1 **Prenatal manipulation of yolk androgen levels affects <u>egg size but not</u>**

2 **<u>egg colour</u> 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 exposition 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 maternal exposure to yolk androgens during her own embryonic development. Our research calls for further long-term research on the influence of pre-natal androgens on the fitness mechanisms regulating reproductive investment and its potential signalling role in a perspective of sexual selection.

Keywords: maternal effects, androgens, biliverdin, egg coloration, post mating sexual selection

Data availability

67 All data underlying the findings are hosted on the Spanish National Research Council

68 (CSIC) digital repository: [insert] / The datasets generated during and/or analysed during

69 the current study are available from the corresponding author on request.

70

71 **Compliance with ethical standards**

72 **Ethical approval**

73 All applicable international, national and/or institutional guidelines for the care and use

74 of animals were followed (RD 53/2013). Capture and manipulation of birds were

75 authorized by the Consejería de Medio Ambiente (Comunidad de Madrid, Spain) under

76 licence from the Spanish institu- tional authorities (Consejería de Medio Ambiente and

77 Centro de Migración de Aves de SEO/BirdLife). Permission to work in the area was

78 granted by both the Parque Regional de la Cuenca Alta del Manzanares and the

79 Ayuntamiento de Soto del Real, Spain.

80 **Consent to participate**

81 Not applicable.

82 **Consent for publication**

83 Not applicable.

84 **Conflict of interest**

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

86 **Significance statement**

87 In birds, mothers prepare the offspring to the environment they will encounter at hatching

88 by depositing a mixture of compounds into the eggs. Androgens specifically exert a

89 variety of effects during embryo development and chick growth, but some of these may

90 be negative in adult life. In particular, early androgen may be detrimental for females in

91 the long-term, decreasing maternal investment and fitness. Using the spotless starling as

4

92　　study species, we tested whether an experimental increase in yolk androgens negatively

93　　affects breeding investment, measured by egg colour (a sexual signal) and egg size. We

94　　found no effects of the treatment on egg colour. However, females exposed to high

95　　androgen levels during their development reduced egg size along the laying order more

96　　strongly than controls. This may affect nestling survival, and indicates that prenatal yolk

97　　androgens may influence some egg characteristics with known relevance for offspring

98　　viability.

99

100

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 imply 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 the 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 decisive 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 (Saino et al. 2006; von Engelhardt et al. 2006; Ruuskanen and Laaksonen 2010; Muriel et al. 2015b, 2017). Long-term effects of systemic androgen levels on reproduction point out 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 of 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

151 oxidative status of the laying female and eggshell coloration (Morales et al. 2008, 2011).

152 Indeed, empirical evidences of the connection between female quality and the appearance

153 of their blue-green eggs have been repeatedly reported for a number of species

154 (Siefferman et al. 2006; Moreno et al. 2006; Krist and Grim 2007; López-Rull et al. 2008),

155 suggesting a possible role for egg colour signalling.

156   Female investment and condition can also induce variations in the size of eggs

157 laid (Nilsson and Svensson 1993; Smith et al. 1993; Viñuela 1997; Cunningham and

158 Russell 2000). Investment in egg size by the mother is beneficial for the offspring, since

159 they receive a greater amount of water, nutrients and hormones (Krist 2011). Egg size is

160 thus a key maternal effect for oviparous species, although the fact that it involves a

161 mixture of constituents makes it difficult to pinpoint the exact benefit that a larger egg

162 may imply. Egg size is thus a key maternal effect for oviparous species, although the fact

163 that it involves a mixture of constituents makes it difficult to pinpoint the exact benefit

164 that a larger egg may imply. Nevertheless, nestlings that hatch from bigger eggs have

165 been shown to be heavier and bigger than their siblings born from smaller eggs, even

166 regardless of egg composition (Krist 2011).

167   Despite the wide body of literature testing the costs and benefits of yolk androgens

168 and the interest that has arisen around the role of egg coloration as a signal (Moreno and

169 Osorno 2003), no study so far has tested empirically the connection between prenatal

170 exposure to maternal hormones in females and the colour of the eggs laid by these later

171 in life. However, at least two studies have analysed the effects of egg androgens on egg

172 size, finding in both cases no effects of egg-androgen injections in this variable (Rubolini

173 et al. 2007; Müller et al. 2009).If maternal androgens impose a cost to females, it is to be

174 expected that such a cost could be transferred to the expression of traits that depend on

175 their quality and physiological state, like the intensity of the colour or the size of their

176 eggs.

