FEATURED STUDENT RESEARCH PAPER

Prenatal manipulation of yolk androgen levels affects egg size but not egg colour in a songbird

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Abstract

Maternal allocation of androgens to the egg yolk allows mothers to adaptively manipulate offspring phenotype. Increases of egg androgen levels have often been shown to induce sex-specific effects. Some previous studies suggest that females specifically may suffer a fitness reduction after early exposure to high androgen levels. In this study, we explored whether female birds that developed exposed to high yolk androgen concentrations would reduce their maternal investment later in life. We explored two different aspects of female reproduction in spotless starlings (*Sturnus unicolor*): egg coloration (a post mating sexual signal in this species) and egg size. We manipulated androgen yolk levels in a large sample of clutches of this species. We monitored the reproduction of the females that hatched from these eggs, measuring the size and colour of the eggs they laid throughout their life (from 1 to 7 years of age). We found no overall difference in egg colour in relation to treatment, clutch number or age. However, females exposed to high egg androgen levels showed a steeper decrease in egg size along the laying order than controls. This pattern likely results in a more unbalanced distribution of resources within the clutch, possibly favouring brood size reductions in experimental females. In addition, control and experimental females differed in how egg volume changed with age. These results show that some egg characteristics may be affected by the female's exposure to yolk androgens during her own embryonic development. Our research calls for further long-term research on the influence of prenatal androgens on the fitness mechanisms regulating reproductive investment and its potential signalling role in a perspective of sexual selection.

Significance statement

In birds, mothers prepare the offspring to the environment they will encounter at hatching by depositing a mixture of compounds into the eggs. Androgens specifically exert a variety of effects during embryo development and chick growth, but some of these may be negative in adult life. In particular, early androgen may be detrimental for females in the long term, decreasing maternal investment and fitness. Using the spotless starling as study species, we tested whether an experimental increase in yolk androgens negatively affects breeding investment, measured by egg colour (a sexual signal) and egg size. We found no effects of the treatment on egg colour. However, females exposed to high androgen levels during their development reduced egg size along the laying order more strongly than controls. This may affect nestling survival, and indicates that prenatal yolk androgens may influence some egg characteristics with known relevance for offspring viability.

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Introduction

Parental effects occur when the expression of the phenotype of one generation is modified as a consequence of the environment experienced by the parents, especially by the mother (Mousseau and Fox 1998a). Maternal effects involve a variety of mechanisms with high potential to influence individual fitness and the adaptive ability of organisms to survive in variable environments (Mousseau and Fox 1998a). One of the most direct pathways of maternal effects in birds is via transfer of certain compounds to the eggs. Schwabl (1993) demonstrated that androgens found in avian eggs have maternal origin and have effects on the offspring. In birds, the amount of hormones that mothers deposit in the yolk varies according to a whole suite of factors, including laying order (Schwabl 1993; Groothuis and Schwabl 2002; Muriel et al. 2019), female quality and immune capacity (Müller et al. 2002; Pilz et al. 2003; Groothuis et al. 2005; Gil et al. 2006), but also breeding density (Groothuis and Schwabl 2002) and parasite infection (Tschirren et al. 2004).

These maternal androgens exert several effects on offspring. Early research focused on effects that confer benefits for the offspring, including chick development and growth (Eising et al. 2001; Navara et al. 2006; Müller et al. 2007), begging behaviour (Schwabl 1996; Eising and Groothuis 2003; Müller et al. 2007) or early survival (Sockman and Schwabl 2000; Müller et al. 2009). However, it has been shown that negative effects are also possible, and research has shown that yolk androgens can also impose a cost and impair individual viability (Ruuskanen et al. 2012; Treidel et al. 2013). Costs can include negative effects, e.g. on the immune system (e.g. the cell-mediated immune response, Muriel et al. 2017) or on the resistance to oxidative stress (Alonso-Alvarez et al. 2017). Following the logic of previous research on androgen-associated trade-offs (Folstad and Karter 1992), it has been suggested that there may be similar trade-offs between costs and benefits of maternal androgens (Groothuis et al. 2005; Muriel et al. 2015a). Most of this research has focused on early life stages (Gil 2008), and studies on the long term (i.e. months to years) are still scarce (Nilsson et al. 2011; Ruuskanen et al. 2012, 2013; Tobler et al. 2013). To our knowledge, no study to date has covered the entire lifespan of individuals. Still, these kind of studies are critical to evaluate the overall effects of maternal hormones on fitness and their adaptive role, especially in the perspective of their sex-specific activity (Muriel et al. 2017). Although the strength and direction of effects vary depending on the species, many of the studies involving egg androgen manipulation suggest that female nestlings do not benefit from elevated androgen levels, whereas males do (von Engelhardt et al. 2006; Saino et al. 2006; Ruuskanen and Laaksonen 2010; Muriel et al. 2015b, 2017). Long-term effects of systemic androgen levels on reproduction point to androgen-treated

females suffering negative consequences on their fitness in terms of number of eggs laid and chicks raised compared to control females, although such effects have been mostly seen in experiments in which adult androgen levels have been manipulated, rather than egg androgens (Rutkowska et al. 2005; Veiga and Polo 2008). The physiological mechanisms linking prenatal egg androgens with adult female reproductive investment are however still unclear.

