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ORIGINAL PAPER

Biliverdin-based egg coloration is enhanced by carotenoid supplementation

Judith Morales · Alberto Velando · Roxana Torres

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Abstract Avian eggshell color is remarkably variable among and within species and its possible function has long been controversial. Female birds use biliverdin pigment to color their eggs blue and green. Although evidence is accumulating that blue-green eggshell coloration is costly to produce, the exact mechanisms underlying its expression are little studied. Biliverdin pigment is involved in important maintenance functions such as immune and antioxidant defenses and shows similar properties to carotenoid pigments. Carotenoids play a role as immunoenhancers and have the potential to ameliorate the impact of oxidative processes, although their antioxidant function could be minor for some bird species. Important life-history components can be limited by carotenoid availability, including sexual displays and fecundity. Here we explored if biliverdin-based eggshell pigmentation was affected by carotenoid availability in a seabird, by performing a carotenoid supplementation experiment in female blue-footed boobies (*Sula nebouxii*) after laying their first egg. In this species, blue eggshell color is associated with incubation patterns, and carotenoid availability affects the crucial trade-off between female foot ornamentation and fecundity. We found that under natural conditions, there was a decline in eggshell color with laying order, suggesting pigment limitation for females. However, carotenoid-supplemented females had enhanced second egg coloration compared to controls, and the color increase was independent of their previous pigment allocation to the first egg. Our results suggest that biliverdin-based eggshell coloration is costly to produce and can be alleviated by current carotenoid availability.

Keywords Biliverdin · Eggshell coloration · Carotenoids · Life-history trade-offs · Pigments · *Sula nebouxii*

Introduction

Avian eggshell color is remarkably variable among and within species and its possible function has long been debated. Traditionally, predation and brood parasitism have been proposed as major selective pressures on eggshell pigmentation (Wallace 1889). Other hypotheses invoke structural functions (such as protection against solar radiation, reinforcement of eggshell strength and thermal protection of the embryo; see Reynolds et al. 2009 for a recent review), and a post-mating signaling role to induce male parental effort (Moreno and Osorno 2003). Within species, the variation of eggshell color between clutches may indicate that the allocation of pigment to the eggshell is physiologically constrained, an assumption that underlies some of the hypotheses mentioned above. Accordingly, regarding blue-green eggshell pigmentation, there is evidence on the cost of biliverdin allocation to the eggshell in terms of antioxidant defenses (Hanley et al. 2008; Morales et al. 2008). However, the exact mechanisms limiting biliverdin-based egg coloration remain little explored.

Biliverdin is formed intracellularly during the degradation of heme in the liver (Bauer and Bauer 2002), although

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J. Morales (✉) · A. Velando
Departamento de Ecoloxía e Bioloxía Animal,
Universidade de Vigo,
36310 Vigo, Spain
e-mail: jmorales@uvigo.es

J. Morales · R. Torres
Departamento de Ecología Evolutiva,
Laboratorio de Conducta Animal,
Universidad Nacional Autónoma de México UNAM,
México, DF 04510, Mexico

recently, it has been proposed that eggshell biliverdin can be directly produced in the shell gland (Zhao et al. 2006). Biliverdin pigment possesses important physiological properties. It is a potent in vitro anti-viral (Nakagami et al. 1992) and free-radical scavenger (e.g., Stocker et al. 1987; McDonagh 2001; Kaur et al. 2003), and a key modulator of cell transduction pathways and gene expression (Maines 2005). Also, it has been suggested that biliverdin, together with bilirubin, mediates immunological responses in mammals (e.g., Otterbein et al. 2003; Sedlak et al. 2009), and, although birds, amphibians, and reptiles do not produce bilirubin, the pigment biliverdin itself has been found to favor embryo development in amphibians (Falchuk et al. 2002). Even though biliverdin can be directly produced in the organism, its deposition in the eggshell may not be cost-free and may impair other physiological processes, as proposed by Moreno and Osorno (2003). Accordingly, recent findings in wild passerines indicate that biliverdin-based egg coloration is constrained when females suffer impaired body condition (Soler et al. 2008) and is traded-off with antioxidant defenses when the female's reproductive effort is experimentally increased (Morales et al. 2008). Thus, biliverdin-based eggshell pigmentation is probably limited by stressful processes that may be alleviated by certain nutrients, as found for egg coloration based on porphyrin pigments (e.g., see Odabasi et al. 2006, in poultry).