177   In this study, we test whether experimentally increased yolk androgen levels affect

178 the size and the eggshell colour of eggs produced by females hatching from androgen-

179 manipulated eggs. We used as a study model the spotless starling (*Sturnus unicolor*), a

180 medium sized passerine in which the intensity of eggshell blue-green colour reflects its

181 biliverdin content (López-Rull et al. 2008) and correlates with body condition and feather-

182 ornament size in females (López-Rull et al. 2007, 2008). We evaluated the effect of an

183 egg androgen manipulation on the colour and on the size of the eggs laid throughout the

184 life of the females that hatched from the experimentally-injected eggs. Previous studies

185 in this species have shown that egg androgen injection enhanced embryonic development

186 and post-hatching growth, regardless of embryo sex, but at the cost of depressing some

187 components of the immune system in clutches laid at the end of the breeding season, when

188 environmental conditions become harsher (Muriel et al. 2015a, b). Assuming that egg

189 coloration and size reliably reflect female quality (Christians 2002; Moreno et al. 2004;

190 López-Rull et al. 2008), and considering the potential negative effects of increased

191 androgen levels for adult females (Rutkowska et al. 2005; Veiga and Polo 2008), we

192 expect the eggs laid by females hatched from androgen-treated eggs (hereafter "treated

193 females") to be less colourful and smaller than those laid by females hatched from control

194 eggs (hereafter "control females").

195

196 **Materials and methods**

197 Study area and species

198 The study colony is composed of 250 nest boxes distributed across an open woodland

199 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]; D. Gil, unpublished), 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),

225 where the same dosage induced a stimulatory effect on hatching nestling body mass and

226 skeletal growth. *In ovo* injections were performed in the field using a standard U-50

227 insulin syringe (Terumo Corporation, Tokyo, Japan), following a standard protocol and

228 before incubation started (Muriel et al. 2015b).

229

230 Data collection

231 We monitored the breeding attempts of experimental recruiting females throughout their

232 lives (2012-2018), from the age of 1 to 7 years old. During the laying period of these

233 breeding seasons, we visited nests on a daily basis from 2012 to 2014, and every other

234 day from 2015 to 2018. This monitoring schedule allowed us to determine laying date

235 with great accuracy, although not the exact laying order for all brood in each year. We

236 marked eggs with a nontoxic waterproof pen and measured their length and width at the

237 widest point with digital callipers (Mitutoyo Absolute, Kawasaki, Japan, precision = 0.01

238 mm). On the fifth day after the first egg had been laid, egg colour reflectance of the entire

239 clutch was measured using a Minolta spectrophotometer CM-2600d in the range 360-700

240 nm. Each egg was measured on a cleaned circular spot of 5 mm diameter on the blunt end

241 of the egg. Reflectance values were obtained at 10 nm steps (see López-Rull et al. 2007).

242 Intra-specific nest parasitism is common in this species (Calvo et al. 2000; Monclús et al.

243 2017). We assumed that a nest had been parasitized when two new eggs were found in a

244 nest on a given day (starlings lay one egg per day) or when a single egg differed strikingly

245 in appearance or size from the other eggs in a clutch. In this case, the egg that differed

246 from the rest of the clutch was recorded as parasitic and excluded from the analyses, to

247 avoid interferences. The same criteria has been used for detecting parasitic eggs before in

248 a study of the European starling, *Sturnus vulgaris* (Pilz et al. 2005). Although the use of

249 visual discrimination to identify parasitic eggs has been questioned by some authors (e.g.

250   Gronstol et al. 2006), we followed a conservative approach to minimize potential errors

251   and discarded from the data base those eggs where the identity of the laying female was

252   doubtful. After performing such conservative selection of available data, we finally

253   considered for the analysis a total sample of 1040 eggs from 231 clutches (169 belonging

254   to the first clutch waves, and 62 to the second) laid between 2012 and 2018 by the females

255   hatched from yolk androgen-treated eggs in 2011 and recruited thereafter (n = 77). Due

256   to technical problems with the spectrophotometer, we lost the data from 160 eggs,

257   corresponding to 23 first and second clutches of 2014 and 4 first clutches of 2015.

