

Maternal Effects Mediated by Antioxidants and the Evolution of Carotenoid-Based Signals in Birds

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ABSTRACT: Bright yellow to red signals used in mate choice or intrasexual competition are based on carotenoid pigments that are hypothesized to be traded between physiological functions and coloration. These signals have recently been shown to be influenced by maternal effects. Indeed, yolk-derived carotenoids are essential for embryos to develop efficient carotenoid metabolism in posthatching life. Maternal effects facilitate adaptation to environmental variability and influence the evolution of phenotypic traits such as secondary sexual signals. Here we propose that maternal investment in yolk carotenoids promotes the evolution of carotenoid-based ornaments. We conducted a comparative analysis of lipid-soluble antioxidants (carotenoids and vitamins A and E) in the eggs of 112 species of bird. Species with large clutch sizes deposited higher yolk concentrations of the three antioxidants. There was a significant positive relationship between yolk carotenoids and the expression of male carotenoid-based signals, but not between yolk carotenoids and sexual dichromatism in these signals. These relationships were specific to carotenoids, as they were not found for vitamins A and E. This provides evidence consistent with the hypothesis that maternal effects mediated by yolk carotenoids play a role in the evolution of carotenoid-based signals as a response to sexual selection, likely based on organizational effects of carotenoids during embryo development.

Keywords: carotenoids, egg composition, sexual dichromatism, sexual selection, vitamin A, vitamin E.

Introduction

Maternal (or parental) effects occur when the phenotype of a mother or the environment she experiences influences the phenotype and fitness of her offspring (Mousseau and Fox 1998b). Conditions experienced during early stages of development may have long-lasting effects on an individual's phenotype and fitness (Lindström 1999; Metcalfe and Monaghan 2001), and maternal effects can modulate these early developmental conditions (Mousseau and Fox 1998a; Price 1998). In particular, maternal effects may affect the development of sexual signals in the offspring (Strasser and Schwabl 2004; McGraw et al. 2005; Eising et al. 2006; Rubolini et al. 2006). Given the potential for maternal effects to influence the evolution of phenotypic traits and facilitate adaptation to environmental variability (Wade 1998; Wolf et al. 1998; Räsänen and Kruuk 2007), maternal effects might promote or constrain the evolution of secondary sexual traits. In addition, maternal effects on the development of condition-dependent secondary sexual characters provide another source of additive genetic variance that can reinforce genetic variation in such signals (Miller and Moore 2007). If genetically based, maternal effects that cause offspring to develop new or more intensely colored signals would evolve more readily than zygotic genes and might thereby promote comparatively rapid evolution of these signals (Brodie and Agrawal 2001). A recent comparative analysis provided the first evidence in line with these hypotheses, showing that the evolution of sexual dichromatism in birds was linked to maternal deposition of immune factors in eggs (Saino et al. 2007).

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Bright yellow-red signals based on carotenoid pigments have repeatedly been shown to be used in mate choice or intrasexual competition (reviewed in Hill 1998; Møller et al. 2000; Hill and McGraw 2006*b*). Carotenoid pigments may be a limiting resource to vertebrates because of environmental variability in their availability, differences in foraging ability, or differences in absorption or metabolizing efficiency (reviewed in Olson and Owens 1998; Møller et al. 2000). The involvement of these pigments in functions related to self-maintenance through the immune and detoxification systems (reviewed in Bendich 1993; Edge et al. 1997; Møller et al. 2000; Surai 2002) has led to the hypothesis of a trade-off between physiological functions and signaling. Such a trade-off could ensure the reliability of sexual displays that depend on carotenoids as pigments for signal production (Lozano 1994; Shykoff and Widmer 1996; von Schantz et al. 1999). However, the mechanism for maintaining reliability in carotenoid-based traits based on the antioxidant properties of these pigments has recently been questioned. A meta-analysis of the association between carotenoid concentration and antioxidant function revealed no clear evidence of a major antioxidant effect (Costantini and Møller 2008). The advertising role of carotenoids has instead been proposed to reveal availability of more important nonpigmented antioxidants (such as antioxidant enzymes or vitamin E and C) that would protect carotenoids from oxidation and consecutive bleaching and thereby allow development of bright signals (Hartley and Kennedy 2004).

Apart from their role in development of sexual signals, carotenoids also play a central role in reproduction, especially during embryo development and at hatching (reviewed in Surai et al. 2001*b*). This period of intense growth is indeed associated with elevated oxidative stress, to which avian embryos are very susceptible (Surai et al. 1999; Surai 2002). Experiments in wild birds in natural and captive conditions have shown that yolk carotenoids efficiently protect embryos against oxidative stress (Blount et al. 2002; McGraw et al. 2005). Female birds allocate large amounts of lipid-soluble antioxidants, carotenoids, and vitamins A and E to their eggs, relative to their circulating levels and body stores (Blount et al. 2000). Thus, carotenoids have been hypothesized to mediate maternal effects through egg composition as well as costs of reproduction in females (Blount et al. 2000). Yolk carotenoid concentration decreases with laying order in several species (Royle et al. 2001, 2003; Saino et al. 2002), and it has been hypothesized that maternal deposition of carotenoids in eggs may be an adaptive strategy related to hatching asynchrony and/or brood reduction (Royle et al. 2001, 2003). Maternal deposition of carotenoids to egg yolk may be modulated as a function of the embryo sex (Verboven et al. 2005; Badyaev et al. 2006; but see Saino et al. 2003*b*; Groothuis

et al. 2006; Romano et al. 2008). Carotenoid concentration in egg yolk may vary as a function of female condition and male attractiveness (Saino et al. 2002; Williamson et al. 2006; Szigeti et al. 2007). Finally, yolk-derived carotenoids modulate offspring phenotypic traits, such as color (Hörak et al. 2000; Biard et al. 2005, 2007; McGraw et al. 2005; Isaksson et al. 2006) and immune response (Saino et al. 2003*a*; Berthouly et al. 2007; Biard et al. 2007; Koutsos et al. 2007; Romano et al. 2008). Therefore, there is growing evidence that maternal allocation of antioxidants, in particular carotenoids, to eggs is a conditional maternal strategy of adaptive value.

