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ORIGINAL PAPER

Egg pigmentation reflects female and egg quality in the spotless starling *Sturnus unicolor*

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Abstract It has been proposed that blue colouration in eggs has evolved as a signal of female quality that males can use to modulate their parental investment. This hypothesis is based in the antioxidant properties of biliverdin whose costly deposition in the eggshell is expected to signal female antioxidant capacity and egg quality. Since maternally derived androgens are costly to produce and may adaptively affect offspring phenotype, high-quality females may benefit by signalling their androgen investment through egg colouration. Our aim was to investigate whether egg colour variation in the spotless starling reflected the amount of pigments on the eggshell and whether egg pigmentation was related to female and egg quality. Chromatography analyses revealed that spotless starling eggshells contained two different pigments: biliverdin and protoporphyrin IX with no correlation between them. Biliverdin contents correlated positively with egg colouration indicating that darker eggs with a higher peak in the blue–green segment of the spectrum contained higher amounts of biliverdin. Eggs containing more biliverdin were laid by high-quality females and contained higher yolk testosterone levels. However, despite the strong correlation between biliverdin and colorimetric variables, egg colouration did not reflect accurately female and egg quality. Our results provide evidence that eggshell pigmentation in the spotless starling is related to female and egg quality as shown by the yolk testosterone levels. However, the lack of relation between egg colour and female condition and egg quality do not provide evidence to support the signalling function of egg colouration.

Keywords Egg pigments · Biliverdin · Yolk androgens · Spotless starling

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Introduction

Egg colouration results from pigments accumulated in the surface of the eggshell such as porphyrins and biliverdin (Miksik et al. 1996). Porphyrins are responsible for reddish and brown egg colours while biliverdin is associated with blue and green colouration (Kennedy and Vevers 1973, 1975). Recently, it has been proposed that blue egg colouration has evolved as a signal of female genetic quality or condition that males could use to modulate their parental investment in their offspring (Moreno and Osorno 2003). The hypothesis is based on the antioxidant properties of biliverdin whose deposition in the eggshell may be costly, thus signalling female capacity to control free radicals and functioning as a handicap (Zahavi 1975) during the laying period. Supporting this hypothesis, variation in egg colouration has been shown to predict female quality or condition in two different passerine species (Morales et al. 2006; Siefferman et al. 2006). Furthermore, in the pied flycatcher (*Ficedula hypoleuca*), egg colouration is related to the amount of maternal antibodies in the yolk, thus reflecting the quality of the

eggs themselves and providing a reliable indication of offspring survival (Morales et al. 2006). As well as maternal antibodies, maternally derived androgens, particularly testosterone (T), is known to adaptively affect offspring phenotype by increasing chick growth, begging behaviour and dominance (Schwabl 1993; Lipar and Ketterson 2000; Sockman and Schwabl 2000; Eising et al. 2001). Since yolk androgens are costly to produce, inter-female variation in yolk androgen deposition suggests that differential hormone transmission may represent an adaptive maternal strategy (Gil et al. 1999; Reed and Vleck 2001; Groothuis and Schwabl 2002; Verboven et al. 2003; Pilz et al. 2004), so its allocation to eggs may function similarly to maternal antibody allocation, and thus high-quality females may also benefit by signalling their androgen investment through egg coloration. To date, there has been no demonstration of an association between egg colour and yolk androgen content.

Another trait that commonly denotes egg quality is eggshell thickness. It has been suggested that in species with brood parasitism the evolution of thicker eggshell may have been selected if aggression by host females forces parasite females to lay eggs quickly or causes eggs to get jostled (Mallory and Weatherhead 1990). Similarly, increasing levels of pollutants affect the thickness of eggshells, increasing the likelihood of egg breakage and reducing reproductive success (Eeva and Lehikoinen 1995). The calcified layer of the shell is largely responsible for its mechanical strength. This layer constitutes the main part of avian eggshell and consists of elongated structures of calcium carbonate and magnesium that are perpendicular to the shell surface. Since the pigmented layer covers the outer surface of the calcitic shell, it is possible that egg colouration could be affected by eggshell thickness. Furthermore, a positive association between egg colouration and shell thickness may appear if both two traits depend on female quality.