258   For the analysis, we considered two dependent variables: blue-green chroma

259   (BGC) as a measurement of egg colour, and egg volume as an estimate of egg size. BGC

260   is the proportion of reflectance in the blue-green part of the spectrum, over the total

261   reflectance ($R_{400-570}/R_{360-700}$). Biliverdin peak of reflectance falls in the same region of the

262   spectrum (Falchuk et al. 2002) and biliverdin content has been found to correlate

263   positively with BGC in eggs of this species (López-Rull et al. 2008). From the

264   measurements of length and width of each egg, we calculated egg volume (mm³) using

265   the formula: 0.51 x length x width² (Hoyt 1979).

266

267   Data analysis

268   Analysis was done in the R language, version 3.4.2 (R Development Core Team 2017).

269   We built linear mixed models to test whether egg characteristics (BGC and volume) were

270   affected by the hormonal treatment, using the lmer function in the "lme4" package (Bates

271   et al. 2015). We visually explored the data distribution to assess normality and

272   homoscedasticity before proceeding with parametric analysis.

273   Since broods were checked daily until 2014 and every other day from 2015, exact

274   laying order was not available for the entire dataset. We therefore created an ordinal

275 variable with three levels depending whether the eggs were found in the first two days

276 from the onset of laying, on the central two days, or on the last two days. Since all

277 individuals were born in the same year, the effect of age is indistinguishable from the

278 effect of the year itself. Moreover, sample size decreased progressively every year due to

279 mortality, sample size was highly unbalanced with respect to female age. Thus, we

280 categorized female age as a factor with four levels: 1) first year; 2) second year; 3) third

281 and fourth years; and 4) fifth year and older. This categorization reduces the unbalanced

282 sample size of the different age categories, while still allows us to capture variation in

283 age. To account for possible selective changes in the population, we considered the age

284 of first and the age of last measurement (AFM and ALM). Selective appearance or

285 disappearance in or from the population of individual with a certain phenotype could

286 change the phenotypic mean of the population itself and this could erroneously interpreted

287 as an aging effect (van de Pol and Verhulst 2006; van de Pol and Wright 2009). Lastly,

288 as the effect of yolk androgens may interact with environmental conditions experienced

289 in early development (Muriel et al. 2015b), we considered in the analyses the female's

290 birth clutch wave (first and replacement versus second wave of clutches), the brood size

291 of the female's birth clutch (i.e. the number of the chicks in the female's birth clutch) and

292 the clutch wave of the actual egg data (first and replacement versus second clutches).

293 Replacement clutches were scarce in our dataset and were always laid a few days after

294 the first clutches. Given that these two clutches experience very similar environmental

295 conditions (in contrast to second wave clutches), we consider first and replacement

296 clutches as "first wave clutches". Egg colour fades progressively after laying (Moreno et

297 al. 2011; unpublished data), and since all eggs were measured at clutch completion, the

298 effects of laying order in colour simply reflect the fact that later eggs have been exposed

299   to the light for a shorter time, making the laying order an unsuitable variable to test further

300   effects or interactions on the colour.

301   Random effects for the volume model included year, female identity, and clutch, coded

302   so that each breeding attempt is identified individually. In both models clutch identity

303   was nested in female identity, as every female mothered several clutches. Both models

304   considered the following fixed factors: treatment, age (factor with four levels), clutch

305   wave (first or second), laying order, AFM, ALM, mother brood size (the brood size from

306   which the mother hatched) and mother clutch wave (the clutch wave from which the

307   mother hatched, first or second). In addition, the following interactions were included:

308   treatment and age, treatment and clutch wave, treatment and mother clutch wave, clutch

309   wave and age, AFM and ALM, brood size of the clutch of origin of the mother and mother

310   clutch wave and a triple interaction between treatment, clutch wave and age (see Table

311   S1 and S2 in Supplementary material). In the model for the volume, we also considered

312   further interactions with the laying order: laying order and age; laying order and clutch

313   wave and laying order and treatment. We then performed a backward selection in R,

314   removing the interactions and the variables resulting non-significant and report final

315   models fitted by restricted maximum likelihood. In all cases, P values were calculated by

316   Satterthwaite's approximation (lmerTest package).

317   We also performed two additional analyses to further validate our main

318   hypothesis. First, we run a linear mixed model to explore the potential relationship

319   between BGC and volume. The model considered the BGC as dependent variable and

320   just the volume and the laying order as covariate, to compensate for the artefact effect we

321   already found in the previous analysis (see above), and the same random effects as the

322   model built for the volume: year, clutch identity and female identity. Lastly, we calculated

323   the repeatability of both the BGC and the volume for the female identity. This was

324   performed in R through the "rptR" package (Stoffel et al. 2017).