It has been proposed that, in species that lay blue-green eggs, the colour of the shell may act as an honest signal of female quality ("sexually selected egg coloration", Moreno and Osorno 2003). This is based on the fact that the pigment responsible for the colour, biliverdin, has antioxidant properties (McDonagh 2001), and it may play a role in the oxidative equilibrium in the organism of birds. Such role may be particularly important in the case of a female during the breeding season, when an unbalanced oxidative status may represent a constraint to reproduction (Costantini et al. 2015, but see Alonso-Alvarez et al. 2017). If this is the case, biliverdin may provide a link between the oxidative status of the laying female and eggshell coloration (Morales et al. 2008, 2011). Indeed, empirical evidence of the connection between female quality and the appearance of their blue-green eggs have been repeatedly reported for a number of species (Moreno et al. 2006; Siefferman et al. 2006; Krist and Grim 2007; López-Rull et al. 2008), suggesting a possible role for egg colour signalling.

Female investment and condition can also induce variation in the size of eggs laid (Nilsson and Svensson 1993; Smith et al. 1993; Viñuela 1997; Cunningham and Russell 2000). Investment in egg size by the mother is beneficial for the offspring, since they receive a greater amount of water, nutrients and hormones (Krist 2011). Egg size is thus a key maternal effect for oviparous species, although the fact that it involves a mixture of constituents makes it difficult to pinpoint the exact benefit that a larger egg may imply. Nevertheless, nestlings that hatch from bigger eggs have been shown to be heavier and bigger than their siblings born from smaller eggs, even regardless of egg composition (Krist 2011).

Despite the wide body of literature testing the costs and benefits of yolk androgens and the interest that has arisen around the role of egg coloration as a signal (Moreno and Osorno 2003), no study so far has tested empirically the connection between prenatal exposure to maternal hormones in females and the colour of the eggs laid by these females later in life. However, at least two studies have analysed the effects of egg androgens on egg size, finding in both cases no effects of egg androgen injections in this variable (Rubolini et al. 2007; Müller et al. 2009). If maternal androgens impose a cost to females, it is to be expected that such a cost could be transferred to the expression of traits that depend on their quality and physiological

state, like the intensity of the colour or the size of their eggs.

In this study, we test whether experimentally increased yolk androgen levels affect the size and the eggshell colour of eggs produced by females hatching from androgen-manipulated eggs. We used as a study model the spotless starling (*Sturnus unicolor*), a medium-sized passerine in which the intensity of eggshell blue-green colour reflects its biliverdin content (López-Rull et al. 2008) and correlates with body condition and feather-ornament size in females (López-Rull et al. 2007, 2008). We evaluated the effect of an egg androgen manipulation on the colour and on the size of the eggs laid throughout the life of the females that hatched from the experimentally injected eggs. Previous studies in this species have shown that egg androgen injection enhanced embryonic development and post-hatching growth, regardless of embryo sex, but at the cost of depressing some components of the immune system in clutches laid at the end of the breeding season, when environmental conditions become harsher (Muriel et al. 2015a, b). Assuming that egg coloration and size reliably reflect female quality (Christians 2002; Moreno et al. 2004; López-Rull et al. 2008), and considering the potential negative effects of increased androgen levels for adult females (Rutkowska et al. 2005; Veiga and Polo 2008), we expect the eggs laid by females hatched from androgen-treated eggs (hereafter "treated females") to be less colourful and smaller than those laid by females hatched from control eggs (hereafter "control females").

Materials and methods

Study area and species

The study colony is composed of 250 nest boxes distributed across an open woodland located in central Spain (Soto del Real, Madrid). The spotless starling is a cavity nesting species that shows a facultative polygynous breeding strategy and a high breeding synchrony (Moreno et al. 1999). In our study area, spotless starlings usually show a bimodal distribution of broods across the season, with a first wave of clutches between mid-April and the beginning of May, and a second one (often corresponding to second clutches of the same females) at the end of May. When the first clutch is lost (predation, conspecific sabotage, etc.), a replacement clutch is laid a few days later. Usually, the same female does not lay a second clutch afterwards if this replacement clutch is successful. The quality of the offspring fledged typically decreases from the first to the second wave of broods (Muriel et al. 2015b), partly due to the harsher environmental conditions at the end of the breeding season (Gil et al. 2019).