A particularly important consequence of maternal investment of carotenoids in eggs for the development of carotenoid-based signals is that the availability of yolk-derived carotenoids is essential for embryos to develop an ability to acquire and use carotenoids in posthatching life (Koutsos et al. 2003). Indeed, embryonic exposure to maternally derived carotenoids determines the subsequent efficiency of carotenoid metabolism and the capacity of offspring to assimilate dietary carotenoids and to use and accumulate these at the cellular level in different tissues, independently of actual availability of dietary carotenoids: in immune organs (Koutsos et al. 2003), epithelium (Koutsos et al. 2003; McGraw et al. 2005), and feathers (Hörak et al. 2000; Biard et al. 2005, 2007; Isaksson et al. 2006). Yolk carotenoid concentration may thus have long-term effects on expression of carotenoid-based signals in adulthood (McGraw et al. 2005). Embryonic exposure to yolk carotenoids also modulates the development of immune function (Biard et al. 2005, 2007; Koutsos et al. 2007) as well as the potential costs of immune response, because chicks hatched from carotenoid-poor eggs showed an impaired ability to regulate systemic inflammation (Koutsos et al. 2006). Similar long-term organizational effects of early developmental conditions on physiological functions are well documented for early nutrition, even when a shortage of dietary availability of nutrients occurs during a short period in crucial stages of the development (e.g., Birkhead et al. 1999; Ohlsson et al. 2002; Blount et al. 2003). As a consequence, embryos that develop under poor conditions (i.e., low yolk carotenoid content) may be less efficient in adulthood at acquiring and using carotenoids for physiological or signaling functions, or they may be more in need of carotenoids as a result of early deficiency. Conversely, maternally derived carotenoids increase the efficiency of carotenoid metabolism in posthatching life, and thus increased maternal investment of carotenoids in eggs may confer an advantage to offspring in development of carotenoid-based signals. Therefore, if such signals are under directional selection, a positive genetic covariance between the particular maternal effect of yolk carotenoid content and offspring performance due to the expression

of the signal may enhance the rate of evolution of carotenoid-based signals (Moore et al. 1998; Wolf et al. 1998).

In light of the long-term enhancing effects of maternally derived yolk carotenoids on the ability to develop carotenoid-based signals, we hypothesize that, through this mechanism, such maternal effects may promote the evolution of carotenoid-based ornaments as a response to sexual selection. Under this hypothesis, maternal effects should mediate a positive relationship between yolk carotenoid concentration and expression of carotenoid-based signals at the interspecific level. However, under the hypothesis that the effects of carotenoids on expression of carotenoid-based signals are mediated through or reveal the effects of antioxidants other than carotenoids per se (Hartley and Kennedy 2004), we would expect a positive relationship between carotenoid-based coloration and these other, more important antioxidants in eggs. We tested these predictions in a comparative analysis of the yolk biochemical composition of 112 bird species from 39 families and 13 orders, for which we collected eggs and determined antioxidant composition in terms of carotenoids and vitamins A and E.

Material and Methods

Egg Collection and Biochemical Analyses

With the help of colleagues we collected fresh eggs from nonincubated clutches during the breeding seasons 2000–2003 in Europe, North America, and South Africa ($n = 93$ species). For ethical reasons we restricted the number of clutches to the minimum that would allow quantification of the amount of variance within and among species. In addition, we gathered data already available from the literature ($n = 22$ species), for which biochemical analyses were conducted in the same laboratory and with the same protocol and equipment. A full list of species and number of eggs and clutches sampled is reported in table A1 in the online edition of the *American Naturalist*. Antioxidants were extracted from 0.100–0.200 g of yolk, and carotenoid, vitamin A, and vitamin E concentrations were determined according to previously published procedures (Surai 2000; Hörak et al. 2002). Mean (\pm SE) antioxidant concentration ($\mu\text{g/g}$) for each species and details on the biochemical analyses are given in the appendix in the online edition of the *American Naturalist*.

Carotenoid-Based Color Scores

To score carotenoid-based colors of plumage and bare parts, we adapted the method of Yezerinac and Weatherhead (1995), using color plates and species descriptions from handbooks (Brown et al. 1982–2004; Cramp et al.

1982–1994; del Hoyo et al. 1992–2004; Poole and Gill 1992–2004). Two of the regions defined by Yezerinac and Weatherhead (1995) were subdivided to increase scoring precision, and the body was divided into the following 10 regions (corresponding percentage of body surface): bill (3%), eye and eye ring (1%), chin, cheeks, and above eye (8%), breast (23%), belly (10%), crown and nape (11%), back (15%), wing (20%), tail (6%), and legs and feet (3%). The proportion of the surface covered with carotenoid-based color was recorded for each body region, as were color hue and brightness.

Color hue was scored as 1 for green, excluding glossy and iridescent green structural colors, 2 for yellow, 3 for orange, and 4 for red colors. Color hue scores ranged from 1 to 4 for plumage and from 2 to 4 for bare parts. Score values increased from yellow to red hues to reflect an increase in the concentration of pigments deposited (Inouye et al. 2001; Saks et al. 2003; Andersson et al. 2007) and/or an increase in the proportion of red pigments metabolically derived from yellow dietary pigments (Hill 1996; Inouye et al. 2001; McGraw and Schuetz 2004; Andersson et al. 2007), both potentially leading to an increase in production costs of the signal. The last score estimated color brightness and saturation and was coded as 1 for dull/pale, 2 for medium, and 3 for bright/intense colors.

