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Short communication

Experimentally impaired female condition does not affect biliverdin-based egg colour

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It has been proposed that blue-green egg coloration is a condition-dependent female sexual trait that may modify paternal care in a post-mating sexual selection scenario. This pattern may arise because the pigment responsible for eggshell colouration (biliverdin) may be a costly and limited resource, whose availability is linked to female health state. Thus, it can be predicted that females whose condition is compromised should be constrained in their capacity to deposit biliverdin in the eggshell, thus producing paler clutches. To test this hypothesis, we performed a handicapping experiment by clipping some feathers of female spotless starlings before egg laying and measuring the colour of their clutches. We expected the handicapping treatment to increase flying costs, impairing female overall condition and resulting in paler clutches. Our experiment was successful in lowering the weight gain of handicapped with respect to control females. However, in contrast to our expectations, we found no effect of the treatment on eggshell colouration. Eggshell colour varied along the laying order, with initial eggs of the laying sequence being relatively paler than the rest of the clutch, but this pattern was not different between experimental groups. Despite a very similar methodology, our results differ from a previous study on the same species and offer no support to the post mating sexual selected hypothesis, questioning the general applicability of the sexual selection role of eggshell coloration.

Keywords: eggshell coloration, handicapping manipulation, oxidative stress, sexual selection, *Sturnus unicolor*

Introduction

The existence of brightly coloured eggs in many bird species is a long-standing unsolved question for evolutionary biologists, who have proposed several explanations for their existence, including thermoregulation, camouflage or the coevolution with brood parasites (Kilner 2006, Cassey et al. 2011, Wisocki et al. 2020). In the last twenty years, the debate about the adaptive meaning of egg coloration has mostly revolved around the 'sexually selected egg coloration hypothesis' (SSECH) (Moreno



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and Osorno 2003). The SSECH proposes that colourful eggs might have evolved as post-mating sexual signals whereby females inform about their quality to their mates to trigger higher paternal investment after egg laying (Moreno and Osorno 2003). A key requirement of the SSECH is the existence of a functional link between female condition and deposition of pigments in the eggshell, which results in eggshell colour mirroring the quality of the female. Specifically, in the case of species laying blue-green eggs, the SSECH proposes that this functional link involves oxidative stress and the alleged antioxidant properties of biliverdin, which is the pigment responsible of this colouration (Moreno and Osorno 2003). The source of biliverdin deposited in the eggshell by female birds is thought to be in the uterus (Hargitai et al. 2017). However, the exact connection between the biliverdin destined to colour the eggshell and that derived from the systemic degradation of the erythrocytes has yet to be described (Homsher et al. 2018). Several studies on different species have found a link between eggshell colouration and female body condition, some of which provide support for a condition-dependence of biliverdin-based egg colour by following an observational approach (Moreno et al. 2005, 2013, Siefferman et al. 2006, Hanley et al. 2008). On the other hand, most of the experimental tests of the hypothesis, involving antioxidant or food supplementations or food limitation, revealed a positive effect of the manipulation on egg colour intensity (Moreno et al. 2006b, Krist and Grim 2007, Avilés et al. 2008, Soler et al. 2008, Morales et al. 2011, Butler and McGraw 2013, Hargitai et al. 2018). Nevertheless, the relation between biliverdin-based eggshell colour and female quality is not always straightforward. Thus, some observed relationships are sometimes found with some specific indicators of female somatic state (i.e. antioxidant status; Giordano et al. 2015, Hargitai et al. 2016), but not with more integrated measures of overall condition (comprising genotype or epigenetic state, Hill 2011). In the last years, a rising number of studies have identified genes linked to blue-green egg coloration in domestic and wild species (Wang et al. 2013, Liu et al. 2021), but the following step, relating the quantitative expression of such genes to the level of expression of the eggshell colour and its relative impact on fitness, has yet to be made. Other studies found a relationship with some specific, instead of the expected overall, difference in colour among clutches (e.g. a change in colour only in the second egg of the clutch, Morales et al. 2011), or the observational relationships compatible with the predictions that were unsupported by experimental manipulations (Hargitai et al. 2018). Given the ongoing uncertainty on the subject, experimental studies are needed.

