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ORIGINAL PAPER

High levels of liver antioxidants are associated with life-history strategies characteristic of slow growth and high survival rates in birds

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Abstract Antioxidants have a large potential to coevolve with life-histories because of their capacity to counteract the negative effects of free radicals on fitness. However, only a few studies have explored the association between antioxidant levels and life-history strategies comparing a large number of species. Here we used an extensive dataset of 125 species of birds to investigate the association between concentrations of antioxidants (carotenoids and vitamin E) in the liver, which is the main storage organ for fat-soluble antioxidants, and life-history and morphology. We found that high liver antioxidant concentrations were associated with life-history strategies characterized by "live slow, die old", in clear contrast to previous studies reporting a relationship between high plasma antioxidants and life-histories characterized by "live fast, die young". Thus, high carotenoid concentrations were present in species with large body, brain and egg sizes, high absolute metabolic rate and a resident lifestyle, while high vitamin E concentrations were present in species with large brain size and long life span and incubation period. Our results indicate that antioxidants and life-histories coevolve, and that this may be mediated by positive fitness consequences of the accumulation of liver antioxidants, as species with higher antioxidant levels live longer.

Keywords Carotenoids · Life-history evolution · Oxidative damage · Senescence · Vitamin E

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Introduction

Oxidation is a ubiquitous phenomenon on earth, and all organisms are thus exposed to its damaging effects on cells (Finkel and Holbrook 2000). The increase in the oxidation state of biomolecules mediated by free radicals produced by cellular respiration or by exogenous factors can negatively impact functionality, which ultimately leads to deterioration of phenotypes (i.e., senescence) and to the death of individuals (Finkel and Holbrook 2000). A diversity of antioxidant mechanisms has thus evolved because of their capacity to counteract the damaging effects of pro-oxidant molecules on cells (McCord 2000). Therefore, variation in oxidative stress, defined as the imbalance between the production of pro-oxidant substances and the state of the antioxidant and repair machinery, is considered a major determinant of the evolution of life-history strategies (Dowling and Simmons 2009; Metcalfe and Alonso-Alvarez 2010), although empirical evidence for these suggestions is lacking.

The relationship between life-history traits and antioxidant capacity is not well understood, probably due to the complexity of the antioxidant machinery (Cohen et al. 2009a). Current knowledge of the effects of antioxidants on

life-history evolution identifies several life-history traits associated with antioxidant capacity at an intraspecific level (reviewed in Metcalfe and Alonso-Alvarez 2010). This relationship arises because exogenous oxidative stress appears to determine growth conditions and characteristics such as diet and spatial distribution, while endogenous oxidative stress appears to determine reproductive strategies (Metcalfe and Alonso-Alvarez 2010; Garratt et al. 2011). Ultimately, these influences of oxidative stress determine the survival and fecundity of individuals (Metcalfe and Alonso-Alvarez 2010; Garratt et al. 2011). Comparative studies of species with a broad range of ecological characteristics and high phylogenetic diversity are required for obtaining a general view of the influence of antioxidants and oxidative stress on the evolution of life-history strategies.

Nevertheless, only few comparative studies on antioxidants and life-history strategies exist. These studies have shown that life-history strategies characterized by "live fast, die young" (i.e., small body size, high metabolic rate, large clutch size and low survival rates) and certain diet characteristics are associated with high levels of circulating (i.e., plasmatic) antioxidants (Tella et al. 2004; Cohen et al. 2009a, b). Interspecific variability in the levels in egg yolks of antioxidants has also been analyzed in an extensive comparison of species, which found that antioxidant levels in the egg yolk are positively related to clutch size (Biard et al. 2009). In addition, the function and activity of antioxidants varies with tissues or systems where they are present (e.g., del Val et al. 2009a), which makes it difficult to generalize conclusions from studies that analyze antioxidants in different systems (e.g., serum and egg yolk, see above). Furthermore, associations between life-history strategies and variability in the levels of individual antioxidants have not been successfully put into an evolutionary context due to the complexity of such associations (Cohen et al. 2008). This complexity is probably due to the fact that ecologists neglected the relationship between cellular respiration and molecular pathways that form the antioxidant machinery of animals. Importantly, it is the balance between pro-oxidants and antioxidants rather than individual antioxidants separately considered what might explain biological effects (Hõrak and Cohen 2010). At an interspecific level, however, levels of different antioxidants can also explain an important amount of variance in life-history traits (Tella et al. 2004; Cohen et al. 2008; Biard et al. 2009). This covariation between antioxidants and life-history may depend upon the complexity of the antioxidant systems (Galván et al. 2010). Clearly, further comparative investigation is needed to determine which particular life-history strategies evolve in relation to interspecific variability in antioxidant levels.

Antioxidants such as carotenoids and vitamin E are sourced from the diet, subsequently absorbed through the gut, and in some cases metabolized and then stored for later use as fat-soluble antioxidants in the liver prior to transport via the blood to the sites of free radical production (Latscha 1990; Surai 2002). Therefore, the liver represents the main long-term store of fat soluble antioxidants such as vitamin E and carotenoids, while the blood antioxidants reflect those required for immediate use. Thus, studies of antioxidants should preferably consider the amounts that are stored if it is these amounts that reflect the requirements for reproduction and survival.