In the species included in this study, yellow, orange, and red feathers have always been found to be, at least partly, based on carotenoids (see appendix). Noniridescent green or olive-green feather colors correspond to carotenoprotein complexes (Ong and Tee 1992) and/or a combination of carotenoids and structural colors (Prum et al. 1999) or a combination of carotenoids and brown melanins (Hill and McGraw 2006a). In accordance with biochemical analyses (McGraw and Wakamatsu 2004; McGraw et al. 2004a, 2004c), red-brown, or “rufous,” feather colors were not scored as carotenoid based (i.e., *Aptenodytes* spp., *Gallus* spp., *Hirundo* spp., *Sialia sialis*, *Taeniopygia guttata*). In the case of bare parts, “fleshy” pinks and brown of legs and feet, as well as “horn” bill colors were not scored as carotenoid based, as these colors may mainly be determined by blood (Negro et al. 2006) and keratin, respectively. Yellow and orange skin colors may be produced by carotenoids alone, by a combination of carotenoids and structural color (Prum and Torres 2003), or by a combination of carotenoids and blood hemoglobin (Negro et al. 2006). Determination of eye color, however, is mechanistically more complex and is reported to be likely due to the presence of purin and pteridin pigments and hemoglobin, whereas the importance of carotenoids is unclear (Hill and McGraw 2006a). A review of information on the biochemical and structural nature of colors used for scoring the species included in this study is provided in table A2 in the online edition of the *American Naturalist*.

The carotenoid-based color score of each body region was calculated as the percentage of the surface colored by carotenoids, multiplied by hue and brightness-saturation scores. The final score was calculated as the sum of the scores of all body regions weighted by the corresponding proportion of the body surface covered (Yezerinac and Weatherhead 1995). Results presented here are based on color scores including feathers, skin, and bill but excluding eye color because of the uncertain role of carotenoids in its determination. Color scores are reported in table A1. Analyses based on color scores calculated on plumage color alone gave qualitatively similar results. Sexual dichromatism in carotenoid-based signals was calculated as the difference between male and female scores.

Carotenoid-based color scoring was done by C.B. blindly with respect to yolk antioxidant data. In order to test the reliability of the scoring method, all species were additionally scored by a naive observer. Repeatability of scores among observers, calculated as the intraclass correlation coefficient (Lessells and Boag 1987) was highly significant (male score: $r = 0.89$; female score: $r = 0.87$; sex difference in score: $r = 0.88$; all $P < .0001$). In order to check whether the results were dependent on the method used to score color and/or on using color plates in handbooks, the analyses were run with male carotenoid-based coloration and sexual dichromatism in carotenoid-based coloration coded as binary variables (Birkhead and Møller 1994). The results were qualitatively similar (data available from the authors upon request).

Male and female carotenoid-based color scores were strongly positively correlated ($r = 0.83$, $P < .0001$), preventing them from being used together as predictors in multiple-regression analyses because of problems of collinearity. Male color scores and sexual dichromatism were also positively correlated ($r = 0.61$, $P < .0001$). Therefore, variation in yolk antioxidant concentration was investigated in relation to the extent of carotenoid-based color by use of male color score or sexual dichromatism in carotenoid-based color scores.

Comparative Data

Data on female body mass and clutch size were collected either from handbooks (Brown et al. 1982–2004; Cramp et al. 1982–1994; del Hoyo et al. 1992–2004; Dunning 1992; Poole and Gill 1992–2004; Hockey et al. 2005) or from our own field data, and where several estimates were available, mean values were used (data are reported in table A1). Female body mass was used to control for allometric effects because information on egg or yolk mass was not available for all species and because female body mass and egg mass were strongly positively correlated ($r = 0.98$, $P < .0001$, $n = 102$). Animals cannot synthesize caroten-

oids, which are therefore obtained from the diet (Goodwin 1984). High prevalence of carotenoid-based color across families of birds has been shown to be associated with carotenoid-rich diets, although diet was not the best ecological predictor of interspecific variation in carotenoid-based color (Olson and Owens 2005). We did not find any indication of correlated evolution between yolk antioxidants and diet. Furthermore, including diet in the analyses did not change the conclusions of the study (these results are reported in the appendix). Any relationship between clutch size and yolk antioxidant concentration might reflect the underlying relationship between clutch size and latitude. Mean latitude was taken as the midpoint of the northernmost and southernmost limits of each species' geographical breeding range (Garamszegi et al. 2005*b*; table A1). Including latitude in all models yielded results qualitatively similar to those presented here (data available from the authors upon request).

Statistical Analyses

Patterns of antioxidant investment in egg yolk in relation to female body mass, clutch size, and carotenoid-based signals were first investigated via generalized linear models with log-transformed mean concentrations for species and the GLM procedure in SAS v8.2 (SAS Institute, Cary, NC). Tests of residuals for normality and homoscedasticity were used to check the validity of the model.

However, phenotypic mean values for species cannot be considered statistically independent observations because cases of convergent evolution are mixed with cases of similarity due to common ancestry (Felsenstein 1985; Harvey and Pagel 1991). Therefore, we used a comparative method based on a composite phylogeny compiled from recent molecular phylogenies. Sources of phylogenetic information are detailed in the appendix, and the topology of the phylogenetic tree is shown in figure A1 in the online edition of the *American Naturalist*. Information on branch lengths was unavailable for this composite phylogeny, and thus all distances between nodes and nodes and tips were set to 1. Using alternative phylogenies or branch lengths based on molecular data gave qualitatively similar results (appendix).

