

Pigment allocation to eggs decreases plasma antioxidants in a songbird

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Abstract Although evidence is accumulating on the adaptive function of female ornamentation, very little is known about maternal allocation decisions involving sexual signaling and other reproductive functions. Blue egg coloration has been suggested as a sexually selected signal of female quality to males, and some recent studies are in accordance with this hypothesis. Blue eggshell coloration results from the deposition of biliverdin pigment by laying females, which is a potent antioxidant. Thus, egg pigmentation should be costly in terms of antioxidants, an assumption of the signaling hypothesis that has not been tested yet. We induced increased reproductive effort in a set of female pied flycatchers *Ficedula hypoleuca* through nest removal and measured egg pigmentation and plasma antioxidant levels in relation with a control group. Experimental females showed a negative association between egg color and plasma antioxidant levels, while there was no relationship for control birds. This supports that egg

pigmentation is costly in terms of general antioxidant defenses and suggests a tradeoff between the allocations to both traits. Furthermore, experimental females with more colorful eggs raised more fledglings, especially when breeding early. Controls did not show a relationship between egg color and reproductive success. Females laying more colorful eggs could have shifted their allocation decisions towards current reproduction, at the expense of their own antioxidant defenses. Our results highlight that blue egg coloration is a life-history trait, subject to tradeoffs with other attributes, and seems to be especially informative under harsh breeding conditions.

Keywords Antioxidants · Blue-green egg coloration · Female ornamentation · Life-history tradeoffs · Sexual selection

Introduction

Understanding the evolution of female ornamentation in species with conventional sex roles is becoming a target in behavioral ecology studies (e.g., Cunningham and Birkhead 1998; Amundsen 2000; Bonduriansky 2001). Most evidence for an adaptive function of sexual signaling in females has been based on positive relationships between ornament expression and traits indicative of quality (see Kraaijeveld et al. 2007 for a review in different taxa). However, compared to male ornaments, very little attention has been paid to the selection pressures that are likely to act on female signals and to the hypothetical tradeoffs that may emerge (LeBas 2006). Nevertheless, because the costs derived from offspring production must be mainly assumed by the females, the investment in ornamentation may more strongly influence the outcome of reproductive tradeoffs in

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females than in males (Chenoweth et al. 2006; LeBas 2006).

An interesting set of sexual signals are those external to the body itself, the so called extended ornaments (Dawkins 1982). Recently, it has been suggested that the blue eggshell coloration in species with biparental care may function as a post-mating sexual signal of female quality to their mates (Moreno and Osorno 2003). As a result, more intensely colored eggs would induce higher paternal care (Moreno and Osorno 2003). Evidence is accumulating in agreement with the hypothesis predictions. Thus, blue egg color intensity may indicate female immunocompetence and condition (Moreno et al. 2005, 2006b; Siefferman et al. 2006; Krist and Grim 2007; Soler et al. 2008) and appears to be positively associated with the degree of paternal effort, both in comparative (Soler et al. 2005) and intraspecific studies (Moreno et al. 2006a; Soler et al. 2008; but see Krist and Grim 2007 and López-Rull et al. 2007). The hypothesis is based on the fact that blue egg coloration results from the deposition of biliverdin pigment by laying females (Kennedy and Vevers 1976; Miksik et al. 1996), which has a strong antioxidant capacity among other functions (McDonagh 2001; Falchuk et al. 2002; Kaur et al. 2003; Maines 2003). Blue color intensity of pied flycatcher eggs correlates positively with biliverdin contents in the eggshell (Moreno et al. 2006b) and decreases in the laying sequence (Moreno et al. 2005), as in the closely related collared flycatcher, *Ficedula albicollis* (Krist and Grim 2007). Moreover, females suffer high levels of oxidative stress while laying eggs (von Schantz et al. 1999). For these reasons, pigment deposition might be costly in terms of antioxidant availability (Moreno and Osorno 2003). Yet, evidence for this assumed link has not been explored.

It has been repeatedly shown that tradeoffs can most efficiently be revealed through experimental phenotypic manipulation, as otherwise differential resource availability may blur the expected negative associations between traits (van Noordwijk and de Jong 1986; Partridge et al. 1991; Zera and Harshman 2001). Enhanced reproductive effort induces energetic costs (Velando and Alonso-Álvarez 2003 and references therein), which decrease the resistance to oxidative stress (Alonso-Álvarez et al. 2004) and affect the allocation to ornament expression (von Schantz et al. 1999; Doutrelant et al. 2008). In line with this idea, Soler et al. (2008) found that feather clipping prior to laying negatively affected blue egg color intensity in female spotless starlings (*Sturnus unicolor*). Increased reproductive effort in females may thus bring to light latent maternal tradeoffs involving ornamentation.