The aim of our study was to investigate whether variation in egg colouration in the spotless starling (*Sturnus unicolor*) reflects the amount of pigments deposited on the eggshell and whether egg pigmentation is related to female quality and to egg quality in terms of maternally derived androgens and eggshell thickness. A main prediction of the sexually selected egg colour hypothesis is that there is a direct relationship between colouration and eggshell pigmentation, particularly biliverdin. All studies to date have used data from reflectance spectra in order to avoid reliance on human colour perception. However, different methods have been used to analyse the spectral shape (analysis of tristimulus colour variables, principal components analysis (PCA) and analysis by segment classification), resulting in different colorimetric variables that hamper comparisons between studies. Moreover, only one study in the pied flycatcher (*F. hypoleuca*) has analysed eggshell biliverdin content and related it to egg colouration (Moreno et al. 2006). Thus, it becomes necessary to compare the different methods of quantifying egg colouration and discuss them on the basis of how colorimetric variables reflect biliverdin concentration.

Materials and methods

General procedures

We studied a ringed population of spotless starling in Soto del Real, near Madrid, in central Spain (40° 44' N, 3° 48' W), from March to July 2006. The colony consisted of 150 nest boxes distributed in deciduous mixed woodland of oak (*Quercus rotundifolia*) and ash (*Fraxinus angustifolius*). Nest boxes were checked daily throughout the breeding season to determine laying dates and eggs were marked with a non-toxic marker as they were found in order to determine laying sequence.

Before incubation started, approximately 25 mg of yolk were obtained from every egg in the clutch in order to analyse yolk steroids. Biopsies were done by inserting the 25-gauge needle of a winged infusion set into the yolk, while the egg was being candled. After sample removal the hole was sealed with a tiny strip of flexible wound dressing (Opsite, Smith&Nephew, Hull, UK) so the embryo could continue its normal development. The hatching success of biopsied eggs was 73.5%. Yolk samples were individually weighed in the laboratory, homogenised in 1 ml of distilled water and frozen at −70°C for future androgen analysis. On the fourth day of incubation, we measured egg colour, collected two eggs from each clutch (the first and the second egg laid) and captured females in their nests. Measurements of egg colour were taken using an Avantes spectrophotometer (AvaSpec-2048FT), which covered reflectance in the human visible spectrum and the ultraviolet range (300 to 700 nm). Spectral data were truncated at 700 nm since this is the upper limit of the visible range of birds (Endler and Mielke 2005). The data output consisted of 40 reflectance values in steps of 10 nm. A reference calibration with a white standard was taken prior to the colour measurements according to the apparatus specifications. Biometric data were recorded from each female and three feathers were randomly collected from the central region of the throat by pulling them near the base. Feathers were kept in plastic bags until length measurements were performed with a digital calliper to the nearest 0.01 mm. Because variance in throat feather length is greater between females than within females ($F_{77,\ 155}=18.51$, $P<0.001$; repeatability $r=0.73$), three feathers are enough to give an accurate representation of average feather length.

