwright et al. 1992, Freeman-Gallant et al. 2003, 2006). Exact ages and detailed breeding histories are known for most of the individuals at the main study site, and all breeding adults are marked with both colored bands and US Fish and Wildlife Service bands.

Sampling

We drew blood from 56 Leach's storm-petrels between 18–25 June 2005. Forty-two petrels were caught when they were inactive during the daytime by reaching into burrows; the other 14 birds were captured at night in mist nets. Day-caught birds were known to be incubating, probably during the first half of incubation given the time of year. Night-sampled petrels were generally not banded and of unknown history, but may have been pre-breeders or adults returning from feeding. Sex of petrels was generally not known. We also drew blood from 41 nesting Savannah sparrows (24 female and 17 male) captured in mist nets between 07:00 and 17:00 between 18–25 June 2005. All Savannah sparrows sampled bred in 2005. We were able to confirm precise breeding status for most females sparrows by weight (laying females generally weigh more than 20 g) and observation of hatch date. Out of 24 females, 11 were sampled post-hatch date (presumably feeding nestlings), two were incubating, two were sampled on the hatch date, one appeared to be laying (>20 g), and the rest were of unknown status but were less than 20 g, and thus likely incubating. Of the 17 males, four were feeding nestlings, two had nests being incubated, and 11 had nests at unknown stages.

Blood samples for both species were taken by pricking the brachial (wing) vein with a needle and collecting the blood into unheparinized microcapillary tubes. Samples were kept on ice for less than 1 h, centrifuged, and the serum was frozen in liquid nitrogen, then later stored at −80°C following Cohen et al. (2007). All samples were

taken within 3 min of capture to avoid effects of the hypothalamo-pituiary-axis-activated stress response on antioxidant concentrations (Cohen et al. 2008a). Trolox-equivalent antioxidant capacity (TEAC) of serum and uric acid were measured using spectrophotometric methods following Cohen et al. (2007). Vitmain E and carotenoids were measured using HPLC following previously published methods (McGraw and Parker 2006). Details of these methods can be found in the Supplementary material Appendix 1.

Measures of reproductive rate, age and body size

In both study species, reproduction is higher in older birds; in the petrels this is apparently due to higher mortality of low-quality individuals at young ages (Wheelwright and Schultz 1994, Mauck et al. 2004). However, in many species there is also evidence for a tradeoff between reproduction and survival, or old individuals may be senescent (Daan et al. 1996). The positive association between reproduction and age in our species allows us to use a measure of reproduction – average annual offspring production, up to current age, or 'reproductive rate' – that would be confounded by a tradeoff or by strong effects of senescence in other species. We could not use lifetime reproductive success, which includes all offspring produced in a lifetime, because most of our individuals were not near the end of their lifespans. Because we did not have data on fledging for many Leach's storm-petrels, we used the percentage of all eggs that hatched up to present during the bird's lifetime as an index of reproductive rate (n = 18 birds). Hatching was considered the presence of a chick where once there was an egg, and since fledging rate is very high (∼93%), hatching rate (∼76%) is a reasonable reflection of reproductive success (Huntington et al. 1996). For Savannah sparrows, we used fledglings produced per year (excluding 2005, when reproductive data were incomplete). For neither species did we have the ideal measure of fitness: genetic offspring surviving to reproductive age. Nonetheless, knowledge of our species' natural history gives us confidence that we have reasonable measures of reproductive output.

Because natal philopatry is low, Leach's storm-petrels are generally banded on their first breeding attempt, usually at age 4–5 year (Huntington et al. 1996). Age is thus calculated as years since first breeding attempt, when birds are ringed (n = 24 birds). Because of strong natal and breeding philopatry, Savannah sparrows were considered to be one-year old at their first breeding attempt (Wheelwright and Rising 1993), and for most of the individuals this could be confirmed because they were banded as nestlings or juveniles.