Interspecific variability in liver antioxidants has only been investigated in relation to ecological attributes by a previous study showing that high liver antioxidant levels favor the invasion of urban areas by birds (Møller et al. 2010). However, the role of the liver in antioxidant storage during periods of need might be of central importance for the evolution of life histories in birds, thus potentially constraining life-history strategies that are related to activities that demand high levels of energy and antioxidant resources such as reproductive investment or foraging activity (Alonso-Alvarez et al. 2004; van de Crommenacker et al. 2011). Indeed, during periods of high energy demand liver antioxidants may play a significant role in combating oxidative stress in birds and other vertebrates (e.g., Morales et al. 2004; Dvorska et al. 2007; Garratt et al. 2011). Furthermore, the liver is the site for conversion of yellow carotenoids into red carotenoids (del Val et al. 2009a), which seem to provide a greater antioxidant activity (Martínez et al. 2008) and to be involved in the expression of visual sexual signals more frequently than other pigments (Hill 1996). The aim of this study was to make an extensive comparison of antioxidant levels in birds belonging to a broad phylogenetic spectrum in relation to their life-history strategies. With this aim we analyzed variation in the levels of fat-soluble antioxidants (carotenoids and vitamin E) in relation to a range of life-history traits for 125 species. The fat-soluble antioxidants investigated here (i.e., carotenoids and vitamin E) are prime agents for controlling oxidative stress (Møller et al. 2000; Surai 2002; Halliwell and Gutteridge 2007). Despite some results suggesting a weak antioxidant role of carotenoids (Costantini and Møller 2008), it is clear that these molecules also have immunostimulatory functions (Møller et al. 2000; Saino et al. 2003), which are important for combating the inhibitory effects of oxidative stress on immune responsiveness (Kurien and Scofield 2008; Costantini and Møller 2009). Thus, liver carotenoids and vitamin E have the potential to represent antioxidant resources that shape the evolution of life-history traits in birds.

We hypothesize that life-history strategies that imply energy-demanding activities should evolve in association with high levels of liver antioxidants as such levels would be indicative of high antioxidant capacities that may counteract negative effects on fitness of activities that generate high oxidative stress levels. Although all activities imply

the production of free radicals as a consequence of normal cellular metabolism, life-history strategies that impose strong nutritional constraints (i.e., demand high amounts of energy) are the most likely candidates to evolve according to availability of liver antioxidants. Thus, we predicted that levels of liver carotenoids and vitamin E should increase with the following traits: (1) Duration of the incubation period, because incubation requires large amounts of energy (Thomson et al. 1998; Ghalambor and Martin 2002). (2) Clutch size, because large clutches require more energy for incubation than small clutches (Moreno and Sanz 1994; Thomson et al. 1998). (3) Egg mass, because producing large eggs is energetically costly (Viñuela 1997). (4) Number of broods in a breeding season, because producing several broods is energetically costly (Martin 1995). (5) Annual fecundity, because total reproductive output should be directly related to the costs of different reproductive phases (Martin 1995). (6) Duration of the breeding season, because the maintenance costs of reproductive activity should increase with time (Martin 1995). (7) Basal metabolic rate (BMR), because this is directly related to energy expenditure (Ricklefs et al. 1996). (8) Brain mass, because, although the brain forms only a small fraction of the total mass of metabolically active tissues, brain development, and maintenance demand large amounts of energy and antioxidants (Hoffman and Heinz 1998; Sewalk et al. 2001). (9) Migration distance, because migration is energetically costly (Wikelski et al. 2003). (10) Degree of habitat generalism, because specialists exclude generalists through more efficient use of resources, thus leading to lower levels of interspecific competition in the former (Futuyma and Moreno 1988), and competition demands energetic resources (Kodric-Brown and Brown 1978). (11) Longevity, because oxidative damage mediated by free radicals that are produced as a consequence of normal cellular respiration accumulates with age (Finkel and Holbrook 2000). Additionally, we predicted that (12) liver carotenoids and vitamin E levels should be related to mode of development and thus be higher in precocial than in altricial species, as the energy costs of development is probably higher in the former where the developmental period takes longer (Vleck and Vleck 1980), although altricial species have faster growth rates (Weathers 1996).

In addition, we hypothesized that levels of liver antioxidants are associated with the expression of integumentary traits produced by two families of pigments: carotenoids and melanins. In the first case, the association should arise because the amount of carotenoids that are deposited in integumentary structures to be part of visual signals depends on availability of carotenoids (e.g., Tella et al. 2004; Biard et al. 2009). Thus, we predicted that liver carotenoid levels should be positively related to the extent of integument colored by carotenoids. In the case of melanins, the association could arise because melanogenesis leads to the synthesis of eumelanin (i.e., the darkest form of melanin) under low levels of a key intracellular antioxidant (i.e., glutathione, GSH), and to the synthesis of pheomelanin (i.e., the lightest form of melanin) under high levels of GSH (Ozeki et al. 1997). GSH levels are highly dependent on environmental influences (e.g., Isaksson et al. 2005; Galván and Alonso-Alvarez 2009), and they are consumed during pheomelanogenesis (Meyskens et al. 1999; Galván et al. 2011). Since depletion of liver GSH caused by oxidative stress is counteracted by other antioxidants such as vitamin E (Barón and Muriel 1999), we predicted that liver antioxidant levels should be higher in species with large proportions of integument colored by pheomelanin and small proportions colored by eumelanin.

Last, we investigated the capacity of dietary characteristics to explain interspecific variability in liver antioxidant levels, as diet is an important factor determining levels of both circulating (Tella et al. 2004; Cohen et al. 2009b) and stored antioxidants in birds (Møller et al. 2010). We predicted that liver carotenoid levels should be especially high in species feeding on ants (Stradi 1995; Møller et al. 2010), while vitamin E levels should be especially low in species feeding on ants and terrestrial invertebrates (Møller et al. 2010). All our hypotheses were tested controlling for the potentially confounding effects of body size and breeding latitude, because these parameters determine several life-history traits through effects on growth rate (Wikelski and Ricklefs 2001) and predation risk (Martin et al. 2000).