Biliverdin properties are similar to those of carotenoid pigments. Carotenoids are well known to exert an immunostimulatory role (Chew 1993). In birds, variation of dietary carotenoids has been shown to enhance cellular and humoral immune responses (McGraw and Ardia 2003; Velando et al. 2006). Also, carotenoids are involved in antioxidant defenses, protecting DNA, proteins, and lipids from oxidation (Edge et al. 1997), although their antioxidant role could be minor for some bird species (Costantini and Møller 2008). Carotenoids transferred to eggs by laying females can enhance the survival of embryos and chicks (Blount 2000). Furthermore, carotenoid pigments are also responsible for ornamental displays in many bird species (see Hill and McGraw 2006, for a review). However, they are suggested to be a limiting resource to vertebrates because they are only obtained through the diet (i.e., not synthesized de novo by the organism; Olson and Owens 1998). Consequently, there may be tradeoffs in the allocation of carotenoids between competing somatic demands, such as sexual displays and immune and antioxidant functions (Lozano 1994; von Schantz et al. 1999), which has received a strong body of empirical support (e.g., Blount et al. 2003; reviewed by Pérez-Rodríguez 2009). All these suggest that both carotenoids and biliverdin pigments may be involved in similar physiological functions. Thus, we hypothesized that carotenoid availability could affect biliverdin deposition in the eggshell, since carotenoid consumption may allow females to deflect biliverdin from other physiological processes, presumably alleviating the immunological or oxidative costs of egg pigmentation.

Resource-allocation tradeoffs are of special interest in long-lived species. According to life-history theory, the amount of parental investment of long-lived species should be influenced by the relationship between current reproductive effort and future adult survival (Stearns 1992). Hence, long-lived species should be more restrictive in current parental effort than are short-lived ones. Here, we studied if biliverdin-based egg pigmentation was constrained by carotenoid availability in the blue-footed booby, *Sula nebouxii*, a long-lived seabird where both sexes devote intense parental care (Nelson 1978). Fresh eggs show a distinctly blue shell color, and pairs with more colorful eggs are more coordinated during incubation (Morales et al. 2010). The pigment responsible for the color of blue-footed booby eggs is biliverdin (Morales et al. 2010), and the eggshells do not contain carotenoid pigments (Authors, unpublished data). Previous evidence in this species highlights the importance of carotenoid availability in reproductive functions, as it modulates carotenoid-based blue foot pigmentation (Velando et al. 2006), a mutual sexual ornament (Torres and Velando 2003, 2005). Furthermore, an experimental increase of dietary carotenoids resulted in enhanced immunocompetence (Velando et al. 2006). We performed a carotenoid supplementation experiment in wild female blue-footed boobies after they laid their first egg. This experiment has been previously reported with regard to the role of carotenoid availability in the trade-off between female ornamentation and fecundity traits, such as egg mass and volume (Morales et al. 2009). Here, we dealt with the potential effects of carotenoid availability on eggshell coloration. The experimental design enabled us to investigate the effect of dietary carotenoids on second egg color, while controlling for the color of the first egg. We predicted that supplemented females would lay more colorful second eggs compared to controls.

