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TESTING AN ASSUMPTION OF THE SEXUAL-SIGNALING HYPOTHESIS: DOES BLUE-GREEN EGG COLOR REFLECT MATERNAL ANTIOXIDANT CAPACITY?

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Abstract. The presence of blue-green egg pigmentation has puzzled naturalists for more than 100 years. One hypothesis on the function of this pigmentation proposes that blue-green egg chroma signals female quality and that males respond to this signal by increased provisioning to presumably higher-quality clutches. The hypothesized mechanism is that blue-green egg color signals female antioxidant capacity, because the eggshell pigment responsible for blue-green coloration, biliverdin, has antioxidant properties in the mother. Our study is the first to examine this mechanism, which is critical to this hypothesis. We found that female Gray Catbirds with higher total antioxidant capacity laid eggs with higher blue-green chroma. In addition, we found that males provided more care to nestlings from clutches with higher average blue-green egg chroma. This shows an intriguing potential link between female antioxidant capacity and blue-green egg chroma. Interpreting the variation in females' antioxidant capacity will require a better understanding of the relative importance of dietary intake of antioxidants, oxidative stress, and the cost of depositing biliverdin into eggs.

Key words: *antioxidant capacity, biliverdin, egg coloration, parental effort, pigment, sexual signaling.*

Probando una Suposición de la Hipótesis de Señalización Sexual: ¿Refleja el Color Azul-Verde de los Huevos la Capacidad Antioxidante de la Madre?

Resumen. La pigmentación verde-azul de los huevos ha asombrado a naturalistas por más de cien años. Una hipótesis propone que la coloración verde-azul es una señal de la calidad de la hembra y que los machos responden a esta señal proporcionando mayor atención a nidadas de presunta mayor calidad. El mecanismo detrás de esta hipótesis es que la coloración verde-azul de los huevos indica la capacidad antioxidante de las hembras debido a que el pigmento responsable del color de la cáscara, la biliverdina, tiene propiedades antioxidantes en la madre.

Nuestro estudio es el primero en analizar este mecanismo, que resulta crítico para la validez de la hipótesis. Encontramos que las hembras de *Dumetella carolinensis* con mayor capacidad antioxidante ponen huevos con mayor coloración verde-azul. Además, encontramos que los machos dan más cuidado a crías que provienen de nidadas con huevos de alta coloración verde-azul. Esto expone un potencial nexo entre la capacidad antioxidante de las hembras y la coloración verde-azul de los huevos. Para interpretar la variación en la capacidad antioxidante entre distintas hembras se requiere de un mejor entendimiento de la importancia relativa del consumo de antioxidantes en la dieta, del estrés oxidativo y del costo de incluir biliverdina en los huevos.

The enormous natural variation in avian eggshell pigmentation is largely unexplained (Underwood and Sealy 2002, Kilner 2006). Blue-green eggs are particularly curious because they appear to be conspicuous to some predators (Blanco and Bertellotti 2002, but see Aviles, Stokke et al. 2006) but are found across many families. Why do so many birds produce blue-green eggs?

Adaptive hypotheses for blue-green egg pigmentation have included aposematism (Swynnerton 1916, Cott 1948), thermoregulation (McAldowie 1886, Bakken et al. 1978), egg recognition (Victoria 1972, Jackson 1992, Soler and Møller 1996), and crypsis (Lack 1958). Few of these hypotheses have gained widespread support (Underwood and Sealy 2002, Kilner 2006), and some have been discredited altogether (Lack 1958, Kilner 2006). A recent hypothesis that is gaining support is the sexual-signaling hypothesis (Moreno and Osorno 2003). This hypothesis suggests that egg coloration signals female quality and that males make postmating investment decisions based upon these signals. The cost associated with this signal is proposed to be maintained by the in vitro antioxidant-like properties (Kaur et al. 2003) of the pigment responsible for blue-green egg coloration in birds, biliverdin (Kennedy and Ververs 1976). Maternal deposition of biliverdin into eggshells during the oxidatively stressful period of reproduction should be costly (Wang et al. 2001, Wiersma et al. 2004). Therefore, only the highest-quality females should be able to afford to produce highly chromatic blue-green eggs.

