ORIGINAL ARTICLE

Age-related patterns of yolk androgen deposition are consistent with adaptive brood reduction in spotless starlings

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Abstract

Female birds can influence offspring development by adjusting egg size or by a differential allocation of egg resources. Such maternal effects can be expected to be shaped by natural selection, given the costs connected to the allocation of maternal resources. Among egg components, yolk androgens play an important role in affecting offspring life-history traits. Despite their relevance for nestling development, factors accounting for the observed within- and between-clutch variation are still poorly known. By using a cross-sectional sampling approach, we tested the effect of female age, laying order and laying date on the deposition of yolk androstenedione (A4) and testosterone (T), since young and older females could maximize their fitness differently according to breeding conditions. We found a remarkable lack of differences in overall yolk androgen levels across different ages. However, comparing first-year with older females, our results showed that age did not influence yolk T levels at the beginning of the breeding season, whereas at the end, first-year females transferred lower hormone levels than older females. Within clutches, both androgens increased across the laying sequence, suggesting that late nestlings benefit from an increased allocation that could compensate hatching asynchrony. However, when considering A4, we found that, whereas older females always increased A4 levels across the laying sequence, first-year females did not increase it at the end of the breeding season, thus increasing the likelihood of brood reduction when environmental conditions became hardest. These findings suggest that yolk A4 variation may be particularly important at the within-clutch scale, by providing females with a tool to modify nestling hierarchies.

Significance statement

The study of maternal effects shows that avian mothers can adjust offspring development to environmental conditions by altering egg composition. The effects of maternal age on such egg allocation process depend on the balance of benefits and costs that affects each age class. We examine if young and older mothers differ in terms of yolk androgen deposition and egg quality, since age may influence offspring fitness, and could represent a costly maternal investment. We found an absence of overall age-dependent patterns in yolk androgen deposition, except when we considered laying order and laying date. In late clutches, when breeding conditions are harsher, 1-year-old females carried out a lower deposition of both resources (yolk mass) and modifiers (androgens) than older mothers did. We suggest that such a pattern would reinforce the effects of hatching asynchrony, favouring brood reduction at the end of the breeding season by first-year females.

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Introduction

In birds, as in other oviparous species, development of the embryo occurs within a self-contained egg environment, whose characteristics are influenced by maternal condition at the time of egg formation (Price 1998; Poisbleau et al. 2009). For example, it has been shown that females in good nutritional status produce larger eggs than those in poor nutritional status (Smith et al. 1993; Viñuela 1997). The indirect influence of the maternal genotype or environment is generally referred to as a maternal effect (Mousseau and Fox 1998). Avian maternal effects are largely mediated via egg size and quality (Rubolini et al. 2011), as well as other factors such as efficiency of incubation, nestling feeding and level of predator defence (Krist 2011). One of the most studied aspects of prenatal maternal investment is egg size (Bernardo 1996; Christians 2002), which has effects on growth and survival of offspring (e.g. Williams 1994; Reed et al. 2009). Additionally, mothers may adjust the development and the phenotype of their offspring to environmental conditions by an adaptive allocation of resources into the egg (Groothuis et al. 2005a). These maternally derived components include nutrients, antioxidants, immunoglobulins, albumen lysozymes, and hormones (e.g. Schwabl 1993; Saino et al. 2002; Biard et al. 2005; Gil 2008; Ruuskanen and Hsu 2018), whose concentrations can be related to egg size (Williams 1994; Reed et al. 2009). Although these resources have benefits on offspring fitness and survival (Williams 1994; Reed et al. 2009; Hipfner 2000), they can be physiologically costly for the female in both the short (Carey 1996; Monaghan and Nager 1997) and long term (Trivers 1974; Winkler 1993; Godfray 1995). Therefore, high-quality females would be expected to deposit higher amounts of resources than low-quality females (e.g. Gil et al.2004; Gasparini et al. 2007). Beyond maternal condition, the concentration of these components may vary depending on an adaptive pre-adjustment linked to the environmental information perceived by the mother, such as temperature, food availability or mate quality (Kaplan 1992; Sheldon 2000; Benton et al. 2005; Michl et al. 2005).

Maternal hormones transferred from the mother to the offspring during egg production are important epigenetic mediators of prenatal maternal effects (Schwabl 1993; Love et al. 2005; Moore et al. 2019). Particularly, androgen-mediated maternal effects can exert both short- and long-term effects on various offspring traits, with potential fitness consequences (reviewed in Gil 2008; Ruuskanen 2015; Groothuis et al. 2019). Most studies on hormone-mediated maternal effects have focused on androstenedione (A4) and testosterone (T),