Material and methods

Experimental procedure

We studied a breeding colony of blue-footed boobies at Isla Isabel, Nayarit, México, from January to March 2007. We combed the study area in search for freshly laid first eggs. Fresh eggs are easily detected because they appear clean, wet and show a distinctly blue-green color. The eggshell dries very fast, usually during the following hour after laying. Also, in the blue-footed booby, as in other sulids, the shell is covered with a chalky layer that starts fading after laying and gradually becomes whitish; in some cases, this is perceived visually throughout the laying day (Morales et al. 2010). Thus, it is possible to detect if an egg is fresh when it is wet, or at least, if it has been laid a few hours ago if it looks mainly blue (Morales et al. 2010). Although the outermost eggshell layer soon becomes discolored, the internal layers are also biliverdin-pigmented (even at hatching, the inner side of the eggshell is clearly blue). Interestingly, both parents exhibit their highest rates of nest attendance as laying approaches (Osorio-Beristain and Drummond 1998). Furthermore, females allow males to see freshly laid eggs in a conspicuous manner, sometimes including parading and nest building movements (Morales and Velando, personal observation; see Nelson 1978, in the north Atlantic gannet, *Sula bassana*).

On the laying day of their first egg, 48 females were captured by night-lighting and randomly assigned to receive a single dose of either 1 ml of drinking water (control group, n=22) or a solution of carotenoids in 1 ml of drinking water (experimental group, n=26), given by means of a syringe. The experimental group received 0.2 mg of Xamacol (16 g/Kg of lutein and 1 g/Kg of zeaxanthin) and 0.2 mg of Canthacol (100 g/Kg of canthaxanthin), provided by CaroTech, Carotenoid Technologies S.A., IQF group, Tarragona, Spain. The dose that we chose, 23.4 μg of carotenoids (including lutein, zeaxanthin, and canthaxanthin) in 1 ml of water falls well below the estimated total amount of carotenoids contained in the plasma of blue-footed booby females (on average, 1,200 μg of carotenoids; estimation based on the average concentration of plasma carotenoids in our females prior to manipulation, 13.57±3.56 μg/ml, and on the volume of plasma in birds, which averages 5 ml/100 g body mass, according to Goldstein and Skadhauge 2000). A higher carotenoid dose has been previously applied to male blue-footed boobies without any apparent adverse effect (Velando et al. 2006). Lutein, zeaxanthin, and canthaxanthin were the most commonly identified among total carotenoids in the plasma of female blue-footed boobies prior to manipulation (Morales et al. 2009). These three carotenoids are commercially available and widely used in poultry feeds to enhance yolk or skin pigmentation. Probably, the prey consumed by seabirds does not contain a single type of carotenoid (see for instance Shahidi et al. 1998, for a detailed description of the carotenoids contained in many fish and crustacean species). Thus, we considered that supplementation with a mixture of carotenoids was appropriate.

Females were individually marked with a numbered metal band, weighed (accuracy 25 g) and sampled for blood (100–500 μL) from the brachial vein to assess plasma carotenoid concentration prior to treatment. We identified and quantified plasma carotenoids by high-performance liquid chromatography (HPLC) following the protocol described by Pérez et al. (2008). Carotenoids were determined at 445 nm with a UV-detector and quantified by the use of external standards (astaxanthin, canthaxanthin, ß-carotene, ß-cryptoxanthin, echinenone, lutein, zeaxanthin; for details on HPLC protocol, see Pérez et al. 2008 and Morales et al. 2009). The concentration of unknown carotenoids was calculated in relation to a lutein standard. We summed the concentration of known and unknown carotenoids to estimate total carotenoid concentration (μg/ml) prior to treatment.

Nests were examined at least twice a day in search of second eggs. In our population, egg laying mostly occurs between 0900 and 1600 hours (Morales and Velando, personal data from 2006 to 2007 based on 106 females monitored hourly). The maximum interval between second egg laying and color measurement was 3 or 4 h in most nests. Second eggs were measured in the same fashion as first eggs (see the following section). Females lay a mode of two eggs with an average interval of 4 days. Thus, the number of days elapsed (mean±SE: 4.1±1.8) from supplementation to second egg laying in our study was taken into account in statistical analyses. Only nine females laid a third egg (two controls and seven experimentals; Morales et al. 2009), but we could not measure its color on the laying day because of time constraints. Twelve females abandoned prior to laying the second egg (25%, four controls and eight experimental females). In this species, on average 30% of clutches are abandoned during incubation. Thus, the proportion of abandoned clutches in our experiment is within the natural range. Two additional females did not lay a second egg. Thus, a total of 34 females (17 in each experimental group) laid a second egg.