We also took body size measurements (mass and tarsus) for both species. We were unable to use a body-mass index (BMI) as a measure of condition in incubating Leach's storm-petrels because oils stored in the proventriculus contribute significantly to mass, making BMI problematic (Huntington et al. 1996). Mass measurements in Leach's storm-petrels were only available for night-caught birds. We did use BMI in Savannah sparrows, calculated as $100 \times mass/tarsus^2$, but interpret it cautiously in light of theore-

tical criticisms of such indices (Green 2001, but see Schulte-Hostedde et al. 2005). Male Savannah sparrows are larger than females, and we accordingly stratify all size analyses by sex. BMI in females could be confounded if some females were laying; only one appeared to be laying, and results did not differ excluding this individual.

Data analysis

Antioxidant data were processed according to Cohen et al. (2007), including calculation of residual antioxidant capacity (Res) controlling for uric acid, i.e. non-uric acid antioxidant capacity. Because uric acid often accounts for 90% or more of the variation in TEAC, this allows us to see whether overall antioxidant levels are driven simply by uric acid (Cohen et al. 2007). Data were checked for normality using Q–Q plots and the Shapiro–Wilk test in R ver. 2.5.1. All subsequent analyses were conducted in SAS ver. 9.1. For most variables, simple correlations between antioxidant variables and reproductive rate, age, and size were used (proc corr). Further analyses were conducted using general linear models (proc glm) to control for covariation and possible confounding variables, especially age. Antioxidants have been observed to change in birds with time of day (Hõrak et al. 2004); Savannah sparrow data were checked for such variation. Vitamin E increased later in the day (r = 0.50, p = 0.001), but no other effects were observed. Time of capture is thus not included in models presented in the Results; vitamin E results did not change when linear and/or quadratic time-of-capture terms were added to models (data not shown).

Because there are multiple antioxidant variables, we ran a large number of correlations for these analyses, and it is common to correct for multiple testing in such situations. However, Bonferroni corrections are appropriate when one looks for any significant result out of several tests; in this case, we are also reporting a lack of significance for each correlation when we find it, so each correlation should be viewed as a separate test (Perneger 1998, Moran 2003). In particular, Bonferroni corrections are inappropriate because the tests are separate but not fully independent – antioxidants measures are correlated. We are aware of no statistical methodology for dealing with multiple testing in this situation, and we have taken the approach of presenting the raw correlations. In almost all cases of significance, there are multiple highly significant associated measures confirming the pattern. When the data are ambiguous, we treat them as such rather than rely on an arbitrary value for significance. In other words, we present raw p-values, but interpret the data considering multiple testing issues rather than with $\alpha = 0.05$.

Results

Leach's storm-petrels

All antioxidant variables except TEAC and uric acid (UA) were normally distributed or nearly so; these two were log-transformed. However, TEAC was log-normal only if day and night samples were analyzed separately; accordingly,

these data were always separated for analysis. One night-caught bird was an outlier with high TEAC (4.16, $=$ mean $+6.2$ SD) and low Res (-1.19, $=$ mean -8.7 SD) and was excluded from all analyses of these variables. (These standard deviations were calculated excluding the outlier. If it is included, they become 2.9 and 3.2 SDs from the mean respectively.) Only two carotenoids, lutein and zeaxanthin, were detected in Leach's storm-petrels. All measures of antioxidants except possibly lutein differed markedly between day and night, with much higher values at night (Table A1). Day- and night-caught birds did not differ in body size. Correlations among antioxidants are shown in Fig. 1, but are not strong or general enough to justify multivariate characterization of antioxidants (Cohen and McGraw 2009).

In day-caught birds, average annual reproductive rate was negatively associated with TEAC (Fig. 2) and with UA after controlling for breeding age, but was not associated with Res, vitamin E concentration, or concentration of either carotenoid (Table 1). The TEAC association remained significant when the lowest-reproducing individual (0% success) was excluded (p $=0.04$), and when we weighted for breeding age (our effective sample size, p $=$ 0.007). Results were identical to correlations without age adjustment (data not shown). There was a clear negative correlation between age and UA (Fig. A1) and a marginal one between age and Res, but other antioxidant measures were unassociated with age or body size in Leach's storm-petrels (Table 1).

Figure 2. Relationship between TEAC and hatching rate in Leach's storm-petrels.

