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Evidence for the signaling function of egg color in the pied flycatcher *Ficedula hypoleuca*

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A recent hypothesis proposes that the bright colors, especially blue and green, of many avian eggs may function as signals of female or offspring phenotypic quality or condition to males in species with biparental care, inducing them to allocate more effort to their offspring. The pigment determining blue and green egg colors is an antioxidant whose availability for eggshell coloring may be limited. To test the signaling function on a species with blue eggs, the pied flycatcher *Ficedula hypoleuca*, we measured egg color with a spectrophotometer on the day of laying and obtained two principal components from their reflectance spectra that together explained 99% of variation and represented shell lightness, and hue and saturation, respectively. We also measured female immunocompetence during the nestling period through the response to phytohemagglutinin as a measure of cell-mediated immunity and the response to a tetanus vaccination as a measure of humoral immunity. The total amount of immunoglobulins in blood of females and of nestlings before fledging was also estimated. Mean within-clutch egg darkness was positively associated with both measures of female immunocompetence, while better female condition was associated with colors tending away from intermediate and toward short wavelengths. Ageing female laid lighter eggs. The mean within-brood level of nestling IgY was also associated with mean within-clutch egg colors tending away from intermediate and toward short wavelengths. Mean egg darkness decreased linearly during the laying sequence, suggesting pigment limitation. Males were observed frequently visiting nests during the laying period, allowing them to observe eggs before the start of incubation. These results support the signaling hypothesis for explaining bright colors of avian eggs. *Key words:* antioxidants, egg color, female signaling, immunocompetence, sexual selection. *[Behav Ecol 16:931–937 (2005)]*

Egg coloration has until recently been interpreted as a response to the selective pressures imposed by nest predators or brood parasites (for a recent review see Underwood and Sealy, 2002). However, a recent hypothesis has proposed that the blue and green colors of the eggs of many species with biparental care may in fact constitute signals of females to their mates about their phenotypic quality in order to induce a higher allocation of paternal care (Moreno and Osorno, 2003). This hypothesis is based on the antioxidant qualities of the blue-green eggshell pigment biliverdin (Kaur et al., 2003; McDonagh, 2001) and on the differential allocation version of sexual selection proposed by Burley (1986) as applied to female signals. The deposition of biliverdin in the eggshell may signal antioxidant capacity during the laying period, when progesterone action may induce an especially high level of oxidative stress (von Schantz et al., 1999). Although the implied relationships between immune function, biliverdin deposition, and egg color variation contain several untested assumptions, the signaling hypothesis offers a viable explanation for previously unexplained interspecific variation in egg coloration (Soler et al., 2005).

If avian egg color is a sexually selected trait signaling female quality to mates (Moreno and Osorno, 2003), we should expect intraspecifically that females with more intensely blue or green eggs should show a higher immune response, given the sensitivity of the immune system to oxidative stress (von Schantz et al., 1999). Also, females with more colorful eggs should show a better condition at laying, if condition is somehow related to antioxidant capacity. Furthermore, if females are signaling not only their own quality but also that of the eggs themselves, as mediated through maternal effects or heritable traits (Moreno and Osorno, 2003), we could expect that nestlings resulting from more intensely colored eggs should show a better condition or a higher immune capacity. The assumption that males visit nests during the laying period, when recently laid eggs can be observed at leisure, has to be met for the hypothesis to be viable. A further assumption is that biliverdin is actually a limiting factor for females, which should be expressed in reduced allocation throughout the laying sequence.

A previous study of pied flycatchers *Ficedula hypoleuca* (Moreno et al., 2004) showed that the chromaticity of the characteristic blue eggs of this species was associated with male provisioning rate to resulting nestlings. Furthermore, nestling condition was positively correlated with male effort, thus supporting a link between signal intensity and fitness benefits for the signaling females. Although these results were not experimental and can therefore be interpreted differently, they at least offer tentative support for the signaling hypothesis. However, in that study no association was sought between egg coloration and female or offspring immune capacity. In the present study we have measured egg coloration as well as female and nestling immune response. For females, we have used as measures of immune capacity the response to phytohemagglutinin (PHA) injection, a standard assay to estimate T-cell–mediated immune response (Lochmiller et al., 1993; Smits et al., 1999), and the response to a tetanus vaccine as a specific measure of humoral immune response (Ilmonen et al., 2000), as well as the total amount of immunoglobulins (IgY) in the blood (Morales et al., 2004), and related these traits to egg color as measured with a spectrophotometer.