Materials and methods

Study samples

JE received specimens for taxidermy, and he collected a homogeneous sample of fresh liver with a maximum mass of 1 g for biochemical analyses after having weighed the liver on a precision balance to the nearest 0.001 g. For all specimens JE also recorded sex, age, date, year, site, and cause of death upon receipt. Almost all specimens died from window strikes, road kills, predation by cats, or hunting. More than 90 % of the samples derive from a small area in Southern Denmark during the period 2000–2006. JE has more than 50 years of experience as a taxidermist, and thus we are certain that all specimens were fresh when samples were taken. The total sample consisted of 666 individuals belonging to 125 species.

Antioxidant analyses

Fat-soluble antioxidants were determined in μg/g. Livers were frozen immediately after collection and then

maintained at –20 °C until analysis. Any livers that were not absolutely fresh were discarded from the present study. Vitamin E concentration was determined using a Shimadzu prominence full HPLC system (Sil -20A Autosampler; LC-20AD solvent delivery system; RF-10 A_{XL} Spectrofluorometric detector, CBM-20Alite system controller; Cto-100AS*vp* column oven) fitted with a Spherisorb, type S30DS2, 3 µm C-18 reverse phase HPLC column (15 cm × 4.6 mm; Phase Sepa-rations, UK). Chromatography was performed using a mobile phase of methanol/water (97:3, v/v) at a flow rate of 1.05 ml/min. Fluorescence detection of vitamin E used excitation at 295 nm and emission at 330 nm. Peaks of δ-, μ- and α-tocopherol were identified by comparison with the retention time of standards of tocopherols (Sigma, Poole, UK). All sampled livers were analyzed for vitamin E concentration. Vitamin E was calculated as the summed concentrations of δ-, μ- and α-tocopherol. Concentrations and not quantity of vitamin E were used as the variable of interest in statistical analysis because concentration is the main factor in determining physiological action of antioxidants at the level of tissues (Surai 2002). The inter-assay CVs for α-tocopherol determination are typically 3.9 % (Surai et al. 1999).

Total carotenoid concentration of liver was determined using the same HPLC system with a diode array detector at 444 nm, fitted with a Waters Spherisorb type NH2 column (25 cm × 4.6 mm; phase separation) with a mobile phase of methanol-distilled water (97:3), at a flow rate of 1.5 ml/min as described by Hõrak et al. (2002). The HPLC was calibrated using lutein standards (Sigma). All analytic detections were performed at 30 °C in column oven and constant temperature in room temperature at 24 °C controlled by air-conditioning.

There was statistically significant consistency in estimates of total carotenoids and total vitamin E among individuals of the same species (Møller et al. 2010). We tested for effects of sex and season (breeding or non-breeding) on concentrations, but neither effect reached statistical significance in models that also included species as a factor.

Log_{10}-transformed concentration of vitamin E in liver was not significantly related to log_{10}-transformed mean liver mass in a model that also included log_{10}-transformed body mass as a predictor [partial effect for liver mass: $F_{1, 122} = 2.15$, $P = 0.14$, coefficient (SE) = −0.73 (0.50)]. Likewise, log_{10}-transformed concentration of total carotenoids in liver was not significantly related to log_{10}-transformed mean liver mass in a model that also included log_{10}-transformed body mass as a predictor [partial effect for liver mass: $F_{1, 122} = 2.16$, $P = 0.14$, coefficient (SE) = −0.54 (0.37)]. Thus, there was no reason to include liver mass in the statistical analyses. However, we also performed the PLSR models to investigate the variability in liver carotenoid and vitamin E concentrations among species (see below) adding liver mass (log_{10}-transformed) as a continuous predictor variable to compare these results with those obtained with PLSR models not including liver mass. As we could not obtain liver mass for two species, and probably because of this the models including liver mass explained a smaller proportion of variance in carotenoid and vitamin E levels (see "Results"), and because the results of the models including liver mass were almost identical to those excluding this predictor variable (see "Results"), we only provide the details of the analyses excluding liver mass.

Melanin-based coloration

Using color plates in Cramp and Simmons (1977–1992) and Cramp and Perrins (1993–1994), we obtained information on melanin-based integumentary coloration of the 125 species of birds included in this study. Several authors have used this method previously, and it has been shown to be a reliable method of quantifying different components of color that is even correlated with the avian perception of color (del Val et al. 2009b; Seddon et al. 2010).

Eumelanic and pheomelanic traits generally consist of distinctive colors. Eumelanic pigments associate with black and gray colors and pheomelanic pigments with yellowish, reddish, chestnut, and brown colors (McGraw and Wakamatsu 2004; Toral et al. 2008). While both pigments occur simultaneously in the tissues (Ozeki et al. 1997), the darker colors conferred by eumelanin (Toral et al. 2008) are indicative of the lower content of this pigment in chestnut and brown colors as compared with black and gray colors (Galván and Alonso-Alvarez 2009). Furthermore, many bird species have feather melanin contents of high purity (>90 % of either eumelanin or pheomelanin, McGraw and Wakamatsu 2004; JJ Negro pers. comm.). Therefore, we considered that black and gray colors in the integument were predominantly generated by eumelanin, while chestnut and brown colors were predominantly generated by pheomelanin. We did not include conspicuous yellow or red colorations that are assumed to be generated by other pigments (i.e., carotenoids), unless chemically identified as melanin-based by Toral et al. (2008). Owls (Order Strigiformes) were not scored because their feathers contain both melanins and porphyrins and because some species have a high degree of color polymorphism in which the abundance of morphs strongly varies geographically (e.g., Antoniazza et al. 2010). Although a rough approximation to the real proportion of eumelanic and pheomelanic integument, the assumption that black-gray colors are eumelanic and brown-chestnut colors are pheomelanic should be adequate for comparative purposes.