Savannah sparrows

Five carotenoids – lutein, zeaxanthin, β-cryptoxanthin, β-carotene, and α-cryptoxanthin – were detected in Savannah sparrow serum. TEAC, UA, α-cryptoxanthin, and β-cryptoxanthin levels were log-normally distributed and were transformed accordingly. Compared to females, males were heavier and had higher serum levels of xanthophyll carotenoids (Table A2). No other measure differed between the sexes. No antioxidant measure differed between birds incubating and feeding nestlings (t-tests; p >0.2). Correlations among antioxidants are shown in Fig. 1. Due to the variability of correlations among carotenoids, we did not condense these into a single axis as we have elsewhere (Cohen et al. 2008b). TEAC and UA were not correlated with reproductive rate, and Res had a marginal positive correlation that was not robust in alternative analyses such as controlling for age (Table 2). Reproductive rate was not correlated with vitamin E or carotenoid concentrations except for β-carotene. No antioxidant measure was associated with age, but BMI was positively associated with TEAC, UA, Res, and possibly β-carotene after controlling for sex.

Although most carotenoids showed no clear association with reproductive rate in Savannah sparrows, β-carotene showed some highly suggestive patterns. There were four independent lines of evidence – each weak by itself – pointing to a role of β-carotene in associations between life histories and physiology. First, there was a positive correlation between β-carotene and lifetime fledging rate through 2004 (r $=0.68$, p $=0.03$, n $=10$, Table 2). The association remained when confining the analysis to the eight females in the sample (r $=0.70$, p $=0.05$). Given the small sample size, the marginal p-value, and multiple testing considerations, little can be made of this correlation without corroboration. Second, among 11 antioxidant measures in a limited study on 2005 reproduction, β-carotene showed the strongest association with fledging success (p $=0.007$). Because the 2005 data were not included in our reproductive measure, this is an independent line of evidence. Third, β-carotene was the only carotenoid even marginally associated with mass or BMI. Lastly, there are strong a priori reasons to suspect that of all
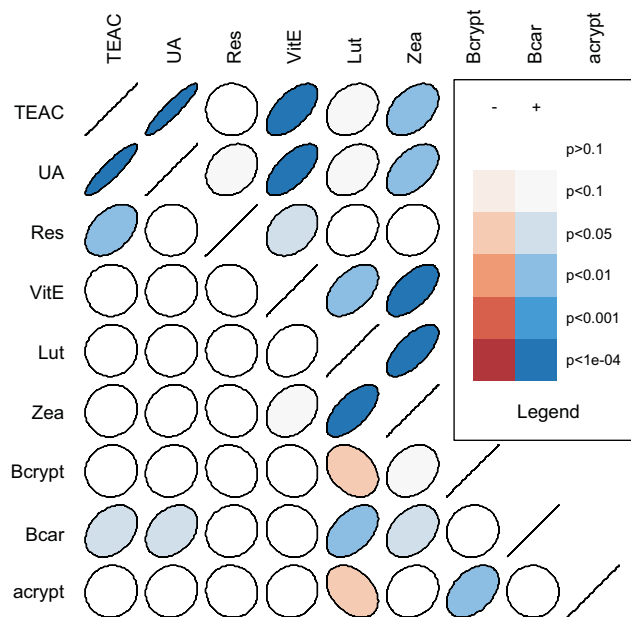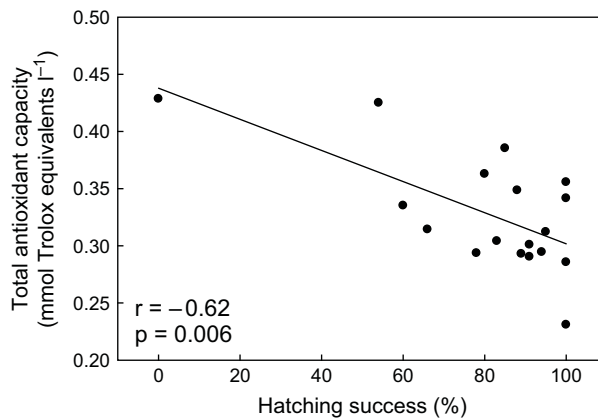