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For nestlings, we have used the PHA response and the total amount of IgY in blood as measures of immunocompetence. We have also tested the assumptions that males visit nests frequently during the laying period and that color intensity decreases during the laying sequence of clutches due to limitation of pigment allocation to eggs by females.

MATERIALS AND METHODS

Study population

We studied egg coloration in a population of pied flycatchers breeding in Valsaín, central Spain, which has been subjected to a long-term study since 1991 (Sanz et al., 2003). All individuals are individually ringed, and their reproductive success and survival between years is known. On first capture, females were classified as of one or more years according to Svensson (1984). We have assumed for all recruited females of more than 1 year who were not raised in the study area a minimum age of 2 years (Sanz and Moreno, 2000). For some females exact age was known as they were ringed as nestlings in the study area. However, for most females age as analyzed here represents a minimum estimate. Clutch size ranges from 4 to 7 eggs, with a mode of 6 eggs.

Egg color measurement

Eggs were weighed with a portable electronic balance to the nearest 0.1 g on the day of laying as well as measured with a portable battery-driven Minolta spectrophotometer CM-2600d, which covered the reflectance spectrum above 360 nm. We assume that there may be unmeasured variation in egg color in the UV range below 360 nm that may be associated to signaling capacity and that should be considered in future studies. Eggs were placed directly with their broad pole on a target mask of the spectrophotometer with a diameter of 1 cm, so that eggs completely filled the space covered by the specimen measuring port. Reflectance spectra for each egg were obtained as means of three sequential measurements of each egg by changing the position of the egg with respect to the apparatus. Reference calibrations against a white standard were performed periodically according to apparatus specifications.

Principal components analysis (PCA) was performed on reflectance spectra for each egg according to Bennett et al. (1997) and Cherry and Bennett (2001). PCA transforms reflectance at 20-nm intervals into a few orthogonal variables, the principal components (Cuthill et al., 1999). As in other studies of animal coloration, the first principal component (PC1) has similar weightings for all wavelengths and thus describes variation in mean or total reflectance, essentially brightness or lightness (Bennett et al., 1997; Endler, 1990). As mean reflectance comprises most of the variation between spectra, PC1 usually explains more than 90% of the variation in natural spectra (Endler and Thery, 1996; Hurlbert, 1986). The coefficients relating PC1 to the original reflectance data are all negative and of similar magnitude, so the correlation between PC1 and the mean reflectance across all wavelengths is −1.0 (Cuthill et al., 1999). Thus higher PC1 values denote a lower mean reflectance and vice versa. Principal components 2 and 3 (PC2 and PC3 respectively) represent variation in spectral shape and are therefore indirectly related to hue and saturation (Endler, 1990; Endler and Thery, 1996). According to Cuthill et al. (1999), such a method objectively describes variation in reflectance spectra.

Blood sampling and immunization

On day 1 of the nestling period (hatching day = day 0), females were captured at the nest, weighed to the nearest 0.1 g with a Pesola spring balance, and sampled for blood by venipuncture. On day 11, females were recaptured, blood sampled, measured (tarsus and wing length according to Svensson, 1984), and weighed. They were subsequently subjected to the single-wing PHA injection protocol (see Morales et al., 2004 for details) and released. On day 12 most of them were recaptured, weighed, and measured with respect to their inflammatory response to the PHA injection. Repeatabilities of preinjection measurements of wing-web thickness was 0.9 ($F_{50,102} = 28.6$, $p < .001$) and of postinjection measurements was 0.85 ($F_{37,76} = 17.5$, $p < .001$). Blood was centrifuged in the field with a portable centrifuge, and samples were cold-stored in the field before being frozen in the laboratory at the end of the day. From the serum fraction of both initial (day 1) and final (day 11) blood samples, we obtained the total amount of immunoglobulins (IgY) according to the protocol by Martínez et al. (2003). IgY are the functional equivalent of mammalian IgG. Total serum immunoglobulins in pied flycatcher females remain constant throughout the breeding season, respond to parasitism, and stress and are positively associated with PHA response when controlling for these other factors (Morales et al., 2004). High levels of IgY have been interpreted as a sign of a good immune capacity (Morales et al., 2004), although they may also reflect current levels of infection. Given that only PHA response was associated to female IgY levels during the nestling stage (Morales et al., 2004), we have not corrected for blood parasites or stress variables.