Illustrations of both resting and flying adults in breeding plumage were examined. To obtain estimates of the proportion

of eumelanic and pheomelanic color present in the plumage of each species, scores that ranged from 0 (total lack of melanic color) to 5 (all melanic) were assigned. When a species was sexually dichromatic for melanin-based coloration, eumelanic and pheomelanic scores were the average obtained for males and females. When a species had different subspecies differing in extent or type of melanin-based coloration, we used the nominate subspecies. It must be noted that eu- and pheomelanic color patches can coexist in the same feathers, and thus the sum of both color scores in a species with both color types is not always necessarily five because higher values are possible.

Carotenoid-based coloration

The proportion of integument covered by carotenoid-based coloration was determined with the same method used for melanin-based coloration described above. Following Tella et al. (2004), the presence of bright yellow, red, and non-iridescent green colors was considered to be due to carotenoids, unless chemically identified as melanin-based by Toral et al. (2008). We did not consider the yellow head coloration of the gannet *Morus bassanus* because it may be produced by skin secretions whose content is still unknown (Cramp and Simmons 1977–1992).

Life-history traits

We retrieved information on life history traits from Cramp and Simmons (1977–1992) and Cramp and Perrins (1993–1994) for incubation period, clutch size, egg mass, maximum number of broods per year, and duration of breeding season in number of 10-day periods. For longevity we used the maximum longevity records reported by EURING (http://www.euring.org; see Møller 2006). If there were several estimates reported, we used the estimates based on the largest sample size. If only a range was reported, we used the mean value in the analyses.

Migration distance

We estimated migration distance as the difference in latitude between the mean of the northernmost and the southernmost breeding distribution and the mean of the northernmost and the southernmost wintering distribution, relying on information in Cramp and Simmons (1977–1991), Cramp and Perrins (1993–1994) and del Hoyo et al. (1992–2008).

Developmental mode

We used a dichotomous index of developmental mode as precocial (1) or altricial (0), aggregating precocial and semi-precocial species into a single category, using information in Cramp and Simmons (1977–1991) and Cramp and Perrins (1993–1994).

Habitat specialization index

We used the habitat specialization index described by Julliard et al. (2006) as a measure of habitat specialization. In brief, this index of the variance in the distribution of species across breeding habitats reflects the extent to which species vary in habitat use.

Brain mass

Information on brain size was obtained from data reported by Mlikovsky (1989), Iwaniuk and Nelson (2003) and JE (unpublished data). The highly significant repeatabilities among studies indicate that information on brain mass can be combined across sources (Garamszegi et al. 2005).

Basal metabolic rate

We used information on BMR by relying on the two most recent compilations of estimates (McKechnie et al. 2006; McNab 2009).

Diet

We classified the diet of all species with respect to eight food categories, using Cramp and Simmons (1977–1992) and Cramp and Perrins (1993–1994) as sources. These were regular presence (scored as 1) or absence (scored as 0) of seeds, fruit, other plant material, vertebrates, insects, ants, other aquatic invertebrates, and other terrestrial invertebrates.

Body mass

We obtained body mass from Cramp and Simmons (1977–1992) and Cramp and Perrins (1993–1994), or if data were unavailable, from Dunning (1993).

Summary statistics for all variables are provided in Online Resource 1.

Data analyses

We analyzed the relationships between response variables (liver carotenoid and vitamin E concentrations) and life-history and morphological traits of the species (predictor variables) by means of partial least squares regressions (hereafter PLSR; Carrascal et al. 2009), using the species as the sample unit ($n = 125$). This statistical tool is an extension of multiple regression analysis where associations are

established with factors extracted from predictor variables that maximize the explained variance in the dependent variable. The relative contribution of each variable to the derived factors can be estimated with PLSR models, a very useful possibility that allows determination of the magnitude of the different effects (Carrascal et al. 2009). We calculated the relative contribution of each variable to the derived factors by means of the square of the predictor weight. This method is extremely robust to the effects of sample size and degree of correlation between predictor variables, which makes PLSR especially useful when the sample size is small and in cases of severe multicollinearity (Carrascal et al. 2009). This makes PLSR particularly convenient for analyzing associations between antioxidant capacity and several life-history traits, since the latter present high levels of collinearity (e.g., Wikelski and Ricklefs 2001).

To analyze variability in liver carotenoid and vitamin E concentrations ($\log_{10}$-transformed), we introduced all predictor variables as continuous variables ($\log_{10}$-transformed) in the PLSR models, except mode of development and diet characteristics, which were introduced as categorical factors (mode of development: factor scores precocial-1 vs. altricial-0; diet: factor scores presence-1 vs. absence-0 of a given dietary element). Since liver carotenoid and vitamin E concentrations are positively correlated (Møller et al. 2010), when we used one as a response variable, we used the other as a predictor variable. Because there was a large variability among species in the number of individuals sampled (see Online Resource 1), we weighted the PLSR models by number of individuals examined per species, thus giving more importance to estimates of antioxidant concentrations in species for which we had information for many individuals. However, we also show the results of PLRS models excluding species with sample size of one individual only. Since body mass was included as a predictor variable in the PLSR models, the original variables were used for brain size and BMR instead of residuals of their regression against body mass. We selected the most important predictor variables that resulted from the PLSR models (i.e., those with predictor weights that retained >5 % of the information content of the PLSR axes).