Figure 1. Pearson correlations among antioxidant types within species. Day-caught Leach's storm-petrels are shown above the diagonal; Savannah sparrows are below the diagonal. Because all antioxidants are much higher in night-caught petrels, including them would have caused a spurious appearance of strong correlations. Narrowness of ellipses is proportional to r-values. Right-tilted (blue) ellipses indicate positive correlations; left-tilted (red) ellipses indicate negative correlations. VitE $=$ vitamin E; Lut $=$ lutein; Zea $=$ zeaxanthin; Bcrypt $=$ β-cryptoxanthin; acrypt $=$ α-cryptoxanthin; Bcar $=$ β-carotene.

Table 1. Associations between antioxidant variables and quality measures by time of day in Leach's storm-petrels.

		Reproductive rate[a]	Age	Tarsus		Mass
		Day	Day	Day	Night[b]	Night[b]
TEAC	r	**−0.63**	−0.14	**0.50**	0.21	**−0.51**
	p	**0.007**	0.53	**0.02**	0.50	**0.08**
Uric acid (mg dl^{-1})	r	**−0.48**	**−0.50**	0.38	0.15	**−0.52**
	p	**0.05**	**0.01**	0.08	0.62	**0.07**
TEAC-UA residual	r	0.00	−0.40	0.00	−0.29	0.14
	p	0.99	**0.05**	0.98	0.33	0.66
Vitamin E (abs. units)	r	0.06	0.05	**−0.61**	−0.39	−0.10
	p	0.82	0.81	**0.02**	0.29	0.79
Lutein (µg ml^{-1})	r	0.19	0.05	**−0.54**	−0.11	−0.04
	P	0.48	0.81	**0.05**	0.78	0.91
Zeaxanthin (µg ml^{-1})	r	0.25	0.12	−0.46	0.19	0.04
	p	0.32	0.57	0.10	0.63	0.92

Bold indicates $p < 0.1$. [a] adjusted for age. [b] excluding an outlier with high TEAC, low res, and long tarsus.

carotenoids, β-carotene would be the one associated with individual life-history parameters. It is an important vitamin A precursor and tends to be limiting in the xanthophyll-rich diets of granivores (Goodwin 1980, 1984). β-carotene is present in Savannah sparrows at much lower levels than any of the other carotenoids, consistent with a limiting role in physiology. Taken together, these lines of evidence suggest a link between β-carotene levels and reproductive rate in Savannah sparrows.

Discussion

We detected associations between antioxidant measures and reproductive rate in two sympatric, distantly related species, but the associated measures and the directions of the associations differed between the species. In Leach's storm-petrels, TEAC (and perhaps uric acid as well) correlated negatively with hatching rate. In Savannah sparrows, there appeared to be a positive association between β-carotene and fledging rate. Considered in light of Wiersma et al.'s (2004) and Alonso-Alvarez et al.'s (2004) experimental demonstration of the oxidative costs of reproductive effort in domesticated zebra finches, these results suggest that antioxidant physiology is linked to reproduction in wild birds, but that the nature of the association is species-specific rather than general. Savannah sparrows are small, fast-lived birds compared to Leach's storm-petrels. Their varied diet of seeds and insects allows acquisition of many carotenoids, some of which are used in their yellow facial plumage pigmentation, and this could explain why β-carotene level is apparently associated with reproductive rate in this species even though it was not detected in Leach's storm-petrels.

Although no broad generalizations can be made on the basis of a two-species comparison (Garland and Adolph 1994), the antioxidant levels of these two species are consistent with their position in the short-lived/long-lived continuum presented by Cohen et al. (2008b): larger, longer-lived species generally have lower antioxidant levels.

Table 2. Pearson correlations and partial correlations between antioxidant variables and reproduction, age, and size in Savannah sparrows.