We tested for correlated evolution of continuous characters, using generalized least squares models implemented in the software Continuous (Pagel 1997, 1999). These models control for similarity due to common ancestry and estimate evolutionary changes along the phylogeny through variance components of traits (Pagel 1997). Hypothesis testing relies on likelihood ratio statistics, by comparing the fits of two nested models. Phylogenetic trees should be bifurcating for Continuous to fit these models optimally. Polytomies were therefore resolved to bifurcations by excluding three species from the phylogeny (*Va-*

nellus vanellus, *Turdus philomelos*, and *Cercotrichas coryphaeus*). Species to be excluded were chosen at random among those involved in a polytomy, if none had carotenoid-based coloration. If some species in a polytomy had carotenoid-based colors, species were chosen at random so that the resulting bifurcating node included one species with and one species without carotenoid-based color. We first constructed our models controlling for potentially confounding variables and assessed the contribution of scaling parameters. In the absence of information on branch length in the phylogeny, the branch length scaling parameter κ and the overall path length scaling factor δ were set to their default value of 1. Another reason for setting the path length scaling factor δ to 1 is that its estimation has been shown to be biased (Freckleton et al. 2002). The importance of phylogenetic relationships is estimated through the phylogeny scaling parameter λ (Pagel 1999). At its default value of 1, λ yields a constant-variance (Brownian motion) model of evolution, while a value of $\lambda = 0$ would suggest that the trait evolved as if species were independent. Values of $\lambda < 1$ indicate that the tree topology overestimates the covariance among species. The contribution of λ was assessed by comparing the log-likelihood ratio of a model in which λ was constrained to its default value with that of an alternative model in which λ was estimated and took its maximum likelihood value. The estimation of λ was used in the final model if doing so improved the log likelihood; otherwise, the default value of 1 was kept. We then tested for correlated evolution between traits by comparing the log likelihood of the model of independent evolution, in which covariances between traits are forced to 0, with that of a model allowing correlated evolution, in which covariances are estimated. In all analyses, the better model was that allowing for correlated evolution, which was thus subsequently used to estimate correlation and covariances between traits. From the correlation coefficients we calculated phylogenetic partial correlation coefficients, and we used their associated Student's t_s values to derive corresponding P values. According to our directional predictions, we used unilateral tests at $\alpha = 0.05$.

Sample sizes differ among tests because information was not available for all species on all three antioxidants, the number of clutches sampled, and clutch size (table A1).

Results

There was significantly more variation in carotenoid concentration among than within species ($F_{92,631} = 20.60$, $MS_{\text{within}} = 283.75$, $MS_{\text{among}} = 5,845.57$, $P < .0001$), and this difference accounted for 75% of the total variance in yolk carotenoids (this test does not include species for which data were collected from the literature and for which

only mean values were available; see table A1). Similarly, there was more variance in vitamin A and vitamin E concentration in egg yolk among than within species (vitamin A: $F_{92,547} = 5.08$, $MS_{\text{within}} = 1.79$, $MS_{\text{among}} = 9.08$, $P < .0001$, $R^2 = 0.46$; vitamin E: $F_{92,547} = 7.77$, $MS_{\text{within}} = 3,772.14$, $MS_{\text{among}} = 29,322.58$, $P < .0001$, $R^2 = 0.57$). In addition, for carotenoids and vitamins, respectively, there was 13 and three times as much variation in yolk antioxidant concentration among species as among females (clutches) within species (carotenoids: among species: $MS = 5,082.59$, $F_{71,335} = 22.42$, $P < .0001$, females within species: $MS = 394.23$, $F_{207,335} = 1.74$, $P < .0001$; vitamin A: among species: $MS = 7.59$, $F_{71,292} = 4.28$, $P < .0001$, females within species: $MS = 2.70$, $F_{166,292} = 1.52$, $P = .001$; vitamin E: among species: $MS = 23,517.77$, $F_{71,292} = 13.52$, $P < .0001$, females within species: $MS = 8,536.47$, $F_{166,292} = 4.91$, $P < .0001$). Therefore, the relatively low number of eggs that we could ethically or practically sample in some species should not have affected the outcome of the comparative analyses. Mean carotenoid and vitamin A and E concentrations for the species were used in subsequent analyses. Variance in mean yolk antioxidant concentration across species is illustrated in frequency distributions given in figure A2 in the online edition of the *American Naturalist*.

Mean carotenoid concentration (\pm SE) in egg yolk was $40.43 \pm 3.82 \mu\text{g/g}$, ranging from 1.50 to $162.09 \mu\text{g/g}$ across 112 species. Mean vitamin A concentration was $1.66 \pm 0.16 \mu\text{g/g}$ (range 0.12–7.07 $\mu\text{g/g}$, $n = 104$), and mean vitamin E concentration was $97.31 \pm 9.28 \mu\text{g/g}$ (range 9.30–387.57 $\mu\text{g/g}$, $n = 110$).

Mean carotenoid and vitamin A concentrations were both positively correlated with mean vitamin E concentration (log-transformed data; $r = 0.30$, $P = .001$, $n = 110$ and $r = 0.27$, $P = .005$, $n = 104$, respectively), while carotenoid and vitamin A concentrations were not significantly correlated with each other (log-transformed data; $r = 0.06$, $P = .56$, $n = 104$). When similarity among species due to common descent was taken into account, all three egg components were significantly positively correlated (carotenoids and vitamin A, correlation at $\lambda = 0.763$: $r_{\text{phy}} = 0.21$, $P < .05$, $n = 100$; carotenoids and vitamin E, correlation at $\lambda = 0.796$: $r_{\text{phy}} = 0.40$, $P < .001$, $n = 106$; vitamins A and E, correlation at $\lambda = 0.671$: $r_{\text{phy}} = 0.30$, $P < .005$, $n = 100$).

Antioxidants in Relation to Female Body Mass and Clutch Size

In an analysis based on species, yolk carotenoids and vitamin A were significantly positively related to clutch size after female body mass was controlled for, but that was not the case for vitamin E (table 1). When similarity

among species due to common descent was taken into account, yolk carotenoids (partial correlation at $\lambda = 1$: $r_{\text{phy1}} = 0.33$, $P < .001$, $n = 108$), vitamin A (partial correlation at $\lambda = 1$: $r_{\text{phy1}} = 0.34$, $P < .001$, $n = 100$), and vitamin E (partial correlation at $\lambda = 1$: $r_{\text{phy1}} = 0.35$, $P < .001$, $n = 106$) showed significant positive correlation with clutch size after female body mass was controlled for.