325

326   **Results**

327   We found no effect of the androgen treatment on the colour of the eggs as measured by

328   BGC (p > 0.1). The model investigating BGC (Table 1) only revealed a positive effect of

329   laying order (p < 0.001) and age (p = 0.007, Fig. 1), but no effect of treatment, clutch

330   wave, the clutch wave and clutch size of birth of the mother, nor any of the interactions

331   (all p > 0.1). As expected, eggs increased in colouration along the laying order due to the

332   difference in time of exposure to the air between the laying and the measurement.

333   Moreover, we found that colour decreased as females age, as shown by the age estimate

334   decreasing progressively with age class (Table 1). Neither AFM, nor ALM were kept in

335   the model, supporting the age-specific decrease on egg colour (p > 0.1) (Table S1,

336   Supplementary material).

337   In the case of egg volume, we found significant interactions between treatment

338   and age (p = 0.04). Analyses of these interaction estimates showed that this was due to

339   differences between treatments showing opposite trends in different age classes, being

340   stronger and negative for treated females in the second class than in the other classes (Fig.

341   2). In addition, we found significant effects of the interaction between treatment and

342   laying order, showing a steeper decrease of the volume along the laying order in treated

343   females as compared to controls (p =0.03; see Table 2 and Fig. 3). We also found an

344   overall significant reduction in volume between the first to the second brood wave (p =

345   0.015). No other variables were significant in the model for egg volume.

346   Finally, we found a positive but very weak relationship between BGC and volume

347   (p = 0.038, marginal R^2 = 0.02) (Fig. 4), revealing that bigger eggs were also more

348   colourful. BGC and volume had both a strong positive repeatability within female identity

349   across years (BGC: R = 0.69 ± 0.04 SE; volume: R = 0.49 ± 0.06 SE).

350

351   **Discussion**

352   We conducted a large long-term experiment to test the hypothesis that yolk androgens

353   have long-lasting effects on the individual's phenotype throughout their lives, possibly

354   influencing future generations (i.e. transgenerational effects). In this study, we tested one

355   particular component of the female phenotype: reproductive investment as measured by

356   egg colour and egg size. Our results indicate that the colour of the eggs was not affected

357   by the androgen treatment, although it did get lighter in females of older age. In the case

358   of egg volume, we found a negative effect of the interaction between the treatment and

359   the laying order (Table 1). The volume of the eggs decreased along laying order in a

360   steeper way in treated than in control females. Moreover, there was a difference in volume

361   between first and second clutch waves, with eggs of the second clutch wave being smaller

362   than first wave ones (Table 2).

363   BGC has been shown to reflect biliverdin content and then, according to our

364   hypothesis and previous literature, it could be used as a signal of female quality (Moreno

365   and Osorno 2003; Moreno et al. 2005, 2006; Costantini et al. 2015). Eggshell coloration

366   in this species has been shown to reflect egg testosterone and vitamin E content, as well

367   as body condition and telomere dynamics of fledglings (López-Rull et al. 2008; Navarro

368   et al. 2011; Soler et al. 2018). In a similar way, egg volume has been widely used as a

369   proxy of female reproductive ability, being related to offspring viability (Williams 1994;

370   Krist 2011). According to our hypothesis, females developing in androgen-treated eggs

371   would experience some physiological costs that may ultimately impair their breeding

372   capacity, resulting in paler or smaller eggs when they become adults (Rutkowska et al.

373   2005; Veiga and Polo 2008; López-Rull et al. 2008; Muriel et al. 2015b). Contrary to our

374   expectations, our results show that BGC values were similar in eggs laid by control and

375   treated females. The sexually selected egg coloration hypothesis is specifically based in

376   a physiological connection between the oxidative stress experienced by the female during

377   egg laying and biliverdin deposition in the eggshell (Moreno and Osorno 2003). Thus,

378   our results suggest that pre-hatching exposure to androgens as manipulated in our

379   experiment does not lead to breeding oxidative costs on the females in this species in the

380   long term, or that such costs are not reflected in a reduced investment in the colour of the

381   eggs. The control over the coloration of the eggs may imply mechanisms different from,

382   or more complex than, the androgen-oxidative stress connection (Alonso-Alvarez et al.

