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Experimental evidence that egg color indicates female condition at laying in a songbird

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The signaling hypothesis of eggshell coloration in birds is based on the assumption that females of species with blue-green eggs signal their phenotypic quality to their mates through deposition of the antioxidant biliverdin as pigment. Egg pigmentation may be an expression of the condition of females at laying or of genetic linkages between egg color and female performance variables. We have supplemented 16 pied flycatcher, *Ficedula hypoleuca*, females with mealworms before and during laying and compared the mass and color of their eggs as measured on the day of laying to those of 16 control females with the same nest construction and laying dates and clutch sizes. Supplemented females laid significantly heavier and more intensely blue-green eggs than control females. Egg blue-green chroma was significantly associated with the amount of biliverdin in eggshells. Egg color, and thus biliverdin content, is an expression of female condition at laying. *Key words:* biliverdin, color analysis, egg coloration, eggshell pigments, female condition, food supplement, nutrition, phenotypic quality, sexual selection, signaling. *[Behav Ecol 17:651–655 (2006)]*

A recent hypothesis (Moreno and Osorno 2003) posits that the blue-green color of the eggshells of many avian species with biparental care of young represent signals of female phenotypic quality to their mates. The pigment responsible for these colors, biliverdin, is a potent antioxidant (Stocker et al. 1987; Kaur et al. 2003), whose allocation to eggshells may indicate traits related to the antioxidant capacity of laying females. According to the differential allocation hypothesis (Burley 1986; Sheldon 2000), males would contribute differentially more to parental care of offspring of females with exaggerated signals (eggs with a higher blue-green chroma), and thus of high quality, and females would benefit from signaling quality through improved offspring condition. This application of sexual selection theory (Darwin 1871; Andersson 1994) to postmating investment is based on the crucial assumption that eggshell color is significantly associated to female condition, immunocompetence or stress (Moreno and Osorno 2003). Moreno et al. (2005) detected significant associations between female immunocompetence and degree of eggshell pigmentation in pied flycatchers *Ficedula hypoleuca*. They also found a significant increase in egg lightness with the laying sequence, suggesting pigment limitation in females (Moreno et al. 2005). Recently, Morales et al. (2006) have found that egg blue-green chroma reflects egg maternal antibody contents and nestling survival probability in the same species. Siefferman et al. (2006) have found a positive association of egg blue-green chroma (henceforth egg BGC) with female condition in eastern bluebirds *Sialia sialis*.

These field studies were observational and thus only suggest that eggshell color has informational content regarding female quality (Moreno et al. 2005). Experimental evidence is needed to fully support these associations. The actual mechanism through which egg color is linked to female immunocompetence remains to be elucidated. Eggshell color could be a condition-dependent trait and therefore express female immune capacity as mediated through body condition and food availability at laying in general, or, in particular, through availability of antioxidants and their effect on immune responses (Alonso-Álvarez et al. 2004; Horak et al. 2004). Otherwise, the relationship between immune capacity of females and coloration of their eggs could result from a genetic linkage between egg pigment synthesis and immune response. If food and/or antioxidant availability at laying is of paramount importance, the genetic quality content of the signal may be less important than the maternally derived products expected to be added to the eggs in relation to female condition and thereby affecting offspring quality (Blount et al. 2002; Grindstaff et al. 2003; Morales et al. 2006). Another unverified assumption of the hypothesis is that egg BGC is associated with eggshell pigment content.

In the present study, we have experimentally provisioned female pied flycatchers with supplementary food before and during laying to establish the importance of food availability at laying for eggshell pigmentation. The study has been conducted in the same population as our earlier correlative study (Moreno et al. 2004, 2005). We predict that if eggshell coloration is related to female condition at laying, provisioned females will lay more intensely blue-green eggs than control females. We have also analyzed eggshell biliverdin content and related it to egg BGC.