There is some support for the assumption that higher-quality females deposit more biliverdin to their eggs (Moreno et al. 2005, Moreno, Lobato et al. 2006, Siefferman et al. 2006). Previous

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research has shown that females who mount larger immune responses lay brighter eggs (Moreno et al. 2005), and females with supplemented diets produce eggs with higher blue-green chroma (Moreno, Lobato et al. 2006). There is also some evidence for a relationship between female body condition (size-adjusted mass) and egg coloration (Moreno et al. 2005, Siefferman et al. 2006). However, a direct test of the relationship between egg pigmentation and a female's antioxidant capacity is lacking. This mechanistic link between maternal antioxidant capacity and blue-green egg pigmentation is critical to the sexual-signaling hypothesis, because differences between females in antioxidant capacity are the presumed basis for differences in females' ability to afford pigment deposition into eggshells.

Another limitation of empirical support for the sexual-signaling hypothesis is that most tests of it to date have focused on cavity-nesting species (Moreno et al. 2004, Soler et al. 2008), with only one exception (Cassey et al. 2008). In these birds, the ability to assess relative mate quality may be limited by few opportunities to inspect other birds' nests to assess the relative quality of one's own eggs (Moreno and Osorno 2003). Thus, the sexual-signaling hypothesis may be more relevant to open cup-nesting species, where physical inspection of other clutches is possible (Moreno and Osorno 2003). We studied an open cup-nesting species, the Gray Catbird (*Dumetella carolinensis*), to test the relationship between blue-green egg chroma and female antioxidant capacity, female body condition, and provisioning of nestlings by the female's mate. To our knowledge, this is the first study to directly assess the relationship between blue-green egg chroma and female antioxidant capacity.

METHODS

STUDY SPECIES AND STUDY SITE

The Gray Catbird is socially monogamous, sexually monomorphic, and exhibits biparental care. Males establish territories and sing to attract females. Shortly after pair bonds are established, nests are created by females with some male assistance (Cimprich and Moore 1995). The Gray Catbird is double brooded and generally lays four blue-green eggs per clutch (Baicich and Harrison 1997). The female develops a brood patch and incubates the eggs, while the male will occasionally bring her food. During the early nestling period, the male contributes most of the nestling feeding while the female does most of the brooding. Parents may continue to feed their young for 12 days after fledging; however, if the female begins building another nest, only the male will feed the offspring (Zimmerman 1963). The Gray Catbird practices intraspecific egg predation (DH, pers. obs.) as well as occasional polygyny (Johnson and Best 1980, Hanley et al. 2007), making it possible for males to physically inspect other eggs, and therefore making this species an ideal candidate for studying the sexual-signaling hypothesis.

We conducted this study in East Buffalo Township, Pennsylvania over the course of two summers (2005 and 2006). The study site is located in the area surrounding Dale's Ridge Trail, under the stewardship of the Merrill W. Linn Conservancy. The study area was 24 ha and was comprised of forested, grassy, and edge habitat inaccessible to the public. We have limited our analysis to data from 2006 because the egg reflectance data collected in 2005 were limited and incomplete. We present data with and without two cases in which females' antioxidant capacity was measured in 2005 and clutch chroma was measured in 2006.

EGG-COLOR QUANTIFICATION

Egg color was measured in the field, 6.0 ± 2.3 days prior to hatching, using a portable spectroradiometer (Spectra Scan PR 650, Photo Research Inc., Chatsworth, California), tungsten light source, and an opal-white glass plate at nine nests. Although this spectroradiometer omits important reflectance information from the ultraviolet region (Aviles, Soler, and Perez-Contreras 2006, Aviles, Soler, Perez-Contreras et al. 2006), previous work has shown that blue-green chroma calculated without UV wavelengths is an adequate proxy for blue-green chroma calculated across the entire avian visual range (Moreno, Lobato et al. 2006). In addition, many studies have used similar methods to assess egg coloration (Moreno et al. 2004, 2005, Moreno, Morales et al. 2006, Lopez-Rull et al. 2007).

We designed a light-tight blackened box to control for incident light and to measure each egg from the same angle. The eggs were opaque; thus, translucence interference was not an issue. We measured the reflectance of each egg three times and averaged these values to represent the average egg coloration. Blue-green chroma was calculated as the proportion of the total reflectance (380–700 nm) that was in the blue-green region (400–576 nm) of the spectrum. Gray Catbird eggs are characterized by a peak of reflectance at ~500 nm (Fig. 1), which corresponds to the peak reflectance of biliverdin (Ding and Xu 2002, Falchuk et al. 2002). We then calculated clutch blue-green egg chroma as the mean of all eggs in a clutch.