In this study, we tested a key assumption of the SSECH, namely that eggshell colour is a condition-dependent trait that reflects female quality. As a model species we used the spotless starling *Sturnus unicolor*, a facultative polygynous passerine that lays biliverdin-rich blue eggs (López-Rull et al. 2008). Following a previous study on the same species (Soler et al. 2008), we manipulated female condition by increasing their wing load through a feather-clipping treatment, thus

handicapping their flight ability. The increase in wing load is a common experimental manipulation that results in a higher metabolic costs that in turn causes a reduction in body mass (Senar et al. 2002) and condition dependent traits, such as carotenoid-based integument colour (Leclaire et al. 2011). Increases in workload also result in impaired oxidative status (Costantini et al. 2012, Nikolaidis et al. 2012); nevertheless, the effect of feather clipping treatments on the oxidative state does not necessarily have an effect on the overall success of the reproduction (Casagrande and Hau 2018). One of the previous studies on spotless starlings found that the clipping of two primary feathers weeks before laying resulted in poorer condition and paler eggshell colour (Soler et al. 2008). Here we followed the same experimental protocol and therefore predicted that females whose condition was experimentally worsened would lay paler clutches, i.e. eggs with less biliverdin content in their eggshells (Soler et al. 2008). Although relative body mass has been considered an insufficient indicator of condition (Hill 2011), one of our purposes was to replicate a previous experiment, and this required using comparable measurements in the same trait. Moreover, we controlled for the interaction of the manipulation with laying order, since previous studies have shown a variation of the colour along the laying order in this species (López-Rull and Gil 2009), and it is possible that the treatment could affect only some eggs within the laying sequence (e.g. the final ones) rather than the full clutch.

The rationale of replicating this experiment was to test possible relationships between eggshell colour and condition, assuming that the experimental manipulation would indeed lead to a change in eggshell colour.

Methods

This study was carried out in Soto del Real, central Spain, in an open woodland used for cattle pasture, where since 2003, a population of spotless starlings breeds in 250 nest boxes. Starlings lay between 2 and 6 eggs per clutch (modal clutch size=4), and nest building and parental care are shared between the pair members. However, males vary widely in their effort, from 50% to 5% of total feeds (D'Arpa et al. unpubl.).

In March 2020, about a month before laying, adult individuals were captured while sleeping in the nest-boxes or using traps attached to the nest box entrance. We recorded body mass, tarsus and wing length, and individually marked all individuals with unique numbered aluminium rings and PIT tags (unless they were already marked from previous years). Finally, we carried out the handicapping procedure following Soler et al. (2008). Every captured female was randomly assigned to either the handicapping treatment or the control group. The handicapping treatment consisted in the clipping the fourth and fifth primary feathers of each wing at their base with a nail-clipper. This treatment has been found to cause a 5% increase in wing loading (Soler et al. 2008). Control females were subjected to regular manipulation,

including the same wing measurement and handling as experimental females. A total of 45 females were captured before laying and assigned either to the handicap ($n=22$) or the control group ($n=23$). Of these, 41 finally succeed to lay a clutch (20 handicapped and 21 controls) and have been considered for the analysis of eggshell colour. A fraction of these females were recaptured and measured during the brood-rearing period (when nestlings were between 6 and 9 days old) to test the effect of the handicapping treatment on their body mass. Eventually, we eliminated from the analyses on body mass and egg coloration the data of one female that had been captured too late the first time (i.e. only two days before she started laying), as the forming egg in the oviduct likely affected her pre-laying weight. As a result, we had pre-treatment body mass data of 40 females (21 controls and 19 handicapped) captured between 39 and 14 days before the onset of egg laying (mean = 30.8 days, SD = 5.9 days). We were also able to record post-treatment body mass data of 25 of these females (11 handicapped and 14 controls) that were recaptured between 20 and 23 days after the onset of egg laying (mean = 22.1 days, SD = 1 day). We recorded eggshell coloration from 160 eggs laid by 41 females (handicapped, $n=20$; control, $n=21$).