Our aim was to investigate if blue egg color intensity was related to the antioxidant capacity of females, as assumed by the signaling hypothesis (Moreno and Osorno 2003). If egg pigmentation is costly in terms of antiox-

idants, we may expect a negative relationship between egg color and antioxidant defenses otherwise used for a different function, which may be more easily detectable under stressful conditions. We conducted a nest-removal experiment in a wild population of pied flycatchers, a species in which egg coloration seems to work as a sexually selected signal (Moreno et al. 2004, 2006a) and nest building is mainly performed by females (Lundberg and Alatalo 1992; Moreno et al. 2008b). Nest building has been reported to be energetically expensive and time consuming in other species (e.g., Lens et al. 1994; Fargallo et al. 2001; de Neve and Soler 2002). Furthermore, the rate of nest construction in female pied flycatchers has been shown to be associated with higher physiological stress as measured by the levels of stress protein HSP60 (Moreno et al. 2008b), which is indicative of oxidative stress and high metabolic rate (Sørensen et al. 2003). Then, by forcing a set of females to build a second nest prior to laying, we prolonged the period of heightened physiological stress compared to controls. If elevated HSP60 levels indicate increased oxidative stress, experimental females presumably experienced a prolonged period of enhanced antioxidant demand compared with controls. As a measure of body antioxidant defenses, we assessed the concentration of total antioxidants in plasma (Erel 2004). We predicted that repeated nest-building would affect the relationship between blue egg coloration and plasma antioxidant levels in females, by showing a negative association between egg pigmentation and plasma antioxidants after laying. In such case, it still pays for males to trust in the signal if egg color honestly reflects the clutch reproductive value (Morales et al. 2006) or the females' disposition to invest in reproduction. Further, egg coloration could be especially informative under harsh conditions (see Doutrelant et al. 2008 for plumage traits). Hence, egg color could be more strongly related to reproductive success in experimental females than in controls.

Materials and methods

In a previous correlative study, we found a non-significant relationship between maximum egg color and female plasma antioxidants, measured at the nestling stage (Beta = -0.45, $F=3.46$, $P=0.084$, $n=16$). The experimental study was conducted in 2006 in a population of pied flycatchers breeding in nest boxes close to the village of Lozoya, central Spain (40°58' N, 3°48' W). The study area is a montane Pyrenean oak *Quercus pyrenaica* forest on an east-facing slope at 1,500 m elevation, where 100 nest boxes were erected in 2001. Pied flycatchers occupy approximately half of the nest boxes each year after most individuals of other non-migratory species have occupied a nest box and started

to breed. Clutch size ranges from four to seven eggs with a mode of six eggs. Nest boxes were checked daily from arrival of the first males to the study area (April 15) in order to detect the first indications of nest construction by pied flycatchers. The process of nest construction was followed through daily visits until nests showed the rounded and closely knit nest cup indicating completion, when they were extracted from the nest box and either placed back in the nest box (control nests) or removed (experimental nests). The same experiment has been described in another study (Moreno et al. 2008b), where only the association of nest construction with physiological stress was covered and where no data concerning egg coloration or antioxidants were used. The same observer made the subjective decision about nest completion, so any error of appreciation was probably systematic (for obvious reasons nest removal can only be carried out some time before laying, which means as soon as there is a cup in the nest). Nests are sufficiently compact to allow removal without deterioration. Treatments were randomly allocated to 48 nests (24 to each treatment) during each day on which completed nests were found. In all experimental nest boxes, nest construction was resumed during the next 2 days after removal of the first nest. Takeover by another female in this short time and initiation of a new nest remains possible but not plausible due to the stiff competition among females for nest boxes in the study area (which may be lethal, J. Moreno pers. obs.). We, therefore, assume that resumption of nest construction in such a short interval implied a second attempt by the same female. Specific data on nest construction in our study, such as nest size, construction rate, and interval between nest construction and laying have been already reported in another study (Moreno et al. 2008b). Four late experimental nests have been excluded as females did not build a complete nest with nest cup before laying eggs.