Hormone analysis

Yolk steroids were extracted by adding 3 ml of diethyl ether to the sample, vortexing for 15 min and centrifuging for 10 min (4°C, 2,000 rpm). This procedure was repeated a second time. The ether phases were decanted after snap-freezing tubes in an alcohol bath at −30°C, and the two combined phases evaporated under a stream of air. The dried extract was redissolved in 1 ml of 90% ethanol, kept at 4°C for 12 h and centrifuged for 10 min to further remove proteins (4°C, 2,000 rpm). Supernatants were dried and extracts were redissolved in 400–300 μl of steroid-free serum (DRG Labs, USA) depending on the amount of yolk in each sample. Yolk concentrations of testosterone (T) and androstenedione (A4) were determined by two different enzyme-linked immunosorbent assay kits highly specific for each hormone (DSL Labs, USA for T and DRG Labs, USA for A4). According to the manufacturers, cross-reactivity of the A4 antibody was less than 1% for all hormones tested and cross-reactivity of the T antibody was less than 1% for all hormones tested, except for 5α-dihydrotestosterone, which was 6.6%. Assays were performed according to manufacturer's instructions. Samples were analysed in duplicate with respect to a standard curve. For T, the intra-assay coefficient of variation was 7.4% and the inter-assay coefficient of variation was 7.71%. For A4, the intra-assay coefficient of variation was 6.62% and the inter-assay coefficient of variation was 6.03%. Variation in yolk androgens was greater between clutches than within clutches (T: $F_{39,\ 40}=2.85$, $P<0.001$; A4: $F_{39,\ 40}=3.01$, $P<0.001$), indicating that this measure was repeatable within females (repeatability T $r=0.49$; A4 $r=0.47$).

Pigment analyses

Egg pigmentation was analysed in 80 eggs belonging to 40 different clutches. Based on previous chromatographic studies on eggshell pigments in European starlings (*Sturnus vulgaris*; Miksik et al. 1996), we assayed the amount of the two main eggshell pigments: biliverdin and protoporphyrin IX. These pigments were determined as methyl esters by a slightly modified published method (for details, see: Miksik et al. 1996). Briefly, eggshells were cleaned and washed by distilled water and then solubilised (and esterified) in 15 ml methanol containing 8.5% concentrated sulphuric acid at room temperature in the dark for 2 days. The arising solutions were filtered (to remove shell membranes) and 7.5 ml chloroform and 5-ml distilled water were added then shaken. The lower chloroform phase was washed with 5 ml 10% NaCl, followed by distilled water, until the washing water had neutral pH (typically twice). The extract was evaporated to dryness and reconstructed in 1 ml chloroform. Standards for the quantification protoporphyrin IX and biliverdin (Sigma, St. Louis, MO, USA) were treated using the same procedure. Porphyrins were analysed by reversed-phase high-performance chromatography using Agilent 1100 LC system (Agilent, Palo Alto, CA, USA) consisting of a degasser, binary pump, autosampler, thermostatted column compartment and multiwavelength and fluorescence detectors. Chromatographic separation was carried out on a Gemini 5u C18 110A column (250×2 mm I.D.; Phenomenex, Torrance, CA, USA). The sample (20 μl) was injected into the column and eluted with a gradient consisting of (a) methanol water–pyridine 35:65:0.25 *v/v* and (b) methanol–acetonitrile–pyridine 90:10:0.25 *v/v* (flow rate 0.3 ml/min, temperature was 55°C). The gradient started at a–b 80:20 reaching 10:90 ratios after 15 min. For the next 10 min, the elution was isocratic followed by another 10 min isocratic elution at 100% b. Elution was monitored by absorbance at 410 nm and by fluorescence at 405ex–620em nanometer.

Data analyses

Egg thickness was measured using a Quanta 200 scanning electron microscope under low-vacuum conditions. Egg colouration was described in two different ways commonly used in previous studies of blue egg colouration: by means of PCA and by calculating the blue–green chroma index (BGC). (1) PCA performed on the reflectance data allowed an objective description of the colour spectra by the reduction of the large number of correlated variables into a few orthogonal variables that summarise most of the variation (Cuthill et al. 1999; Cherry and Bennett 2001). In the PCA, the first two principal components explain together 96.9% of the variance in the spectra. The first principal component (PC1) describes the variance in mean reflectance; it is flat throughout the spectrum of wavelengths and consequently represents achromatic brightness (89.0% of the variance; eigenvalue=36.5). The second principal component (PC2) shows variation in spectral shape thus measuring aspects of the egg's chromatic variation such as hue and saturation (7.9% of the variance; eigenvalue=3.2): high scores in PC2 are mainly characterised by low UV wavelengths (around 360 nm), high green wavelengths (around 550 nm) and relatively low red wavelengths (around 660 nm; López-Rull et al. 2007). Since PC1 scores are positively associated with brightness, we expect them to be negatively associated with the amount of pigments present in the egg (Moreno et al. 2006). (2) BGC was calculated as the proportion of total reflectance in the blue–green region (R400–570/R300–700) of the spectrum. BGC describes egg reflectance data in the blue–green region of the spectra, which is the region of greatest reflectance of biliverdin (Falchuk et al. 2002) and matches the region where spotless starling eggs reflect light maximally (López-Rull et al. 2007). BGC was correlated