Yolk Antioxidants and Carotenoid-Based Signals

Across species, yolk carotenoid concentration was significantly positively related to the extent of carotenoid-based color: the model controlling for female body mass and clutch size explained 28% of the total variance (table 2; fig. 1A). In addition, carotenoid concentration increased with sexual dichromatism in carotenoid-based signals: the model controlling for female body mass and clutch size explained 17% of the variance (table 2). Vitamin A concentration was not significantly related to the extent of carotenoid-based color (table 2; fig. 1B) but also increased with sexual dichromatism, although less strongly than did carotenoid concentration (table 2). In contrast, vitamin E concentration was not significantly related to carotenoid-based color of feathers and bare parts (table 2; fig. 1C) or to sexual dichromatism in these signals (table 2). When similarity among species due to common descent was taken into account, models controlling for female body mass and clutch size confirmed the significant positive relations between yolk carotenoids and extent of carotenoid-based signals (partial correlation at $\lambda = 1$: $r_{\text{phy1}} = 0.36$, $P < .0005$, $n = 108$) and between yolk carotenoids and sexual dichromatism (partial correlation at $\lambda = 1$: $r_{\text{phy1}} = 0.18$, $P < .05$). When incorporating effects of phylogeny, models controlling for female body mass and clutch size confirmed the absence of a significant relationship between vitamin A or vitamin E and extent of carotenoid-based color (vitamin A, partial correlation at $\lambda = 1$: $r_{\text{phy1}} = 0.02$, $P > .05$, $n = 100$; vitamin E, partial correlation at $\lambda = 1$: $r_{\text{phy1}} = 0.03$, $P > .05$, $n = 106$). Sexual dichromatism was confirmed to be positively related to vitamin A (partial correlation at $\lambda = 1$: $r_{\text{phy1}} = 0.18$,

$P < .05$, $n = 100$) but not to vitamin E (partial correlation at $\lambda = 1$: $r_{\text{phy1}} = 0.10$, $P > .05$, $n = 106$).

In a multiple regression of male carotenoid-based color score as a function of female body mass, clutch size, and the three antioxidants, only carotenoid concentration explained a significant part of the variance (table 3). The same result was found when similarity among species due to common descent was taken into account (carotenoids, partial correlation: $r_{\text{phy1}} = 0.40$, $P < .0005$; vitamin A, partial correlation: $r_{\text{phy1}} = 0.0003$, $P > .05$; vitamin E, partial correlation: $r_{\text{phy1}} = -0.13$, $P > .05$; at $\lambda = 0.965$, $n = 100$). In a multiple-regression model based on species, sexual dichromatism in carotenoid-based color was positively related to yolk carotenoids and vitamin A but not to vitamin E (table 3). These results were confirmed only for yolk carotenoids when the effects of similarity due to common phylogenetic descent were controlled for (carotenoids, partial correlation: $r_{\text{phy1}} = 0.18$, $P < .05$; vitamin A, partial correlation: $r_{\text{phy1}} = 0.16$, $P > .05$; vitamin E, partial correlation: $r_{\text{phy1}} = -0.02$, $P > .05$; at $\lambda = 1$, $n = 100$).

Among species that were sexually dichromatic for carotenoid-based signals, sexual dichromatism was strongly positively related to male color scores ($F_{1,21} = 40.72$, $P < .0001$, $R^2 = 0.67$, slope estimate \pm SE = 0.80 ± 0.13). In order to determine whether the previously found relationship between sexual dichromatism and yolk carotenoids actually reflected an underlying relationship between male carotenoid-based color score and yolk carotenoids, we used the residuals from the phylogenetic regression as an index of the extent of sexual dichromatism, independent of male color, in analyses based on species and controlling for phylogeny. In analyses based on species and including female body mass and clutch size, residual sexual dichromatism did not explain significant variation in yolk carotenoids ($F_{1,18} = 2.24$, $P = .16$), vitamin A ($F_{1,17} = 0.45$, $P = .51$), or vitamin E ($F_{1,18} = 0.01$, $P = .92$; fig. 2). The same results were obtained after phylogeny was controlled for (carotenoids, partial correlation at $\lambda = 1$: $r_{\text{phy1}} = 0.014$, $n = 22$; vitamin A, partial correlation at $\lambda = 1$: $r_{\text{phy1}} = -0.03$, $n = 21$; vitamin E, partial correlation at $\lambda = 1$: $r_{\text{phy1}} = -0.007$, $n = 22$; all

Table 1: Regression models with log-transformed yolk antioxidant concentrations as dependent variables and clutch size as independent variable, with log-transformed female body mass controlled for, from species-specific data

Variable	Female body mass			Clutch size		
	Slope \pm SE	F (df)	P	Slope \pm SE	F (df)	P
Carotenoids	-.02 \pm .04	.15 (1, 108)	.70	.09 \pm .03	6.99 (1, 108)	.01
Vitamin A	.09 \pm .02	20.28 (1, 100)	<.0001	.04 \pm .02	6.83 (1, 100)	.01
Vitamin E	.004 \pm .034	.01 (1, 106)	.91	.01 \pm .03	.19 (1, 106)	.66

Note: See text for results of phylogenetic analyses (r_{phy1}).

Table 2: Regression models with log-transformed yolk antioxidant concentrations as dependent variables and log-transformed male carotenoid-based color scores or sexual dichromatism in carotenoid-based color as independent variables, with female body mass and clutch size controlled for

Variable	Male color			Sexual dichromatism		
	Slope \pm SE	F (df)	P	Slope \pm SE	F (df)	P
Carotenoids	1.18 \pm .21	32.01 (1, 107)	<.0001	1.31 \pm .35	13.45 (1, 107)	.0004
Vitamin A	.11 \pm .11	1.04 (1, 99)	.31	.36 \pm .17	4.21 (1, 99)	.04
Vitamin E	.06 \pm .20	.09 (1, 105)	.76	.28 \pm .31	.82 (1, 105)	.37

Note: Results are given for analyses performed on species-specific data. See text for results of phylogenetic analyses (r_{phy}).