383   2007). Although circulating biliverdin is a product of the catabolism of the heme group

384   of the erythrocyte, the specific amounts of this pigment to be deposited in the eggshells

385   are most likely synthetized in the shell gland (Wang et al. 2007) and it may not affect the

386   concentration of systemic pigment. Most of the studies linking oxidative status (Morales

387   et al. 2008, 2011; Moreno et al. 2013), or body condition (Siefferman et al. 2006; Soler

388   et al. 2008), to egg coloration take into account proximate agents affecting the short-term

389   quality of the female (i.e. nutritive status). Our work predicted in the first place that it

390   would be possible to detect an effect in adult life, long after the manipulation. However,

391   the empirical support of the impact of maternal androgens on oxidative stress during early

392   development is mixed (Galván and Alonso-Alvarez 2010; Noguera et al. 2011; Tobler et

393   al. 2013; Treidel et al. 2013; Giraudeau et al. 2017; Parolini et al. 2018). This evidence

394   suggests that females may buffer the imprint of maternal hormones in adulthood. Also,

395   the effect of maternal androgens in the eggs may be modulated during the development

396   by the expression of androgen receptors in the extraembryonic membranes (Kumar et al.

397 2019), opening the possibility of an active role of the embryo towards maternal allowance

398 of compounds (Groothuis et al. 2019).

399 We found a negative effect of laying order on the BGC. This effect most likely

400 reflects the colour fading that can be explained by the difference in days elapsed between

401 laying and measurement of each particular egg (Moreno et al. 2011). Biliverdin fades

402 right after oviposition, possibly because of photodegradation, with a 1% decrease in the

403 few days elapsed between oviposition and clutch completion. Since the protocol we

404 followed implied measuring the colour after the last egg of the clutch had been laid, the

405 first eggs were also the ones most exposed to light and then the less pigmented of the

406 clutch. This artefact did not allow us to further investigate interactions that involved

407 laying order and thus we cannot exclude subtler mechanisms controlling eggshell

408 coloration. This limitation does not allow us to definitely reject the hypothesis that egg

409 colour, perhaps through within-clutch variation, may not signal female quality.

410 We did observe a decrease in the colour of the eggs in older females. This could

411 be a sign that the colour of the eggs is dependent on the overall condition of the mother,

412 and as the quality of the breeding females generally declines after the third or fourth year

413 of life, our results seem to be in agreement with this. Previous studies that found a

414 difference in BGC described mainly a positive association between BGC and age

415 (Siefferman et al. 2006; Krist and Grim 2007) or no association (Hargitai et al. 2008;

416 Honza et al. 2011). We considered the age of first measurement (AFM) and the age of

417 last measurement (ALM) to control for selective female appearance or disappearance

418 affecting longitudinal age differences. Our results showed no effects given by either AFM

419 or ALM, suggesting that the pigmentation of the eggshell decreased due to within-

420 individual changes.

421   Egg size showed a response to the treatment in interaction with the laying order,

422   showing a steeper decrease in androgen-treated females than in controls. To our

423   knowledge, this is the first time that a long-term effect on a fecundity-related trait is

424   observed as a consequence of an egg androgen modification. We already expected from

425   previous studies in this and other species (Gibson and Williams 2017; Monclús et al.

426   2017; personal obervations) that the volume of the eggs would decrease along laying

427   order, but our results also show that this decrement is greater in treated females. Early

428   exposure to androgens may interact with oogenesis patterns perhaps affecting hormonal

429   regulation processes (Williams 2012). For example, Williams (2012) reported a reduction

430   in egg size after injecting females (not eggs) with follicle-stimulating hormone (FSH),

431   which is fundamental in the regulation of follicle development. If androgens interact with

432   FSH (as observed in humans, Dewailly et al. 2016), they may also alter egg size

433   regulation. Anyway, we cannot exclude that other kinds of costs affected the treated

434   females. Given that egg size affects hatchling size (Krist 2011), this effect could translate

435   to transgenerational consequences if the future fitness of these individuals resulted

436   impaired. Given that the starling shows strong hatching asynchrony (1-2 days) (personal

437   observations), this larger reduction in egg size along the laying order should promote

438   brood reduction. This would allow first-hatched hatchlings to outcompete their younger

439   siblings, possibly resulting in a reduction in fledging number.

440   Our results showed an effect of the treatment on the egg size in interaction with

441   the age of the females. The direction of effect was not linear and it is difficult to

442   understand from an aging perspective (Fig. 2). We would argue that year-specific events

443   might explain this odd pattern. All females in this study were bred in 2011, and thus all

444   experienced in 2013 a particularly harsh season that may have increased adult mortality

445   and reduce nutrients for egg formation (Gil et al. 2019). Although we are not able to

446   provide evidence of this, due to the complete overlapping of the year effect with the age,

447   it is the most likely explanation that we can provide for such pattern.