since they are the main androgens present in avian yolks (reviewed in Groothuis et al. 2005a, b; Gil 2008; Groothuis and Schwabl 2008; Ruuskanen 2015). Experimental studies consider both T and A4 as the most biologically relevant yolk androgens, because during embryo and nestling development, the increase of these hormones can lead to effects on growth and survival, while they simultaneously could carry immunological costs (reviewed in Muriel et al. 2015a). Differential allocation of these yolk androgens to eggs within a clutch may allow mothers to shape the development and the competitive asymmetries within their brood (Schwabl et al. 1997). In this way, increased androgens in the last egg of asynchronously hatching clutches could compensate for age- and size-related disadvantages of the chick hatching from that egg (Lipar and Ketterson 2000; Muller and Groothuis 2013; but see Braasch and Becker 2019). In line with this idea, yolk androgen concentrations are often found to increase with laying order within clutches (Schwabl 1993; Pilz et al. 2003). To maximize offspring survival, and therefore female fitness, maternal yolk androgen concentrations not only vary across the laying sequence within clutches (Schwabl et al. 1997; López-Rull and Gil 2009) but also exhibit an even larger variation between clutches (Groothuis et al. 2005a; Gil 2008). Three main sources of variation could explain differences between clutches in yolk androgen levels: (1) female condition (e.g. Pilz et al. 2003; Tschirren et al. 2004; Tanvez et al. 2008); (2) maternal environment, e.g. food availability (Verboven et al. 2003; Gasparini et al. 2007; Benowitz-Fredericks et al. 2013), photoperiod (Schwabl 1996a) or ectoparasite abundance (Tschirren et al. 2004; Postma et al. 2014); (3) social factors affecting the mother, like aggressive interactions (Whittingham and Schwabl 2002), mate attractiveness (Gil et al. 1999; Gwinner et al. 2013) or breeding density (Groothuis and Schwabl 2002; Pilz and Smith 2004). These factors often vary across the breeding season and can influence yolk androgen deposition, either by imposing physiological constraints on female allocation capacity or because they indirectly influence the external context where offspring will develop, resulting in changes in the optimal yolk composition that may allow maximizing offspring fitness (e.g. Martínez-Padilla et al. 2014; Muriel et al. 2015b).

Generally, reproductive performance of individuals improves with age until a point in which senescence leads to a decrease (Stearns 1992). This variation across ages could depend on both age-specific individual variation and permanent effects of individual quality (Ardia and Clotfelter 2007; McCleery et al. 2008). In fact, several studies have shown that age and body condition are positively correlated with the number of fledglings (Stearns 1992). On the one hand, the overall

improvement of reproductive performance with age could be due to a progressive disappearance of poor quality phenotypes from the population (Forslund and Pärt 1995; Mauck et al. 2004). Additionally, this increase in reproductive abilities could be derived from age-related improvements of competence related to previous breeding experience, foraging skills or social status (Curio 1983; Stearns 1992; Forslund and Pärt 1995), although in a general way this could be related to differences in reproductive experience and individual quality (Forslund and Pärt 1995). A recent study conducted in a long-lived species found an age-specific pattern in yolk lutein and vitamin A concentrations, but not in testosterone levels, where middle-aged females allocated more of these substances to yolk compared to young and old mothers (Urvik et al. 2018). These results can be explained by age-related differences in foraging skills, where first-time breeding birds could be less proficient foragers than older breeders (Jansen 1990; Desrochers 1992). On the other hand, few studies have focused on the physiological mechanisms underlying this age-related variation in reproductive performance, which could be determined, for example, by endocrinological factors (e.g. Hector et al. 1990; Nisbet et al. 1999) or by the variability in oxidative stress levels (Alonso-Álvarez et al. 2010).

This cross-sectional study examines if young and older female spotless starlings (*Sturnus unicolor*) differ in terms of egg mass and androgen composition, since age is expected to influence traits that play a role in the development and survival of the offspring and whose consequences could lead to an energetically demanding form of maternal investment (Gil et al. 1999; Müller et al.). We measured A4 and T, which we consider to be physiologically key yolk androgens in this species: A4 is the androgen with a highest concentration in starling eggs (Pilz et al. 2003; López-Rull and Gil 2009) and both yolk A4 and T are known to influence growth in spotless starlings (Müller et al. 2007; Muriel et al. 2013). In addition, since the spotless starling is a multi-brooded passerine, we explore the effect of changes in the environmental context as the breeding season progresses (e.g., harsher meteorological conditions, decline in food availability). These changes imply an increase in reproductive effort that may exert differential effects depending on female age (Laaksonen et al. 2002; Barbraud and Weimerskirch 2005). We designed a sampling scheme that not only allows us to test for age-dependent patterns, but also to analyse differences due to the environmental context. A previous study in the European starling (*Sturnus vulgaris*) assumed that androgen allocation was costly as a way to explain why older, better quality females invested higher levels of egg androgens than young females (Pilz et al. 2003). Based on these previous findings, we make the prediction that a quadratic relationship (an inverted U-shaped relationship) could arise between female age and maternal investment, with an increase in resource allocation over the first years, that tendency becoming negative due to senescence

processes after a certain middle-age plateau (e.g. Balbontín et al. 2012; Urvik et al. 2018). In addition, we expect that differences in age-related maternal investment could be more evident, and therefore, the quadratic relationship may become more pronounced, when breeding conditions become harsher (i.e. last clutches in the breeding season), because of different breeding strategies or limitations associated with individual quality (Forslund and Pärt 1995).