The day of clutch initiation was detected in all cases. Eggs were weighed on the day of laying with a portable electronic balance, and eggshell color was measured with a MINOLTA CM-2600d portable spectrophotometer (Minolta Co. Ltd., Osaka, Japan) and placed back after marking for identification. Egg color measurement assumptions and procedures with the MINOLTA spectrometer are described by Moreno et al. (2006a, b). From reflectance spectra elaborated by the apparatus, we calculated blue-green chroma (hereafter called “egg color”) as the proportion of reflectance in the blue-green part of the spectrum ($R_{400-570}/R_{360-700}$). Although the apparatus does not cover wavelengths below 360 nm, we have previously shown that egg color measured here correlates significantly with a measure with a spectrophotometer covering the full UV range and with biliverdin content of the eggshell (Moreno et al. 2006b). We know that males of the study species respond to clutches with higher peak values rather than with higher

mean values (Moreno et al. 2006a), so we have estimated the maximum value within the clutch as well as the mean clutch color. We measured the color of all eggs in some clutches and when there was time limitation, we measured all except the last egg or the last two eggs (in a previous study, among 92 nests of a neighboring population, no clutch showed the maximum color in the last egg and only two showed it in the penultimate egg). Thus, we were confident that we knew the maximum egg color in almost all clutches. In addition, egg color is highly repeatable within pied flycatcher clutches (Moreno et al. 2005). Accordingly, in the present study the mean egg color of clutches where all eggs were measured (0.583 ± 0.005) was not different from the mean egg color of clutches where all except the last egg were measured (0.589 ± 0.003) or all except the last two (0.589 ± 0.003 ; $P=0.54$). Since male pied flycatchers respond to the maximum egg color we analyzed this variable, but similar results are achieved when we analyzed the mean egg color (data not shown, but available upon request). We registered hatching success (number of hatched eggs divided by clutch size) and reproductive success (absolute number of fledglings).

One week after clutch completion, most incubating females were captured in the nest box during daytime without traps as they usually sit very tight on the eggs. We did not capture them at an earlier stage given the increased risk of nest desertion. They were banded if necessary, identified, weighed to the nearest 0.25 g with a PESOLA spring balance. Females were sampled for blood (80 μ l maximum) by venipuncture of a brachial vein. Many females continued incubating after being placed back on the nest. Blood was centrifuged in the field with a portable centrifuge and separated into plasma and cellular fractions. Plasma fractions were maintained in a cool box until being frozen on the same day at -80°C for plasma antioxidant analyses. Body antioxidant defenses were measured using the method described by Erel (2004). Basically, it consists in the use of the molecule 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate), which is decolorized by antioxidants according to their concentration and antioxidant capacity. The change in color was measured as the change in absorbance at 655 nm. The concentration of total antioxidants (hereafter called “plasma antioxidants”) is expressed as nanomole of Trolox equivalent/l.

We used SAS software (SAS Institute 2001) for all statistical analyses. First, we used general linear models (GLMs) to test whether the experiment affected female plasma antioxidants controlling by laying date, female body mass, and all interaction terms. Second, we performed a GLM to investigate whether the experiment affected the relationship between maximum egg color and plasma antioxidants. Laying date, clutch size, female body mass, egg mass (of the egg with maximum egg color), and all

interaction terms were included as covariates. Finally, a generalized linear model (GENMOD procedure in SAS) with Poisson distribution was used to investigate whether the experiment affected the relationship between maximum egg color and reproductive success or hatching success. Laying date was included as covariate and all interaction terms were performed. Data dispersion was corrected using the Pearson scale parameter. Final models were obtained by a backward deletion procedure. Differences in sample sizes reflect missing values due to, for instance, reduced blood volume.

Results

Experimental females delayed laying by 2.4 days and prolonged nest construction by almost 5 days on average compared with controls (Moreno et al. 2008b); clutch size was not affected by treatment (Moreno et al. 2008b). In the present study, we found that female plasma antioxidant levels were not significantly affected by treatment (treatment: $F_{1,30}=1.25$, $P=0.27$; female mass: $\text{Beta}=0.31$, $F_{1,30}=3.13$, $P=0.087$).