with PC1 (Pearson's $R=-0.61$, $p<0.001$, $n=80$) and PC2 (Pearson's $R=-0.52$, $p<0.001$, $n=80$). Female quality was estimated by measuring female throat feather length, a trait that increases with age in both males and females (Hiraldo and Herrera 1974) and is positively related to female reproductive success as measured by fledgling number (López-Rull et al. 2007). Additionally, we calculated body condition as body mass to the cube of tarsus length. To test for an association between egg colour, eggshell pigments and yolk androgen content, we used variance component models including the nest as a random factor and eggshell thickness as a covariate. Non-significant terms were removed from each model ($P>0.05$). To test for an association between egg colour and female quality, we related mean egg colour to female feather length and female body condition. All variables were normally distributed, as shown by Shapiro–Wilk tests for normality (all $P>0.1$). Statistical analyses were performed with SPSS v.12 and STATISTICA.

Results

Egg pigmentation and egg colour

Chromatography analyses revealed that spotless starling eggshells contained the two different pigments that we expected to find, although biliverdin content made up for 99% of all pigment content: biliverdin (mean±SD 96.88± 42.52 nmol/g) and protoporphyrin IX (0.18±0.10 nmol/g). No correlation between these two pigments was found (Pearson's $R=0.03$, $P=0.77$, $n=80$). Biliverdin content was significantly related to measures of egg colouration: it was positively associated with BGC ($F_{1,\ 39}=14.20$, $P=0.0005$, $R=00.66$; Fig. 1b) and negatively with PC1 ($F_{1,\ 39}=33.70$, $P<0.0001$, $R=-0.69$; Fig. 1a). No association was found between biliverdin and PC2 ($F_{1,\ 39}=0.18$, $P=0.68$). Protoporphyrin IX was not related to any of our measures of egg colouration (BGC: $F_{1,\ 39}=0.007$, $P=0.93$; PC1: $F_{1,\ 39}=0.58$, $P=0.55$; PC2: $F_{1,\ 39}=0.95$, $P=0.33$).

Egg pigmentation and female quality

A model relating egg biliverdin content to measures of female quality revealed that the amount of biliverdin was positively associated with female body condition but not significantly associated with throat feather length (full model: $F_{2,\ 32}=5.83$, $P=0.008$, $R^2=0.24$; throat feather length: $t=1.84$, $P=0.07$, Fig. 2a; body condition: $t=2.68$, $P=0.012$, Fig. 2b). Despite the previously shown relationship between biliverdin and egg colouration, none of these female traits were significantly related to egg colouration as measured by either BGC, PC1 or PC2 (BGC full model: $F_{2,\ 28}=1.62$, $P=0.21$; throat feather length: $t=0.10$, $P=0.92$; body condition: $t=1.78$, $P=0.08$; PC1 full model: $F_{2,\ 28}=1.02$, $P=0.37$; throat feather length: $t=-0.42$, $P=0.66$; body condition: $t=-1.32$, $P=0.19$; PC2 full model: $F_{2,\ 28}=0.05$, $P=0.95$; throat feather length: $t=0.01$, $P=0.99$; body condition: $t=0.32$, $P=0.75$). Similarly, protoporphyrin IX was not related to female feather length or to female body condition (both $P>0.48$).