To estimate humoral immune response, on day 1 of the nestling period we injected females intraperitoneally with 100 μl of diphtheria-tetanus vaccine (National Public Health Institute, Helsinki, Finland), which contains two antigens novel to the birds (diphtheria 38Lf and tetanus 10Lf, mixed with the adjuvant aluminum phosphate at 1.0 mg/ml). By using killed pathogens (human diphtheria-tetanus vaccine) we excluded the direct negative effects of parasites and, thus, tested only for the effects of activating the host immune defense (Ilmonen et al., 2000). In previous studies on blue tits (*Parus caeruleus*) there is no evidence that diphtheria-tetanus vaccination would have any toxic effects on survival (Råberg et al., 2000). The females were blood sampled prior to injection and on day 11 of the nestling period, when peak primary antibody levels are expected (i.e., 10–14 days postimmunization for the primary response) (Roitt et al., 2001; Svensson et al., 1998). We measured female humoral immune responsiveness as the antigen-specific antibody levels in the sera by means of an enzyme-linked immunosorbent assay (ELISA) described by Kilpimaa et al. (2004), with the only difference that we have measured the specific humoral immune response to the tetanus toxoid only (Berna Biotech España S.A.), as the diphtheria toxoid was unavailable. The linear range of this antibody-antigen response, as well as the optimal serum dilution of these samples (1:40) had been previously determined. This assay has proved to work as shown by higher post- than preinjection antibody levels against the tetanus toxoid (paired *t*-test: $t_{55} = -4.83$, $p < .0001$). The preinjection serum samples from each female were run to measure the background level of each individual, and thus, in further analyses, we controlled for the effect of this initial background level (Ilmonen et al., 2000). Postinjection serum samples were added in duplicate, and the average of these was the measure of specific antibody levels. In order to compare specific antibody levels of samples run on the same day but in different plates, we used pooled serum from immunized females as standard (Kilpimaa et al., 2004; Råberg et al., 2000; Svensson et al., 1998). The standard was within the linear range of response and was run four times in each plate. We calculated the factor of correction in each plate by dividing the average value of the standard in all the plates by the average value of the

standard in each plate. As the final specific antibody level of each sample we used the mean optical density multiplied by the corresponding factor of correction.

Nestlings were weighed and measured on day 12 in the same way as adults, and a blood sample was obtained by brachial venipuncture. Blood was processed in the same way as for females. Nestling IgY level was obtained as for females. Nestlings were subjected to the same PHA protocol as females (Morales et al., 2004; Moreno et al., 2005). Ectoparasites were estimated given their strong effect on nestling fitness (Merino and Potti, 1995; Moreno et al., 1999). The main ectoparasites of pied flycatcher nestlings in our study population are the mite *Dermanyssus gallinoides* and larvae of the parasitic fly *Protocalliphora azurae* (Moreno et al., 2002). Scores of mite infestation were estimated according to Merino et al. (1998). The numbers of pupae of the parasitic fly were counted after nestlings fledged by dismantling nests and extracting parasites from the nest material.

Male behavior

On the day the fourth egg was laid, 26 nests were filmed for 3 h with video cameras placed in front of the nest-box entrance. Visits of males to the nest and the total time spent by them inside the nest-box were computed from the films.

Statistics

To perform statistical analyses, means of egg reflectance spectral data for each clutch were computed. To do that we estimated repeatabilities within clutches of egg weights and color measures. Nestling condition, IgY level, and PHA response were significantly repeatable within broods ($p < .001$), so we will use brood means in statistical analyses. Sample sizes differ because not all females could be recaptured at the correct time for immunocompetence tests.