Material and methods

Study area and study species

The study was done between mid-April and late June 2012 in a nest-box colony of spotless starlings located in a mixed oak and ash woodland in central Spain (Soto del Real, Madrid). The spotless starling is a medium-sized multiple-brooded and hole nesting passerine (Moreno et al. 1999; Veiga and Polo 2016), which shows high breeding synchrony. It is a moderately long-lived species with a maximum lifespan of 8–9 years (Veiga and Polo 2016). Modal clutch size is five eggs, although it is typically lower at the end of the breeding season (López-Rull et al. 2007; this study). Regarding breeding effort, females usually show a higher nestling feeding rate than males (Jimeno et al. 2014), although paternal care varies widely (Moreno et al. 1999). In our study population, this species is usually double brooded, laying a first clutch around mid-April and a second clutch towards the end of May (Muriel et al. 2015a), but the egg-laying period is continuous throughout the breeding season, since females lay a replacement clutch when the first one fails (Müller et al. 2007; Muriel et al. 2015a). Our study area exhibits a Continental Mediterranean climate (Köppen-Geiger climate classification: Csb category; reviewed in Peel et al. 2007) with hot and dry summers. Thus, breeding conditions become harsher as the season advances (Salaberria et al. 2014), where late breeding conditions are characterized by higher temperatures and scarcer food. The daily average maximum temperature (mean ± SE) rose from 18.24 ± 0.98 °C in mid-April to 27.39 ± 0.64 °C in early June, while precipitation (mean ± SE) dropped from 1.51 ± 0.43 L/m^2 in mid-April to 0.05 ± 0.03 L/m^2 in early June (data provided by the Spanish Meteorological Agency (AEMET)). This rise in temperatures and decrease in precipitation could lead to an increase in the aridity and soil compaction, thus reducing food availability.

Field procedure

The colony has been monitored since 2002, and accurate information about box ownership and age is available for most individuals. During the pre-laying period (from early March until the first egg of the colony was laid), starlings were caught

by spring traps placed inside nest-boxes. Starlings were individually marked with numbered metal bands and with a passive integrated transponder (PIT) tag (Trovan Ltd., Douglas, UK). We recorded body mass with a precision digital pocket scale to the nearest 0.01 g (Pesola AG, Switzerland) and tarsus length with a digital calliper (Mitutoyo Absolute, Japan, accuracy = 0.01 mm). An index of body condition was estimated using the residuals from a regression of body mass on a linear measure of body size (tarsus length) (Schulte-Hostedde et al. 2005). Female's body condition was used as predictor in the analyses for all birds except one individual which was trapped less than 1 week before the first egg was laid (to avoid biases due to the effect of egg formation on body mass). From early March to early July, we verified nest ownership by means of data-logging PIT-readers attached to the nest-box for two consecutive days (Trovan, EID Iberica, Madrid, Spain). In addition, we used handheld PIT-readers (GR-250, Trovan Ltd., Douglas, UK) to identify all incubating females at night. Therefore, it was not possible to record blind age data because our study involved females of a certain age in the field. However, this allowed us to randomly select for the study clutches laid by females of known age in order to cover a wide range of ages (from 1 to 8 years, Supplementary Fig. 1S).

From mid-April onwards, nest-boxes were checked daily to determine laying date and laying sequence. Eggs were marked with a non-toxic waterproof marker as they were laid and measurements of length and width were taken with digital callipers (Mitutoyo Absolute, Japan, precision = 0.01 mm). Egg volume (mm^3) was calculated by the formula: $0.45 \times$ length $\times$ width^2 (Worth 1940). We collected the first and the fourth egg from 75 different clutches throughout the breeding season. To reduce disturbance to females, both eggs were collected on the day that the fourth egg was laid, just when females begin incubation and eggs are still cold. We chose the first and the fourth egg in order to be able to quantify the variation of the androgen levels throughout the laying order. During the collection day, all the eggs were kept at 4 °C in a fridge bag before being stored at − 80 °C, where they were kept until being processed.

Yolk processing and androgen assays

Each egg was slowly defrosted to isolate the yolk and take its weight by a precision balance (A-2005, Sartorius Analytical Balance, Goettingen, Germany, accuracy = 0.1 mg). Subsequently, we took a small sample of yolk (mean ± SE = 210 mg ± 11.6) and resuspended it in 1 mL of MilliQ® autoclaved water. We extracted steroids by adding 3 ml of a mixture of petroleum and diethyl ether (40:60) to the diluted yolk sample, vortexing for 15 min and centrifuging for 10 min (4 °C, 2000× RPM). The ether phase was decanted after snap-freezing the tube in an alcohol bath at − 30 °C. This procedure was repeated a second time, and both ether phases were combined in a single tube and evaporated to dryness. In order to remove protein residues, this dried extract was resuspended in 1 ml of 90% ethanol and kept at − 20 °C overnight to be subsequently centrifuged for 10 min (4 °C, 2000× RPM). Samples were dried under a stream of nitrogen and subsequently resuspended in 300–350 ml of steroid-free serum (DRG Labs, Germany), depending on the specific amount of yolk used for each sample.