ASSESSING PARENTAL QUALITY

In 2006, we caught Gray Catbirds in mist nets 0 to 15 days before they laid their first egg (mean = 8 ± 5) and calculated body-condition index ($100 \times [\text{mass}^{-3} / \text{tarsus length}]$) for each adult bird captured. We also drew 100 μl of blood from the bird's brachial vein, using a 26 ga needle. We kept these whole blood samples on ice while in the field and then placed them in a -80°C freezer within a few hours after collection. In the lab, we thawed the samples, centrifuged them to separate the plasma, and then measured plasma antioxidant levels using a commercially available assay of total antioxidant capacity (Cayman Chemical 709001, Ann Arbor, Michigan). This kit evaluates the ability of a sample to inhibit the oxidation of ABTS⁺, a radical cation, compared to that of a Trolox standard. Trolox is a water-soluble tocopherol analogue of known antioxidant capacity. Therefore, we present the antioxidant capacities of these samples as millimolar Trolox

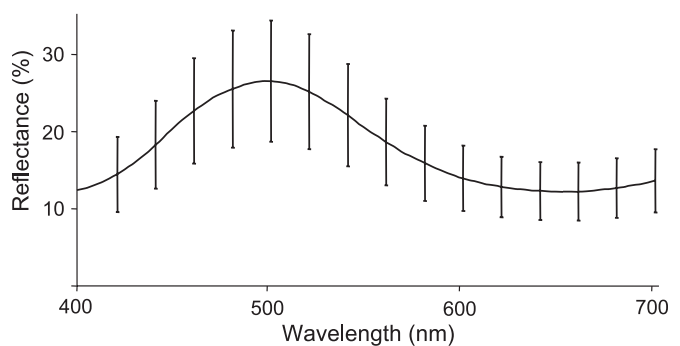


FIGURE 1. Mean reflectance value of Gray Catbird eggs sampled in 2006, in East Buffalo Township, Pennsylvania ($n = 36$). To illustrate the population level variation, we include SD at every 20 nm between 420 and 700 nm.

equivalents. Parallelism with the standard curve was demonstrated using two pooled, serially diluted samples of blood plasma. Our small sample size enabled us to include all of our samples in triplicate on a single assay; therefore there is no interassay variability to report. The average intraassay variability, as calculated by the coefficient of variation, was 4.0%. An initial dilution series was analyzed to determine the plasma:buffer dilution that kept the sample (10 μ l of this mixture) in the most robust range of the kit's sensitivity. The optimal dilution was 1:2048 for males and 1:1024 for females.

PARENTAL EFFORT

We assessed paternal effort at eight Gray Catbird nests through approximately 1.5 hr observations of feeding visits using digital video cameras (DCR-TRV22, Sony, San Diego, California). Film observations have previously been shown to correspond with direct observations (Moreno et al. 2004), and Gray Catbirds acclimate relatively quickly to cameras in the vicinity of the nest (Dolby et al. 2005; DH, pers. obs.). A feeding visit was recorded when a male fed at least one offspring during a visit. Average paternal feeding rate is positively related to nestling age in the Gray Catbird (Johnson and Best 1982). A general linear model showed that in 2006, paternal investment increased with chick age (whole model: $F_{1,16} = 32.4$, $P < 0.001$; day: $F_{9,16} = 2.6$, $P = 0.04$; nest: $F_{7,16} = 7.4$, $P < 0.001$). Therefore, we restricted our measures of paternal investment to when the chicks were six or seven days old, which reduced our sample size to seven nests. We controlled for brood size at time of observation, and therefore future references to "paternal effort" refer to feeding visits per chick per hour. We similarly quantified female investment per nest during the same observation period (hereafter "maternal effort").

STATISTICAL ANALYSES

Linear regression was used to detect any potential relationship between female antioxidant capacity and the number of days prior to egg laying that blood was sampled. We used linear regression to determine the relationship between female body-condition index and average clutch blue-green egg chroma, while polynomial regression was used to analyze the relationship between female antioxidant capacity and average clutch blue-green chroma. We used stepwise regression to create a model to predict paternal effort and included average clutch blue-green egg chroma and female effort as predictor variables (setting the probability of removal at $P = 0.10$). We also used Pearson correlation to determine the relationship between female body condition and antioxidant capacity. We addressed the potential alternative that Gray Catbirds in our population are mating assortatively based on parental quality, by using Pearson correlations to determine if there was an association between parental effort of males and females. We examined the association between male body-condition index and average clutch chroma to assess whether high quality males (who obtain high quality territories) father clutches of higher blue-green chroma. We consider findings with $\alpha \leq 0.05$ significant. All analyses were conducted with SPSS for Windows 12.0.0 (2003).