RESULTS

Egg color

Pied flycatcher eggs appear greenish-blue in the human visible spectrum, with a mean peak of reflectance at 510 nm (Figure 1). The first two principal components together explain 99% of the variance in spectra (Figure 2). PC1 explains 93.7% of the spectral variance and shows constant and negative coefficients for all wavelengths (Figure 2). Consequently PC1 scores represent the exact opposite of mean reflectance or achromatic brightness (Cuthill et al., 1999) as expressed by its strong negative relation with lightness L in the CIELAB color space ($r_{1,69} = -0.99$, $p < .001$). Thus, PC1 scores are positively associated with egg darkness and amount of pigmentation and should accordingly reflect female phenotypic quality. PC2 explains 5.3% of the total variance and 83.6% of the color variance (when removing variation due to mean reflectance), and so represents aspects of the egg's chromatic (hue and saturation) variation. The remaining factors explain too little variance and will not be considered further. PC2 coefficients vary according to wavelength, being low and negative at intermediate wavelengths (blue-green colors) and high and positive at short (UV) and long wavelengths (Figure 2). Given the higher coefficients at short wavelengths, it can be interpreted as a contrast between UV and green reflectance, with more positive values denoting a tendency toward UV reflectance and more negative values a trend toward more green colorations. PC1 and PC2 were significantly repeatable within clutches (PC1: $r = .38$, $F_{73,337} = 4.8$, $p < .001$; PC2: $r = .30$, $F_{73,337} = 3.6$, $p < .001$), so we will use clutch means in further analyses.

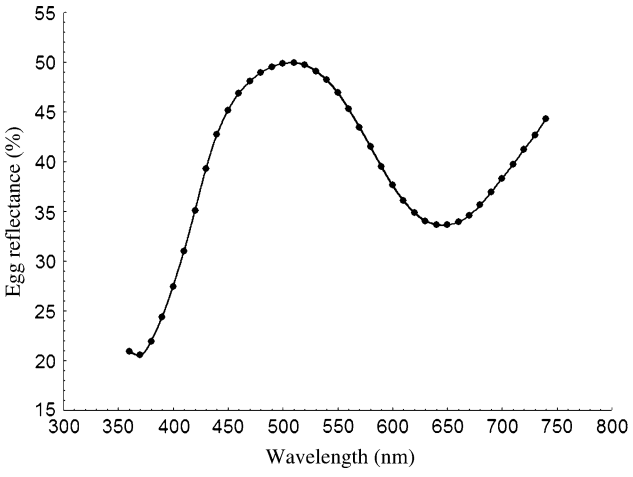

Figure 1
Mean reflectance spectrum of pied flycatcher eggs. So that each clutch contributes equally, values are the means of the within-clutch means.

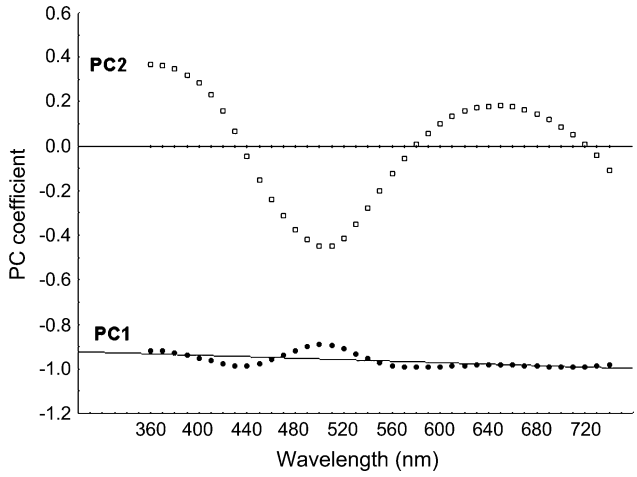

Figure 2
Principal components as a function of wavelength. Derived from reflectance spectra from eggs in each clutch ($n = 74$). PC1 indicates principal component 1, and PC2 indicates principal component 2.

Egg color and female condition and immunity

We first related mean egg color of clutches to reproductive variables that could affect color, like laying date, clutch size, and mean egg weight (within-clutch repeatability: $r = .60$, $F_{73,336} = 9.9$, $p < .001$). None of these variables showed any significant association with PC1 or PC2 in multiple regression analyses ($p > .10$). Female age was negatively associated with PC1 ($F_{1,70} = 4.1$, $p = .048$) but not with PC2 ($F_{1,70} = 0.58$, $p = .45$). The decline in PC1 and thus in pigmentation occurred only after age 4 (4 years or less versus 5 or more years: $F_{1,70} = 5.8$, $p = .019$). Thus, egg lightness is associated to senescence. Female condition at first capture expressed as mass divided by tarsus length was significantly associated with PC2 when controlling for female minimum age and condition at the end of the nestling period (Figure 3; initial condition: $F_{1,53} = 5.1$, $p = .028$; final condition: $F_{1,53} = 1.7$, $p = .20$; age: $F_{1,53} = 2.8$, $p = .10$). Females in better initial condition laid eggs with a tendency away from green and toward higher UV reflectance. No association of condition with PC1 was found ($p > .20$).