Yolk androgen manipulation

Details of the experimental manipulation are provided in Muriel et al. (2015b). During the 2011 breeding season, all clutches in the population were injected either with an androgen treatment ($N = 185$, 128 belonging to the first clutch wave and 57 to the second) or with a control solution ($N = 160$, 113 belonging to the first clutch wave and 47 to the second), amounting to a total of 345 clutches in the experiment. All eggs of each clutch received the same treatment. The androgen treatment consisted of 24 ng of testosterone and 68 ng of androstenedione—the two main androgens found in eggs of this species—diluted in 10 μl of sesame oil. This corresponds to four standard deviations of the mean amount of testosterone and androstenedione found in eggs in this population in a breeding season (testosterone: 14 ng per yolk [SD = 6.0], androstenedione: 50 ng per yolk [SD = 17.1]; DG, unpublished data), adjusted for mean yolk mass (average yolk mass 1.4 g). The control treatment consisted of 10 μl of sesame oil alone. The concentration of hormones was selected based on a previous study on the same population by Muriel et al. (2015a), where the same dosage induced a stimulatory effect on hatching nestling body mass and skeletal growth. *In ovo* injections were performed in the field using a standard U-50 insulin syringe (Terumo Corporation, Tokyo, Japan), following a standard protocol and before incubation started (Muriel et al. 2015b).

Data collection

We monitored the breeding attempts of experimental recruiting females throughout their lives (2012–2018), from the age of 1 to 7 years old. As these females were focal individuals, it was not possible to record data blind. During the laying period of these breeding seasons, we visited nests on a daily basis from 2012 to 2014, and every other day from 2015 to 2018. This monitoring schedule allowed us to determine laying date with great accuracy, although not the exact laying order for all broods in each year. We marked eggs with a nontoxic waterproof pen and measured their length and width at the widest point with digital callipers (Mitutoyo Absolute, Kawasaki, Japan, precision = 0.01 mm). On the fifth day after the first egg had been laid, egg colour reflectance of the entire clutch was measured using a Minolta spectrophotometer CM-2600d in the range 360–700 nm. Each egg was measured on a cleaned circular spot of a 5-mm diameter on the blunt end of the egg. Reflectance values were obtained at 10 nm steps (see López-Rull et al. 2007). Intraspecific nest parasitism is common in this species (Calvo et al. 2000; Monclús et al. 2017). We assumed that a nest had been parasitized when two new eggs were found in a nest on a given day (starlings lay one egg per day) or when a single egg differed strikingly in appearance or size from the other eggs in a clutch. In this case, the egg that

differed from the rest of the clutch was recorded as parasitic and excluded from the analyses. The same criteria has been used for detecting parasitic eggs before in a study of the European starling, *Sturnus vulgaris* (Pilz et al. 2005). Although the use of visual discrimination to identify parasitic eggs has been questioned by some authors (e.g. Gronstol et al. 2006), we followed a conservative approach to minimise potential errors and discarded from the data base those eggs where the identity of the laying female was doubtful. After performing such conservative selection of available data, we finally considered for the analysis a total sample of 1009 eggs from 231 clutches (169 belonging to the first clutch waves, and 62 to the second) laid between 2012 and 2018 by the females hatched from yolk androgen–treated eggs in 2011 and recruited thereafter ($N = 77$). Due to technical problems with the spectrophotometer, we lost the data from 160 eggs, corresponding to 23 first and second clutches of 2014 and 4 first clutches of 2015.

For the analyses, we considered two dependent variables: blue-green chroma (BGC) as a measurement of egg colour, and egg volume as an estimate of egg size. BGC is the proportion of reflectance in the blue-green part of the spectrum, over the total reflectance ($R_{400-570}/R_{360-700}$). Biliverdin peak of reflectance falls in the same region of the spectrum (Falchuk et al. 2002) and biliverdin content has been found to correlate positively with BGC in eggs of this species (López-Rull et al. 2008). From the measurements of length and width of each egg, we calculated egg volume (mm^3) using the formula: $0.51 \times \text{length} \times \text{width}^2$ (Hoyt 1979).

Data analysis

Analysis was done in the R language, version 3.4.2 (R Core Team 2020). We built linear mixed models to test whether egg characteristics (BGC and volume) were affected by the hormonal treatment, using the lmer function in the "lme4" package (Bates et al. 2015). We visually explored the data distribution to assess normality and homoscedasticity before proceeding with parametric analysis.