Yolk androgen concentrations were determined by two different EIA kits highly specific for A4 (DRG Labs, Germany) and T (DSL Labs, USA). According to the manufacturers, cross-reactivity of the A4 and T antibody was less than 1% for all hormones tested. Samples were analysed in duplicate with respect to a standard curve. For A4 the intra- and inter-assay coefficients of variation were 10.17% and 15.72%, respectively, and for T the intra- and inter-assay coefficients of variation were 8.57% and 5.2%, respectively. To minimize observer bias, blinded methods were used when all androgen data were recorded and/or analyzed.

Statistical analysis

All analyses were conducted in the R language v. 3.4.0 (R Core Team 2017). Egg volume, yolk weight, clutch size and the different androgen levels were analysed applying general linear mixed models with the *lmer* function in the 'lme4' package (Bates et al. 2017). Based on data distribution, we used the Gaussian link function for all variables, except for clutch size that we use a Poisson link function. Total A4 and T yolk contents were calculated by multiplying the yolk weight by the A4 and T concentration, respectively. We also calculated the ratio A4 /A4 + T, since it has been suggested that high yolk A4 concentrations could be associated with higher fitness, contrary to what is expected of T (Tschirren et al. 2014). Female age, laying order (first or fourth egg) and laying date were the fixed factors (predictor variables). In addition, clutch size was considered as an additional predictor in the main models to control for a possible effect of clutch size on androgen deposition levels along the laying sequence. To reduce its clumped distribution, laying date was transformed by using ranked values, where the first laying date was coded as 1, the second as 2, and so on up to 14. The analyses were ran firstly considering age as a continuous variable (from 1 to 8 years) to allow testing for complex trends (age-related increases, decreases or quadratic relationships). After examining these patterns, we included a new set of analyses where age was defined as a categorical variable (1-year-old vs. older females). We entered the plate identity in which the ELISA was performed as a random factor to control for between-plate differences. We considered laying order as an ordinal variable nested within nest ID, the latter being included in the model as a random effect affecting the intercept. All biologically meaningful double and triple interactions were also included

in the main models (Supplementary Table 2S, 3S, 4S). When age was incorporated in the model as a continuous variable, its quadratic term was also tested. Values represented are means ± SE. Starting from the saturated model, a backward stepwise procedure was used to remove terms with $P > 0.05$. Assumptions of normality and homoscedasticity were confirmed by checking the residuals of the models. Yolk androgen concentrations were normalized using the Box-Cox transformation since they were non-normally distributed variables. Individual repeatability (R) of the different maternal investment variables was calculated for those females that achieved two reproductive events throughout the breeding season (13 females older than one year) (Supplementary Table 1S). R was quantified using the function '*rptGaussian*()' of the package *rptR* (Bates et al. 2017), with individual identity as random effect. Fixed effects were not included when calculating the reported repeatability (R) values.

Results

Yolk weight and egg volume

Age as a continuous variable

We firstly examined variation in egg characteristics declaring female age effect as a continuous variable (from 1 to 8 years old). Yolk weight showed a significant increase with age, but it did not have any association with female condition (Table 1A, Supplementary Table 2S A). Laying date also exerted a significant effect on this variable, showing heavier yolks as the breeding season progressed. Although egg volume and yolk weight were positively correlated ($r = 0.434$, $P < 0.001$), egg volume was not associated to female age as did yolk weight, but in turn it was positively affected by body condition (Table 1A, Supplementary Table 2S A). Egg volume was influenced by a two-way interaction between laying

date and the egg position in the laying order, decreasing in volume throughout the laying order only in clutches laid at the end of the reproductive period. Egg volume was marginally affected by clutch size, so that eggs laid in larger clutches tended to have a smaller volume.

Age as a categorical variable

When considering age as a two-level categorical variable (first-year or older females), yolk weight was explained by a two-way interaction between age class and laying date (Table 1B, Fig. 1). Thus, yolk weight for first-year females was lighter as breeding season progressed, but did not vary for older females. Given that age was not retained in the final model for egg volume, the results for this variable are the same as reported with the previous approach (Table 1A, B; Supplementary Table 2S A, B). Regarding individual quality, our results showed that first-year females had worse body condition (mean ± SE − 0.153 ± 0.151) than older females (mean ± SE 0.039 ± 0.042) ($\chi^2 = 4.55$, $P = 0.032$).

