



Blue-green eggshell coloration reflects yolk antioxidant content in spotless starlings *Sturnus unicolor*

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The hypothesis that eggshell colouration is a sexually selected trait of female birds is based on the fact that biliverdin, the pigment responsible for blue-green colours of the eggshell, is a potent antioxidant and that only females with high antioxidant capacity can deposit higher concentrations of biliverdin as eggshell pigment. Antioxidants (e.g. carotenoids, vitamins) are also abundant in the egg yolk, which serve as nutrient reserves for the developing embryo, and eggshell colour intensity may reflect maternal investment in yolk antioxidants. Here, we test the relationship between blue-green eggshell colour intensity and concentration and amount of carotenoids, vitamin A, and vitamin E in the egg yolk of spotless starling *Sturnus unicolor*, a species for which we have previously shown good evidence of sexual selection driving egg coloration. As could be extrapolated from the hypothesis of sexual selection driving the evolution of blue-green eggshells, we found that eggshell colour intensity was positively related to the concentration and amount of carotenoids and vitamin E in the yolk. Thus, mothers may use egg colour intensity to signal to fathers the antioxidant status of their offspring. Moreover, we provide evidence suggesting that maternal yolk investment in more coloured eggs can also explain the detected association between feeding decisions of males and egg colour intensity that we have found previously in this species.

The hypothesis that eggshell colouration is a sexually selected trait of females (EC hypothesis) in species with biparental care suggests that colour intensity of the eggshells of birds reveals the condition of females at the time of laying and is used by males as a cue to determine their parental effort (Moreno and Osorno 2003, Soler et al. 2008). This hypothesis is based on the antioxidant capacity of eggshell pigments, such as biliverdin (Kennedy and Vevers 1976, Stocker et al. 1987). Because females are thought to be under high levels of oxidative stress during breeding (von Schantz et al. 1999, Alonso-Alvarez et al. 2004), only those with a high antioxidant capacity would be able to deposit higher concentrations of biliverdin in eggshells (Moreno and Osorno 2003).

Three main predictions emerge from the EC hypothesis. First, the intensity of blue-green coloration should reflect female condition at the time of laying. This prediction has received empirical and experimental support (Moreno et al. 2005, Siefferman et al. 2006, Krist and Grim 2007, Hanley et al. 2008). Second, the hypothesis posits that males should adjust paternal behaviour as a function of blue-green eggshell colour intensity. Some findings support that idea that males provision young more when they hatched from more colourful eggs (Moreno et al. 2004, Hanley et al. 2008, Soler et al. 2008, Morales et al. 2010), but this is not always the case (Krist and Grim 2007,

Hanley and Doucet 2009, Reynolds et al. 2009). In spotless starlings *Sturnus unicolor*, Soler et al. (2008) showed that the intensity of egg coloration significantly influenced male effort. Third, the hypothesis predicts that shifts in paternal effort as a result of egg coloration should translate to higher offspring fitness, but this prediction has received indirect support only (Soler et al. 2008).

Egg colouration may also signal egg quality (Morales et al. 2006, Hargitai et al. 2010). Birds provision their egg yolks with nutrients, hormones, antibodies, as well as antioxidants (e.g. vitamin A, vitamin E, and carotenoids) that can aid in development, growth, and health of offspring (Surai et al. 2001a, b, Saino et al. 2003). Studies have shown that blue-green colour intensity of the eggshell can reflect yolk immunoglobulin concentration in pied flycatchers *Ficedula hypoleuca* (Morales et al. 2006), yolk testosterone titer in spotless starlings (López-Rull et al. 2008), and yolk carotenoid concentration in European cuckoos *Cuculus canorus* and great reed warblers *Acrocephalus arundinaceus* (Hargitai et al. 2010). However, Cassey et al. (2008) failed to show such relationships in two thrush (*Turdus*) species.