Since broods were checked daily until 2014 and every other day from 2015, exact laying order was not available for the entire dataset. We therefore created an ordinal variable with three levels depending whether the eggs were found in the first 2 days from the onset of laying, on the central 2 days, or on the last 2 days. Since all individuals were born in the same year, the effect of age is indistinguishable from the effect of the year itself. Moreover, sample size was highly unbalanced with respect to female age due to mortality. Thus, we categorised female age as a factor with four levels: (1) first year, (2) second year, (3) third and fourth years and (4) fifth year and older. This categorisation reduces the unbalanced sample size of the different age categories, while still allows us to capture variation in age. Selective appearance or disappearance in or from

the population of individuals with a certain phenotype could change the phenotypic mean of the population itself and this could erroneously be interpreted as an ageing effect (van de Pol and Verhulst 2006; van de Pol and Wright 2009). Thus, to account for these possible selective changes in the population, we considered the age of first and the age of last measurement (AFM and ALM) in the analyses. Lastly, as the effect of yolk androgens may interact with environmental conditions experienced in early development (Muriel et al. 2015b), we considered in the analyses the female's birth clutch wave (first and replacement versus second wave of clutches), the brood size of the female's birth clutch (i.e. the number of the chicks in the female's birth clutch) and the clutch wave of the actual egg data (first and replacement versus second clutches). Replacement clutches were scarce in our dataset and were always laid a few days after the first clutches. Given that these two clutches experience very similar environmental conditions (in contrast to second wave clutches), we consider first and replacement clutches as "first wave clutches". Egg colour fades progressively after laying (Moreno et al. 2011; LP-R, unpublished data), and since all eggs were measured at clutch completion, the potential effects of laying order in colour simply reflect the fact that later eggs have been exposed to the light for a shorter time, making the laying order an unsuitable variable to test further effects or interactions on the colour.

Random effects for the volume model included year, female identity and clutch, coded so that each breeding attempt is identified individually. In both models, clutch identity was nested in female identity, as every female mothered several clutches. Both models considered the following fixed factors: treatment, age (factor with four levels), clutch wave (first or second), laying order, AFM, ALM, mother brood size (the size of the brood from which the mother hatched) and mother clutch wave (the clutch wave from which the mother hatched, first or second). In addition, the following interactions were included: treatment and age, treatment and clutch wave, treatment and mother clutch wave, clutch wave and age, AFM and ALM, brood size of the clutch of origin of the mother and mother clutch wave, and a triple interaction between treatment, clutch wave and age (see Table S1 and S2 in Supplementary material). In the model for the volume, we also considered further interactions with the laying order: laying order and age; laying order and clutch wave and laying order and treatment. We then performed a backward selection in *R*, removing the interactions and the non-significant variables and report final models fitted by restricted maximum likelihood. In all cases, *P* values were calculated by Satterthwaiet's approximation (lmerTest package, Kuznetsova et al. 2017).

We also performed two additional analyses to further validate our main hypothesis. First, we run a linear mixed model to explore the potential relationship between BGC and volume. The model considered the BGC as dependent variable and just the volume and the laying order as covariate, to

compensate for the artefact effect we already found in the previous analysis (see above), and the same random effects as the model built for the volume: year, clutch identity and female identity. Lastly, we calculated the repeatability of both the BGC and the volume for the female identity. This was performed in R through the "rptR" package (Stoffel et al. 2017).

Results

We found no effect of the androgen treatment on the colour of the eggs as measured by BGC ($p > 0.1$). The model investigating BGC (Table 1) only revealed a positive effect of laying order ($p < 0.001$) and age ($p = 0.007$, Fig. 1), but no effect of treatment, clutch wave, the clutch wave and clutch size of birth of the mother, nor any of the interactions (all $p > 0.1$). As expected, eggs increased in colouration along the laying order due to the difference in time of exposure to the air between the laying and the measurement. Moreover, we found that colour decreased as females age, as shown by the age estimate decreasing progressively with age class (Table 1). Neither AFM nor ALM were kept in the model, supporting the age-specific decrease on egg colour ($p > 0.1$) (Table S1, Supplementary material).