We monitored the nests of the experimental females in their first breeding attempt to determine the onset of egg laying. Once the first egg was detected in a nest, it was marked with a non-toxic permanent marker, and its colour was measured in duplicate with a portable spectrophotometer in the range 360–700 nm at 10 nm steps. Nests were visited daily afterwards, allowing us to identify and measure the colour of all the eggs in a clutch in their laying day. This close monitoring protocol also allowed us to detect and exclude intra-specific parasitic eggs, which are relatively common in this species, and can be easily recognized by comparing the suspect eggs with the colour and shape of the rest of the clutch (Monclús et al. 2017). In the clutches of the females included in this experiment we identified and removed two parasitic eggs. From the colour spectra we calculated the blue-green chroma (BGC), which is the proportion of reflectance in the blue-green part of the spectrum relative to the total reflectance ($R_{400-570}/R_{360-700}$). Eggshell BGC is a good proxy of the biliverdin content in the eggshells in this species (López-Rull et al. 2008). The two measurements collected from each egg were highly repeatable ($r=0.96$, $t=47.8$, $p < 0.001$), so we used the mean values per egg in the analyses.

Data analysis

Analyses were carried out in the R language, ver. 3.4.2 (<www.r-project.org>). Our analysis consisted of three parts as follows:

- 1) We first tested the effect of the handicap treatment on body weight. We used an ANCOVA analysis where post-laying weight was the dependent variable, the treatment the independent variable and pre-laying weight a covariable. We considered one measurement of weight

pre-laying and post-laying for each female. When more than one measurement of the same female was available, we used the first one in time.

- 2) We then tested the effect of the experimental treatment on the BGC of each egg with a linear mixed model using the lme4 package (Bates et al. 2015). We included treatment, laying order as a quadratic term (after checking its distribution) and their interaction as predictors. For laying order, given the low number of clutches with six eggs ($n=2$), we encoded these together with the fifth egg of these clutches, treating it as a continuous variable. Laying order was also considered as a random term nested in female identity, affecting the slope and the intercept of the model. We chose this random effect structure because it provided a lower AIC value than a simpler model using the mother ID as random intercept only and more complex structures comprising the treatment nested in mother ID (Schielzeth and Forstmeier 2009).
- 3) Lastly, we also explored the possibility that the signal value of eggshell coloration depended on the most colourful egg within each clutch, that might act as a superstimulus (Moreno et al. 2008), or on the general colour of the eggs within the nest. To do so, we performed two linear models where the response variables were the maximum BGC of each clutch, in the first case, and the mean BGC of the clutch in the second. For both models, treatment was the only fixed factor.

Results

There were no differences in body weight between experimental groups before females were manipulated ($F_{1,38} = 0.14$, $p = 0.07$). Four females deserted before or during the egg laying (2 handicapped and 2 control), suggesting that there was no effect of the treatment on the likelihood to attempt breeding. After controlling for initial weight, we found an effect of the treatment on body mass at the second capture (estimate \pm SE: -2.35 ± 0.61 , $F_1 = 14.7$, $p < 0.001$, Fig. 1): control females showed a higher increase in body weight than handicapped females did (Cohen's $d = 1.17$).

Egg BGC was not affected by our experimental treatment (Fig. 2a): handicapped females laid eggs that were similar in colour to control females (estimate \pm SE: 0.22 ± 0.35 , $F_{1,39,53} = 0.39$, $p = 0.534$). We found an effect of laying order on egg BGC (Fig. 2b), which was mostly caused by a marked increase in BGC from the first egg to the middle of the laying sequence, being relatively stable or slightly decreasing in the last laid eggs (laying order: estimate \pm SE = 0.91 ± 0.15 , $F_{1,119,4} = 37.2$, $p < 0.001$; laying order²: estimate \pm SE = -0.13 ± 0.03 , $F_{1,118,2} = 26.4$, $p < 0.001$). This variation in colour along the laying sequence was not affected by treatment (no significant interaction between treatment and the quadratic effect of laying order: estimate \pm SE: -0.01 ± 0.01 , $F_{1,40,4} = 0.42$, $p = 0.519$).