		Age	Reproductive rate	Mass	Tarsus	BMI
TEAC	r	0.001	0.35	**0.40**	−0.18	**0.52**
	p	0.99	0.29	**0.01**	0.27	**0.0006**
Uric acid	r	−0.02	−0.02	**0.37**	−0.09	**0.42**
	p	0.88	0.96	**0.02**	0.57	**0.007**
TEAC-UA residual	r	−0.05	0.55	0.26	−0.19	**0.39**
	p	0.74	0.10	0.11	0.24	**0.01**
Vitamin E	r	−0.12	−0.47	0.17	0.07	0.09
	p	0.49	0.17	0.31	0.68	0.57
Lutein	r	−0.07	−0.10	0.15	0.06	0.07
	p	0.68	0.80	0.39	0.72	0.69
Zeaxanthin	r	−0.12	−0.20	0.10	0.18	−0.07
	p	0.50	0.58	0.53	0.26	0.65
β-cryptoxanthin	r	−0.04	0.24	−0.12	0.15	−0.24
	p	0.82	0.50	0.44	0.36	0.13
β-carotene	r	−0.05	**0.68**	**0.30**	−0.03	**0.28**
	p	0.77	**0.03**	**0.08**	0.86	**0.09**
α-cryptoxanthin	r	−0.16	−0.06	0.02	0.01	0.004
	p	0.35	0.86	0.90	0.95	0.98
Total carotenoids	r	−0.08	−0.02	0.19	0.16	0.02
	p	0.63	0.95	0.27	0.35	0.89

Age and reproductive rate statistics are from Pearson correlations; size measure statistics are from general linear models controlling for sex. Reproductive rate is fledglings per year. Bold indicates $p < 0.1$.

1858

If associations between antioxidants and reproductive rate are generally dependent on life history strategy, long-lived species may have higher reproduction when overall antioxidant levels are low. In contrast, in shorter lived species, many of which have carotenoid-rich diets, reproductive rate may depend more on carotenoids (Blount et al. 2004, McGraw et al. 2005), and overall antioxidant levels might indicate stress even without strong effects on reproductive rate. However, it is equally plausible that differences in antioxidant-reproduction associations here are mediated by other factors such as diet (marine organisms versus insects and seeds), habitat (land vs sea), energetics, or phylogenetic constraints. For example, in a comparative study of many avian species, carotenoid concentrations depended on species differences in diet, phylogeny, and body size (Tella et al. 2004).

It is tempting to interpret our results as showing associations between antioxidant levels and 'individual quality' or fitness, but our reproductive measures do not quantify total reproduction over a lifetime. High reproductive output could reflect quality, but it could also reflect a tradeoff strategy valuing current over future reproduction. Both patterns have been observed in studies of various bird species (Daan et al. 1996, Mauck et al. 2004). Observed reproductive output could also reflect the balance between these forces. Because Leach's storm-petrels and Savannah sparrows both show increasing reproductive output with age, high reproductive output is likely more indicative of quality than of a strategy favoring early reproduction; nonetheless, we were unable to follow offspring until adulthood, and we may have failed to pick up variation in fledging success (storm-petrels) or feeding of fledglings (sparrows) that may have confounded 'quality'-antioxidant associations.

Regardless of whether antioxidant-reproduction associations reflect variation in individual quality, alternative reproductive strategies, or some other physiological link, the persistence of the associations in our dataset is striking. Leach's storm-petrels had been breeding for as long as 28 years and an average of 8 years, so current antioxidant levels presumably reflect something about reproduction that happened many years before. Even after controlling for age, current TEAC levels explain 36% of the variation in hatching rate. In the shorter-lived Savannah sparrows, we did not use breeding data for the year of sampling, so current β-carotene levels were also reflective of past breeding success. Because these relationships could be confounded by so many things – diet, stress, season, and temporal change within individuals, to name a few – it is surprising that we were able to detect any relationship at all. It is thus possible that the associations found are indicative of mechanistic links that would show up even more clearly in associations with current reproduction.

Our data here and current knowledge of antioxidant physiology are insufficient to say whether reproduction affects long-term antioxidant levels, or whether antioxidant levels affect reproductive rates, or whether both are associated with some third factor. Nonetheless, our results support the idea that physiological systems such as oxidative balance may serve as a currency for evolutionary tradeoffs. Even if the antioxidant-reproduction associations within species are attributable to variation in individual quality rather than tradeoffs and alternative strategies, selection would likely still be able to act on the association over time to produce patterns of tradeoffs across species.