$P > .05$). The relationship between sexual dichromatism and egg carotenoids was therefore not supported when the effect of carotenoid-based color was controlled for. Indeed, in a multiple regression, none of the three antioxidants explained a significant part of the variation in residual sexual dichromatism (model including female body mass and clutch size; carotenoids: $F_{1,15} = 1.92$, $P = .07$; vitamin A: $F_{1,15} = 0.81$, $P = .43$; vitamin E: $F_{1,15} = 2.79$, $P = .12$). This result was confirmed when phylogeny was taken into account (carotenoids, partial correlation: $r_{\text{phy}} = 0.20$; vitamin A, partial correlation: $r_{\text{phy}} = -0.10$; vitamin E, partial correlation: $r_{\text{phy}} = -0.27$; all $P > .05$; at $\lambda = 0.741$, $n = 21$).

Discussion

The main results of this comparative analysis of egg yolk antioxidants in birds were that concentration of all three egg components increased with clutch size across species and that there were significant positive relationships between yolk carotenoids, but not vitamins A or E, and the extent of carotenoid-based signals. Maternal deposition of antioxidants in eggs was not significantly related to the extent of sexual dichromatism in carotenoid-based colored signals.

Maternal deposition of all three antioxidants in eggs increased with clutch size, which is somewhat unexpected if they are limited resources for females. Clutch size has been found to coevolve with adult immunocompetence (ability to efficiently raise defenses against parasites) across species, reflecting natural selection due to parasite-induced mortality (Martin et al. 2001). Given the importance of early availability of antioxidants for development of the immune system, we might speculate that the increase in maternal deposition of antioxidants in eggs with increasing clutch size favors increased immunocompetence. Increased levels of antioxidants with clutch size might also reflect a maternal allocation strategy to reduce the costs of oxidative stress due to increased sibling competition in the early posthatching phase. At that time, nestlings still rely mainly on yolk-derived antioxidants, which have been

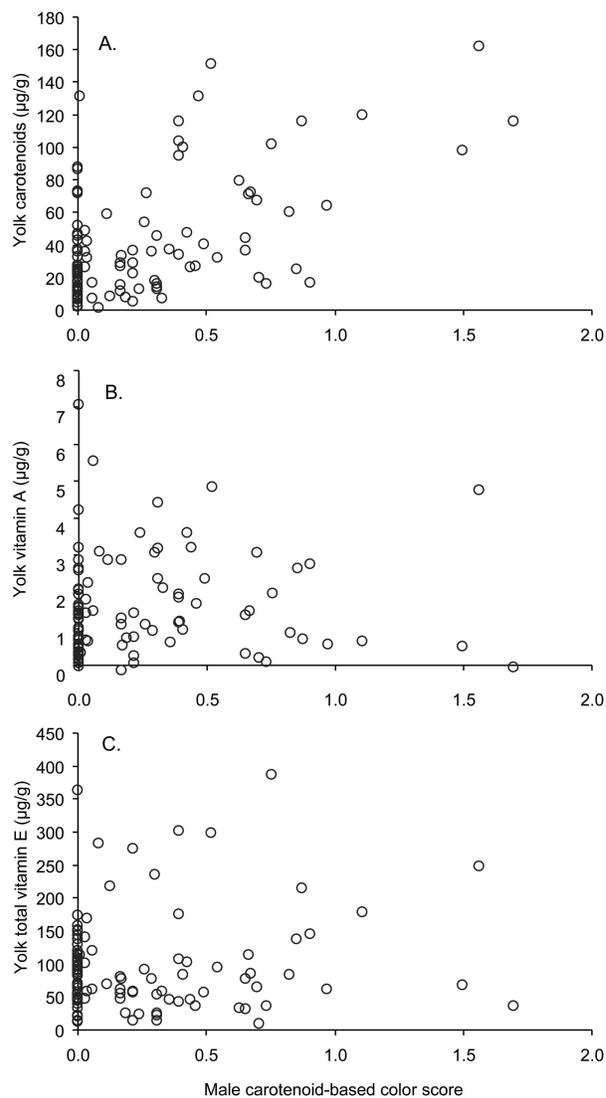


Figure 1: Mean concentrations of egg yolk antioxidants ($\mu\text{g/g}$) as a function of male carotenoid-based color scores presented on a log scale ($\log(\text{score} + 1)$): A, carotenoids ($n = 112$); B, vitamin A ($n = 104$); and C, vitamin E ($n = 110$).

Table 3: Multiple-regression model of male carotenoid-based color score and sexual dichromatism in carotenoid-based color scores as a function of yolk carotenoid and vitamin A and E concentrations, with female body mass and clutch size controlled for

Variable	Male color			Sexual dichromatism		
	Slope \pm SE	<i>F</i> (df)	<i>P</i>	Slope \pm SE	<i>F</i> (df)	<i>P</i>
Female body mass	-.02 \pm .02	.74 (1, 97)	.39	-.03 \pm .01	4.67 (1, 97)	.03
Clutch size	-.02 \pm .01	2.88 (1, 97)	.09	-.01 \pm .01	2.89 (1, 97)	.09
Carotenoids	.21 \pm .04	30.44 (1, 97)	<.0001	.09 \pm .02	13.75 (1, 97)	.0003
Vitamin A	.1 \pm .08	1.53 (1, 97)	.242	.11 \pm .05	4.45 (1, 97)	.04
Vitamin E	-.08 \pm .05	2.63 (1, 97)	.11	-.03 \pm .03	.72 (1, 97)	.40

Note: All variables were log transformed except clutch size. Results are given for analyses performed on species-specific data. See text for results of phylogenetic analyses (r_{phyt}).

shown to reduce oxidative stress during development (Blount et al. 2002; Surai 2002; McGraw et al. 2005).