In the case of egg volume, we found significant interactions across treatment and age ($p = 0.04$). Analyses of these interaction estimates showed that this was due to differences between treatments showing opposite trends in different age classes, being stronger and negative for treated females in the second class than in the other classes (Fig. 2). In addition, we found significant effects of the interaction between treatment and laying order, showing a steeper decrease of the volume along the laying order in treated females as compared to controls ($p = 0.03$; see Table 2, Fig. 3). We also found an overall significant reduction in volume between the first to the second brood wave ($p = 0.015$). No other variables were significant in the model for egg volume.
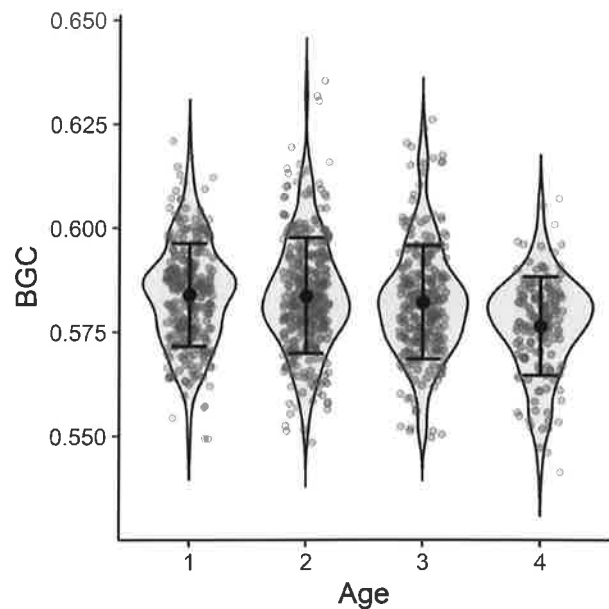
Fig. 1 Distribution of egg colour (BGC, blue-green chroma) between the age categories; black points represent the mean and bars 1 SD and grey shapes reflect point density along y-axis

Finally, we found a positive but very weak relationship between BGC and volume ($p = 0.038$, marginal $R^2 = 0.02$) (Fig. 4), revealing that bigger eggs were also more colourful. BGC and volume had both a strong positive repeatability within female identity across years (BGC: $R = 0.69 \pm 0.04$ SE; volume: $R = 0.49 \pm 0.06$ SE).

Discussion

We conducted a large long-term experiment to test the hypothesis that yolk androgens have long-lasting effects on the individual's phenotype throughout their lives, possibly influencing future generations (i.e. transgenerational effects). In this study, we tested one particular component of the female phenotype: reproductive investment as measured by egg colour

Table 1 Linear mixed model showing the effect of treatment, age of the mother (as a factor with four levels) and laying order on the blue-green chroma (BGC) of the eggs. Random effects account for year, mother identity and breeding attempt. Degrees of freedom are calculated via Satterthwaite's method. P-values considered significant ($p < 0.05$) are in bold

Fixed effects		Estimates	SE	χ^2	df	p
Intercept		$5.81\ e^{-01}$	$2.2\ e^{-03}$	69960.87	1	**< 0.001**
Treatment T		$-6.60\ e^{-04}$	$2.78\ e^{-03}$	0.06	1, 74.77	0.812
Age				12.16	3, 2	**0.007**
	Age 2	$-1.92\ e^{-03}$	$1.5\ e^{-03}$			
	Age 3	$-3.66\ ^{-03}$	$1.45\ e^{-03}$			
	Age 4	$-5.22\ e^{-03}$	$1.57\ e^{-03}$			
Laying order		$2.92\ e^{-03}$	$3.64\ e^{-04}$	64.12	1, 856.49	**< 0.001**
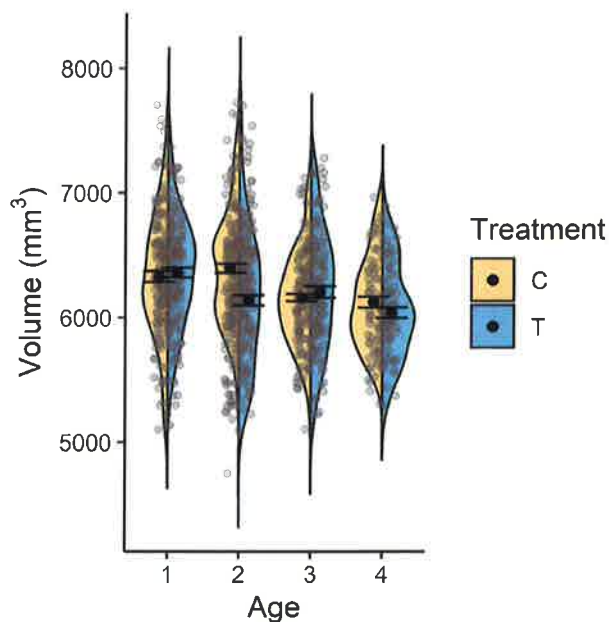
Fig. 2 Distribution of egg sizes across the age categories, for control (yellow-half violins) and androgen-treated females (blue-half violins); black points represent the mean and bars 1 SD and grey shapes reflect point density along y-axis
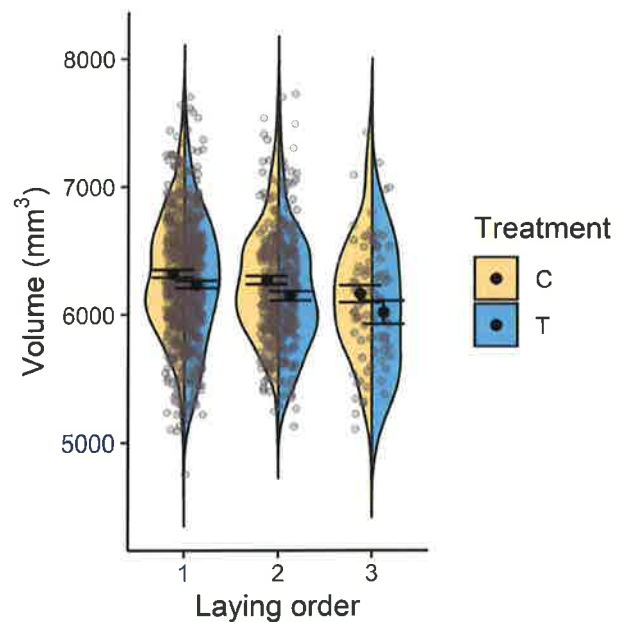
Fig. 3 Distribution of egg sizes across the laying order, for control (yellow-half violins) and androgen-treated females (blue-half violins); black points represent the mean and bars 1 SD and grey shapes reflect point density along y-axis