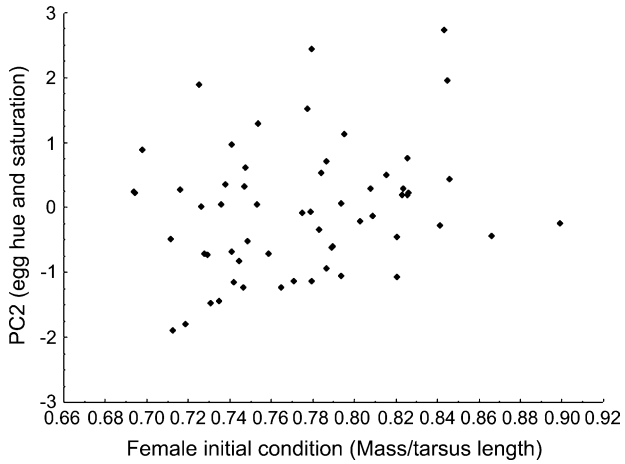

Figure 3
Mean within-clutch egg PC2 (hue and saturation) in relation to female condition (mass/tarsus length) at the beginning of the nestling period.

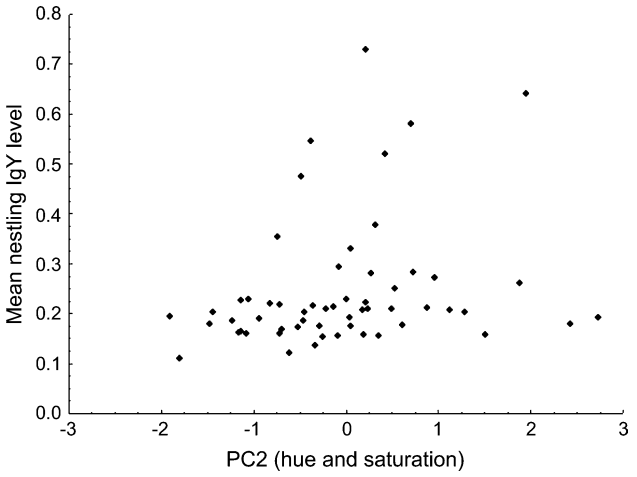

Figure 5
Mean within-brood IgY level in absorbance units in relation to mean within-clutch egg PC2 (hue and saturation).

No associations between total immunoglobulins in blood during either the first or second capture and either PC1 or PC2 were detected ($p > .50$). PC1 was positively associated with female PHA response (Figure 4a), and this remained significant when correcting for female age (age: $F_{1,35} = 1.9$, $p = .17$; PHA response: $F_{1,35} = 4.5$, $p = .041$). No association between PC2 and female PHA response was found ($F_{1,36} = 0.65$, $p = .42$). Female humoral immune response to a tetanus vaccine was positively related to PC1 alone (Figure 4b) and when controlling for individual background level and for female age (initial response: $F_{1,51} = 0.13$, $p = .71$; final response: $F_{1,51} = 4.1$, $p = .049$; age: $F_{1,51} = 1.4$, $p = .25$). No association between humoral immune response and PC2 was found in a similar analysis ($p > .50$).