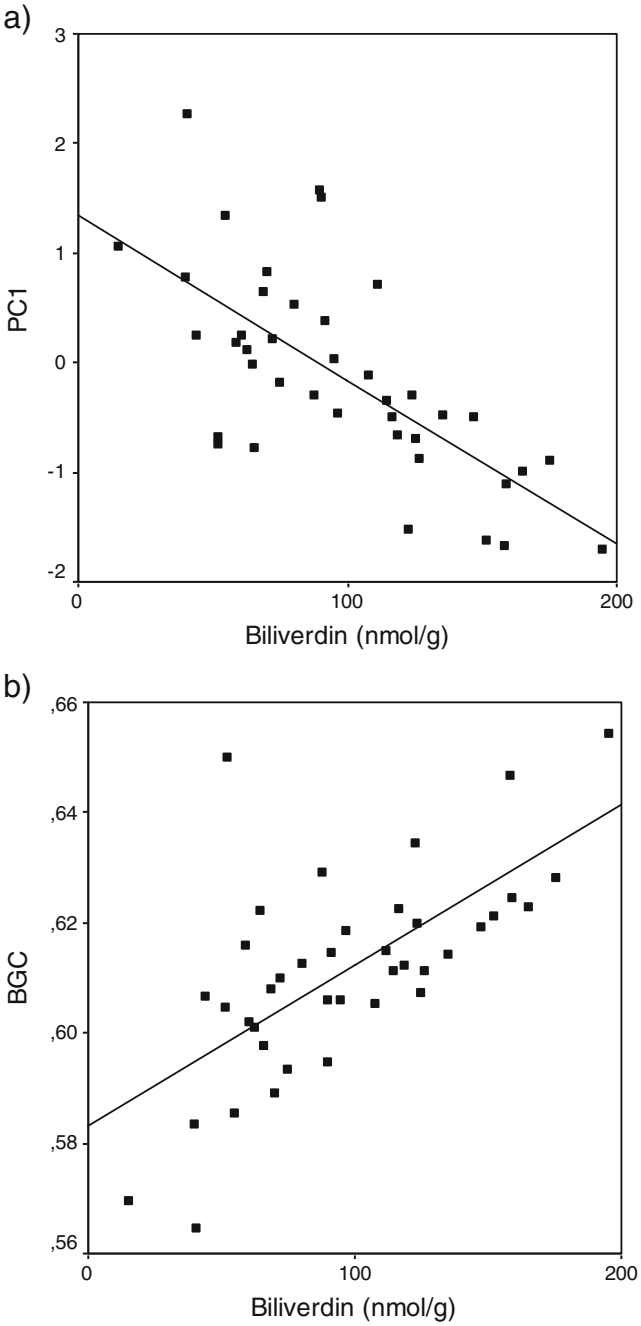

Fig. 1 Egg colouration in relation to biliverdin eggshell content. Darker eggs (**a**) reflecting more blue–green colouration (**b**) presented greater amounts of biliverdin in the eggshell. Presented values correspond to mean scores of PC1 and BGC