We hypothesized that maternal effects might promote the evolution of carotenoid-based signals. A possible mechanism might be the long-term positive effects of embryonic exposure to yolk carotenoids on the ability to develop bright carotenoid-based signals. If that were the case, yolk carotenoid concentration should covary positively with expression of carotenoid-based signals across species. However, a positive relationship between color and antioxidants more important than carotenoids in eggs would instead be expected if the effects of carotenoids were indirect (as suggested by Hartley and Kennedy [2004]). Yolk carotenoid concentration increased with the extent of carotenoid-based signals, whereas that was not the case for the other two major antioxidants in egg yolk, vitamins A and E. This result is consistent with the hypothesis of a direct relationship between yolk carotenoids and development of carotenoid-based signals. In addition, the relationship between yolk carotenoids and carotenoid-based color was unaffected by inclusion of vitamins A and E in the analyses, contrary to what would be expected under the hypothesis that the relationship with carotenoids was mediated through or revealed by the role of other antioxidants (Hartley and Kennedy 2004). In fact, statistically controlling for levels of vitamins A and E in eggs even increased the strength of the relationship between yolk carotenoids and carotenoid-based color. This relationship was not confounded by any potential underlying relationship with diet or latitude and was also consistently found within lower phylogenetic taxa (see “Material and Methods” and the appendix).

Maternal investment of carotenoids in eggs may thus have evolved as an indirect result of selection for carotenoid-based signals in offspring when they reach adulthood. An increase in yolk carotenoid concentration enhances the ability of offspring to acquire and use carotenoids later in life (Hörak et al. 2000; Koutsos et al. 2003), thereby allowing offspring, when reaching adulthood, to develop bright carotenoid-based signals (McGraw

et al. 2005) that might increase fitness. Such a mechanism may also apply in an interspecific context and was specific to the properties of carotenoids, as vitamins A and E did not increase significantly with the extent of carotenoid-based signals across species. This may be due to the fact that vitamins A and E have physiological roles somewhat different from that of carotenoids, independent of their antioxidant functions. Carotenoids are involved in activation and regulation of the immune system (reviewed in Bendich 1993; Møller et al. 2000; Surai 2002), including inflammatory response relying on the production of reactive oxidative molecules (Rafi and Shafaie 2007; Kim et al. 2008), gap-junction and cell communication, and gene expression and protection against mutation (reviewed in Burri 2000; Surai 2002), and they are precursors of vitamin A (reviewed in Surai 2002). However, there is only limited evidence suggesting that they play a role *in vivo* as major antioxidants in birds (Costantini and Møller 2008). In contrast, vitamin A is involved in immune function, but to a lesser extent than carotenoids (Bendich 1993), and vitamin E is involved in membrane stabilization and cellular signaling (reviewed in Brigelius-Flohé and Traber 1999; Surai 2002). Carotenoids are simultaneously involved in various physiological and signaling functions, which is not the case for vitamins A and E. Carotenoids are thus potentially at the center of a multiple trade-off that might be modulated by maternal effects during early development. Maternal investment of these pigments in eggs has indeed been shown to exert long-lasting organizational effects (reviewed in “Introduction”). Maternally derived carotenoids increase the efficiency of carotenoid metabolism in posthatching life, through the ability of offspring to assimilate and use carotenoids, and thereby have long-lasting positive effects on the ability to develop bright carotenoid-based signals. Embryonic exposure to yolk carotenoids also modulates the development of immune function as well as the costs of its use in relation to regulation of systemic inflammation. Maternally derived carotenoids may therefore allow offspring to change the trade-off between sexual signaling and physiological func-

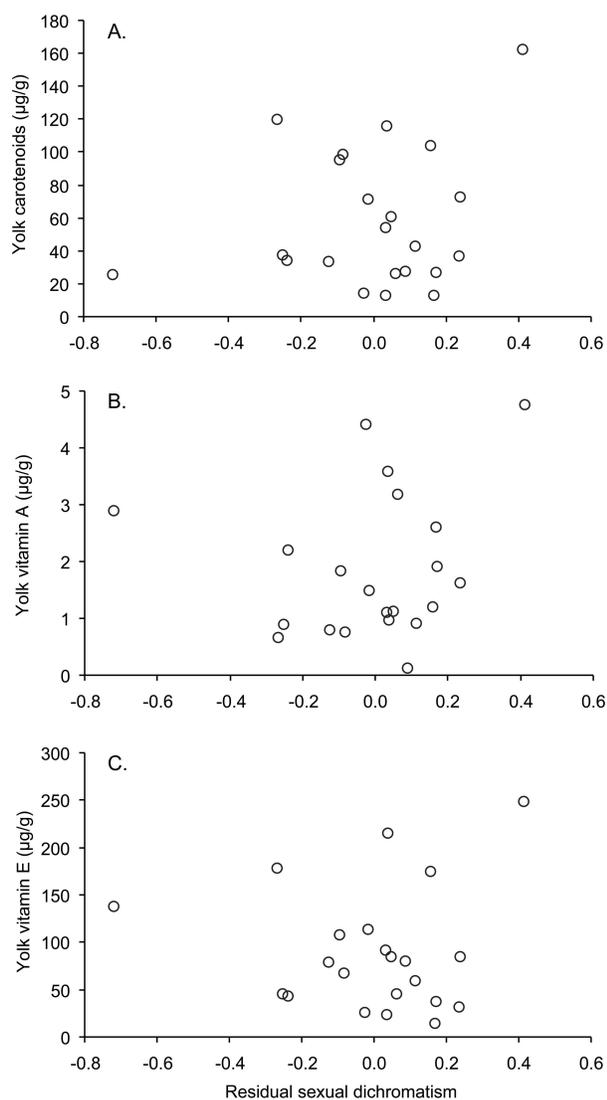


Figure 2: Mean concentrations of egg yolk antioxidants ($\mu\text{g/g}$) as a function of residual sexual dichromatism in carotenoid-based color scores: A, carotenoids ($n = 22$); B, vitamin A ($n = 21$); and C, vitamin E ($n = 22$).