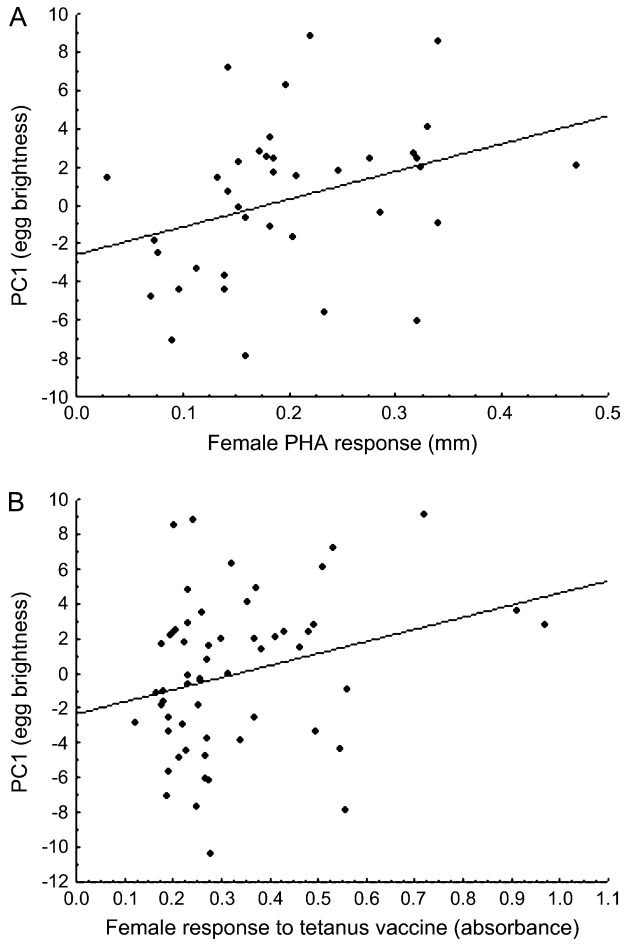

Figure 4
Mean within-clutch egg PC1 (opposite of brightness) in relation to (A) female PHA response ($y = -2.6 + 14.5x$, $r = .33$, $p = .037$) and (B) female humoral response to tetanus vaccine ($y = -2.3 + 6.9x$, $r = .27$, $p = .038$).

Egg color and nestling condition and immunity

Mean nestling condition in broods was marginally nonsignificantly and positively associated with PC2 when correcting for the presence of mites and fly pupae (presence of mites: $F_{1,51} = 0.29$, $p = .59$; number of pupae: $F_{1,51} = 0.82$, $p = 0.37$; condition: $F_{1,51} = 3.2$, $p = .079$). No tendency was detected for PC1 ($p > .70$). Mean level of IgY in nestlings was positively associated with PC2 when correcting for the number of fly pupae and for mite score (Figure 5; mites: $F_{1,49} = 0.01$, $p = .92$; number of pupae: $F_{1,49} = 0.83$, $p = .37$; nestling IgY: $F_{1,49} = 5.1$, $p = .029$). Thus, nestlings derived from eggs reflecting less toward the green part of the spectrum and more toward the UV part had higher levels of immunoglobulins at the age of 12 days. The association with PC1 was weaker and marginally nonsignificant (mites: $F_{1,47} = 0.42$, $p = .74$; number of pupae: $F_{1,47} = 0.02$, $p = .90$; nestling IgY: $F_{1,47} = 3.3$, $p = .075$). In this case, darker eggs produced nestlings with higher levels of IgY. No association between mean within-brood nestling PHA response and either PC1 or PC2 was found ($p > .50$).

Egg color and laying sequence

PC1 declined linearly with laying order (Figure 6a). This trend explained more than a third of variation in egg lightness. PC2 showed a nonlinear association with laying order (Figure 6b). There was a slight initial decline, which can be interpreted as a shift toward blue-green, and a subsequent marked increase after the fourth egg, indicating a shift away from blue-green colors for the last eggs laid.

Male nest visits during laying

Of 26 males, 22 perched on the nest-box during the 3-h observation period and did so for on average 7.4 ± 8.1 (SD) times (range 1–36). Of these, 13 males entered the nest-box