Egg measurements

As soon as a first or second egg was detected, egg color was measured with a CM-2600d portable spectrophotometer (Minolta Co. Ltd., Osaka, Japan). Before obtaining the reflectance spectra of each egg, we gently wiped a small area of the outer layer of the shell with a cotton cloth moistened with water to remove dirt or small sand pieces that usually get stuck to the shell. Eggs were placed directly on a target mask (diameter 1 cm). The reflectance spectra for each egg were automatically obtained as means of three sequential measurements of each egg by changing the position of the egg with respect to the apparatus. Eggs completely filled the measuring port. Blue-green chroma (hereafter called "egg color" or BGC) was calculated as the proportion of reflectance in the blue-green part of the spectrum ($R_{400-570}/R_{360-700}$), following Siefferman et al. (2006). Blue-footed boobies show maximum visual sensi-

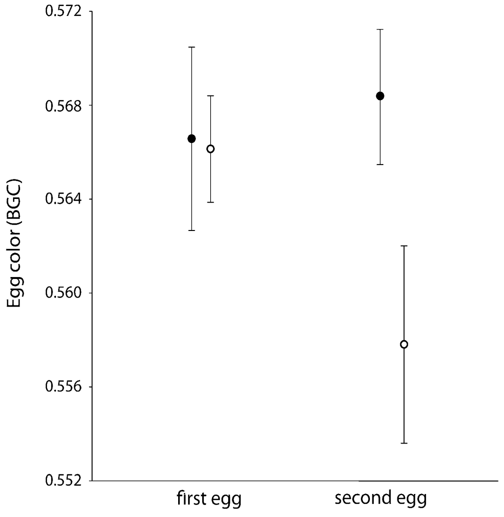

Fig. 1 Natural decline in blue-green eggshell coloration (proportion of blue-green chroma, BGC) with laying order and effect of carotenoid supplementation on the color of second eggs. *Full* and *empty dots* represent, respectively, the carotenoid-supplemented and control groups. Values are means±SE

tivity between 460 and 620 nm (Reed 1987), almost coinciding with the curve of egg color. Using the proportion of reflectance between 460 and 620 nm as a measure of egg color led to similar results (not presented but available upon request). Egg length (L) and width (W) were measured with a caliper to the nearest 0.01 mm, and egg volume (V) was calculated with the formula $V = 0.51LW^2$ (Hoyt 1979). Egg measurement and color estimation were completed in 2 min in all cases.

Statistical analyses

We used SAS software (SAS institute 2001) for statistical analyses. A general lineal model with normal distribution was fitted to test whether the color of second eggs was affected by carotenoid supplementation, while controlling for the color of first eggs (laid prior to treatment). Additionally, the following covariates and interactions of interest were included in the maximal model: number of days elapsed from supplementation to second egg laying, second egg volume, female body mass prior to manipulation, the total carotenoid concentration in plasma prior to manipulation, first egg color × treatment, total carotenoid concentration in plasma × treatment. The final model was obtained by a backward deletion procedure. First, the interaction terms and then the other covariates were removed from the full model when the variance explained did not significantly improve the model ($\alpha=0.05$). We show the *F* and *P* values of each non-significant variable before removing it from the full model. The difference in sample size between the full ($n=30$) and the final model ($n=34$) is due to four blood samples that were not of sufficient quality to assess carotenoid concentration. No outliers were detected, as indicated by Cook's distance >1 in all cases.

Results

The color of first eggs (laid prior to manipulation) did not differ between experimental treatments ($F_{1,45}=0.02$, $P=0.89$). Similarly, laying date, mass, and volume of first eggs, pre-manipulation values of female body mass and plasma concentration of carotenoids did not differ between experimental treatments (reported in Morales et al. 2009). The coefficient of variation of first eggshell color in the study population was 2.5%.