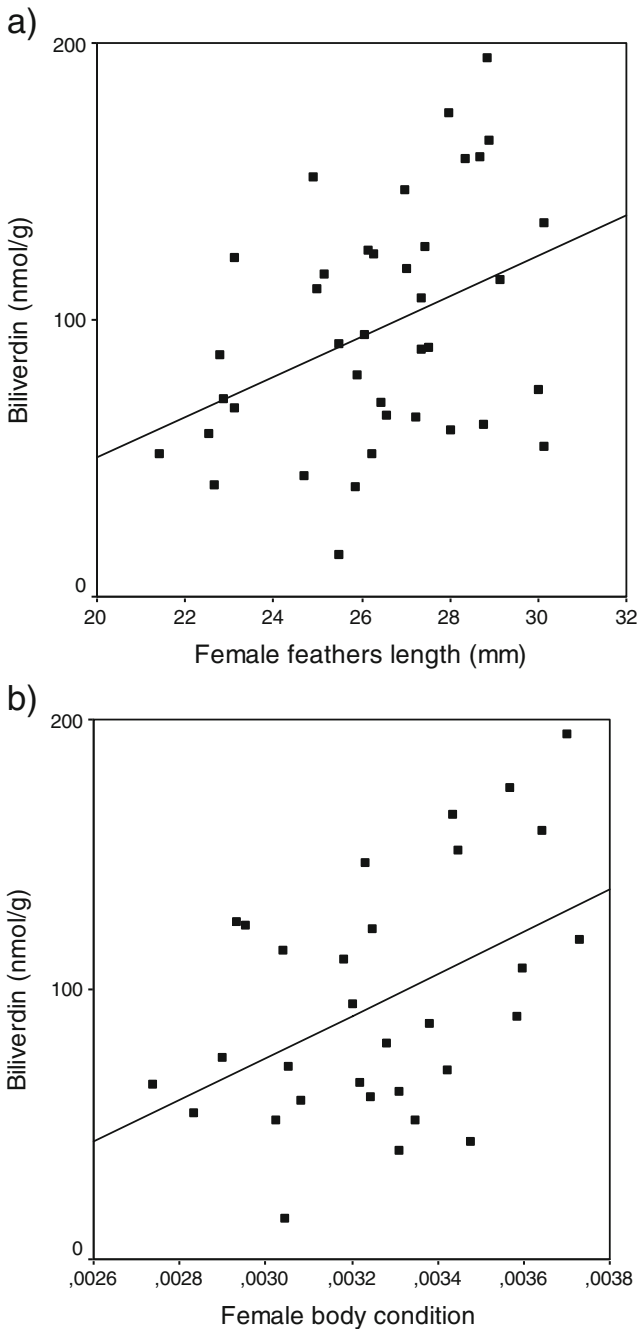

Fig. 2 Mean biliverdin eggshell content in relation to female throat feathers length (**a**) and female body condition (**a**)

Egg pigmentation and egg quality

Biliverdin was positively associated with the amount of testosterone in the yolk ($F_{1,\ 39}$=5.49, P=0.022, R=0.27; Fig. 3) but showed no association with A4 ($F_{1,\ 39}$=0.39, P=0.53). We repeated this analysis excluding one possible outlier that could have biased this relationship (see Fig. 3), finding that the correlation between biliverdin and yolk testosterone remained significant ($F_{1,\ 38}$=4.07, P=0.048, R=0.20). Yolk testosterone was not related to egg colouration (BGC: $F_{\ 1,\ 39}$=0.82, P=0.37, PC1: $F_{1,\ 39}$=0.65, P=0.42, PC2: $F_{1,\ 39}$=0.01, P=0.93). Eggshell thickness was not related to egg pigmentation either (biliverdin: $F_{1,\ 39}$=0.42, P=0.84; protoporphyrin IX: $F_{1,\ 39}$=0.32, P=0.57) or to egg colouration (BGC: $F_{1,\ 39}$=0.11, P=0.74; PC1: $F_{1,\ 39}$=1.43, P=0.23; PC2: $F_{1,\ 39}$=0.01, P=0.98). We found no significant association between female quality and yolk androgen deposition neither in yolk testosterone (full model: $F_{2,\ 19}$=0.14, P=0.87; throat feather length: t=0.19, P=0.84; body condition: t=−0.51, P=0.61) nor in yolk A4 (full model: $F_{2,\ 19}$=1.88, P=0.18, throat feather length: t=−0.53, P=0.60; body condition: t=1.91, P=0.08).