Yolk androgens

Age as a continuous variable

Within clutches, the analysis showed that A4 and T concentration, and total A4 and T yolk contents, increased from the first to the fourth egg and that this pattern was not affected by female age or laying date (Table 2). Mean clutch egg A4 and T concentration decreased progressively from the beginning to the end of the breeding season, while this effect was not observed for A4 and T total yolk contents. On the other hand, age and female condition were not retained in the models for any of these measurements (Supplementary Table 3S). In the case of A4/A4+T ratio, we observed a significant positive effect of age (Table 2, Fig. 2). None of the measures of egg androgen that we studied were affected by an age quadratic

Table 1 Summary of final linear mixed models for egg volume and yolk weight considering female age as a continuous variable (A) and as a categorical variable with two levels (first-year vs. older females) (B). P values considered significant ($P < 0.05$) are in italics

| | A. Continuous age | | | | | | B. Categorical age | | | | | |
| | Yolk weight | | | Egg volume | | | Yolk weight | | | Egg volume | | |
	df	χ^2	P	df	χ^2	P	df	χ^2	P	df	χ^2	P
Age	1	5.390	*0.020*	–	–	–	1	3.089	0.078	–	–	–
Clutch size	–	–	–	1	3.211	0.073	–	–	–	1	3.211	0.073
Laying order	–	–	–	1	0.246	0.619	–	–	–	1	0.246	0.619
Laying date	2	2.909	*0.088*	1	0.020	0.886	1	4.540	*0.033*	1	0.020	0.886
Age * laying date	–	–	–	–	–	–	1	8.715	*0.003*	–	–	–
Laying order * laying date	–	–	–	1	4.242	*0.039*	–	–	–	1	4.242	*0.039*
Female body condition	–	–	–	1	11.98	*< 0.001*	–	–	–	1	11.98	*< 0.001*
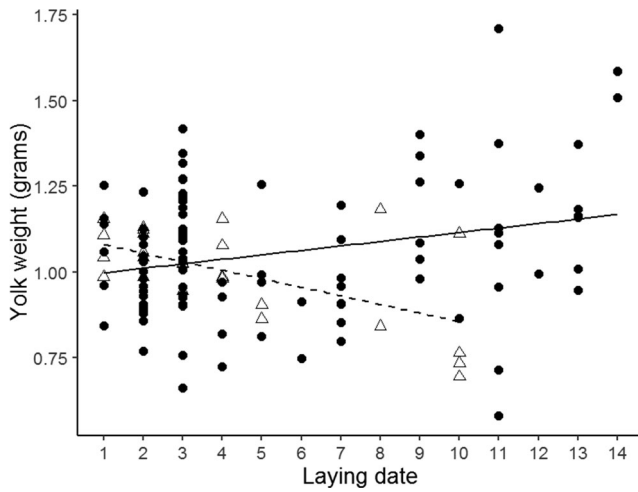
Fig. 1 Differences in yolk weight according to the laying date and female age (white triangles and dashed line correspond to first-year females, black circlesand solid line correspond to older females)
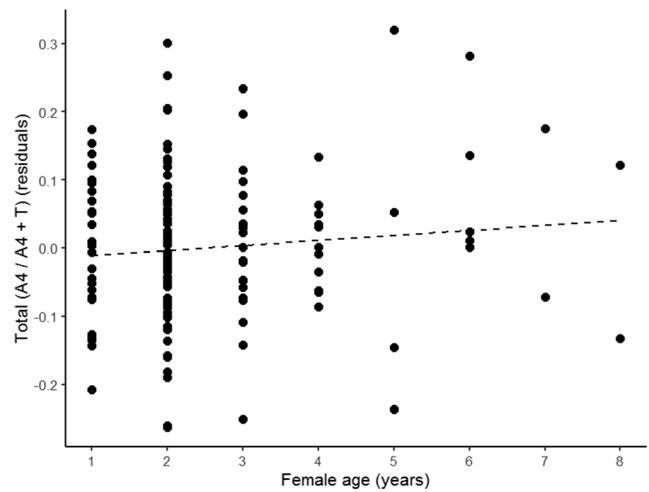

Fig. 2 Relationship between the A4/androgen total ratio, shown as residuals from statistical models, and female age treated as a continuous variable

term (Supplementary Table 3S). A4 and T total yolk contents showed a positive correlation ($r = 0.396$, $P < 0.001$). Neither A4 nor T concentrations were correlated with egg volume (A4: $r = 0.019$, $P = 0.815$; T: $r = -0.042$, $P = 0.602$) or yolk weight (A4: $r = -0.105$, $P = 0.200$; T: $r = 0.031$, $P = 0.703$).

Age as a categorical variable

When considering age as a categorical variable (first-year vs. older females), A4 concentration and A4 total yolk content were affected by a three-way interaction between age class, laying order and laying date (Table 3). While females older than 1 year old maintained a significant increase within their clutches between the first and fourth egg regardless of laying date (Fig. 3b), first-year females did not increase A4 levels throughout the laying order from the middle of the breeding season onwards (Fig. 3a).

T concentration and total T yolk content were explained by a two-way interaction between age class and laying date (Table 3), showing that first-year females deposited lower testosterone levels than older females, but only at the end of the breeding season (Fig. 4). Beyond this double interaction, testosterone levels increased significantly from the first to the

fourth egg in the laying order. Neither A4 nor T levels were affected by female body condition (Supplementary Table 4S). Regarding the A4/A4+T ratio, we observed a marginal effect of a two-way interaction between age class and laying date (Table 3): this ratio tended to decrease as breeding season progressed in first-year females, whereas the opposite trend was found in older females.