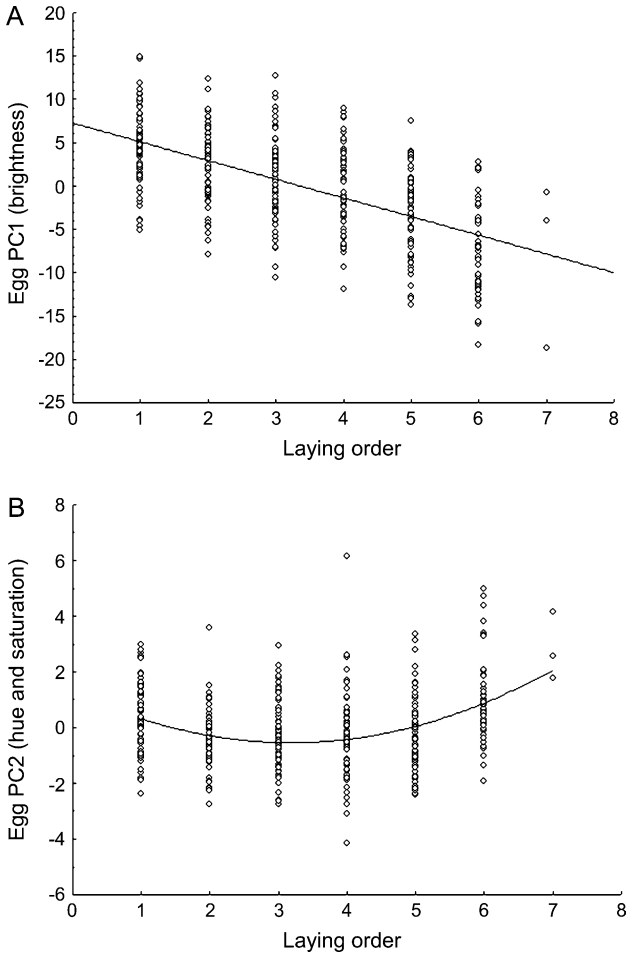

Figure 6
Regressions of (A) egg PC1 (opposite of brightness) ($F_{1,398} = 224.7$, $p < .001$, $r = .60$, $y = 7.24 - 2.15x$) and (B) egg PC2 (hue and saturation) (polynomial regression: *deviance* = 700.4, *loglikelihood* = −679.6, $y = 1.27 - 1.14x + 0.18x^2$, all parameters: $p < .001$) in relation to laying order.

on average 5.0 ± 4.3 times (range 1–15). They spent inside the nest-box on average 42 ± 62 s (range 3–189 s). Males apparently visit the nest-box frequently during the laying period and can presumably observe recently laid eggs at leisure.

DISCUSSION

Our results show that old-age females lay lighter eggs and that higher female immunocompetence is associated to darker and presumably more pigmented eggs, when correcting for female age. Higher female condition is linked to colors tending away from intermediate wavelengths and toward shorter wavelengths. Also, the mean within-brood level of nestling IgY is associated with egg colors tending away from intermediate wavelengths. Mean egg pigmentation decreases linearly during the laying sequence, suggesting pigment limitation. The last eggs show less pronounced blue-green colorations. Finally, males are observed frequently visiting nests during the laying period, allowing them ample time to observe freshly laid eggs. These results offer support for the signaling function of blue and green egg colors in birds.

The hypothesis that females signal their phenotypic quality to males in order to elicit a higher contribution of parental effort from their mates was proposed to cover the explanatory gap concerning the function of bright colorations in avian eggs (Moreno and Osorno, 2003). Sexual selection theory (Andersson, 1994) and the differential allocation hypothesis (Burley, 1986) were applied to ornaments external to the females themselves but synthesized by them, a new application of sexual selection theory. It was also applied to female signaling, a certainly neglected topic in the study of sexual selection (Amundsen, 2000). The physiological basis of the signal was the antioxidant properties of the pigment biliverdin used to color eggs blue and green (Moreno and Osorno, 2003). Blue and green egg background colors are very frequent in birds as can be deduced from any field guide of avian eggs (e.g., Harrison, 1975). Eggs offer a good opportunity for female signaling as they do not compromise their survival during the breeding period through conspicuous morphological ornaments, while at the same time being directed specifically to their mates, the only receivers that may help them in raising young. The signals may not only signal their own quality but also that of the offspring contained in the brightly colored eggshells (Moreno and Osorno, 2003). The fact that this function has not been proposed earlier given its potential advantages may be due to the prevalent focus on nest predation in the study of egg coloration (see Underwood and Sealy, 2002 for a recent review) and the disregard for female signaling in sexual selection studies.

According to the signaling hypothesis (Moreno and Osorno, 2003), females would be allocating more pigment to eggs to indicate their antioxidant capacity and possibly that of their offspring. During laying, the action of progesterone may induce a higher oxidative stress in females (von Schantz et al., 1999), making it even more relevant to signal their capacity to control oxidative stress at this time. The activation of the immune system generates oxidative stress (von Schantz et al., 1999), so we could predict that only females with a strong immune response may be able to allocate more pigments to the eggshell without suffering adverse consequences. Another possibility derived from the hypothesis is that egg coloration in fact signals the phenotypic quality of the offspring as mediated either through traits inherited from the mother, or through maternal allocation of antioxidants and immunoglobulins to the eggs (Grindstaff et al., 2003; Møller et al., 2000). The hypothesis is based on two assumptions. One is that males are able to observe freshly laid eggs, when egg color is still unaffected by subsequent environmental effects. Another assumption is that the pigment biliverdin in the case of blue or green eggs is limiting for females and thus can become an honest signal. In that case, we would expect a decline in pigment allocation throughout the laying sequence as expressed through color parameters. To our knowledge, none of these assumptions or predictions of the signaling hypothesis has been tested before.