Carotenoids are important for offspring fitness and for regulation of embryonic development, gene expression, and the activity of growth factors and hormones (Hofmann

and Eichele 1994). Carotenoids in eggs also affect the development and the expression of some components of the immune system related to probability of offspring survival (Blount et al. 2003, McGraw and Ardia 2003, Saino et al. 2003), and provide the embryo with defences against oxidative stress in tissues (Surai and Speake 1998). Others compounds present in the egg yolk, such as vitamins A and E, also contribute to antioxidant defences and therefore to offspring development (Surai et al. 2001b). The integrated antioxidant system of developing animals is quite complex (Surai 2002), as different carotenoids can occur (e.g. carotenes, hydroxycarotenoids, ketocarotenoids) with different antioxidant activity and with different interactions with vitamins and other antioxidant components (e.g. enzymes) (Catoni et al. 2008). There are also important ecological and genetic factors underlying inter-specific variability in antioxidant composition (Tella et al. 2004, Cohen et al. 2009), and it is likely that different kinds of antioxidants affect embryonic development in different ways.

Here, we investigate the hypothesis that the intensity of blue egg colouration signals maternal investment in yolk carotenoids and vitamins in a species, the spotless starling, for which support for the three main predictions of the EC hypothesis has been previously found.

Material and methods

Spotless starlings are sexually dimorphic and typically socially monogamous (Navarro et al. unpubl.). Eggs are blue-green in colour, with a small peak in the ultraviolet (300–400 nm) range (Soler et al. 2008). Females lay one egg daily (Cramp 1998), and start incubation before clutch completion, which determines asynchronous hatching (Cramp 1998). In our population, clutch size is 4 or 5 eggs (Soler et al. 2008) and females typically incubate eggs.

The study was carried out in Guadix (37°18'N, 3°11'W), south-eastern Spain, during the breeding season of 2008, on colonies of spotless starlings that breed in nest-boxes. We visited each nest-box every 3–4 d during the laying season. Once we detected egg(s) in the nest, we started to visit the nest every day to determine the end of laying. Intraspecific nest parasitism has been demonstrated in spotless starling (Calvo et al. 2000), but our daily visits allowed us to eliminate eggs laid by other females. Although we have not accounted for paternal identity in statistical analyses, each nest was attended by a single male. Because starlings lay one egg per day, we could then calculate the date on which females started to lay (hereafter, laying date). Two days after the end of laying, we randomly collected 38 eggs, two eggs from 16 nests and one single egg from 6 additional nests. Nests from which we collected one or two eggs did not differ in clutch size ($t=0.67$, $DF=20$, $p=0.51$), egg colour ($t=0.44$, $DF=20$, $p=0.66$) or antioxidant content (PC1 $t=1.46$, $DF=20$, $p=0.16$; PC2 $t=2.03$, $DF=20$, $p=0.06$; PC3 $t=1.01$, $DF=20$, $p=0.32$). To reduce the possible influence of laying date in our analyses, we limited our sample to nests that were initiated over a 6-d period ($n=22$, $SD=1.59$ d) at the peak of reproduction. After collecting

eggs, we immediately transported them to the lab in a portable cooler, and measured eggshell colour in a dark room. Thereafter, following previous work (Borttoloti et al. 2003), we stored eggs in a -20°C freezer.

Egg measurements

All eggs were weighed with a digital balance (accuracy = 0.001 g) and measured with digital callipers (length and width to the nearest 0.01 mm) before being frozen. Egg yolks were weighed from frozen eggs. Egg volume was calculated using Hoyt (1979). Variation in egg mass, egg volume and yolk mass was significantly higher among than within nests (volume of eggs: $R=0.65$, $F_{15,16}=4.71$, $p=0.001$; egg mass: $R=0.51$, $F_{15,16}=3.08$, $p=0.01$; yolk mass: $R=0.46$, $F_{15,16}=2.70$, $p=0.02$), so we used mean values per nest in statistical analyses.

We measured egg colour on five randomly selected areas of the surface of the egg along the long axis (Aviles et al. 2006). Reflectance spectra (300–700 nm) were recorded by using an Ocean Optics S2000 spectrometer connected to a deuterium-halogen light (D2-W, mini) by a co-axial reflectance probe (QR-400-7-UV-vis) and the OOIBase32 operating software (Ocean Optics, USA). Reflectance was measured with the probe placed at a constant distance (2 mm) and at an angle of 45° . Measurements were taken relative to complete darkness and a standard white reference (WS-2).