tions, while the costs of maternal investment in eggs would maintain the reliability of signals. Furthermore, maternal effects increase phenotypic plasticity in offspring traits (Mousseau and Fox 1998b). Therefore, we speculate that maternal effects through yolk carotenoids might increase plasticity and potential for innovation in this metabolism that they influence, making more readily possible the use of newly available dietary carotenoids or the development of new metabolic pathways for transforming carotenoids. Variation among several tanager species (*Piranga* spp.) in metabolic pathways for producing colorful feathers and

their evolution in relation to dietary availability of pigments illustrates how phenotypic plasticity might drive the evolution of colored signals (Price et al. 2003; Grether 2005; Price 2006). Maternal effects through egg carotenoids may thus underlie the relatively greater evolutionary flexibility observed for carotenoid-based coloration, as compared to melanin- or structurally based coloration (Badyaev and Hill 2003; Hill and McGraw 2006b).

Comparative studies of sexual dichromatism and carotenoid-based coloration have shown that sexual dichromatism has a positive correlation with carotenoid-based plumage coloration but not with melanin-based or structural coloration, suggesting that sexual selection has driven the evolution of sexual dichromatism in carotenoid-based signals (in North American passerines: Gray 1996; in cardueline finches: Hill 1996; Badyaev and Hill 2000). We found a positive relationship between sexual dichromatism in carotenoid-based signals and the level of carotenoids deposited in eggs across species. However, this relationship was weak and appeared mainly to reflect that sexual dichromatism in carotenoid-based color strongly increased with the extent of carotenoid-based color. It should be noted that the number of species in our data set that were sexually dichromatic for carotenoid-based signals was relatively low (22 species). Such a sample size is sufficient to detect a strong effect (explaining 25% of the variance). The absence of a significant relationship between yolk antioxidants and sexual dichromatism thus indicates that there was no strong effect, but we cannot rule out the possibility that a weak effect (explaining 1% of the variance) might be detected with a larger sample size. Females did not invest more carotenoids in eggs in dichromatic species than in monochromatic species with the same level of male carotenoid-based color. In sexually dichromatic species, males would have elevated requirements for carotenoids to invest in development of carotenoid-based signals, while females that do not develop carotenoid-rich plumage would have elevated needs for carotenoids to invest in production of carotenoid-rich eggs. We hypothesize that these requirements in males and females may be of similar magnitude, potentially explaining the absence of a significant relationship between carotenoid concentration in eggs and sexual dichromatism in carotenoid-based coloration. The absence of significant covariation between sexual dichromatism and maternal investment of antioxidants in eggs raises a question about the evolution of female ornamentation that is still a subject of debate (Amundsen 2000). Female coloration may have evolved as a by-product of sexual selection due to a genetic correlation with male ornamentation or independently as a response to male mate choice or female contests (Amundsen 2000). The positive relationship between maternal investment of carotenoids in eggs and the devel-

opment of carotenoid-based colors suggests other mechanisms for the evolution of female ornamentation in relation to sex allocation. There is recent evidence that females can differentially allocate carotenoids to eggs as a function of embryo sex (Verboven et al. 2005; Badyaev et al. 2006), although the occurrence of sex allocation of carotenoids might not be a general pattern, even in closely related species (Saino et al. 2003*b*; Groothuis et al. 2006; Romano et al. 2008). In the case where females do not differentially allocate carotenoids to eggs as a function of embryo sex and requirements for carotenoids are equivalent in both sexes, then offspring of both sexes would benefit equally from maternally derived carotenoids, and carotenoid-based color would evolve in the same direction in females as in males, without necessarily implying a genetic correlation. In contrast, if females do invest carotenoids as a function of embryo sex and if males and females do not differ in development of carotenoid-based signals, this would suggest a differential rate of use of antioxidants by the two sexes, with males having higher turnover than females. For example, a differential rate of use of antioxidants by males may be related to increased susceptibility of males to parasites (Møller et al. 1998).

The hypothesis that maternal effects through egg composition may influence the evolution of secondary sexual characters has recently been tested for two other important maternal effects, immune compounds and hormones (Garamszegi et al. 2007*a*; Gil et al. 2007; Saino et al. 2007). Although there was evidence of a positive relationship between maternal investment of lysozymes in eggs and sexual dichromatism in plumage brightness across species (Saino et al. 2007), no evidence for such a relationship was found when two yolk androgens, testosterone and androstenedione, were considered (Gil et al. 2007). However, maternal testosterone has been shown to impose constraints on the evolution of song characteristics (Garamszegi et al. 2007*a*). This suggests a differential effect of sexual selection on different aspects of maternal effects and that the role of particular maternal effects in development of sexual signals relies on specific physiological mechanisms and constraints. In addition, this result suggests complex maternal effects based on multiple pathways, which might have evolved independently to optimize a cocktail of specific egg components as a function of prevailing selective forces and physiological constraints.

In conclusion, a comparative analysis of the antioxidant contents in bird eggs revealed a positive relationship between yolk carotenoids and relative size of carotenoid-based sexual signals but not between yolk carotenoids and sexual dichromatism in these signals. These findings provide evidence consistent with the hypothesis, based on the specific role of carotenoids during embryo development, that maternal effects mediated by yolk carotenoids may

promote the evolution of carotenoid-based signals as a response to sexual selection. Maternal effects may therefore contribute to the evolution of secondary sexual characters and to the maintenance of additive genetic variation for these traits.

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Females of the pied crow (*inset*) lay carotenoid-poor eggs, whereas females of the vividly colored southern masked weaver lay eggs that are particularly rich in carotenoid pigments (photographs by Mark Anderson).