Discussion

Our cross-sectional approach examined how female investment in eggs changed with age in the spotless starling. Beyond the potential long-term costs (such as an increase in parental effort or a decrease in survival or fecundity of the focal offspring, e.g. Müller et al. 2007), several studies have suggested that the deposition of resources into the yolk can be costly for females in the short term (such as costs related to the immune system, e.g. Gil et al. 2006). In contrast to this prediction, we found that female spotless starlings did not show age-related differences (from 1 to 8 years old) in androgen levels (T and A4) or egg volume. Thus, our main finding does not support the prediction that would arise if yolk hormones

Table 2 Summary of final linear mixed models, considering female age as a continuous variable (1–8 years old). P values considered significant ($P < 0.05$) are in italics

	Concentration A4			Total A4			Concentration T			Total T			Total (A4/A4+T)		
	df	χ^2	P	df	χ^2	P	df	χ^2	P	df	χ^2	P	df	χ^2	P
Age	–	–	–	–	–	–	–	–	–	–	–	–	1	3.916	*0.047*
Laying order	1	79.80	*< 0.001*	1	71.20	*< 0.001*	1	71.22	*< 0.001*	1	60.80	*< 0.001*	–	–	–
Laying date	1	8.99	*0.0027*	–	–	–	1	4.108	*0.042*	–	–	–	–	–	–
Assay plate	–	–	–	4	12.97	*0.011*	4	28.68	*< 0.001*	4	42.47	*< 0.001*	4	18.05	*0.001*

Table 3 Summary of final linear mixed models, considering female age as a categorical variable (first-year vs. older females). *P* values considered significant (*P* < 0.05) are in italics

	Concentration A4			Total A4			Concentration T			Total T			Total (A4/A4+T)		
	df	χ^2	P	df	χ^2	P	df	χ^2	P	df	χ^2	P	df	χ^2	P
Age	1	0.316	0.573	1	0.025	0.873	1	2.535	0.111	1	6.270	*0.012*	1	1.269	0.259
Laying order	1	9.882	*0.001*	1	9.640	*0.001*	1	74.35	*< 0.001*	1	59.85	*< 0.001*	–	–	–
Laying date	1	0.551	0.457	1	0.001	0.967	1	2.238	0.134	1	10.87	*< 0.001*	1	2.996	0.083
Age * laying order	1	4.172	*0.041*	1	4.334	*0.037*	–	–	–	–	–	–	–	–	–
Age * laying date	1	1.184	0.276	1	0.068	0.793	1	4.207	*0.040*	1	11.57	*< 0.001*	1	2.885	0.089
Laying order * laying date	1	6.309	*0.012*	1	6.490	*0.010*	–	–	–	–	–	–	–	–	–
Age * laying order * laying date	1	6.865	*0.008*	1	7.005	*0.008*	–	–	–	–	–	–	–	–	–
Assay plate	–	–	–	–	–	–	4	3.096	0.078	4	39.79	*< 0.001*	4	17.46	*0.001*

were a costly investment. However, this lack of relationship does not mean that yolk components are not costly, since it is possible that the high to moderate levels of repeatability that we found for most egg investment components impose a limit on plasticity (Christians 2002; Tschirren et al. 2009; Supplementary Table 1S). Nevertheless, yolk weight and A4/A4+T ratio—a measure that is positively related to female quality in the collared flycatcher (Tschirren et al. 2014)—showed signs that maternal inversion could be determined by female quality, since these components reached higher values as females were older. These results may suggest that an age-related constraint may be present for some components. Similarly, several studies have also shown that yolk weight increases with female age in birds (e.g. Marion et al. 1964; Suk and Park 2001). Although egg volume did not change with female age, it was positively related to female body condition, as previously found in our starling population (Monclús et al. 2017). Regarding the increase in the A4/A4+T ratio throughout the female age, Tschirren et al. (2014) suggested that natural selection favours females that allocate high A4 and low T concentrations to their eggs since high A4/A4+T ratios are associated with higher survival. Thus, more experienced spotless starling females increase A4 levels over T levels, which may result in increased offspring fitness.

As found in previous studies with starlings, androgen concentration and total androgen amount increased across the laying sequence (Pilz et al. 2003; López-Rull and Gil 2009). This implies that, in the context of sibling competition, mothers could compensate the competitive disadvantage of late hatching by allocating higher levels of androgens to the last eggs of the laying sequence in most clutches (Muller and Groothuis 2013). This is the core idea of the 'competition hypothesis' of yolk androgen allocation (Schwabl 1993, 1996b, 1997), whose proximate mechanism relies in the positive effect of yolk androgens on embryonic development (Eising and Groothuis 2003; Muriel et al. 2015b), development of the hatching muscle (Lipar and Ketterson 2000),