and egg size. Our results indicate that the colour of the eggs was not affected by the androgen treatment, although it did get lighter in females of older age (Fig. 2). In the case of egg volume, we found a negative effect of the interaction between the treatment and the laying order (Table 1). The volume of the eggs decreased along laying order in a steeper way in treated than in control females (Fig. 3). Moreover, there was a difference in volume between first and second clutch waves,

with eggs of the second clutch wave being smaller than first wave ones (Table 2).

BGC has been shown to reflect biliverdin content and then, according to our hypothesis and previous literature, it could be used as a signal of female quality (Moreno and Osorno 2003; Moreno et al. 2005, 2006; Costantini et al. 2015). Eggshell coloration in this species has been shown to reflect egg testosterone and vitamin E content, as well as body condition and

Table 2 Linear mixed model showing the effect of treatment, age of the mother (as a factor with four levels), laying order, clutch wave and the interactions of treatment with the age of the mother and with the laying order on the volume of the eggs. Random effects account for year, mother identity, breeding attempt and year. Degrees of freedom are calculated via Satterthwaite's method. P-values considered significant ($p < 0.05$) are in bold

Fixed effects		Estimates	SE	χ^2	df	p
Intercept		7266.63	145.53	2493.34	1	**< 0.001**
Treatment T		48.07	123.46	0.15	1, 116.43	0.697
Age				1.60	3, 2	0.660
	Age 2	64.31	185.85			
	Age 3	− 112.14	169.61			
	Age 4	− 119.88	175.84			
Laying order		− 47.75	19.97	5.75	1, 818.96	**0.017**
Clutch wave 2		− 117.64	48.58	5.86	1, 166.75	**0.016**
Treatment T × age				8.21	3, 168.81	**0.042**
	× Age 2	-239.57	116.50			
	× Age 3	57.04	127.64			
	× Age 4	− 133.82	150.87			
Treatment T × laying order		− 67.23	30.43	4.88	1, 820.48	**0.027**
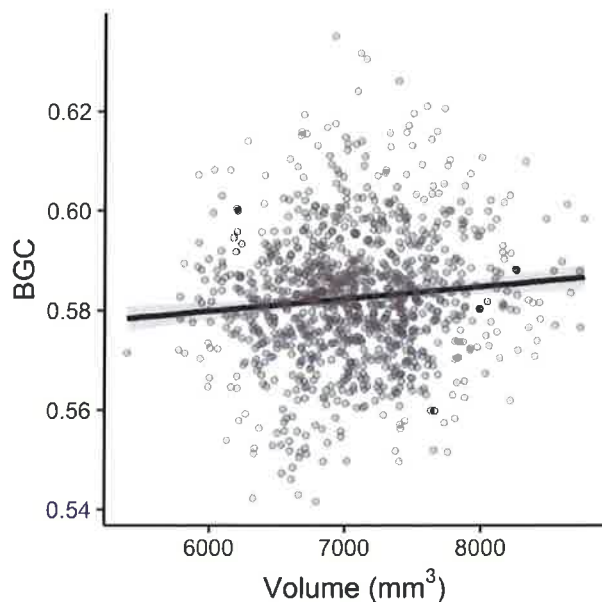
Fig. 4 The linear relationship between egg blue-green chroma (BGC) and volume; the grey area around the black line denotes the 95% confidence interval