Species should not be treated as independent sample units because they are evolutionarily related through common phylogenetic descent (Felsenstein 1985). Thus, the effect of common ancestry among taxa can lead to an overestimation of degrees of freedom if phylogenetic relationships are not taken into account. We used phylogenetic eigenvector regression (PVR; Diniz-Filho et al. 1998) to quantify the amount of phylogenetic signal and to correct for it in the analyses of liver antioxidant concentrations in relation to life-history traits.

We first performed a Principal Coordinates Analysis (PCORD) on the matrix of pairwise phylogenetic distances between the 125 bird species (after a double-center transformation). In a second step we selected the first 10 eigenvectors (EV1–EV10 hereafter) obtained by the broken-stick rule to account parsimoniously for the phylogenetic signal. Eigenvectors extracted from double-centered phylogenetic distance matrices are able to detect the main topological features of the cladogram under different sample sizes or number of taxa used in the analyses (Diniz-Filho et al. 1998). We found that the original matrix of phylogenetic distances between the 125 bird species and the reproduced matrix of distances estimated based on EV1–EV10 were very similar (Mantel test with 999 randomized matrices to estimate significance: $r = 0.768$, $P < 0.0001$; test carried out using PopTools 3.2.3; Hood 2010). EV1–EV10 were used as additional predictor variables in the PLSR models (see above) to control for effects of phylogeny. Thus, the overestimation of degrees of freedom that would occur if phylogenetic relationships were not taken into account is avoided in the PLSR models with the inclusion of EV1–EV10 as covariates.

The phylogenetic hypothesis (see Online Resource 2) was constructed from the species-level supertree constructed by Davis (2008), with additional information from other sources: Grosso et al. (2006), Voelker et al. (2007), Alström et al. (2008), Nguembock et al. (2009), and the phylogeny compiled by Møller (2006). Since we used different phylogenies that employed different methods, we set all branch lengths equal to unity in our compiled phylogeny, thus assuming a speciational model of evolution (e.g., Díaz-Uriarte and Garland 1996).

Results

Carotenoid concentrations in liver

The PLSR model was able to explain a large amount of variance (64.9 %) in liver carotenoid concentration among species of birds. This model generated two components (Components 1 and 2) accounting for 53.9 and 11.0 % of variance, respectively (Fig. 1a). Component 1 was mainly influenced by the positive effect of the presence of ants in the diet, which alone accounted for 18.1 % of variance explained by this component. Since this variable also explained 2.1 % of variance in Component 2, this means that the presence of ants in the diet explained $(53.9 \times 0.181) + (11.0 \times 0.021) = 10.0$ % of total variance in liver carotenoid concentration (Table 1).

The second most important predictor in order of magnitude for Component 1 was migration distance (6.8 % of

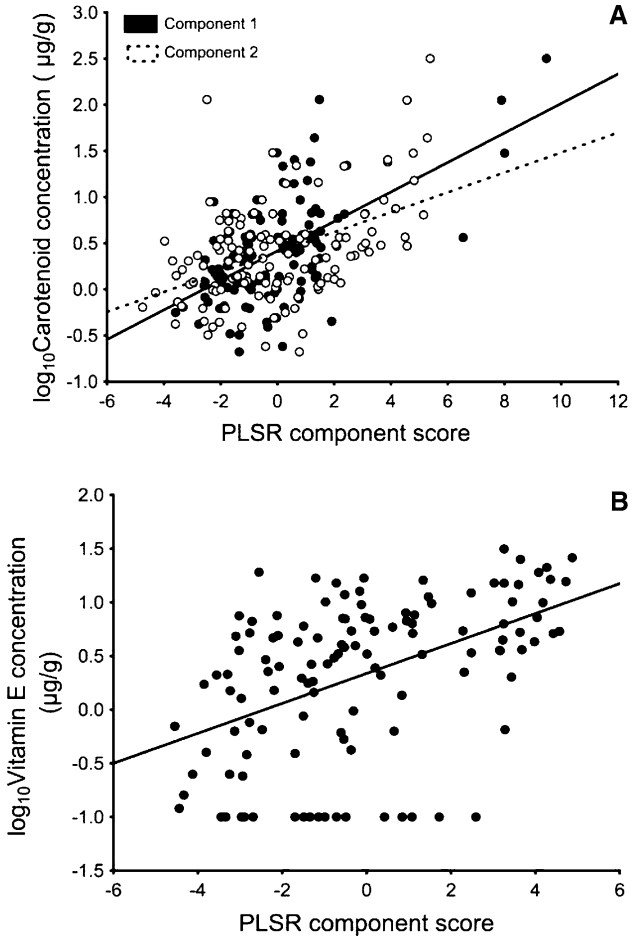

Fig. 1 Relationship between concentration of liver antioxidants (**a** carotenoids; **b** vitamin E) and the scores of PLSR components with information on morphological, physiological, life-history and color traits, and diet characteristics in 125 species of birds. Phylogenetic effects were computed from the first ten eigenvectors (EV1–EV10) obtained from a principal coordinates analysis applied to the matrix of pairwise phylogenetic distances between the 125 bird species. The *lines* are the best fit regression lines. Pearson correlation tests: **a** PLSR Component 1: $r = 0.586$, $n = 125$, $P < 0.0001$, PLSR Component 2: $r = 0.476$, $n = 125$, $P < 0.0001$; **b** $r = 0.476$, $n = 123$, $P < 0.0001$

variance explained by the PLSR component and 3.7 % of total variance in carotenoid concentrations), which was negatively related to carotenoid concentration (Table 1). Vitamin E concentrations also exerted an important influence on Component 1 and it was the most important predictor in Component 2, thus explaining 5.1 % of total variance (Table 1).