METHODS

Food supplementation

We conducted our experiment on a population of pied flycatchers subjected to a long-term study in Valsaín, central Spain, in 2005 (see Moreno et al. 2004, 2005 for details about

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study species and area). Nest-boxes were inspected daily to detect initiation of nest construction. As soon as nest construction by pied flycatchers was observed in any of the 300 nest-boxes erected in the study area, a small green plastic container (semicircular with a radius of 5.4 cm and depth of 3.7 cm) was fixed under the nest-box entrance. Each experiment initiation day, pairs of nests were assigned randomly to the experimental and control treatments. In the experimental nest-boxes, 9 g of live mealworms, *Tenebrio molitor*, was delivered each day into the containers, whereas no mealworms were left at the control nest-boxes. Assuming that the assimilation efficiency of mealworms by insectivorous birds is 0.65 and that their energy content amounts to 8.5 kJ/g (Moreno 1989), a daily ration of 4.5 g (assuming that males and females shared equally) would correspond to supplementary daily energy intakes of 25 kJ. Female pied flycatchers expend 60 kJ per day during incubation (Moreno and Sanz 1994) and 55 kJ per day during nestling care (Moreno et al. 2001). Thus, 25 kJ would cover 40–50% of the daily energy needs of female flycatchers. In the experimental nest-boxes, the amount of mealworms left in the containers was weighed daily with an electronic balance to the nearest 0.1 g, and 9 g was left after the visit. We could thus calculate the daily amount of supplementary food consumed. Control nest-boxes were also visited without leaving any supplementary food. Laying of eggs was followed through daily visits. One day after laying of the last egg in each clutch, the experiment was interrupted and the container removed. Only nest-boxes where more than half of the daily ration was consumed on each visit have been included in the experimental treatment, as some pairs only sporadically used the feeders. This tried to ensure that food consumption was significantly increased above the control level. Control pairs corresponding to excluded experimental pairs have also been excluded. All experimental pairs consumed more than 90 g during the provisioning period. In total, 16 pairs of nest-boxes have been included in the analyses. The date of initiation of experiments is not exactly the same for both groups because some nests could not be paired with one of the same date and were paired with nests initiated 1 day before or after.

To determine food use, video cameras were placed near 12 of the 16 experimental nest-boxes before laying commenced, and films of 1.5- to 3-h duration of the nest-box front were obtained. The numbers of food items collected by male and female territory owners were computed from the films.

Color measurement

The color of eggs was measured in the field with a portable battery-driven MINOLTA spectrophotometer CM-2600d (Minolta Co. Ltd, Osaka, Japan) on the day of laying. Eggs were placed directly with their broad pole on a target mask of the spectrophotometer with a diameter of 8 mm, so that eggs completely filled the space covered by the specimen measuring port. Reference calibrations against zero and a white standard tablet associated with the apparatus were performed periodically according to apparatus instructions. Reflectance spectra for each egg are automatically produced as means of 3 sequential measurements of each egg by changing the position of the egg with respect to the apparatus. The SPECTRAMAGIC software (Minolta Co. Ltd) was used to analyze spectra. The spectrophotometer covers the reflectance spectrum above 360 nm in intervals of 10 nm. BGC was calculated as the proportion of total reflectance that is in the blue-green region ($R_{400–570}/R_{360–700}$) of the spectrum. We used BGC to describe egg reflectance data because this region corresponds to the region of least absorbance (and therefore greatest reflectance) of biliverdin (Falchuk et al. 2002) and because pied flycatcher eggs reflect light maximally in this region (Moreno et al. 2005). This method of color estimation has been successfully employed in another study of a species with blue-green eggs (Siefferman et al. 2006). There is a strong correlation between mean BGC and brightness as derived from principal component analysis (PCA) when including all eggs laid in the population ($r_{82} = 0.85$, $P < 0.001$), eggs with a higher BGC being darker (see Moreno et al. 2005 for calculations of PCA from spectra). There is also a strong correlation with PC2 ($r_{82} = -0.63$, $P < 0.001$) as coefficients of PC2 were negative at blue-green wavelengths (Moreno et al. 2005). BGC and results from PCA are therefore redundant, so we will just analyze BGC.