telomere dynamics of fledglings (López-Rull et al. 2008; Navarro et al. 2011; Soler et al. 2018). In a similar way, egg volume has been widely used as a proxy of female reproductive ability, being related to offspring viability (Williams 1994; Krist 2011). According to our hypothesis, females developing in androgen-treated eggs would experience some physiological costs that may ultimately impair their breeding capacity, resulting in paler or smaller eggs when they become adults (Rutkowska et al. 2005; López-Rull et al. 2008; Veiga and Polo 2008; Muriel et al. 2015b). Contrary to our expectations, our results show that BGC values were similar in eggs laid by control and treated females. The sexually selected egg coloration hypothesis is specifically based in a physiological connection between the oxidative stress experienced by the female during egg laying and biliverdin deposition in the eggshell (Moreno and Osorno 2003). Thus, our results suggest that pre-hatching exposure to androgens as manipulated in our experiment does not lead to breeding oxidative costs on the females in this species in the long term, or that such costs are not reflected in a reduced investment in the colour of the eggs. The control over the coloration of the eggs may imply mechanisms different from, or more complex than, the androgen-oxidative stress connection (Alonso-Alvarez et al. 2007). Although circulating biliverdin is a product of the catabolism of the heme group of the erythrocyte, the specific amounts of this pigment to be deposited in the eggshells are most likely synthetized in the shell gland (Wang et al. 2007) and it may not mirror the concentration of systemic pigment. Most of the studies linking oxidative status (Morales et al. 2008, 2011; Moreno et al. 2013), or body condition (Siefferman et al.

2006; Soler et al. 2008), to egg coloration take into account proximate agents affecting the short-term quality of the female (i.e. nutritive status). Our work predicted in the first place that it would be possible to detect an effect in adult life, long after the manipulation. However, the empirical support of the impact of maternal androgens on oxidative stress during early development is mixed (Galván and Alonso-Alvarez 2010; Noguera et al. 2011; Tobler et al. 2013; Treidel et al. 2013; Giraudeau et al. 2017; Parolini et al. 2018). This evidence suggests that females may buffer the imprint of maternal hormones in adulthood. Also, the effect of maternal androgens in the eggs may be modulated during the development by the expression of androgen receptors in the extraembryonic membranes (Kumar et al. 2019), opening the possibility of an active role of the embryo towards maternal allowance of compounds (Groothuis et al. 2019).

We found a negative effect of laying order on the BGC. This effect most likely reflects the colour fading that can be explained by the difference in days elapsed between laying and measurement of each particular egg (Moreno et al. 2011). Biliverdin fades right after oviposition, possibly because of photodegradation, with a 1% decrease in the few days elapsed between oviposition and clutch completion. Since the protocol we followed implied measuring the colour after the last egg of the clutch had been laid, the first eggs were also the ones most exposed to light and then the less pigmented of the clutch. This artefact did not allow us to further investigate interactions that involved laying order, and thus we cannot exclude subtler mechanisms controlling eggshell coloration. This limitation does not allow us to definitely reject the hypothesis that egg colour, perhaps through within-clutch variation, may signal female quality.

We did observe a decrease in the colour of the eggs in older females. This could be a sign that the colour of the eggs is dependent on the overall condition of the mother, and as the quality of the breeding females generally declines after the third or fourth year of life, our results seem to be in agreement with this. Previous studies that found a difference in BGC described mainly a positive association between BGC and age (Siefferman et al. 2006; Krist and Grim 2007) or no association (Hargitai et al. 2008; Honza et al. 2011). We considered the age of first measurement (AFM) and the age of last measurement (ALM) to control for selective female appearance or disappearance affecting longitudinal age differences. Our results showed no effects given by either AFM or ALM, suggesting that the pigmentation of the eggshell decreased due to within-individual changes.