In addition to vitamin E concentrations, body mass, brain mass, BMR, egg mass, and presence of seeds in the diet were also important predictors of liver carotenoid concentration in Component 2. All these variables exerted positive effects on carotenoid concentrations, except the presence of seeds in the diet, whose effect was negative (Table 1). The magnitude of the effect of the presence of seeds in the diet was the largest among these effects, accounting for 2.6 % of total variance in carotenoid concentrations. The other effects, however, explained less than 1 % of total variance in carotenoid concentration (brain mass 0.88 %, egg mass 0.75 %, body mass 0.75 %, BMR 0.69 %) (Table 1).

Therefore, these results indicate that the magnitude of the effects of dietary characteristics (presence or absence of ants and seeds in the diet, respectively) is relatively stronger than those of life-history traits when predicting interspecific variability in liver carotenoid concentrations. Migration distance was the most important predictor among life-history traits. Excluding species with sample size of one individual yielded virtually identical results (Table 1).

The same PLSR model including liver mass as a predictor variable yielded almost identical results. The model generated two components explaining 53.6 and 10.8 % of the variance in liver carotenoid concentrations. The most important predictors were the same that resulted from the model without liver mass (Table 1), except the presence of seeds in the diet that explained <5 % (4.6 %) of the variance accounted for by the second component. Liver mass only explained 0.05 % of variance accounted for by the first component, but 5.7 % of the variance of the second component, indicating that liver mass was positively associated with liver carotenoid concentrations.

We also compared our measurements of carotenoid concentrations in liver with the levels in plasma that were determined by Tella et al. (2004) for 31 species that were included in our dataset. A PLSR model with liver carotenoid concentration as a response variable and plasma carotenoid concentration and phylogenetic eigenvectors as predictor variables generated a component that explained 18.9 % of the variance in liver carotenoid concentrations. Plasma carotenoid concentration had a positive effect on the PLSR component as shown by a weight of 0.42, meaning that 17.6 % of variance in the PLSR component was explained by the positive effect of plasma carotenoid concentration. Thus, liver and plasma concentrations were positively correlated in the same species.

Vitamin E concentrations in liver

The PLSR model for liver vitamin E concentration only generated one component that accounted for 19.1 % of the variance in this variable (Table 1; Fig. 1b). This model was mainly influenced by the positive effect of carotenoid concentrations, which accounted for 2.2 % of total variance in vitamin E concentration (Table 1). The other important predictors of vitamin E concentration were the presence of vertebrates in the diet, longevity, brain mass, and duration of the incubation period, all exerting positive effects on vitamin E concentrations (Table 1). These variables

Table 1 Predictor weights of the two partial least squares regression (PLSR) analyses explaining the relationship between liver carotenoid and vitamin E concentrations (response variables) and morphological, physiological, life-history and color traits, and diet characteristics of 125 species of birds (predictor variables)

Predictor variable	Carotenoids (Component 1)	Carotenoids (Component 2)	Carotenoids (Component 1, $n > 1$)	Carotenoids (Component 2, $n > 1$)	Vitamin E	Vitamin E ($n > 1$)
Morphological and physiological traits						
Other antioxidant	**0.25**	**0.38**	**0.25**	**0.32**	**0.34**	**0.34**
Body mass	0.02	**0.25**	0.00	**0.28**	0.19	0.18
Brain mass	0.03	**0.27**	0.01	**0.30**	**0.24**	**0.24**
Basal metabolic rate	−0.02	**0.25**	−0.02	**0.29**	0.13	0.12
Life-history traits						
Length of incubation period	−0.13	0.16	−0.15	0.19	**0.22**	0.20
Clutch size	0.09	−0.06	0.10	−0.07	−0.06	0.00
Egg mass	−0.05	**0.24**	−0.07	**0.26**	0.18	0.17
No. broods in a breeding season	0.03	−0.04	0.03	−0.06	−0.03	−0.03
Annual fecundity	0.07	−0.07	0.08	−0.09	−0.06	−0.02
Duration of breeding season	0.02	0.12	0.01	0.12	0.10	0.09
Migration distance	**−0.26**	0.05	**−0.25**	0.04	−0.20	**−0.22**
Habitat specialization index	0.06	0.01	0.05	0.01	0.01	−0.01
Longevity	−0.03	0.18	−0.03	0.21	**0.24**	0.20
Mode of development	−0.00	0.07	−0.01	0.05	0.02	0.00
Coloration						
Carotenoid-based color score	0.20	0.03	0.20	0.02	0.01	0.02
Eumelanin-based color score	0.13	−0.01	0.12	0.01	0.11	0.12
Pheomelanin-based color score	−0.17	−0.05	−0.18	−0.08	−0.01	−0.04
Diet						
Fruits	−0.00	0.09	−0.00	0.05	−0.07	−0.09
Seeds	0.19	**−0.22**	0.22	−0.19	−0.03	−0.03
Ants	**0.43**	0.14	**0.42**	0.19	−0.03	0.05
Vertebrates	−0.07	0.20	−0.10	0.20	**0.27**	**0.24**
Insects	−0.15	0.05	−0.14	−0.00	−0.17	−0.17
Other terrestrial invertebrates	−0.12	0.10	−0.11	0.04	−0.17	−0.19
Other aquatic invertebrates	0.01	0.05	0.01	0.07	0.14	0.17
Other plant material	−0.03	−0.21	−0.01	−0.19	−0.21	**−0.23**
Phylogenetic effects						
EV1	−0.05	0.18	−0.07	0.20	0.18	0.17
EV2	0.13	−0.09	0.14	−0.11	−0.17	−0.13
EV3	−0.09	0.12	−0.09	0.13	−0.01	0.02
EV4	−0.08	−0.02	−0.07	0.03	0.13	0.13
EV5	0.08	−0.20	0.09	−0.15	−0.03	−0.01
EV6	0.07	−0.10	0.08	−0.07	0.05	0.03
EV7	−0.17	0.08	−0.15	0.04	−0.12	−0.13
EV8	−0.19	−0.07	−0.20	0.02	0.06	0.15
EV9	**−0.25**	0.00	**−0.25**	−0.03	0.10	0.05
EV10	0.17	0.07	0.14	0.03	0.11	0.17
% Variance accounted for	53.94	11.00	56.52	13.05	19.10	21.84