Though avian visual perception can be partially modelled in complex ways, by incorporating retinal sensitivities and ambient wavelengths (Endler et al. 2005, Aviles et al. 2008), here we relied on colour variables extracted from spectrophotometric data that have proved successful in revealing individual quality in previous work (Siefferman et al. 2006). So, as a variable indicating intensity of blue-green coloration, we used the proportion of total reflectance within the blue-green region (R400–575/R300–700) of the spectrum (Siefferman et al. 2006, Soler et al. 2008). Biliverdin shows the lowest absorbance (Falchuk et al. 2002) within the blue-green region (400–574 nm), where reflectance of starling eggs reaches its maximum (Fig. 1 in Soler et al. 2008). Furthermore, we know that total reflectance in this region is related to biliverdin concentration in the eggshell of spotless starlings (López-Rull et al. 2008). Colour estimates for the same eggs showed high repeatability ($R=0.93$, $F_{476,1901}=31.14$, $p<10^{-17}$), whereas mean values per egg showed moderate intra-nest repeatability (R400–575/R300–700; $R=0.62$, $F_{15,16}=4.22$, $p=0.003$) (Harper 1994). Thus, we used mean values for each nest in subsequent analyses.

After measuring eggshell coloration, eggs were frozen (-20°C) in individualized hermetic plastic bags and protected in a dark cardboard box. On the following day, yolk and albumen were separated from frozen eggs using a scalpel and blotting paper for later antioxidant analyses. Yolk samples were again frozen (-20°C) in cryotubes and stored in dark cardboard boxes for 165 d, at which point the samples were stored in a -80°C freezer until antioxidant analyses were performed 109 d later.

Antioxidant analyses

Lipid-soluble carotenoids and vitamins were extracted from yolks following McGraw et al. (2005). Based on comparison with standards, we detected retinol (vitamin A), tocopherol (vitamin E), cis-lutein, trans-lutein, zeaxanthin, two unidentified ketocarotenoids (λ -max = 473 and 474 nm), and β -carotene (Table 1). Concentrations of vitamins A and E and of the different carotenoids were calculated based on external curves constructed from known amounts of each purified reference compound. Total amounts of compounds were calculated by multiplying compound concentration \times total yolk mass. All measurements were made by KJM, which reduced variance due to inter-observer variability, and after obtaining repeatable measurements in preliminary tests. Analyses were performed blind with respect to egg colour values. We found moderate within-nest repeatability for concentrations of most of these compounds (vitamin E: $R=0.76$, $F_{15,16}=7.23$, $p=0.0001$; trans-lutein: $R=0.68$, $F_{15,16}=5.30$, $p=0.0009$; cis-lutein: $R=0.62$, $F_{15,16}=4.27$, $p=0.0032$; zeaxanthin: $R=0.70$, $F_{15,16}=5.56$, $p=0.0007$; ketocarotenoids: $R=0.86$, $F_{15,16}=13.30$, $p<0.00001$; and β -carotene: $R=0.66$, $F_{15,16}=4.84$, $p=0.0016$) with the exception of vitamin A ($R=0.03$, $F_{15,16}=1.07$, $p=0.44$). Thus, we used the mean value per nest for the repeatable variables, while for vitamin A we used the value from a randomly selected egg.

Statistical analyses

All variables approximately fitted a normal distribution (Kolmogorov–Smirnov test for continuous variables, $p > 0.2$). To reduce the number of independent variables and problems of collinearity, we performed a principal components analysis (PCA). Amounts and concentrations of total yolk carotenoids and vitamins were highly positively correlated (one-way ANOVAs, $F_{1,36}=526.95$, $p<0.00001$, $R>0.96$) and, thus, we used PCAs on

Table 1. Means (SD) for concentration and total yolk vitamin A, vitamin E and carotenoid (cis-lutein, trans-lutein, zeaxanthin, unidentified ketocarotenoids and β -carotene) levels ($\mu\text{g g}^{-1}$). Factor loadings for the principal components as well as the relationships with different antioxidants are also shown.