448   Average egg volume was lower in second than in first clutches, something that

449   has already been observed in our population (López-Rull et al. 2010). This may be due to

450   the harsher environmental conditions experienced by females during second broods, as

451   well as to the fact that many of them have already raised a first brood weeks before, which

452   may constrain their capacity to allocate resources to produce large eggs (Muriel et al.

453   2019). An alternative adaptive hypothesis is that smaller eggs may be favoured in second

454   broods when climate is warmer and brooding requirements are relaxed. Moreover,

455   although egg size decreased, yolk weight in this population increased in the second clutch

456   wave, which may compensate for the loss in total egg volume (Muriel et al. 2019). Our

457   analysis revealed a significant egg-size repeatability (R = 0.51 ± 0.05), which is lower

458   than previous reported repeatabilities for this population (R > 0.68, Monclús et al. 2017;

459   R = 0.58 for the first egg, R = 0.71 for the fourth egg, Muriel et al. 2019). This indicates

460   that although egg size is a relatively non-plastic trait (Christians 2002; Monclús et al.

461   2017), females facing different environmental conditions vary egg size to some extent.

462   We also found that the size of the eggs and their colour are positively related (Fig.

463   4). The detected relationship was weak ($R^2 = 0.02$). Noteworthy, this positive relationship

464   emerged despite the opposite trend for egg size and BGC to vary across the laying

465   sequence. The strength of the relationship might be arguably higher if the colour of each

466   egg had been measured just after it had been laid. This positive relationship is consistent

467   with our assumption that egg colour and size are positive proxies of female quality. If

468   eggshell colour is a costly post mating sexual signal of female quality, we might expect

469   such a positive relationship between egg colour and egg size. However, factors other than

470   the post mating sexual selection hypothesis may also underpin this positive relationship.

471 For instance, Morales (2020) proposed that biliverdin in the eggshell may play a direct

472 role in protecting the embryo from oxidative stress during development. If female ability

473 to deposit high amounts of biliverdin to bolster offspring development is linked to its

474 condition, the positive association between egg colour and size would emerge as well.

475 In summary, our results show that the potential of females to invest in the

476 colouration of the eggs is not modified by an increased exposure to prenatal androgens,

477 whereas egg size patterns within clutches are affected. Yolk androgens are one of the

478 mechanisms whereby females may modulate offspring phenotype to maximize their

479 fitness (Mousseau and Fox 1998b), though they have been hypothesized to exert negative

480 side-effects. Our findings show that the long term effects of maternal hormones in the

481 offspring are prone to sex-specific patterns that may limit and modulate the potential of

482 these maternal effects to induce adaptive transgenerational effects.

483

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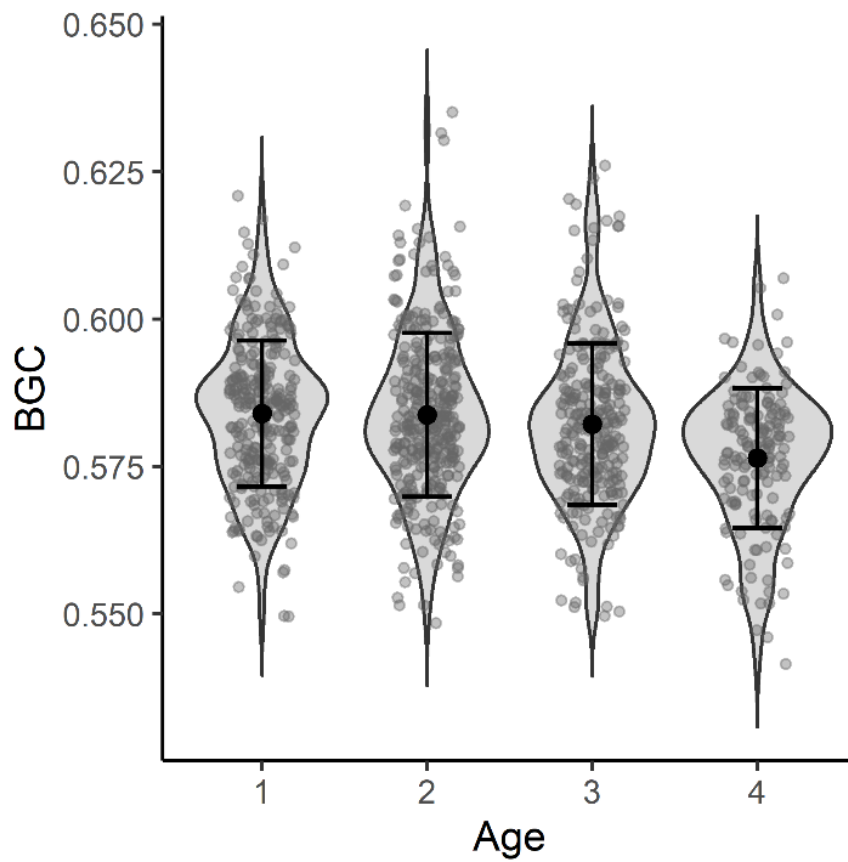729   Princeton, pp 8–51