RESULTS

PARENTAL QUALITY

There was no relationship between antioxidant capacity and number of days before clutch initiation when considering birds caught in 2006 ($r^2 = 0.001$, $P = 0.96$, $n = 5$) or when including all data from both years ($r^2 = 0.002$, $P = 0.91$, $n = 9$); therefore, we

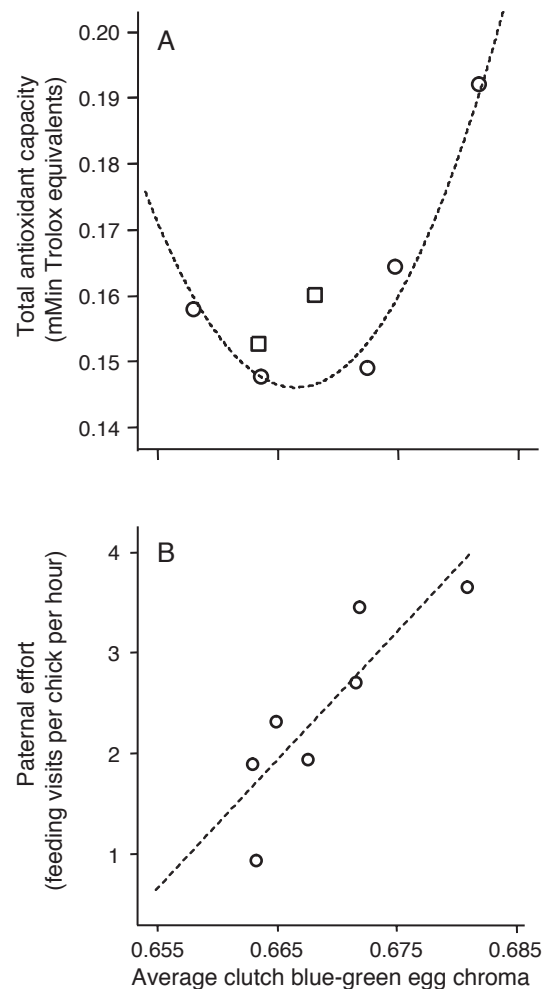


FIGURE 2. Relationship between the average clutch blue-green egg chroma of Gray Catbirds in East Buffalo Township, Pennsylvania and (A) female total antioxidant capacity, or (B) paternal effort. In (A), circles represent data from the 2006 field season, and squares represent two individuals for which antioxidant capacity was measured in 2005 but clutch reflectance was measured in 2006. The dashed line is the cubic relationship fit to the 2006 data only; the relationship when including the 2005 antioxidant data was still significant ($P \leq 0.05$).

did not need to control for sampling date in subsequent analyses. The relationship between average blue-green chroma of the clutch and female antioxidant capacity fit a cubic regression ($r^2 = 0.97$, $P = 0.03$, $n = 5$; Fig. 2A). The relationship remained the same if we included the two birds whose total antioxidant capacities were measured in 2005 ($r^2 = 0.81$, $P = 0.02$, $n = 7$). In 2006, female body-condition index was not related to the average blue-green chroma of her eggs ($r^2 = 0.09$, $P = 0.58$, $n = 5$). Body condition was not correlated with antioxidant capacity ($r = -0.27$, $P = 0.66$, $n = 5$).

PARENTAL EFFORT

Stepwise regression analysis found that the average blue-green chroma of the clutch was a significant predictor of paternal investment in 2006 ($r^2 = 0.74$, $P = 0.01$, $n = 7$; Fig. 2B) and excluded

maternal provisioning from the model ($P = 0.96$). In 2005, one male provisioned two separate nests, complicating how we view paternal investment in this species (Hanley et al. 2007). Although it is possible that some males in 2006 were polygynous, we believe that polygyny is an exception rather than the rule in this species.

ALTERNATIVE CONSIDERATIONS

There was no significant association between paternal and maternal effort ($r = -0.07$, $P = 0.87$, $n = 7$). Although male body-condition index was not significantly associated with average clutch blue-green egg coloration ($r = 0.85$, $P = 0.07$, $n = 5$), we likely were unable to detect any relationship due to limited sample size.