Given the possible reflectance of pied flycatcher eggs in the UV range, we measured in the lab 10 eggs collected for another study both with the MINOLTA spectrophotometer and with an OCEAN OPTICS USB2000 spectrophotometer covering the range 300–800 nm. The OCEAN OPTICS spectrophotometer has ultraviolet (deuterium) and visible (tungsten halogen) lamps and a bifurcated 400-μm fiber-optic probe (Dunedin, Florida), providing a reading area of 1 mm. All measurements were relative to a white "Spectralon" tablet (WS-1-SS), and one reference measurement was made for each egg. The spectral curves were generated by using the OOIBase software. We calculated the proportion of reflectance in the range 400–570 nm for the spectra obtained with both apparatuses ($R_{400–570}/R_{360–700}$ for MINOLTA and $R_{400–570}/R_{300–700}$ for OCEAN OPTICS) in order to find if these proportions were correlated. A positive and significant association would indicate that results would not change if we had used the spectrophotometer covering the UV range in the field.

Table 1

Differences in experimental and breeding variables according to food supplementation treatment for 16 pairs of nests

	Food supplemented	Control		
	Mean ± SD	Mean ± SD	Test	*P*
Mass mealworms (g)	113.5 ± 21.6			
No. exp. days before laying	8.6 ± 3.10	8.9 ± 3.52	0.52[a]	0.60
Laying date	45.1 ± 4.5	46.6 ± 4.6	1.32[a]	0.19
Clutch size	6.0 ± 0.52	5.9 ± 0.70	−0.41[a]	0.68
Egg mass (g)	1.70 ± 0.11	1.62 ± 0.10	2.64[b]	**0.019**
Egg BGC	0.594 ± 0.010	0.586 ± 0.009	2.28[b]	**0.038**

SD, standard deviation. *P*-values in bold < 0.05.

[a] Wilcoxon matched-pairs test.

[b] *t* from paired *t*-test.

Pigment analysis

We collected 31 eggs from 25 pied flycatcher clutches in another study area in 2004 (see Morales et al. 2006 for study area and collection protocol). Biliverdin was extracted from individual eggshells by adding, in this order, 0.6 ml of acetonitrile and 0.5 ml of HCl 3 N to the whole shell in Eppendorf tubes. After 5 min of the addition of reagents, the tubes were capped and subsequently vortexed for 15 s and sonicated for 10 s in an Ultrasons-H (Selecta, Barcelona, Spain). Samples were centrifuged for 10 min at 12 000 r.p.m. in a Biofuge Pico Heraeus (Kendro Laboratory Products, Osterode, Germany), and 0.35 ml of organic supernatant was transferred to glass vials for high-performance liquid chromatography (HPLC) analysis. HPLC analyses were conducted following Mateo et al. (2004) with some modifications. The column was maintained at 63 °C, and the UV detection was done at 377-nm wavelength (the peak of absorbance of biliverdin). The quantification of samples was performed using calibration curves constructed by standard addition of 0, 10, 20, 40, and 80 nmol of biliverdin (Frontier Scientific Europe, Carnforth, United Kingdom) to 0.2 g of white eggshell of domestic hens and processed as samples. Calibrations were injected at the beginning of the analytical sequence and every 12 injected samples. Concentration of biliverdin was expressed as nmol/g of dry weight of eggshell.

Statistics

Paired tests are used throughout given the design of the experiment. Nonparametric tests are used when dependent variables were not normally distributed. Egg parameters are significantly repeatable within clutches in the study population (Moreno et al. 2004, 2005), so clutch means will be used in analyses.

RESULTS

Experimental pairs consumed on average 113 g of mealworms during the approximately 2 weeks of food supplementation (Table 1). In 12 observations of different experimental pairs (26.6 h of films), males consumed 5.6 ± 2.3 mealworms and females 5.0 ± 4.6 mealworms per hour ($t = -0.39$, $P = 0.71$). No birds other than the nest owners were observed at the feeders. Control and experimental females did not differ with respect to duration of the experiment, laying date, or clutch size (Table 1). Egg mass differed significantly between treatments, with supplemented females laying heavier eggs (Table 1). Experimental females laid eggs with a significantly higher BGC than control females (Table 1).