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FIGURES

Fig. 1 Distribution of egg colour between the age categories; black points represent the

mean and bars 1 SD and grey shapes reflect point density along y-axis

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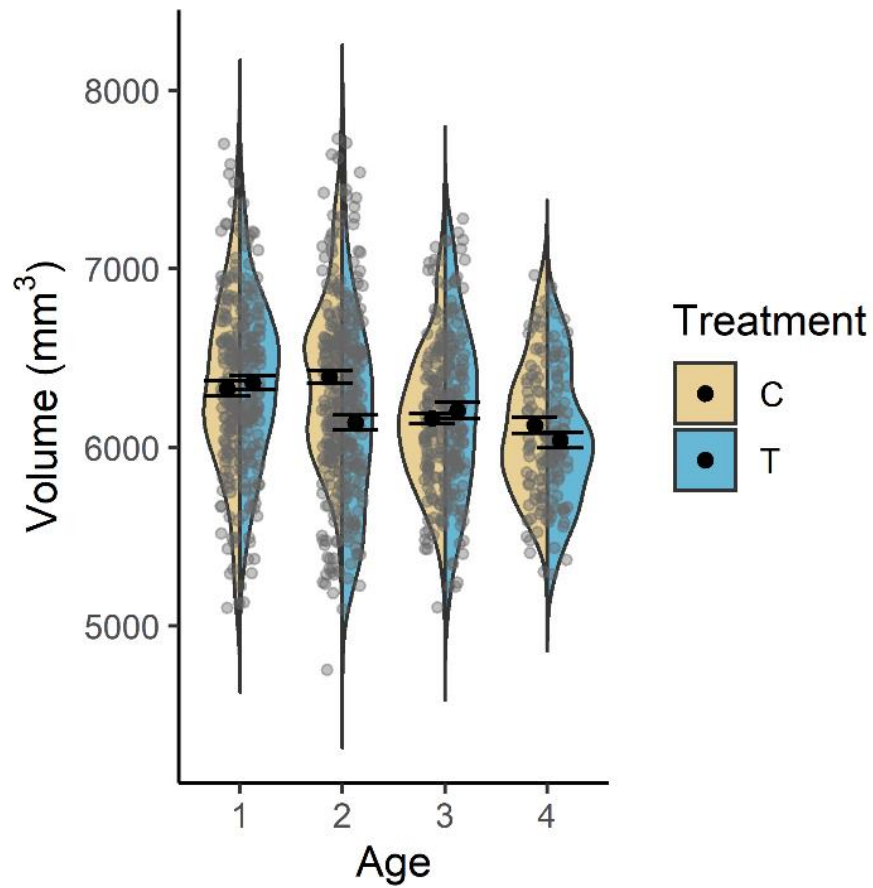Fig. 2 Distribution of egg sizes along age categories, for control (pink half violins) and

androgen-treated females (blue half violins); black points represent the mean and bars 1