Under natural conditions, second eggs were paler than first ones, suggesting pigment limitation throughout laying (Fig. 1). However, the color decline in the laying sequence disappeared with carotenoid treatment, second eggs of carotenoid-supplemented females being more colorful than control second eggs (the effect of treatment was significant per se: $F_{1,32}=4.28$, $P=0.047$; Fig. 1). When controlling for the rest of covariates, second egg color was affected by the interaction between treatment and first egg color (first egg color × treatment: $F_{1,30}=8.31$, $P=0.007$; first egg color: $F_{1,30}=11.50$, $P=0.002$; treatment: $F_{1,30}=8.56$, $P=0.007$). We found a positive association between the color of first and second eggs in control females (beta (β)=0.66, $r^2=0.44$, $P=0.004$; Fig. 2). Contrastingly, there was no association for experimental females, as they laid more colorful second eggs regardless of first egg color ($\beta=0.14$, $r^2=0.02$, $P=0.59$; Fig. 2). The rest of covariates were not

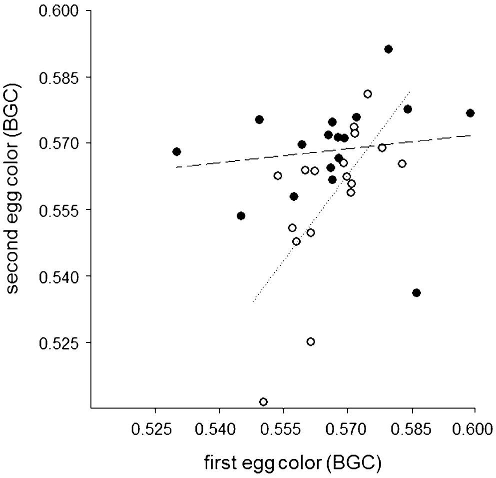

Fig. 2 Effect of the interaction between treatment and first egg color (proportion of blue-green chroma, BGC) on second egg color. *Full dots* and *dashed line* are carotenoid-supplemented females and empty dots and dotted line are controls

significant (total carotenoid concentration × treatment: $F_{1,21}=0.03$, $P=0.87$; second egg volume: $F_{1,22}=0.03$, $P=0.85$; female body mass: $F_{1,23}=0.55$, $P=0.47$; days elapsed from supplementation to second egg laying: $F_{1,24}=2.21$, $P=0.15$; total carotenoid concentration: $F_{1,25}=3.19$, $P=0.09$).

Discussion

We found that females supplemented with a single dose of carotenoids on the laying day of the first egg laid more colorful second eggs than controls. Furthermore, they consistently produced a more colorful second egg independently of their previous investment on egg pigmentation. These results suggest that biliverdin-based blue-green egg pigmentation is costly to produce and is alleviated by carotenoid availability.

Previous evidence in passerines indicates that biliverdin-based eggshell pigmentation can be traded-off against reproductive or maintenance functions. Experimentally increased reproductive effort in pied flycatchers (*Ficedula hypoleuca*) resulted in a negative association between egg coloration and female antioxidant capacity (Morales et al. 2008). In this line of evidence, Soler et al. (2008) found that impaired condition (feather clipping) prior to laying negatively affected blue-green egg color intensity in female spotless starlings (*Sturnus unicolor*). Also, egg color in pied flycatchers was positively influenced by experimentally enhanced nutritional resources (mealworms) at laying (Moreno et al. 2006). Our results indicate that biliverdin-based egg coloration is affected by carotenoid availability and suggest that carotenoids may alleviate reproductive tradeoffs involving eggshell pigmentation. Carotenoid supplementation has been widely used in poultry feeds to enhance yolk color (e.g., Lai et al. 1996) and laying capacity (reviewed in Blount 2004), but its effect on eggshell pigmentation has been little investigated. The only reference that we found on this topic reported that beta-carotene supply did not affect porphyrin-based egg pigmentation in poultry (Odabasi et al. 2006). Egg pigmentations based on biliverdin and porphyrins might be constrained by different mechanisms (see also that porphyrin-based egg pigmentation was neither affected by food supply in Eurasian kestrels, *Falco tinnunculus*; Martínez-Padilla et al. 2010).