aggressiveness (Müller et al. 2009), begging behaviour (Schwabl 1996b) and growth (Eising et al. 2001; Pilz et al. 2004). However, wide among-clutch variation in mean yolk hormone levels shows that females do not allocate maximal levels of yolk androgen to all eggs. Possibly because it could be costly for females themselves (Groothuis et al. 2005a, b) and also because of physiological costs to the nestlings (Sandell et al. 2009; Muriel et al. 2015b). Interestingly, in our analysis, considering age as a categorical variable, we observed that 1-year-old females did not increase A4 levels across the laying order from the middle of the breeding season onwards, while older females maintain a significant difference between the first and fourth egg throughout the breeding season. Since both late clutches and late eggs within a clutch have lower survival probabilities than early ones (Gil et al. 2007), this lack of increase in androgens along the laying order would reinforce the effects of hatching asynchrony. This implies that nestlings hatched from the last-laid eggs of young females and raised under harsher environmental conditions (i.e. late in the season) would be less able to overcome the competitive disadvantage with their earlier siblings, which would facilitate brood reduction. Indeed, brood reduction is much higher in late than in early broods in this population, especially in broods raised by young females (JM et al. unpublished data). As with yolk A4 levels, yolk T levels increased significantly from the first to the fourth egg in the laying order, but in this case, the difference in androgen concentration with respect to the egg position was not conditioned by female age. Although lower androgen levels could be interpreted as a consequence of the reduction in yolk weight observed in late clutches by young females, the lack of a strong correlation between yolk weight and androgen levels does not support this possibility. In a general way, this scenario is consistent with the conclusions derived from a previous study carried out in domestic canaries (Vergauwen et al. 2012). In that study, high food availability and quality led females to deposit higher amounts of testosterone to the last eggs within the clutch. Given the role
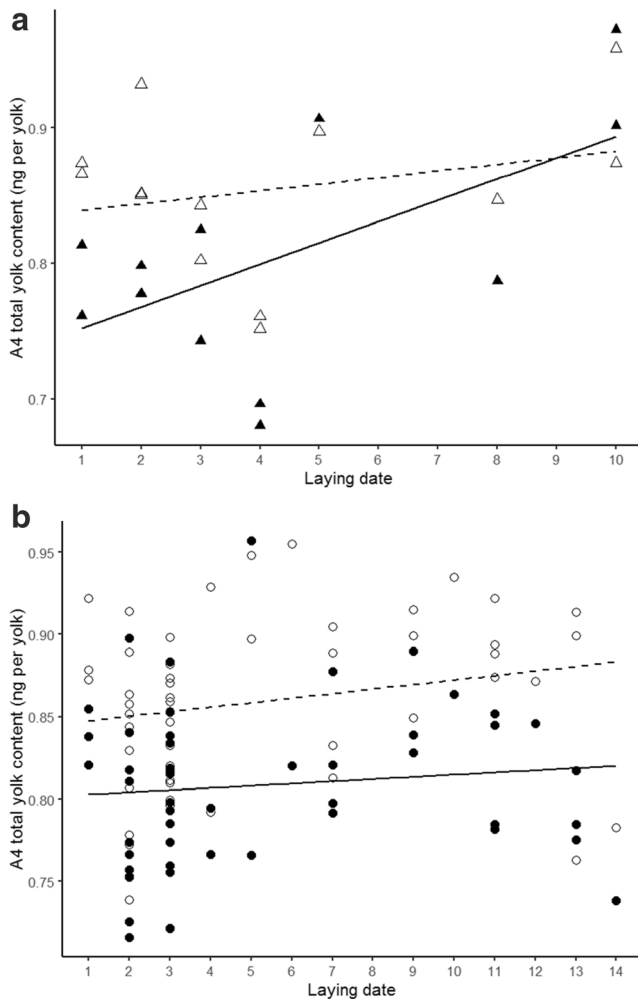
Fig. 3 Total A4 content in egg yolks according to laying date and laying order in (a) first-year and (b) older females. In both cases, white symbols and dashed line correspond to the first egg, whereas black symbols and solid line correspond to the fourth egg.
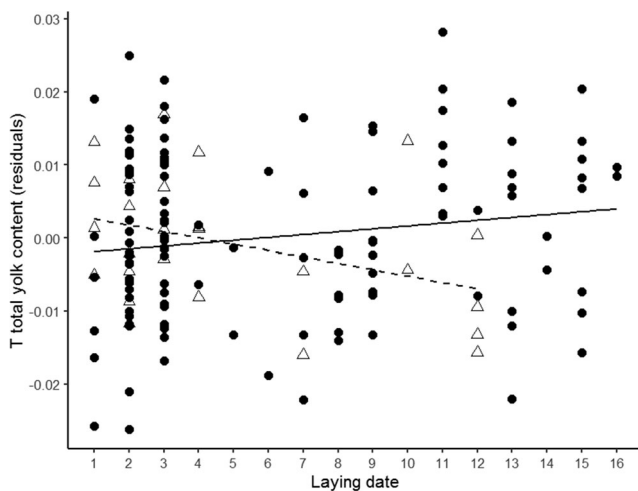

Fig. 4 Differences in total T yolk content, shown as residuals from statistical models, according to laying date and female age (white triangles and dashed line correspond to first-year females, black circles and solid line correspond to older females)

of androgens as epigenetic modifiers, we propose that our results can be interpreted as a mechanism by which less experienced females would reduce their investment in the last eggs under harsh environmental conditions characterized by reduced food availability.