n=22	Mean (SD)	PC1	PC2	PC3
Vitamin A concentration	9.52 (2.18)	0.13	-0.86	-0.31
Vitamin A total	11.88 (3.13)	0.16	-0.81	-0.33
Vitamin E concentration	199.39 (84.94)	0.88	-0.27	-0.08
Vitamin E total	246.76 (106.35)	0.90	-0.25	-0.12
Cis-lutein concentration	26.28 (10.61)	0.97	-0.08	0.04
Cis-lutein total	32.65 (13.22)	0.98	-0.05	0.00
Trans-lutein concentration	135.50 (51.72)	0.98	-0.10	-0.06
Trans-lutein total	168.83 (66.35)	0.96	-0.06	-0.10
Zeaxanthin concentration	23.92 (9.68)	0.96	0.17	-0.03
Zeaxanthin total	29.78 (12.51)	0.95	0.19	-0.06
Ketocarotenoids concentration	10.60 (5.14)	0.21	-0.41	0.88
Ketocarotenoids total	13.09 (6.05)	0.21	-0.41	0.88
β -carotene concentration	0.89 (0.56)	0.52	0.75	0.11
β -carotene total	1.10 (0.70)	0.56	0.74	0.09
Factor loading		0.57	0.22	0.13

amounts and concentration values for the different carotenoids and vitamins analysed. PCA summarized the fourteen variables into three orthogonal principal component axes. The first principal component (PC1) explained 57% of the variation and described variation in concentration and amount of yolk vitamin E, cis-lutein, trans-lutein and zeaxanthin. The second principal component (PC2) explained 22% of the variation and was related to concentration and amount of vitamin A (negatively) and β -carotene (positively) concentration. The third principal component (PC3) explained 13% of the variance and was positively associated with concentration and amount of yolk ketocarotenoid (Table 1).

Blue-green colour intensity was used as the dependent variable and concentrations and amount of vitamins and carotenoids (PC1, PC2 and PC3) as independent variables in general regression models (GRM). All analyses were performed with Statistica 6 software (Statsoft 2001).

Results

The carotenoids and vitamins in the yolk of spotless starling eggs were similar to those found for other species (Cassey et al. 2005). On a $\mu\text{g g}^{-1}$ basis, vitamins A and E represented 2 and 49% respectively of the total chemicals studied in the egg-yolks of spotless starlings. Carotenoids comprised the remaining 49% of total antioxidants analysed in egg-yolks, with the common xanthophyll lutein being the dominant form (Table 1).

We found a significant positive relationship between the intensity of blue-green coloration of eggshells of starlings and concentration and amount of some (PC1: $F_{1,18}=8.10$, $p=0.011$, $\text{Beta(SE)}=0.55(0.19)$), but not other yolk antioxidants (PC2: $F_{1,18}=0.35$, $p=0.56$, $\text{Beta(SE)}=0.12(0.19)$; PC3: $F_{1,18}=0.02$, $p=0.89$, $\text{Beta(SE)}=0.03(0.19)$). The full model explained 21% of variance in egg colour intensity ($R^2=0.21$, $F_{3,18}=2.82$, $p=0.068$). Result did not change when we used one randomly selected egg per nest or included the non-significant effect of laying date in the model (result not shown). The inclusion of the non-significant effect of egg volume ($F_{1,17}=4.38$, $p=0.052$, $\text{Beta(SE)}=0.40(0.18)$) in the model did not affect the significance of the relationship (PC1: $F_{1,17}=6.76$, $p=0.019$, $\text{Beta(SE)}=0.47(0.18)$). Thus egg colour intensity of spotless starling eggs was positively associated with the most abundant antioxidants in the egg yolk (lutein, zeaxanthin, and vitamin E).

Discussion

We found that blue-green colour intensity of eggshells of spotless starlings was positively related to the concentration and amount of vitamin E, and two common hydroxycarotenoids, lutein and zeaxanthin, in egg yolk. Carotenoids and fat-soluble vitamins are valuable micronutrients for developing embryos that can affect hatching success (Surai et al. 2001a, Møller et al. 2008), nestling immunocompetence (Saino et al. 2003), and survival of birds (McGraw et al. 2005). We have previously shown that paternal effort of starlings increased for bluer clutches of eggs (Soler et al.

2008), which was interpreted as the adjustment of males to female condition (i.e. antioxidant, immunological, hormonal status) (Moreno and Osorno 2003). Our results here suggest that this adjustment may also be related to maternal investment in egg yolk and embryos (Morales et al. 2006).