The only association we found between age and antioxidant levels was a negative correlation with uric acid in Leach's storm-petrels. However, our study was cross-sectional, so there may be higher mortality of young individuals with high uric acid rather than changes in age-specific levels within individuals (Haussmann and Mauck 2008). This would be consistent with higher attrition of low reproducing birds found in this species, and with uric acid as an apparent marker of short-term stress in other species (Tsahar et al. 2006, Cohen et al. 2008a). We did not have any very old Savannah sparrows in our sample, and some age effects might be more subtle than we were able to detect. Nonetheless, circulating antioxidant levels appear unlikely to serve as reliable markers of age in birds, and there is some support for the idea that wild birds tend to suffer catastrophic aging, with high mortality of individuals that show any physiological signs of senescence (Ricklefs and Scheuerlein 2001).

An interesting note about Savannah sparrows: they are one of only two species in a larger sample of 78 that were found to accumulate α-cryptoxanthin (Cohen and McGraw 2009). The other species is tree swallows *Tachycineta bicolor*, also sampled on Kent Island. Mainland members of these species did not show serum α-cryptoxanthin, nor the β-carotene or β-cryptoxanthin found in the Kent Island birds (McGraw unpubl.). This strongly suggests that presence of these carotenoids in the diet is related to a particular local food source – midges are thought to be an important shared prey item between the sparrows and swallows on Kent Island (Wheelwright unpubl.). If, as seems possible from our data, high β-carotene levels are indicative of good health in Savannah sparrows, deriving health benefits from β-carotene may either be a new microevolutionary phenomenon or there is a predisposition to benefit from it even in birds that never have access to it. β-carotene is necessary for proper health in humans but was not even detected in Leach's storm-petrels, or in 48 of the 78 avian species in a larger sample, though this could reflect either low dietary intake or efficient hepatic and duodenal conversion to vitamin A (Christian et al. 2000, Wyss et al. 2001, Cohen and McGraw 2009).

Taken together, our data allow postulation of a hypothesis for the evolution of circulating micromolecular antioxidant levels in avian physiology and ecology. Because there are substantial differences across species both in levels of various antioxidants and, as suggested by this study, in fitness consequences of high or low antioxidant levels, physiological strategies for antioxidant utilization likely differ as well (Tella et al. 2004, Cohen and McGraw 2009). For example, some species probably need more β-carotene than other species do: having high levels of a particular antioxidant such as β-carotene may be advantageous, but the presence of many species with negligible levels implies that species can evolve alternative mechanisms to fill that role when β-carotene is not present at high levels in the diet (Cohen and McGraw 2009). Physiological utilization of antioxidants should thus evolve in response to dietary availability. Over time, an antioxidant abundant in the diet may become necessary for proper health as

physiology evolves to rely on it, and it may eventually be incorporated into sexually selected signals. Conversely, foraging habits may also evolve in response to physiological need. In addition to the interplay between diet and physiology, there should be selection in response to trade-offs (along the r–K continuum, for example). But because of the diverse ways that the diet can interact with multiple physiological alternatives, the consequences of this selection on physiological systems (and thereby on antioxidant levels) could vary widely across taxa. A proper understanding of these issues will require much further study, in particular a combination of experimental and comparative approaches tailored to elucidate causes and consequences of variation in antioxidant levels or other aspects of physiology, and how these associations vary across species.

The Supplementary material Appendix 1 contains additional discussion of antioxidant differences by time-of-day in petrels and depending on breeding status in sparrows.

References

Alonso-Alvarez, C. et al. 2004. Increased susceptibility to oxidative stress as a proximate cost of reproduction. – Ecol. Lett. 7: 363–368.

Alonso-Alvarez, C. et al. 2007. Increased susceptibility to oxidative damage as a cost of accelerated somatic growth in zebra finches. – Funct. Ecol. 21: 873–879.