Discussion

Our results provide evidence that pigmentation in eggshells reflects female condition and egg quality. We found that females in better body condition laid eggs containing greater amounts of biliverdin in the eggshell and higher amounts of yolk testosterone. However, despite the strong correlation between biliverdin and egg colour, we found that egg colouration—in contrast with egg pigmentation—did not reflect accurately female condition or egg quality. The fact that females in better body condition allocate greater amounts of biliverdin in their eggs is in accordance with the main assumption of the sexually selected egg colour hypothesis (Moreno and Osorno 2003). This hypothesis proposes that biliverdin deposition in the

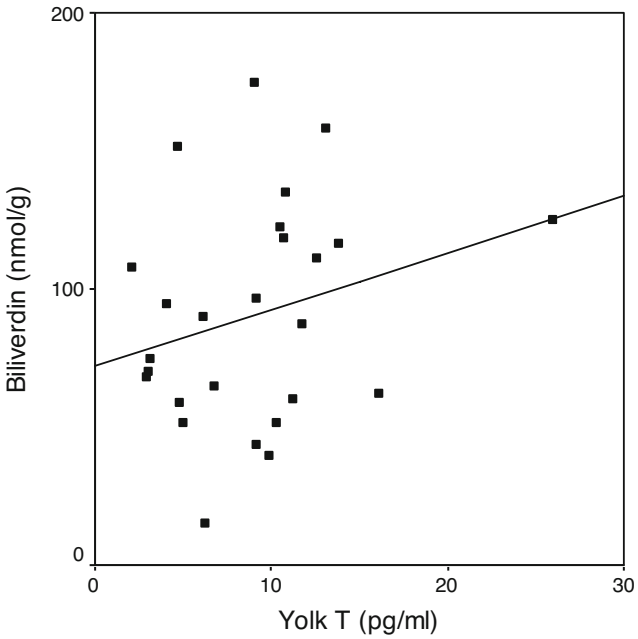

Fig. 3 Mean biliverdin eggshell content in relation to mean yolk testosterone

eggshell signals female capacity to control free radicals. This is because biliverdin and its reduction product, bilirubin, are potent antioxidants (Stocker et al. 1987), and only high-quality females could afford to remove this antioxidant from the system, particularly during egg-laying period when high levels of circulating progesterone can induce oxidative stress (von Schantz et al. 1999). However, due to the lack of a significant relationship between egg colour and female quality, our data do not provide evidence to support the signalling function of egg colouration. Nevertheless, the relationship between female condition and egg pigmentation is in accordance with previous work reporting positive relationships between egg colouration and different female quality traits such as body condition, age and immunological state (Morales et al. 2006; Siefferman et al. 2006).

We found that eggs containing greater amounts of biliverdin also contained greater amounts of maternal yolk testosterone. Yolk androgens are transferred from the mother to the egg (Schwabl 1993) and have been shown to adaptively affect offspring phenotype in both early and adult life. For example, there is evidence that nestlings hatched from eggs experimentally injected with high androgen levels beg more intensively and have shorter incubation periods (Schwabl 1996; Eising and Groothuis 2003), which is particularly relevant in situations of strong competition for food (Pilz and Smith 2004). In addition, when these birds reach adulthood, they develop ornaments earlier than control birds (Strasser and Schwabl 2004). In the spotless starling, recent work in the same population where this study was conducted has shown that, compared to control birds, nestlings hatching from T-injected eggs tended to gain more mass and had wider flanges, a trait that plays a role in food acquisition (Muller et al. 2007). This result provides support to the hypothesis that yolk androgens modulate competitive asymmetries such as those resulting from hatching asynchrony. Although yolk androgens seem to improve nestling growth in the spotless starling, not all females invest the same amount of androgens in their eggs, suggesting that this is a costly investment and that only high-quality females can afford it. It has been previously shown in the house martin (*Delichon urbica*) that immune-challenged females lay eggs with lower amounts of testosterone (Gil et al. 2006). This pattern is consistent with the positive relationship that we found in the present study between biliverdin and yolk testosterone since we expect females with higher levels of antioxidants to have a more efficient immune system (von Schantz et al. 1999) and be able to lay eggs with higher levels of testosterone (Gil 2003). A previous study in the European starling (Pilz et al. 2003) found that yolk androgens were not related with female body condition but with female age: older females allocated more yolk androgens to their eggs than first year females. In our population, we expect a positive relationship between age and phenotypic quality because of a high number of young, inexperienced females (Forslund and Pärt 1995). Since throat feather length is positively related to reproductive success in our population, we expected females with relatively long throat feathers to deposit higher levels of androgens in their eggs than females with relatively short throat feathers. However, we did not find an association between female traits (condition and feather length) and yolk androgens. In several species, females have been shown to increase yolk androgen levels when mated to attractive males (Gil et al. 1999, 2004; Tanvez et al. 2004), but since males were not identified in this study we cannot test a possible relationship with male attractiveness.