The maximum egg color was not related to laying date or female body mass ($P=0.19$ and 0.56 , respectively). However, it was affected by the interaction between egg mass and clutch size (Table 1). Clutches with seven eggs showed a negative association between maximum egg color and egg mass ($\text{Beta}=-0.031$), while the association was positive for smaller clutches ($\text{Beta}=0.24$). Furthermore, as predicted, the relationship between maximum egg color and plasma antioxidants was affected by the experiment (Table 1). Under natural conditions (controls), the maximum egg color was not related to plasma antioxidants ($\text{Beta}=-0.011$; Fig. 1). In contrast, experimental females that laid clutches with higher maximum color showed lower plasma antioxidants ($\text{Beta}=-0.74$; Fig. 1). Interestingly, the interaction was also significant when egg mass and clutch size were removed from the model (represented in Fig. 1). Overall these results suggest that the tradeoff between egg

Table 1 Results of GLM analysis with maximum egg color as dependent variable showing a treatment effect on the association between egg color and the concentration of plasma antioxidants (nmol of Trolox equivalent/l)

| Independent variable | Beta | F | df | P value |
|-------------------------------|-------|-------|-------|---------|
| Treatment | 23.46 | 10.43 | 1, 27 | 0.0033 |
| Clutch size | 4.83 | 8.24 | 1, 27 | 0.0078 |
| Egg mass | 6.16 | 7.96 | 1, 27 | 0.0089 |
| Plasma antioxidants | -0.31 | 4.33 | 1, 27 | 0.047 |
| Treatment*plasma antioxidants | | 10.28 | 1, 27 | 0.0034 |
| Clutch size*egg mass | | 8.01 | 1, 27 | 0.0087 |

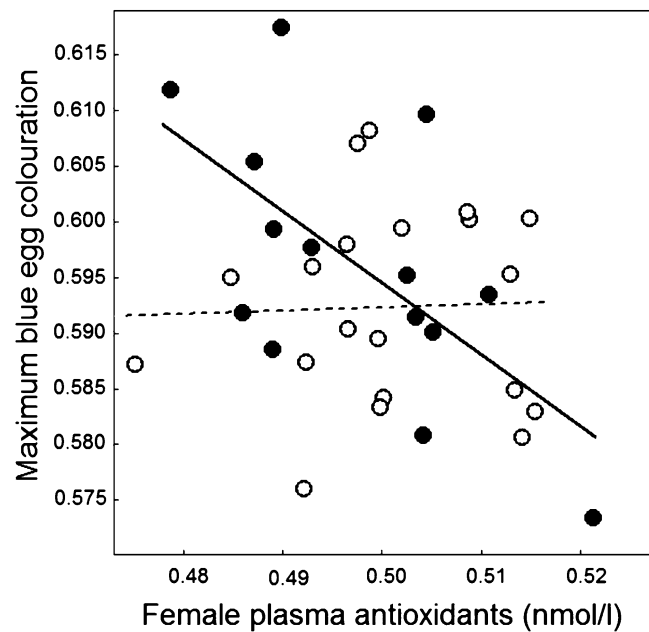


Fig. 1 Effect of treatment on the relationship between the maximum egg color (blue-green chroma) and female plasma antioxidants (nanomole of Trolox equivalent/l) ($F_{1,30}=4.95$, $P=0.034$). Full dots and solid line represent experimental females and empty dots and dotted line represent controls

color and plasma antioxidants is expressed under stressful conditions.

Reproductive success was similarly affected by laying date in interaction with treatment (Table 2). In control females, laying date was not related to reproductive success ($\text{Beta}=0.027$). However, late experimental breeders showed lower reproductive success ($\text{Beta}=-0.33$). Furthermore, the relationship between reproductive success and maximum egg color was affected by the experiment (Table 2). Under natural conditions (controls), reproductive success was not predicted by egg color ($\text{Beta}=-0.045$; Fig. 2). In contrast, experimental females laying more colorful eggs raised more fledglings ($\text{Beta}=0.11$; Fig. 2). Hatching success was affected by laying date in interaction with treatment in the same way than reproductive success (treatment \times laying date: $F_{1,39}=5.59$, $P=0.018$). However, the relationship between hatching success and maximum egg color was not affected by the experiment ($P=0.12$).