The 2 minima and the single maxima of reflectance spectra (Moreno et al. 2005) did not differ significantly between treatments ($P > 0.80$), indicating that the range of colors of the eggs of experimental females were within the natural range for the population. The reflectance spectra of 10 collected eggs obtained with the 2 apparatuses differed depending on the inclusion or not of the range 300–360 nm (Figure 1). However, BGC obtained from both types of spectra was positively and significantly correlated, indicating that the results do not depend on the inclusion of the UV range (Figure 2). Eggshell biliverdin content of 31 eggs collected from another population was positively associated with egg BGC (Figure 3).

DISCUSSION

Pied flycatcher females supplemented prior to and during laying laid heavier and more intensely blue-green eggs than unsupplemented females. Both effects of food supplementation can be attributed to an improved condition of females. Increased egg size in supplemented females has been found in several food supplementation experiments (Wiebe and Bortolotti 1995; Ramsay and Houston 1997; Reynolds et al. 2003). Improved body condition of females has also been found as an effect of supplementary feeding before laying (Schoech 1996; Cucco and Malacarne 1997; Gloutney et al. 1999). We could not ascertain this directly as females cannot be captured at this early stage without risking desertion. However, the assumption that food supplements improved female condition appears reasonable. Siefferman et al. (2006) have also found in a correlative study that females in better condition lay more blue-green eggs in the eastern bluebird. Also, the color of eggs of supplemented females did not differ from the natural color range in the population, indicating that the effect on egg color was an expression of female nutrition rather than a possible direct effect of pigments in the diet.

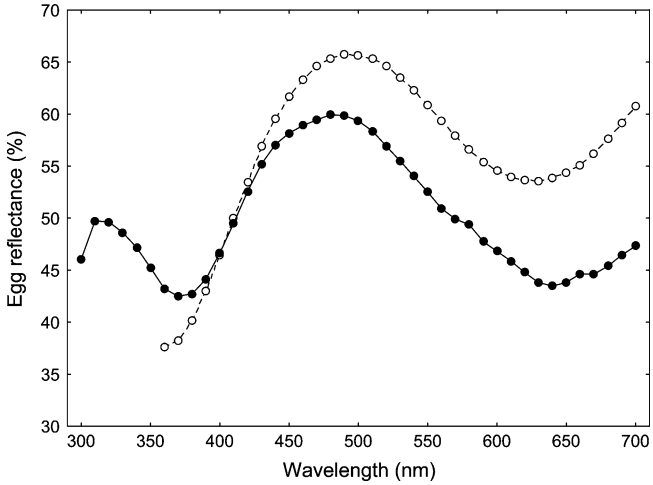

Figure 1
Reflectance spectra of 10 pied flycatcher eggs obtained with a MINOLTA spectrophotometer covering the range above 360 nm (open symbols) and an OCEAN OPTICS spectrophotometer covering the range above 300 nm (filled symbols). Values are means for the 10 eggs.

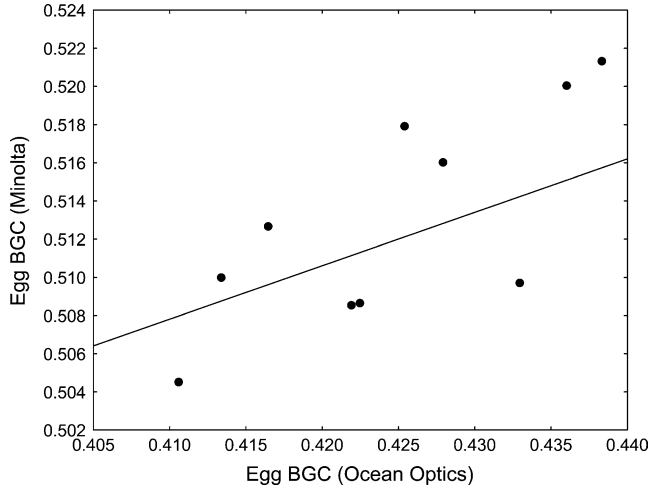

Figure 2
Correlation between egg BGC obtained with an OCEAN OPTICS spectrophotometer ($R_{400–570}/R_{300–700}$) and egg BGC obtained with a MINOLTA spectrophotometer ($R_{400–570}/R_{360–700}$) ($r = 0.75$, $P = 0.012$).