'Other antioxidant' refers to vitamin E concentrations when carotenoid concentrations are the response variable, and to carotenoid concentrations when vitamin E concentrations are the response variable. Additional predictor variables account for variability due to phylogenetic effects, which are computed from the first ten eigenvectors (EV1–EV10) obtained from a principal coordinates analysis applied to the matrix of pairwise phylogenetic distances between the 125 bird species. Predictor weights represent the contribution of each predictor variable to the PLSR axis. Predictor weights that retain >5 % of the information content of the PLSR axis are shown in bold. The results of PLSR analyses excluding species with sample size of one individual are also shown ($n > 1$)

accounted for 1.4, 1.1, 1.1, and 1.0 % of total variance in vitamin E concentration, respectively (Table 1). Excluding species with sample size of one individual yielded virtually identical results (Table 1). The most important predictors remained the same when the model included liver mass as a continuous predictor variable, with a PLSR component explaining 18.4 % of variance in liver vitamin E concentrations and liver mass only accounting for 1.9 % of this variance.

Phylogenetic signal in carotenoid and vitamin E concentrations

Finally, the phylogenetic signal in the response variables, defined as the amount of variance in liver carotenoid and vitamin E concentration exclusively explained by EV1–EV10, was relatively low. Indeed, the only important contribution of eigenvectors to explain variability in liver antioxidant concentrations was exerted by the effect of EV9 on carotenoid concentration (Table 1). Thus, EV1–EV10 accounted for 33.1 and 12.0 % of the variance in carotenoid and vitamin E concentration, respectively, explained by the PLSR models. This represents 21.5 and 2.3 % of total variance in liver carotenoid and vitamin E concentration, respectively.

Discussion

High antioxidant concentrations in the liver were associated with life-history strategies that demand large amounts of energy, as expected. In particular, migration distance was an important predictor of liver carotenoid concentrations. Similarly, egg mass predicted interspecific variation in carotenoid concentrations, and duration of incubation and longevity did so for vitamin E, although with smaller effects. Other morphological and physiological traits predicted variation in antioxidant concentrations including brain mass predicting both carotenoid and vitamin E concentrations, while body mass and BMR only predicted carotenoid concentrations. In sum, our results show that high concentrations of carotenoids in liver have evolved in species with large body, brain and egg sizes, high absolute metabolic rate, and a resident lifestyle, while high concentrations of vitamin E have evolved in species with large brain size, long life span, and long incubation periods. These characteristics are the opposite of those found to be associated with high concentrations of antioxidants in plasma in previous comparative studies, i.e., life-history strategies characterized by high metabolism and short life spans (Tella et al. 2004; Cohen et al. 2008). Additionally, this is the first study showing that vitamin E concentrations are associated with life-history variables, as circulating concentrations of this antioxidant were not found to be related to life-history strategies in a previous study (Cohen et al. 2008). These findings appeared after controlling for the confounding effects of diet characteristics, which have previously been found to be associated with liver antioxidant concentrations (Møller et al. 2010), breeding latitude, and phylogeny.

The discrepancies between analyses based on antioxidants in liver and plasma might be due to differences in activity. Birds use both plasma and liver carotenoids for antioxidant and immune protection (Koutsos et al. 2003; Alonso-Alvarez et al. 2004) and to color their integument (Alonso-Alvarez et al. 2004; McGraw et al. 2006). However, the liver is a storage organ for carotenoids (Goodwin 1950; Koutsos et al. 2003) from which they are transported to their sites of active use by the bloodstream (Castenmiller and West 1998). Similarly, vitamin E is the major lipid-soluble antioxidant protecting lipids against oxidative damage in plasma and red blood cells (Surai 2002), but it is also stored in the liver (Jensen et al. 1998). The association between high concentrations of antioxidants in plasma and life-history strategies that are characterized as "live fast, die young" (i.e., high growth rate, small body size, low survival rate, large clutch size and high metabolic rate; Tella et al. 2004; Cohen et al. 2008) is thus explained because the high rates of free radical production characteristic of these life-history strategies is mirrored in the levels of circulating antioxidants that are used to combat such free radicals (Barja 2004; Cohen et al. 2008). By contrast, the life-history traits found here to be associated with high concentrations of antioxidants in the liver are characteristic of "live slow, die old" strategies, thus suggesting that high liver antioxidant levels have evolved because of their adaptive function as stored antioxidants that can be used in the long term.