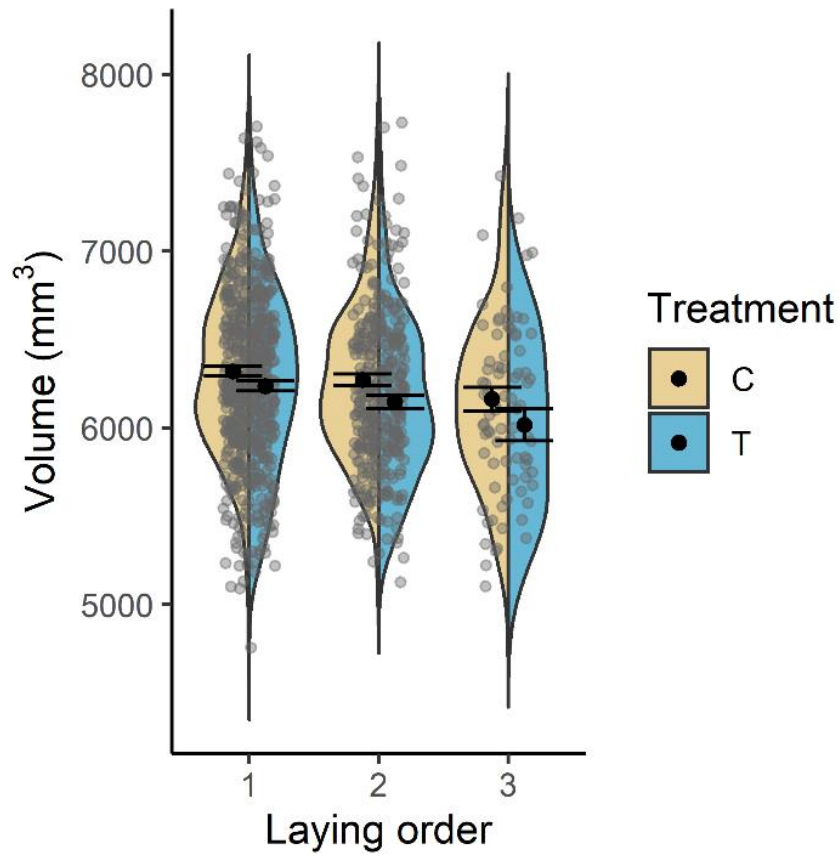SD and grey shapes reflect point density along y-axis

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Fig. 3 Distribution of egg sizes along laying order, for control (pink half violins) and

androgen-treated females (blue half violins); black points represent the mean and bars 1

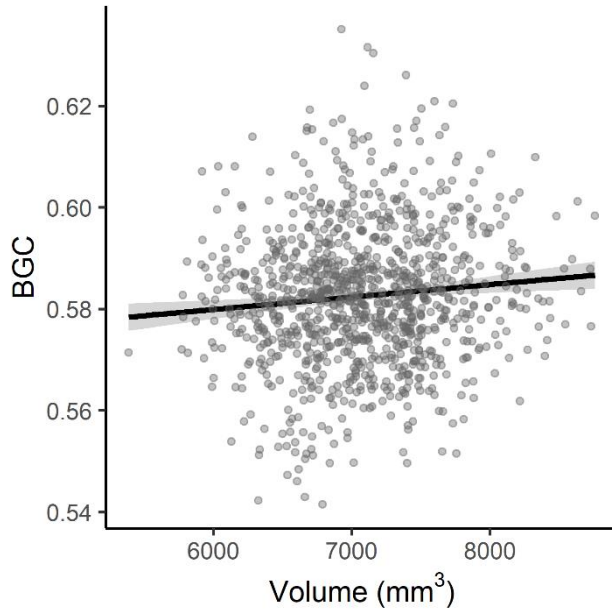SD and grey shapes reflect point density along y-axis

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Fig. 4 The linear relation between egg blue-green chroma (BGC) and volume; the grey

area around the black line denotes the 95% confidence level interval

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Fixed effects	Estimates	SE	$\chi2$	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 2	-1.92 e^{-03}	1.5 e^{-03}	12.16	3,2	**0.007**
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**

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802 **Table 1** Linear mixed model showing the effect of treatment, age of the mother (as a

803 factor with four levels) and laying order on the blue-green chroma (BGC) of the eggs.

804 Random effects account for year, mother identity and breeding attempt. Degrees of

805 freedom are calculated via Satterthwaite's method. P-values considered significant (P <

806 0.05) are in bold.

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Fixed effects	Estimates	SE	χ2	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 2	64.31	185.85	1.60	3,2.00	0.660
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 x Age 2	-239.57	116.50	8.21	3,168.81	**0.042**
Treatment T x Age 3	57.04	127.64			
Treatment T x Age 4	-133.82	150.87			
Treatment T x Laying order	-67.23	30.43	4.88	1,820.48	**0.027**

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820 **Table 2** Linear mixed model showing the effect of treatment, laying order, clutch wave,

821 size of the brood of origin of the mother and the interaction of treatment and laying

822 order the volume of the eggs. Random effects account for year, mother identity,

823 breeding attempt and year. Degrees of freedom are calculated via Satterthwaite's

824 method. P-values considered significant ($P < 0.05$) are in bold

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