Finally, we found no difference between control and handicapped females in the maximum BGC values of their clutch

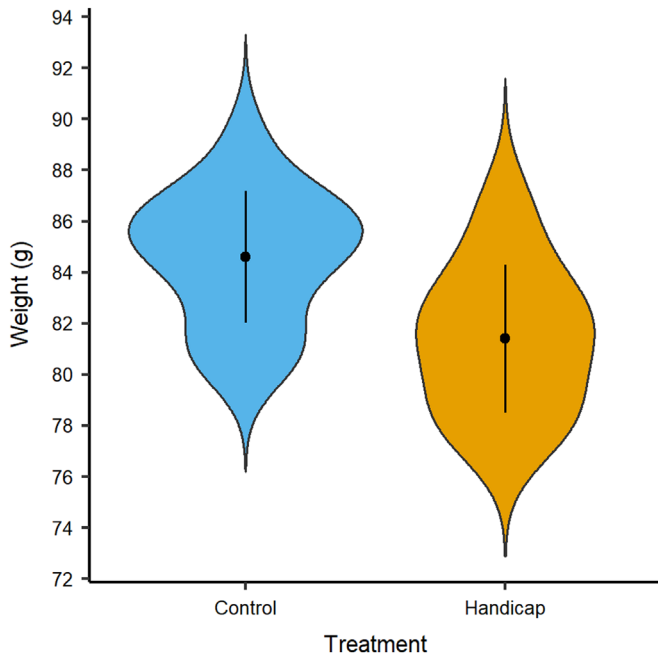


Figure 1. Body weight after experimental manipulation of control and handicapped females. Black dots and whiskers indicate the mean \pm standard deviation for each group.

(handicapped: estimate \pm SE = -0.008 ± 0.17 ; $F_{1,168} = 0.002$; $p = 0.96$), nor in the mean BGC (estimate \pm SE = 0.09 ± 0.16 ; $F_{1,168} = 0.29$; $p = 0.59$).

Discussion

Contrary to our predictions, although our experimental manipulation negatively affected female condition, it did not translate into an effect on the colour of the eggs they laid.

Egg BGC (which is a proxy of the biliverdin content of the eggshell; López-Rull et al. 2008) increased along the laying sequence, reaching its maximum at intermediate eggs, but this pattern was not affected by the treatment. The increasing eggshell coloration in the first part of the clutch, followed by a decrease in the last egg, could be an indication that the pigment is not readily available when laying begins. The transportation or deposition system in the oviduct may not always be active at the same level, but it may need to be activated when the first egg is being produced, being slightly and gradually faded out towards the end of egg laying. This may also suggest that the pigment is limited in its availability (Butler et al. 2017, Morales 2020), but not in a linear way.

We did not find differences between treatments when considering the BGC value of the most colourful egg of each clutch either, as could be expected if some particular eggs in a clutch act as a superstimulus (Moreno et al. 2008), nor in the mean BGC of the clutch, as could be expected if males responded to the overall colour of the clutch (Moreno et al. 2006a).

Our results do not support the hypothesis that the starling eggshell colour is a condition-dependent trait, which runs counter the idea that this trait plays a role as a sexual signal of female condition. Although we followed the same protocol as a previous experiment in the same species (Soler et al. 2008), our results differ. Soler et al. (2008) found that the BGC of the eggs of the handicapped females was significantly lower than that of controls, whereas we did not find such an effect, despite the similarity in the procedure and the higher sample size of our design (41 versus 23 final clutches). Indeed, in both studies, the females were subjected to the handicapping treatment during a similar period of time before egg laying (mean = 30.8 and 32 days, this study and in Soler et al.'s, respectively), which we expected should have produced a similar effect in the colour of the eggs. Still, they