If egg colouration is a sexually selected signal, we expected it to be related to the amount of pigmentation in the eggshell. We quantified egg colour by two different methods, PCA and BGC, and related them to eggshell pigments. Similar to spectral curves found in previous works measuring blue–green egg colouration, spotless starling eggs showed two peaks of spectral reflectance: the greatest spectral peak corresponded to the blue–green segment of the spectrum and the secondary reflectance peak corresponded to the ultraviolet region of the spectrum. We found that two different pigments were present in spotless starling eggshells: biliverdin and protoporphyrin IX. While protoporphyrin IX was not related with any colour measurement (BGC, PC1, PC2), the spectral reflectance of blue–green eggs matched the reflectance spectrum of biliverdin to a large extent (Falchuk et al. 2002). Accordingly, the amount of biliverdin in the eggshell was negatively related with PC1 and positively related with BGC, indicating that darker eggs with a higher peak in the blue–green segment of the spectrum contained higher amounts of biliverdin. PC2 was not related to pigment concentration.

Despite the strong correlation between biliverdin and colorimetric variables, our measures of egg colouration—in contrast with egg pigmentation—did not reflect female condition or egg quality. In contrast, in a previous study carried out in the same breeding population, we found that females with longer throat feathers laid eggs with higher UV and lower green reflectance (López-Rull et al. 2007). The discrepancy between these results could be explained by the fact that colour measurements in the present study were taken 4 days after the onset of incubation, and colour may have partly faded by then. Because egg colour fades soon afterwards the egg has been laid, it is possible that our estimate of colour could have failed to reflect the original signal of female quality or condition.

Interestingly, in two different previous studies, the colour variable PC2—not related to eggshell biliverdin—showed

an association with different aspects of female quality, nestling immunity and differential male investment (Moreno et al. 2006; López-Rull et al. 2007). In a previous study, we found that spotless starling females with longer throat feathers laid eggs with higher UV and lower green reflectance (lower PC2) and received more male feeding contribution than females with shorter feathers who laid eggs with lower UV and higher green reflectance (López-Rull et al. 2007). PC2 is supposed to measure the aspects of the egg's chromatic variation such as hue and saturation: high scores in PC2 reflect less towards the green part of the spectrum and more towards the UV part of the spectrum. So, if PC2 is not related with biliverdin, what does it exactly reflect, and how is its honest signalling function maintained? The sexually selected egg colour hypothesis is based on the antioxidant properties of biliverdin whose allocation signals female antioxidant capacity, but our data show that PC2 is not related to biliverdin. Recently, Siefferman et al. (2006), based on the fact that white and pink eggshells also reflected light in the UV region, proposed that the UV reflection peak of blue–green eggs is coloured by the underlying eggshell (i.e. calcium) and not by biliverdin pigments. As far as we know, no pigments are present in the underlying eggshell, and we found that eggshell thickness did not vary in relation to biliverdin content and egg colour variation. Thus, the UV reflection peak may still be a structural non-pigmented colouration, but further research is necessary to establish the origin of this colour variation.

In summary, our data showed that the amount of biliverdin deposited in the eggshell predicted both female condition and yolk testosterone levels. However, further studies are needed to investigate the signalling function of blue egg coloration because, despite the strong correlation between biliverdin and egg colour, we found no evidence that colorimetric variables reflected female condition or yolk testosterone content in our study population.

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