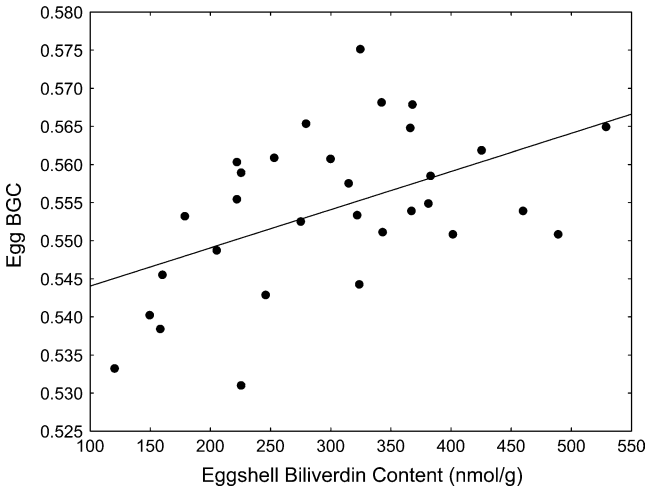

Figure 3
Correlation between egg BGC and eggshell biliverdin content as measured with HPLC ($r = 0.50$, $P = 0.004$).

Experimental eggs expressed a greater proportion of light in the blue-green region than did control eggs. This part of the spectrum corresponds to the maximum reflectance spectrum of biliverdin (Falchuk et al. 2002). Falchuk et al. (2002) clearly show that biliverdin does not reflect in the UV range. The inclusion of the UV range rendered BGC strongly positively correlated with BGC used without including the UV range. Thus, the exclusion of the range 300–360 nm from our measurements in the field does not affect our main result. We have also shown through HPLC analysis of eggshells that pied flycatcher eggshell BGC accurately reflects biliverdin content. This is the first experimental evidence that blue-green eggshell color and biliverdin content are an expression of female nutritional condition in birds as required by the signaling hypothesis of Moreno and Osorno (2003). In a previous observational study in the same population (Moreno et al. 2005), female immunocompetence at hatching of the young was positively associated with egg darkness as derived from PCA, and we know that egg darkness is positively associated with egg BGC as measured in the present study. Egg BGC is also significantly associated to maternal antibody content of eggs and nestling survival probability in pied flycatchers (Morales et al. 2006). The present experimental result supports this association between female condition and egg coloration. The pigment responsible for egg color in the study population appears to be limiting as its deposition markedly decreases throughout the laying sequence (Moreno et al. 2005). However, although females could be signaling present condition and health (antioxidant capacity, immunocompetence) as mediated by nutrition or by the genetic components of such traits (see Soler et al. 2005 for comparative support for the hypothesis), our experiment has no bearing on the signaling aspect of egg color. For this, it has to be shown experimentally that males respond to the signal. In our experiment, males also benefitted from the food supplementation, so females could be in fact responding to male condition by investing more in eggs in a differential allocation scenario (Burley 1986). Although no hypothesis about alternative beneficial effects of eggshell biliverdin on offspring fitness has yet been formally presented, possibilities can be envisaged given the antioxidant and antiviral properties of the pigment (Falchuck et al. 2002; Kaur et al. 2003).

What our results clearly indicate is that the associations between egg color and female immunocompetence and maternal effects found in previous studies (Moreno et al. 2005; Morales et al. 2006) are mediated through general nutritional state. Also, they show that eggshell color, and thus biliverdin content, reflects female condition at laying. Researchers and conservationists can use this knowledge to estimate the condition of breeders in avian populations through egg color analyses without using disruptive and destructive methods.

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