DISCUSSION

In this study, we used Gray Catbirds to investigate two of the assumptions of the sexual-signaling hypothesis: that females with higher antioxidant capacity lay more chromatic eggs, and that males provision more to nestlings from clutches with higher average blue-green chroma. Our main finding shows intriguing support for a critical and previously untested component of the sexual-signaling hypothesis—that females' antioxidant capacity is predictive of average blue-green clutch chroma. Despite having a small sample size, we found a significant relationship between these two measures, which is consistent with the sexual-signaling hypothesis.

In contrast, we found no relationship between a female's body condition and the average blue-green egg chroma of her clutch. Although some studies have found a relationship between female condition and blue-green egg coloration (Moreno, Lobato et al. 2006, Siefferman et al. 2006, Soler et al. 2008), body-condition indices have not generally been predictive of egg coloration (Moreno et al. 2004, Lopez-Rull et al. 2008). Body-condition index did not correlate with antioxidant capacity, suggesting that these two measures of quality may be indicating different aspects of female quality. It is also possible that size-adjusted body mass is not an important signal of quality in this species at this stage of the annual cycle (Moreno and Osorno 2003), potentially increasing the relative value of egg coloration as an indication of quality.

We also found that paternal care was positively related to blue-green egg chroma. Some studies have found a relationship between blue-green egg chroma and paternal investment (Moreno et al. 2004, Soler et al. 2008), while others have not (Lopez-Rull et al. 2007). Previous cross-fostering experiments have shown that in some species, males provide more investment to clutches with more chromatically blue-green eggs (Moreno, Morales et al. 2006), while other cross-fostering studies do not show this relationship (Krist and Grim 2007).

Although these correlational results are an important step forward in studies considering the signaling quality of egg coloration, two key issues remain unresolved: whether biliverdin deposition is truly costly, and whether female antioxidant capacity is more strongly determined by genetic quality or by local environmental factors such as dietary intake (Briviba and Sies 1994). Although it is possible that egg coloration reflects female investment decisions, a strong dietary influence is problematic for the sexual-signaling hypothesis for two reasons. First, dietary antioxidants might reflect territory quality or quality of incubation feeding, such that egg color is more a reflection of male quality than female quality. Second, dietary intake of antioxidants can vary over short time spans, making it difficult to detect a relationship between a female's prelaying antioxidant levels and the average color of eggs in her clutch. Despite these uncertainties about mechanism, our data appear consistent with the sexual-signaling hypothesis in the Gray Catbird.

In this study, we were limited by a small sample size. Although the relationships we found are suggestive, more rigorous work using similar techniques is necessary to definitively establish the link between antioxidant capacity and blue-green egg coloration. Moreover, the correlative nature of this study makes it impossible to definitively rule out several plausible alternatives. It is possible that the relationship we found between paternal care and egg coloration was not due to any signaling function of egg coloration. Instead such a relationship may result from high-quality males mating with high-quality females. If high-quality females lay eggs with high blue-green chroma, a pattern of assortative mating by overall quality could create a correlation between the quality of the male parental care and the color of females' eggs, even though males would not be using egg color as a cue for adjusting their parental effort. Another alternative is that eggs produced in higher-quality territories secured by higher-quality males are more highly chromatic because these territories provide better foraging opportunities for laying females. The small sample size here limited our power, although our results, which approach significance, suggest that a larger sample would likely have produced a significant result. Despite a small sample size, we found a strong trend towards a positive association between male body-condition index and average clutch chroma, making this alternative plausible.

Biliverdin may have a nonsignaling, although equally adaptive function in this species. We might expect results similar to those observed here if biliverdin provides protection from solar radiation (Bakken et al. 1978), or if biliverdin has antiviral, antibacterial, or antifungal properties. If it is costly to produce pigmented eggs, we should find a relationship between female antioxidant capacity and blue-green egg chroma, even if the pigments themselves do not have meaningful antioxidant functions. In this situation, hatchlings from eggs with more biliverdin may be of higher quality, and males may provision more to these high-quality chicks. No signaling mechanism is necessary if the male is assessing the hatchling rather than the egg per se; however, if the effect of biliverdin on chick quality is not easily assessed, a signaling function of egg coloration may still be possible.

In summary, we provide unique support for a key component of the sexual-signaling hypothesis: a relationship between female antioxidant capacity and eggshell pigmentation. We also provide evidence for a connection between egg color and male parental effort. These results are correlative and do not provide a causative link, and therefore should not be taken as definitive support of the sexual-signaling hypothesis. We encourage future research to focus on biliverdin's role in the laying female's antioxidant defense system.

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