The natural decline in blue eggshell color with laying order is suggestive of biliverdin limitation for females. This phenomenon has also been reported in flycatchers (Moreno et al. 2005; Krist and Grim 2007), but not in another seabird laying eggs with blue-green ground color, the ring-billed gull, *Larus delawarensis* (Hanley and Doucet 2009). The low productivity and patchy distribution of prey that blue-footed boobies encounter in tropical waters (discussed by Weimerskirch et al. 2006) may account for this discrepancy. In our study, variation in first egg pigmentation among control clutches explained 44% of the variance in the color of second eggs, suggesting that females vary in their investment on eggshell pigmentation. However, control females consistently produced less colorful second eggs, even after accounting for the variation in first egg color. In contrast, experimental females did not show an association between the color of first and second eggs, indicating that their investment in second eggs was independent of the previous investment. Thus, carotenoid supplementation may have alleviated the natural pigment depletion that was observed throughout laying. Also, biliverdin pigmentation was influenced by current carotenoid intake rather than previous nutritional status, since second egg color was unaffected by female body mass or plasma carotenoid levels prior to supplementation.

The biosynthesis site of eggshell biliverdin is far from clear. Biliverdin deposited in the shell has been traditionally thought to derive from blood (e.g., Kennedy and Vevers 1973), as biliverdin is the final product of heme degradation in birds. Additionally, recent findings reveal that biliverdin can be directly synthesized in the epithelial cells of the shell gland (Zhao et al. 2006). Due to the important role of biliverdin in the immune and antioxidant defenses (e.g., Falchuk et al. 2002), the allocation of this pigment to the eggshell has been hypothesized to impair these physiological processes (Moreno and Osorno 2003). Previous findings revealed that blue-footed boobies enhanced their immune response when they received a supplement of carotenoids in the diet (Velando et al. 2006). Hence, in the present study, carotenoid consumption may have allowed supplemented females to deflect biliverdin from other physiological processes, presumably alleviating immunological or oxidative costs. Another possibility is that supplemented females directly allocated the extra carotenoids to the eggshell, although we have not found traces of carotenoids in the eggshells of females under natural conditions (Authors, unpublished data). Our experiment suggests that biliverdin biosynthesis or deposition may be controlled by the same mechanism that mediates other dynamic physiological processes such as immune responses or foot ornamentation. This may support that eggshell biliverdin derives from blood, rather than being synthesized in a more autonomous and “sealed” system like the shell gland. Alternatively, biliverdin synthesis in the shell gland may be enhanced by carotenoid supplementation.

In a previous survey, we found that a single dose of carotenoids at laying boosted female foot color and egg size, two traits that are traded-off under natural conditions (Morales et al. 2009). Since females enhanced egg color when resources that are critical for ornamentation and fecundity were available, we may speculate that egg pigmentation plays a role in reproduction. Blue eggshell color is related to parental coordination during incubation, probably a key aspect for reproductive success in this species (Morales et al. 2010). Hence, it is plausible that egg color affects the conflict over parental care, although it starts fading in the following hours after laying (Morales et al. 2010). Additionally, eggshell pigmentation could play a role in embryo protection through its anti-bacterial properties, as proposed by Soler et al. (2008), or as a free-radical scavenger against oxidative stress; biliverdin absorption by the embryo could occur through the allantoic membrane (similarly to calcium assimilation; Deeming 2002). To demonstrate a role of eggshell color in reproduction, further studies should investigate whether this trait is related to reproductive success or to the mate's effort in this species. The present study supports the idea that eggshell coloration is costly to produce and is mediated by the current carotenoid availability for laying females.

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