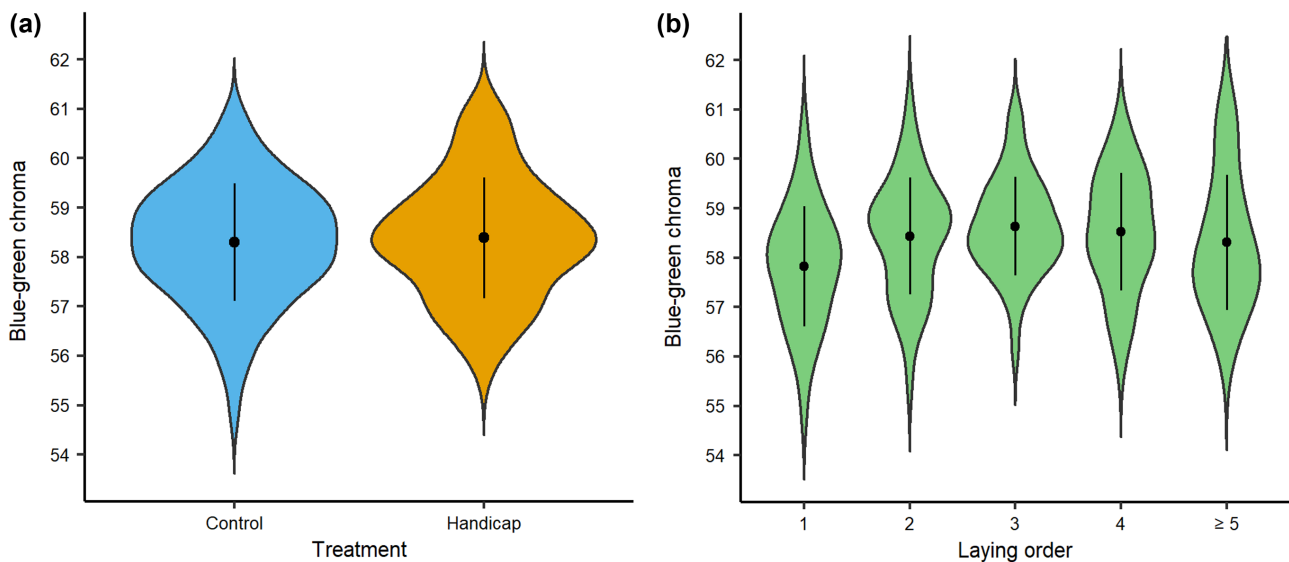


Figure 2. Eggshell blue-green chroma in relation to (a) the experimental treatment of the female (control or handicap) and (b) laying order. In both cases, black dots and whiskers indicate the mean \pm standard deviation for each group.

found a difference of 1.57 BGC units between handicapped and control in their population, whereas ours amounted to just 0.3 units (and, notably, in the opposite direction), which corresponds to a Cohen's d of 0.46 in theirs and of 0.08 in our study. Nevertheless, a calculation of the ideal sample size from means and standard deviations showed that with the standard deviation of our data, we would only have required a total sample of 10 (5 per treatment) to find an effect as big as theirs. Still, we found an effect 5.5 times smaller with a sample size 4 times higher.

One significant difference in our design is the time elapsed between first and second capture. Soler et al. recaptured a subsample of experimental females before egg laying, 1–2 weeks after the first capture, finding a drop in body mass in the experimental birds, while control females gained weight between the first and the second capture. Instead, we opted for a late recapture – i.e. during chick rearing – to minimize the possibility of desertion, finding that both groups increased in weight from capture to recapture, although the increase in controls was higher than that of handicapped females. This small difference in the study protocol is unlikely to explain the lack of effect on eggshell colour in our study. However, it prevents a direct comparison of the effect size of the treatment on female condition between both studies, which could be likely explanation for the inconsistent results. Thus, we can only speculate that differences in environmental conditions (weather harshness), feeding requirements (home range extension) or population dynamics between the populations may partially account for the fact that our clipping treatment was insufficient to generate a handicap strong enough to cause egg differences between groups (Fig. 2a). In this sense, it is remarkable that 2020 was the second worst year since 2012 in female weight (mean 2020 = 79.8 g, SD = 4; mean 2012–2020 = 80.7 g, SD = 4.1, Supporting information). Thus, we would expect that the handicapping procedure might have exerted a particularly strong effect in our study.