Table 2 Results of GENMOD analysis showing the effects of treatment and egg color on the number of fledglings

| Independent variable | Beta | F | df | P value |
|-----------------------|--------|------|-------|---------|
| Treatment | -0.034 | 1.31 | 1, 37 | 0.25 |
| Laying date | -0.15 | 7.06 | 1, 37 | 0.0079 |
| Egg color | 0.032 | 0.69 | 1, 37 | 0.405 |
| Treatment*egg color | | 4.05 | 1, 37 | 0.044 |
| Treatment*laying date | | 9.85 | 1, 37 | 0.0017 |

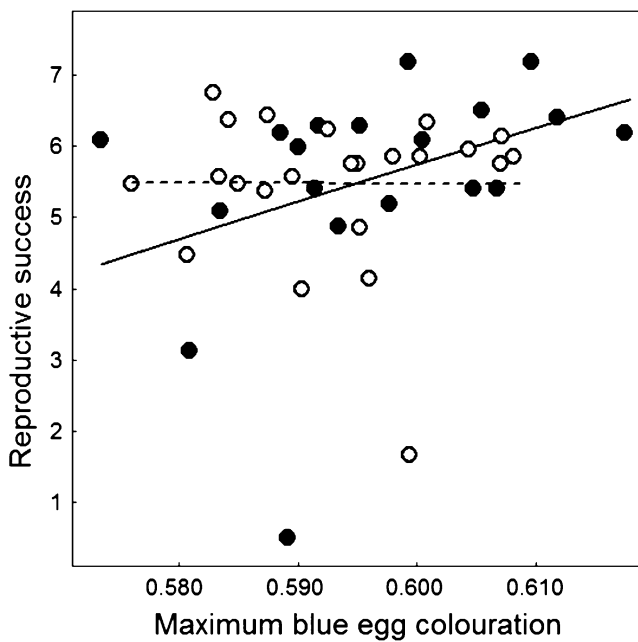


Fig. 2 Effect of treatment on the relationship between the maximum egg color (blue-green chroma) and reproductive success (mean reproductive success plus residuals from a model that controls for laying date). *Full dots and solid line* represent experimental females and *empty dots and dotted line* represent controls. By removing the only case with no reproductive success (Cook's distance > 1), similar results were achieved (treatment*maximum egg color: $F_{1,37}=4.17$, $P=0.041$)

Discussion

Until now, it has not been tested that pigment allocation to blue egg color is costly in terms of antioxidants, one of the main assumptions of the signaling hypothesis of egg coloration (Moreno and Osorno 2003). We found that the relationship between plasma antioxidants and blue egg coloration in the pied flycatcher was affected by the nest removal experiment. Thus, females assuming the extra cost of nest building showed a negative relationship between egg color and plasma antioxidants, although no relationship was detected in controls. This result confirms a nonsignificant relationship found in a preliminary study in the same population in a poor breeding year. Taken together, these findings suggest a tradeoff between antioxidant allocation to sexual signaling and to body defenses, more likely to arise under stressful conditions.

Increasing breeding effort has been found to reduce the resistance to oxidative stress in other species (Alonso-Álvarez et al. 2004, 2006). Similarly, enhanced nest building activity could have increased oxidative stress in experimental females and enhanced their antioxidant demand compared with controls, thus, reducing their capacity to invest antioxidants in both traits. Indeed, nest construction rate is positively related to physiological stress in females after laying (Moreno et al. 2008b). Importantly, the effect of clutch size and egg mass on egg color

highlights that the allocation to egg coloration is probably costly. Thus, large clutches showed a negative relationship between egg mass and maximum egg color. An alternative explanation for the negative association between egg color and plasma antioxidants is that experimental females of high quality were better able to cope with induced oxidative stress, investing more antioxidants in egg pigmentation despite keeping low levels of plasma antioxidants. In this case, we should expect that these experimental females lay paler eggs compared with control females of similar quality, which did not suffer the costs of second nest construction. However, experimental females seemed not to lay paler eggs than controls, their eggs being more colorful in some cases. This suggests that they shifted their allocation decision towards signaling at the expense of their own antioxidant defenses.