Biliverdin, the pigment responsible for the blue-green colour of the eggshell, is a potent antioxidant synthesized by egg-laying females that may combat oxidative stress (Stocker et al. 1987). Other antioxidants like carotenoids and some vitamins (i.e. A, E) cannot be synthesized de novo by vertebrates, and are obtained in food. Carotenoids are therefore limited resources for females to include in eggs (Blount et al. 2004). Carotenoids, however, may not always exhibit direct antioxidant activity in vitro (Costantini and Møller 2008), but may work in concert with other non-pigmented antioxidants, such as some enzymes and vitamins, to boost health (Hartley and Kennedy 2004). This is why some authors have suggested that carotenoids can function as signals revealing the availability of non-pigmented antioxidants in the body (or eggs) (Bertrand et al. 2006, Pike et al. 2007, Perez et al. 2008). Thus, it appears that only females in optimal oxidative balance will be able to lay highly pigmented blue-green eggs that have elevated yolk concentrations of carotenoids and vitamins.

Associations between pigmented and non-pigmented antioxidants, such as vitamin E, are not always detected. In a study of blood plasma antioxidants from 99 bird species, Cohen and McGraw (2009) did not find associations between levels of vitamin E and carotenoids in plasma. Here, we found that blue-green colour intensity was associated with both vitamin E and carotenoid concentrations and, although we did not measure directly the biliverdin content in eggshell, previous work has shown such an association (López-Rull et al. 2008). Moreover, it is known that pigment allocation to eggs decreases plasma antioxidants in collared flycatcher females (Morales et al. 2008). Thus, the relationship between eggshell colour intensity and yolk vitamin E levels could be an example of the use of pigmented antioxidants in the eggshells for signalling both non-pigmented and pigmented antioxidant molecules.

We failed to find a relationship between egg pigmentation and concentration of vitamin A in the egg yolk. Yet we have previously found that vitamin A concentrations in plasma are linked to plasma carotenoid concentrations of adult starlings, and that both plasma vitamin A and carotenoid concentrations covaried with beak colouration of males and females (Navarro et al. 2010). Vitamin A plays an important role in vision, health, and other life history traits of birds (Cohen et al. 2009). However, its role in embryonic development is not so clear, and apparently carotenoids and vitamin E were more important than vitamin A for avian embryos (Blount et al. 2000) (i.e. low levels of antioxidants, such as vitamin E, are associated with a decrease hatching success and slow embryonic development in birds (Wilson 1997)). In sum, the results of our two studies of spotless starlings suggest that females advertise to males their physiological characteristics, including vitamin A status, through the colour of their bills, while

signalling maternal investment in a different vitamin (E) through the colour of their eggs.

As far as we know, only Cassey et al. (2009) and Hargitai et al. (2010) have previously tested the relationship between egg-yolk antioxidants and eggshell colouration. The former studied eggs of two species of thrushes, and failed to detect a relationship, while the later found a relationship with blue-green chroma of eggshells of the European cuckoo and of the great reed warbler and antioxidants. Here, we also found that the strength of the relationships between carotenoids and egg colouration differed for different types of carotenoids. Intensity of blue-green eggshell colouration was positively correlated with yolk concentration and amount of vitamin E, trans-lutein, cis-lutein and zeaxanthin (PC1), but not with other carotenoids. A possible reason for this association between vitamin E, trans-lutein, cis-lutein and zeaxanthin (PC1) could be that zeaxanthin is a stereoisomer of lutein and both accumulate at similar levels in tissues. Also, it has been suggested that these two carotenoids may play analogous functions to vitamin E (α -tocopherol) in the liver of embryos. Later in development, zeaxanthin and lutein are gradually replaced in this function by vitamin E (reviewed by Surai et al. 2001c).

In conclusion, our results support the hypothesis that blue-green eggshell colour intensity is associated with carotenoid and vitamin content of egg yolk in a species, the spotless starling, where crucial predictions of the hypothesis that egg coloration is a sexually selected trait of females has been previously tested experimentally (Soler et al. 2008). Eggs of exaggerated blue-green colouration would be of higher reproductive value for spotless starling males not only because it reflects phenotypic quality of females, but also maternal investment in egg yolk antioxidants.

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