The lower investment carried out by first-year females in the second half of the breeding season was also reflected in yolk weight, confirming that young birds are less able to invest resources in reproduction than older females (Laaksonen et al. 2002; Barbraud and Weimerskirch 2005). Contrary to yolk weight, egg volume was not conditioned by female age. This lack of relationship could be attributed to a higher repeatability of egg volume (Monclús et al. 2017; Supplementary Table 1S) and the relatively weak correlation between egg size and yolk weight found here. This weak correlation could be due to the fact that the egg size is actually more influenced by the amount of albumen than by the size of the yolk (Hussein et al. 1993; Suk and Park 2001). Irrespective of female age, we found that egg volume was reduced from the first to the fourth egg at the end of the breeding season. This decrease across the laying sequence (Henriksen 1995; Viñuela 1997) has been explained as a strategy to increase brood asymmetries, allowing brood reduction under potentially harsh scenarios (Slagsvold et al. 1984). The weak negative effect of clutch size on egg volume could be interpreted as a female compromise between laying few large or many small eggs (e.g. Cichon 1997), especially when resources for egg formation are limiting (Järvinen 1996).

Most studies that examine the relationship between age and reproductive success in birds find an increase in reproductive rates with age (Forslund and Pärt 1995), and so is the case too with our study population (JM et al., unpublished data). This increase with age can be due to several factors, including differential survival, delayed reproduction, effect of learning and experience or life-history optimization strategies (Stearns 1992; Forslund and Pärt 1995). Given the cross-sectional nature of our study, we cannot tell apart these alternatives for the patterns that we found, but our results suggest that yolk androgens are relevant to fine-tuning reproductive output. Life history predicts that reproductive output should increase with age because of improvements in reproductive competence (Charlesworth 1994; Forslund and Pärt 1995). Increases in competence include the effect of breeding experience, learning to forage, and increase in resource holding capacity and have been found in many avian species (e.g. Desrochers·1992). Assuming that these effects are also present in starlings, the differential allocation patterns along the laying sequence and the different broods that we found would allow young females to reduce the reproductive load adaptively with respect to more experienced females.

This interpretation is in line with our previous research in this species. In addition to differences between females, yolk hormone allocation differs too between environmental contexts (Groothuis et al. 2005b). As previously shown in this species (Lopez-Rull et al. 2010), we found a decrease in yolk

A4 and T concentrations as the breeding season advanced. Yolk androgen levels may vary according to the environmental conditions experienced by the mother and that could change during the course of the breeding season, such as nutritional conditions (Verboven et al. 2003; Gasparini et al. 2007; Benowitz-Fredericks et al. 2013), photoperiod (Schwabl 1996a) or parasite abundance (Tschirren et al. 2004; Postma et al. 2014). We have previously shown that yolk androgen-mediated trade-offs experienced by the offspring are context and dose-dependent (Muriel et al. 2015a, b). We found that high yolk androgen levels were detrimental rather than beneficial, for offspring development in clutches laid at the end of the breeding season (Muriel et al. 2015a, b). Thus, the observed decrease in yolk androgens throughout the breeding season is consistent with an adaptive maternal strategy to reduce offspring number, likely maximizing surviving offspring fitness or female survival (Mousseau and Fox 1998).

Our results highlight an absence of overall age-dependent patterns in yolk androgen deposition, which may call into question the existence of a direct cost of the investment in these components for the mother. Nevertheless, females of different ages showed differential deposition patterns when taking into account laying order and laying date. This suggests that females use androgens as epigenetic modifiers to manipulate the sibling developmental hierarchy (see Pilz et al. 2003) in order to maximize their overall fitness. These results are particularly compelling when considering female age and laying date. At the beginning of the breeding season, when breeding conditions are better, there was no difference in maternal investment between age classes. However, at the end of the breeding season, 1-year-old females modified their deposition of both resources (i.e. yolk mass) and epigenetic modifiers (i.e. androgens), showing greater differences in breeding performance between age classes when the breeding conditions are harsher (Laaksonen et al. 2002; Bunce et al. 2005). Older females, typically more experienced and on better condition than younger ones, are expected to forage more optimally when the environmental conditions are adverse, being therefore able to raise larger broods than younger females (Monclús et al. 2017). By modifying androgen allocation patterns across the laying sequence, first-year females would favour within brood asymmetries and, consequently, facilitate adaptive brood reduction. Thus, our results showed an age-specific pattern of reproductive performance in which extrinsic factors, i.e. resource availability, play a main role in shaping costs and benefits derived from egg yolk resource allocation (Stearns 2000). Future studies should assess the underlying physiological mechanism that enables females to regulate yolk androgen allocation into her eggs. Similarly, it is important to assess in which way—if any—young females benefit from these changes in androgen allocation patterns, since both improved female survival and offspring recruiting probabilities are likely options.

Compliance with ethical standards

Ethical approval   All applicable international, national and/or institutional guidelines for the care and use of animals were followed. Our study was in accordance with ethical approval from the Spanish ethics committee since eggs were removed before the incubation and the corresponding embryonic development began (RD 53/2013). Capture and manipulation of birds were authorized 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). Permission to work in the area was granted by both the Parque Regional de la Cuenca Alta del Manzanares 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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