Oxidative stress has been proposed as an important mechanism mediating the optimal investment in life-history traits (Alonso-Álvarez et al. 2006) and, thus, into sexual signaling (von Schantz et al. 1999). The deposition of biliverdin pigment in the eggshell could deplete biliverdin from the female's antioxidant system, presumably impairing essential processes of protection against oxidative damage. It is far from clear where eggshell biliverdin is derived from, although the most likely origin is the shell gland (Zhao et al. 2006). Even in the case that eggshell biliverdin is derived from heme degradation process, this would not mean it is a waste product at no cost. Recent studies have shown that natural "waste" products derived from heme are key modulators of cell transduction pathways and gene expression (e.g., Maines 2005; Florczyk et al. 2008). Moreover, biliverdin is a crucial component of antioxidant defense mechanisms and immune responses (Maines 2003, 2005; Florczyk et al. 2008). Therefore, allocating biliverdin to sexual signaling could be, thus, costly in terms of increased exposure to oxidative stress (Moreno and Osorno 2003). A non-excluding mechanism is that blue egg pigmentation requires that colorless antioxidants are also allocated to eggs in order to prevent biliverdin from bleaching, in a similar fashion as proposed for other signaling pigments like carotenoids (Hartley and Kennedy 2004). In such case, the availability of colorless antioxidants rather than biliverdin would be the key limiting factor that drives the potential tradeoff. Additionally, apart from the passive protection mechanism (Hartley and Kennedy 2004), the allocation of colorless antioxidants to sexual signaling may promote an active mechanism to increase the amount of pigments (Pérez et al. 2008). Since treatment did not affect the relationship between hatching success and egg color, it is not likely that bluer eggs stimulated a higher incubation effort that resulted in a depletion of plasma antioxidants. At the present evidence, the alternative mechanisms based on a limitation of

biliverdin or colorless antioxidants seem more plausible. Future studies are needed to disentangle these possibilities.

Interestingly, the maximum egg color was positively linked to reproductive success only under the stressful conditions undergone by experimental females, as found by Doutrelant et al. (2008) for a plumage female trait. This result was due to higher nestling survival, not to higher hatching success, in experimental broods resulting from highly colored eggs. As the allocation to pigment deposition in eggs seems flexible under varying environmental conditions (Moreno et al. 2006b), females laying more colorful eggs under stressful conditions could have actively shifted their priorities towards current reproduction (Kokko 2001; Velando et al. 2006). Accordingly, an experiment of clutch removal in the house sparrow, *Passer domesticus*, showed that females forced to mount an immune response were more prone to invest in reproduction (Bonneaud et al. 2004). That blue egg coloration indicates the amount of maternally transferred immunoglobulins to pied flycatcher eggs (Morales et al. 2006) could be in line with this argument, egg color reflecting the female's investment in current reproduction. Egg color could also be signaling the maternal allocation of antioxidants to eggs. In addition, males could also have increased parental effort according to signal expression. Hence, they may gain an advantage by working more for a brood of higher reproductive value due to maternal effects (Moreno et al. 2008a), in accordance with the differential allocation theory (Burley 1986). Alternatively, we cannot exclude that experimental females with intensely colored eggs could be of better quality and, thus, better able to handle the costs of building a second nest. However, the reproductive success of these females was not lower than that of controls, the costs of nest construction being only noticeable below a level of 0.590 of egg color intensity. Thus, our results best fit the idea that experimental females that laid colorful eggs invested more in reproduction at the expense of antioxidant defenses (somatic maintenance) or future prospects. Additionally, among experimental females, late breeders showed reduced reproductive success. The fact that the experiment delayed breeding (Moreno et al. 2008b) might have negatively affected brood rearing in very late experimental females, as these individuals probably found poorer conditions.

Our study suggests that blue egg coloration is a life-history trait subject to tradeoffs with other female attributes. According to theoretical models (Kokko 2001), investing in costly sexual signals results in resource allocation decisions that are more complex than expected from the traditional view of the handicap hypothesis of honest signaling (Zahavi 1975), being its principal prediction positive relationships between ornament expression and female traits. Thus, we underline the need to study sexual advertisement under a life-history perspective for a better

understanding of sexual selection (Griffith 2000; Kokko 2001). The potential tradeoff between antioxidant function and egg pigmentation under stressful conditions is fully supportive of the mechanistic basis of the egg color as signal hypothesis (Moreno and Osorno 2003); as to ensure honesty, the ornament should be costly in terms of antioxidants.

To conclude, our findings suggest for the first time that blue egg coloration is costly at the expense of the female antioxidant defenses and add to previous evidence in support of the signaling hypothesis for egg color (Moreno and Osorno 2003). Availability of antioxidants at laying may, thus, constrain egg pigmentation in avian species with blue-green eggs, as has been shown for female integument pigmentation in fish (Grether et al. 2008) and birds (Blount et al. 2002; Bertrand et al. 2006). Also, females laying more pigmented eggs experience higher reproductive success under strenuous conditions, a potential benefit of signaling. We highlight the importance of exploring maternal tradeoffs at the interface of life history evolution and sexual selection (Kokko 2001).

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