Another difference in the experimental protocol between the two studies is the timing of eggshell colour measurements. Soler et al. measured the colour of the eggs within two days after the end of laying, while we measured each egg on the same day it had been laid. This is an important distinction, because as previously found (Moreno et al. 2011, D'Arpa et al. 2021), biliverdin-based egg coloration fades soon after laying. There is no reason to expect a different degree of fading in the eggshell of handicapped and control females, but a late measurement could add noise to the data, especially if the timing of measurement did not follow a strict temporal protocol. Nevertheless, the SSECH does not make any assumption about the timing at which males might evaluate eggshell colour, but given that shared incubation is common in this species, it is very likely that they could evaluate it during the whole incubation period, bypassing the problem of the fading. Another minor difference between both studies is that our BGC values were calculated from spectra ranging between 360 and 700 nm, instead of spectra covering the entire potential UV-visible range of birds (300–700 nm) as in Soler et al. However, measurements using both methodologies

in a sample of 53 eggs reported that both estimates of BGC are virtually identical (Supporting information).

Moreno and Osorno (2003) proposed that the mobilization of biliverdin could be regulated by steroids, progesterone in particular. Progesterone has been found to be related to the concentration of pigments in the shell gland after the ovulation (Soh and Koga 1994), but it is also known to promote oxidative stress (Schantz et al. 1999). Anyway, progesterone may not act at a systemic level, but rather a tissue level (Schantz et al. 1999), meaning that there may be not necessarily a causal connection between the general physical condition and the oxidative equilibrium of the individual. For instance, Wegmann et al. (2015) found that, after a handicapping experiment, great tits *Parus major* lost body mass, but their oxidative status was unaltered, possibly indicating that the reduction in weight could be a strategy to cope with the increased wing load, rather than a consequence of the greater workload they had to sustain. Similar results have been reached in other studies in which handicapped birds responded in complex ways to the manipulation. For instance, feather-clipped house wrens *Troglodytes aedon* reduced the frequency of nestling feeding visits to the nest while maintaining their weight and metabolic rate (Tieleman et al. 2008). A further study in great tits reported that, although handicapped individuals reduced weight, there was no effect on the oxidative status (Wegmann et al. 2015). Thus, a limited impact of increased wing load via feather handicap treatment on individual oxidative status (the proximate mechanism linking individual condition and eggshell pigmentation according to the SSECH) may also explain the lack of effects on eggshell colour of this study.

Another possibility that would explain the lack of effect on egg coloration is that females could be able to respond to their worsened condition by increasing their reproductive investment. Previous studies (Soler et al. 2008, Rivers et al. 2017) showed that handicapped females invested more in the reproduction at the cost of self-maintenance. In a recent essay, Morales (2020) proposed that biliverdin deposited in the eggshell could act as an antioxidant supply, as biliverdin would permeate through the calcified layers and the inner membrane of the eggs during embryo development, bolstering its development. If this is the case, the deposition of biliverdin in the eggshell can be seen as a reproductive investment in itself and handicapped females would have secured the healthy development of their offspring rather than maintaining their own body condition.

In conclusion, we did not find evidence for a fundamental assumption of the hypothetical role of egg coloration as a post mating sexual signal in our population. This work adds to a growing body of literature that question the general applicability of the SSECH. Here we have closely replicated a study that found a clear condition-dependant effect on egg coloration, ourselves finding none, demonstrating that, although very appealing, this hypothesis is based on assumptions whose validity is likely to be highly dependent on the local conditions of the study population.

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Author contributions

Stefania R. D’Arpa: Conceptualization (equal); Data curation (lead); Formal analysis (lead); Investigation (equal); Software (lead); Visualization (lead); Writing – original draft (lead); Writing – review and editing (equal). **Iraida Redondo:** Data curation (equal); Investigation (equal); Writing – review and editing (supporting). **Eduardo Gómez-Llanos:** Data curation (equal); Investigation (supporting); Writing – review and editing (supporting). **Diego Gil:** Conceptualization (lead); Formal analysis (supporting); Funding acquisition (lead); Investigation (equal); Methodology (equal); Project administration (lead); Supervision (lead); Writing – review and editing (lead). **Lorenzo Pérez-Rodríguez:** Conceptualization (lead); Formal analysis (supporting); Funding acquisition (lead); Investigation (lead); Methodology (lead); Project administration (lead); Supervision (lead); Writing – review and editing (lead).

Transparent Peer Review

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Data availability statement

Data are available from the Dryad Digital Repository: <<https://doi.org/10.5061/dryad.f4qrfj6x7>> (D’Arpa et al. 2021).

Supporting information

The supporting information associated with this article is available from the online version.

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