In an earlier study of a Spanish pied flycatcher population, associations of egg color parameters with male parental effort in raising chicks, as well as of male effort with nestling condition before fledging were found (Moreno et al., 2004). This evidence supported the existence of fitness benefits for signaling females. This study was observational, so correlations could be interpreted as due to a third variable affecting both egg color and male provisioning effort. However, it was the first evidence that egg coloration could be affecting male allocation in nestling provisioning in a species with characteristic blue coloration. In that study no attempt was made to relate egg coloration to female immunocompetence. Neither were the assumptions and predictions presented above tested observationally. In the present study we have aimed at filling this gap by measuring both cell-mediated and humoral female immunocompetence through standard assays and by measuring the amount of immunoglobulins in nestlings and mothers, as nestlings receive maternal IgY through the egg yolk

(Grindstaff et al., 2003). We have also used Principal component analyses of reflectance spectra of individual eggs on the day of laying to obtain standard color parameters as recommended by Cuthill et al. (1999) for animal color studies. We have assumed that both PC1 (opposite of lightness) and PC2 (hue and saturation) are related to the amount of pigments deposited in the eggshell. Finally, we have also observed male behavior at the nest-box during laying to test the assumption that males can in fact observe recently laid eggs.

Our results fully support the signaling hypothesis for the blue eggs of pied flycatchers. Female cell-mediated and humoral immune responses during the nestling period as estimated with the standard PHA and tetanus vaccine assays were positively correlated with PC1 and thus with pigmentation. Female condition at hatching of the young was associated with the chromatic PC of the reflectance spectra, indicating a shift away from intermediate wavelengths and toward the UV part of the spectrum in eggs of females in good condition. The chemical composition of the pigments deposited in eggshells may be related to this result, as eggshells that appear blue may have other pigments besides biliverdin (Kennedy and Vevers, 1976). The fact that total IgY levels in females did not relate to egg color may be due to the fact that immunoglobulins have been found to decline from egg laying to hatching of the young (Saino et al., 2001). Low levels of IgY in females may have obscured any association with egg color. Female age was also related to brightness, with senescent females laying lighter and less pigmented eggs. Nestlings hatching from eggs with colors shifted away from green and toward UV had a higher level of IgY, which could be explained by a similar shift in egg color for females in good condition. This could reflect the higher allocation of IgY to the eggs by females in good condition. However, nestling IgY was measured at a late stage of development, when IgY levels may already reflect the nestlings' own IgY production more than the maternal immunoglobulin allocation to eggs (Klasing and Leshchinsky, 1999). Thus stronger effects could have been detected if nestling IgY levels had been sampled at an earlier stage of development. The assumption that the pigments involved may be limiting was reflected in a marked linear increase in lightness throughout the laying sequence. The laying order of the eggs explained a third of the variation in egg lightness. This again suggests that the amount of pigments deposited in the eggshell affects its lightness. The nonlinear association of PC2 with the laying sequence may indicate a shift in the combination of pigments used, as not only biliverdin may be involved in eggshell coloring. Finally, males were observed to visit the nest-boxes frequently on the fourth day of laying (when more than half the clutch has been laid) offering ample opportunities to observe freshly laid eggs. This result validates the assumption that males are actually present at the nest at the appropriate time for assessing egg color. The naive argument that nest-boxes are dark and do not offer the possibility to detect color variation does not consider the visual capacities of birds generally and that of those breeding in cavities specifically. Cavity-breeding birds are apparently able to detect variation in nestling gape color (Hunt et al., 2003) and identify individual nestlings (Gottlander, 1987). The assumption that they cannot observe nuances in egg lightness or color appears unfounded in the light of recent discoveries about avian visual acuity (e.g., Cuthill et al., 2000).

To conclude, the evidence here presented, although observational, strengthens the case for the signaling hypothesis and supports the conclusions in Moreno et al. (2004) concerning pied flycatchers. Comparative evidence shows that egg coloration is related to mating system and duration of the nestling period in European passerines as predicted by the signaling hypothesis (Soler et al., 2005). Field experiments are needed in order to give conclusive support to the hypothesis by Moreno and Osorno (2003), as males could be assessing other characteristics of females as a cue for provisioning decisions. The association between egg color and the amount and composition of eggshell pigments deposited remains to be studied. However, it appears increasingly plausible that female birds can in fact signal their quality or that of their eggs to their mates through eggshell coloration, a possibility that may be applied to all species with bright eggs and male parental care.

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