The different activities of plasma and liver antioxidants may also explain why we did not find an association between liver carotenoid concentrations and proportion of integument colored by carotenoids, in contrast to previous comparative studies of plasma carotenoids (Tella et al. 2004). We can discard the possibility that liver carotenoids are only used for antioxidant protection and not for coloring the integument because it has been shown that they have a pigmenting role (del Val et al. 2009a). However, our study suggests that the pigmenting role of liver carotenoids might be of minor importance as compared with circulating carotenoids, at least for explaining variability among species. Nor did we find a relationship between liver antioxidant concentrations and proportion of integument colored by pheomelanin. Again, this suggests that liver antioxidants are mainly used for storage and their mobilization occurs more slowly than the rate of melanin production.

It is worth noting that the apparently opposite relationships between characteristics of life-histories and circulating and liver antioxidants, respectively, arise even when plasma and liver concentrations of carotenoids are positively correlated at an intraspecific level (McGraw et al. 2006), and our interspecific measurements of liver concentration were positively related to circulating levels reported by Tella et al. (2004). We hypothesize that this difference is due to liver antioxidants mainly representing storage for future needs, while plasma levels are currently used or about to be used in the short term (Barja 2004).

Although liver antioxidant concentrations were positively related to BMR, it must be noted that this parameter does not covary linearly with body size, but follows an exponential function (Lasiewski and Dawson 1967). We analyzed absolute BMRs in our analyses, because PLSR models deal with collinearity between predictor variables and create syndromes among them that explain variation in the response variables (Carrascal et al. 2009). Thus, our PLSR model created a "physiological" syndrome (Component 2 in the model for carotenoid concentrations) that consisted of large body, brain and egg sizes, and high BMRs. Therefore, the positive influence of BMR on this syndrome should not be interpreted as a positive effect of size-corrected metabolic rate on carotenoid concentrations as in general linear models, but as a joint effect of body size and absolute BMR on carotenoid concentrations. Indeed, when we performed the same PLSR model for carotenoid concentrations including residuals of the log–log regression of BMR against body mass instead of absolute BMR as a predictor variable, size-corrected metabolic rate showed a tendency to covary negatively with carotenoid concentrations, although the magnitude of the effect was small, accounting for 2.3 % of variance in the PLSR component (results not shown). Thus, to conclude, liver carotenoid concentrations were associated with large body mass and, as a consequence, high absolute metabolic rate, while there was no clear association with size-corrected BMR.

Our PLSR models allowed us to determine the differential effects of life-history variables on the two antioxidants considered here, as well as the relative contribution of their effects. Thus, brain size was the only important predictor of both carotenoid and vitamin E concentrations. This is not surprising given that brain development is a process that consumes large amounts of energy and antioxidant resources, restricting the production of complex neural structures to species with slow growth rate and large investment in maternal effects (Pagel and Harvey 1988; Iwaniuk and Nelson 2003). Thus, storage of high concentrations of antioxidants in the liver by species with large brains was as predicted.

Although migration distance only predicted carotenoid concentrations, the magnitude of its effect was larger than any other life-history trait predicting carotenoid or vitamin E concentrations. The effect of migration distance on liver carotenoid concentration was opposite to the predicted effect, as the energetic cost of migration (Wikelski et al. 2003) should generate an oxidative cost, especially after prolonged flights characteristic of long migrations (Costantini and Møller 2008). A possible explanation for these contradictory results is that migratory birds not only spend energetic resources during migration, but also enjoy productive and stable environmental conditions year round, which may cause lower levels of oxidative damage than in sedentary species (Møller 2006). Another alternative explanation is that long-distance migrants have developed physiological mechanisms to repair oxidative damage after periods of physical activity (Møller 2006). Last, it should be considered that, although migration is a metabolically costly activity, resident birds face other energetic costs such as thermoregulation (Wiersma and Piersma 1994), which could make resident strategies even more costly than migratory strategies. Future studies should address these possibilities.

Other predictors of liver antioxidant concentrations were egg mass and duration of the incubation period, the former being related to carotenoid concentrations and the latter to vitamin E concentrations. The effect of both variables on concentrations of antioxidants was positive, consistent with the fact that the production of large eggs is energetically costly (Viñuela 1997), large eggs contain large amounts of antioxidants (Biard et al. 2009), and that incubation is a costly activity that demands large amounts of energy (Thomson et al. 1998; Ghalambor and Martin 2002). Large eggs and long incubation periods, as well as the other life-history traits found here to predict antioxidant concentrations, correspond to life-history strategies characterized by slow growth and high survival (Wikelski and Ricklefs 2001; Ricklefs and Wikelski 2002). High metabolic rates related to our measures of antioxidants are also associated with high survival rates in birds (Møller 2008). Indeed, high vitamin E concentrations were here associated with long life spans. Following the free radical theory of aging (Finkel and Holbrook 2000), the proximate cause of senescence is the accumulation of oxidative damage produced by free radicals generated as a consequence of normal cellular metabolism. Therefore, our results indicate that the accumulation of liver antioxidants has important fitness consequences, as species with higher antioxidant levels live longer. This has previously been reported for humans and other mammals with high plasma antioxidants (Cutler 1991; Mecocci et al. 2000), although our results constitute the first time that high antioxidant levels have been related to long life spans in wild animals. Particularly, it was vitamin E that was the antioxidant that accounted for this association as in previous studies of humans and other mammals (Cutler 1991; Mecocci et al. 2000).

In conclusion, our study shows that high liver antioxidant concentrations in birds are associated with life-history strategies characterized by "live slow, die old", in clear contrast to previous studies reporting a relationship between high plasma antioxidants and life-histories characterized by "live fast, die young". The coevolution between antioxidants and life-histories may be mediated by important fitness consequences of the accumulation of carotenoids and vitamin E in the liver that is the main storage organ of fat soluble antioxidants. At this stage, however, we cannot determine whether antioxidants shape the evolution of life-histories or vice versa, something remaining to be explored.

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