Ardia, D. R. 2005. Individual quality mediates tradeoffs between reproductive effort and immune function in tree swallows. – J. Anim. Ecol. 74: 517–524.

Barja, G. 2004. Aging in vertebrates, and the effect of caloric restriction: a mitochondrial free radical-DNA damage mechanism? – Biol. Rev. Camb. Phiolos. Soc. 79: 235–251.

Beckman, K. B. and Ames, B. N. 1998. The free radical theory of aging matures. – Physiol. Rev. 78: 541–581.

Blount, J. D. et al. 2004. Egg-laying capacity is limited by carotenoid pigment availability in wild gulls *Larus fuscus*. – Proc. R. Soc. Lond B. 271: S79–S81.

Bortolotti, G. R. et al. 2003. Carotenoids in eggs and plasma of red-legged partridges: effects of diet and reproductive output. – Physiol. Biochem. Zool. 76: 367–374.

Brand, M. D. 2000. Uncoupling to survive? The role of mitochondrial ineffciency in ageing. – Exp. Gerontol. 35: 811–820.

Christian, P. et al. 2000. Night blindness during pregnancy and subsequent mortality among women in Nepal: effects of vitamin A and beta-carotene supplementation. – Am. J. Epidemiol. 152: 542–547.

Cohen, A. A. and McGraw, K. J. 2009. No simple measures for antioxidant status in birds: complexity in inter- and intraspecific correlations among circulating antioxidant types. – Funct. Ecol. 23: 310–320.

Cohen, A. A. et al. 2007. Measuring circulating antioxidants in wild birds. – Compar. Biochem. Physiol. B 147: 110–121.

Cohen, A. A. et al. 2008a. Stress, metabolis, and antioxidants in two wild passerine bird species. – Physiol. Biochem. Zool. 81: 463–472.

Cohen, A. A. et al. 2008b. Interspecific associations between circulating antioxidant levels and life history variation in birds. – Am. Nat. 172: 178–193.

Costantini, D. and Dell'Omo, G. 2006. Effects of T-cell-mediated immune response on avian oxidative stress. – Compar. Biochem. Physiol. A 145: 137–142.

Daan, S. et al. 1996. Increased daily work precipitates natural death in the kestrel. – J. Anim. Ecol. 65: 539–544.

Freeman-Gallant, C. R. et al. 2003. Social pairing and female mating fidelity predicted by restriction fragment length polymorphism similarity at the major histocompatibility complex in a songbird. – Mol. Ecol. 12: 3077–3083.

Freeman-Gallant, C. R. et al. 2006. Genetic similarity, extra-pair paternity and offspring quality in Savannah sparrows. – Behav. Ecol. 17: 952–958.

Garland, T. J. and Adolph, S. C. 1994. Why not to do two-species comparative studies: limitations on inferring adaptation. – Physiol. Zool. 67: 797–828.

Goodwin, T. W. 1980. The biochemistry of carotenoids. – Chapman and Hall.

Goodwin, T. W. 1984. The biochemistry of carotenoids. – Chapman and Hall.

Green, A. J. 2001. Mass/length residuals: measures of body condition or generators of spurious results? – Ecology 82: 1473–1483.

Haussmann, M. F. and Mauck, R. A. 2008. Telomeres and longevity: testing an evolutionary hypothesis. – Mol. Biol. Evol. 25: 220–228.

Hõrak, P. et al. 2004. Fat soluble antioxidants in brood-rearing great tits *Parus major*: relations to health and appearance. – J. Avian Biol. 35: 63–70.

Hõrak, P. et al. 2006. Antioxidant protection, carotenoids and the costs of immune challenge in greenfinches. – J. Exp. Biol. 209: 4329–4338.

Huntington, C. E. et al. 1996. Leach's storm-petrel (*Oceanodroma leucorhoa*). – In: Poole, A. F. and Gill, F. B. (eds), The birds of North America, No. 233. Acad. Nat. Sci. and Am. Ornithol. Union.

Isaksson, C. and Andersson, S. 2008. Oxidative stress does not influence carotenoid mobilization and plumage pigmentation. – Proc. R. Soc. Lond. B 275: 309–314.