Egg size showed a response to the treatment in interaction with the laying order, showing a steeper decrease in androgen-treated females than in controls. To our knowledge, this is the first time that a long-term effect on a fecundity-related trait is observed as a consequence of an egg androgen modification. We already expected from previous studies in this and other

species (Gibson and Williams 2017; Monclús et al. 2017; SD, personal obervations) that the volume of the eggs would decrease along laying order, but our results also show that this decrement is greater in treated females. Early exposure to androgens may interact with oogenesis patterns perhaps affecting hormonal regulation processes (Williams 2012). For example, Williams (2012) reported a reduction in egg size after injecting females (not eggs) with follicle-stimulating hormone (FSH), which is fundamental in the regulation of follicle development. If androgens interact with FSH (as observed in humans, Dewailly et al. 2016), they may also alter egg size regulation. Anyway, we cannot exclude that other kinds of costs affected the treated females. Given that egg size affects hatchling size (Krist 2011), this effect could translate into transgenerational consequences if the future fitness of these individuals is impaired. Given that the starling shows strong hatching asynchrony (1–2 days) (DG, personal observations), this larger reduction in egg size along the laying order should promote brood reduction. This would allow first-hatched hatchlings to outcompete their younger siblings, possibly resulting in a reduction in fledging number.

Our results showed an effect of the treatment on the egg size in interaction with the age of the females. The direction of effect was not linear and it is difficult to understand from an ageing perspective (Fig. 2). We would argue that year-specific events might explain this odd pattern. All females in this study were bred in 2011, and thus all experienced in 2013 a particularly harsh season that may have increased adult mortality and reduced nutrients for egg formation (Gil et al. 2019). Although we are not able to provide evidence of this, due to the complete overlapping of the year effect with the age, it is the most likely explanation that we can provide for such a pattern.

Average egg volume was lower in second than in first clutches, something that has already been observed in our population (López-Rull et al. 2010). This may be due to the harsher environmental conditions experienced by females during second broods, as well as to the fact that many of them have already raised a first brood weeks before, which may constrain their capacity to allocate resources to produce large eggs (Muriel et al. 2019). An alternative adaptive hypothesis is that smaller eggs may be favoured in second broods when climate is warmer and brooding requirements are relaxed. Moreover, although egg size decreased, yolk weight in this population increased in the second clutch wave, which may compensate for the loss in total egg volume (Muriel et al. 2019). Our analysis revealed a significant egg size repeatability ($R = 0.51 \pm 0.05$), which is lower than previous reported repeatabilities for this population ($R > 0.68$, Monclús et al. 2017; $R = 0.58$ for the first egg, $R = 0.71$ for the fourth egg, Muriel et al. 2019). This indicates that although egg size is a relatively non-plastic trait (Christians 2002; Monclús et al. 2017),

females facing different environmental conditions vary egg size to some extent.

We also found that the size of the eggs and their colour are positively related (Fig. 4). The detected relationship was weak ($R^2 = 0.02$). Noteworthy, this positive relationship emerged despite the opposite trend for egg size and BGC to vary across the laying sequence. The strength of the relationship might be arguably higher if the colour of each egg had been measured just after it had been laid. This positive relationship is consistent with our assumption that egg colour and size are positive proxies of female quality. If eggshell colour is a costly post mating sexual signal of female quality, we might expect such a positive relationship between egg colour and egg size. However, factors other than the post mating sexual selection may also underpin this positive relationship. For instance, Morales (2020) proposed that biliverdin in the eggshell may play a direct role in protecting the embryo from oxidative stress during development. If female ability to deposit high amounts of biliverdin to bolster offspring development is linked to its condition, the positive association between egg colour and size would emerge as well.

In summary, our results show that the potential of females to invest in the colouration of the eggs is not modified by an increased exposure to prenatal androgens, whereas egg size patterns within clutches are affected. Yolk androgens are one of the mechanisms whereby females may modulate offspring phenotype to maximise their fitness (Mousseau and Fox 1998b), though they have been hypothesised to exert negative side effects. Our findings show that the long-term effects of maternal hormones in the offspring are prone to sex-specific patterns that may limit and modulate the potential of these maternal effects to induce adaptive transgenerational effects.

Declarations

Ethical approval All applicable international, national and/or institutional guidelines for the care and use of animals were followed (RD 53/2013). Capture and manipulation of birds were authorised by the Consejería de Medio Ambiente (Comunidad de Madrid, Spain) under licence from the Spanish institutional authorities (Consejería de Medio Ambiente and Centro de Migración de Aves de SEO/BirdLife). The androgen manipulation experiment complies with the animal laws in Spain in 2011 (Real Decreto 1201/2005, 10-10-2005). Permission to work in the area was granted by both the Parque Regional de la Cuenca Alta del Manzanares (Consejería de Medio Ambiente de la Comunidad Autónoma de Madrid: Ref. 10/129049.9/11) and the Ayuntamiento de Soto del Real, Spain.

Conflict of interest The authors declare that they have no conflict of interest.

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