Ketterson, E. D. et al. 1996. Phenotypic engineering: using hormones to explore the mechanistic and functional bases of phenotypic variation in nature. – Ibis 138: 70–86.

Knowles, S. C. L. et al. 2009. Elevated reproductive effort increases blood parasitaemia and decreases immune function in birds: a meta-regression approach. – Funct. Ecol. 23: 405–415.

Lopez-Torres, M. et al. 1993. A comparative study of free radicals in vertebrates-II. Non-enzymatic antioxidants and oxidative stress. – Compar. Biochem. Physiol. B 105B: 757–763.

Mauck, R. A. et al. 2004. Age-specific reproductive success: evidence for the selection hypothesis. – Evolution 58: 880–885.

McGraw, K. et al. 2005. Maternally derived carotenoid pigments affect offspring survival, sex ratio, and sexual attractiveness in a colorful songbird. – Naturwissenschaften 92: 375–380.

McGraw, K. J. 2006. The mechanics of carotenoid coloration in birds. – In: Hill, G. E. and McGraw, K. J. (eds), Bird coloration. I. Mechanisms and measurements. Harvard Univ. Press, pp. 177–242.

McGraw, K. J. and Parker, R. S. 2006. A novel lipoprotein-mediated mechanism controlling sexual attractiveness in a colorful songbird. – Physiol. Behav. 87: 103–108.

Miller, N. J. et al. 1993. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. – Clin. Sci. 84: 407–412.

Moran, M. D. 2003. Arguments for rejecting the sequential Bonferroni in ecological studies. – Oikos 100: 403–405.

Perneger, T. V. 1998. What's wrong with Bonferroni adjustments. – Br. Med. J. 316: 1236–1238.

Ricklefs, R. E. and Scheuerlein, A. 2001. Comparison of aging-related mortality among birds and mammals. – Exp. Gerontol. 36: 845–857.

Ricklefs, R. E. and Wikelski, M. 2002. The physiology/life history nexus. – Trends Ecol. Evol. 17: 462–468.

Schulte-Hostedde, A. I. et al. 2005. Restitution of mass–size residuals: validating body condition indices. – Ecol. 86: 155–163.

Stearns, S. C. 1992. The evolution of life histories. – Oxford Univ. Press.

Tella, J. L. et al. 2004. Ecological, morphological and phylogenetic correlates of interspecific variation in plasma carotenoid concentration in birds. – J. Evol. Biol. 17: 156–164.

Tsahar, E. et al. 2006. The relationship between uric acid and its oxidative product allantoin: a potential indicator for the evaluation of oxidative stress in birds. – J. Compar. Physiol. B 176: 653–661.

Wheelwright, N. T. and Rising, J. D. 1993. Savannah sparrow (*Passerculus sandwichensis*). – In: Poole, A. F. and Gill, F. B. (eds), The birds of North America, No. 45. Acad. Nat. Sci. and Am. Ornithol. Union.

Wheelwright, N. T. and Schultz, C. B. 1994. Age and reproduction in Savannah sparrows and tree swallows. – J. Anim. Ecol. 63: 686–702.

Wheelwright, N. T. and Rising, J. D. 2008. Savannah sparrow (*Passerculus sandwichensis*). – In: Poole, A. F. (ed.), The birds of North America Online. Cornell Lab. Ornithol.

Wheelwright, N. T. et al. 1992. Polygyny and male parental care in Savannah sparrows: effects on female fitness. – Behav. Ecol. Sociobiol. 31: 279–289.

Wheelwright, N. T. et al. 2006. Asymmetrical incest avoidance in the choice of social and genetic mates. – Anim. Behav. 71: 631–639.

Wiersma, P. et al. 2004. Birds sacrifice oxidative protection for reproduction. – Proc. R. Soc. Lond. B 271: S360–S363. Williams, G. C. 1957. Pleiotropy, natural selection, and the evolution of senescence. – Evolution 11: 398–411.

Wyss, A. et al. 2001. Expression pattern and localization of b,b-carotene 15,15′-dioxygenase in different tissues. – Biochem. J. 354: 521–529.