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RESEARCH ARTICLE

Diverse dose–response effects of yolk androgens on embryo development and nestling growth in a wild passerine

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ABSTRACT

Avian egg yolks contain various amounts of maternally derived androgens that can modify offspring phenotype and adjust their development to the post-hatching environment. Seemingly adaptive variation in yolk androgen levels with respect to breeding density conditions or male attractiveness has been found in numerous studies. One important consideration that has been overlooked in previous research is the likely non-linear nature of hormone effects. To examine possible complex dose–response effects of maternal androgens on chick development, we experimentally administered three different androgen doses of the naturally occurring mixture of yolk testosterone and androstenedione to spotless starling eggs (*Sturnus unicolor*). We found that yolk androgens induce a non-linear dose–response pattern in several traits. Androgens had a stimulatory effect on hatchling body mass and nestling skeletal growth, but maximum values were found at intermediate doses, whereas our highest dose resulted in a decrease. However, the opposite U-shaped effect was found on nestling body mass. We also detected linear negative and positive effects on embryonic development period and nestling gape width, respectively. Our results suggest differential tissue responsiveness to yolk androgens, which may result in compromises in maternal allocation to produce adapted phenotypes. Because of the non-linear dose–response pattern, future investigations should carefully consider a wide range of concentrations, as the balance of costs and benefits may strongly differ depending on concentration.

KEY WORDS: Developmental plasticity, Testosterone, Androstenedione, Maternal effects, *Sturnus unicolor*, Hormone transfer

INTRODUCTION

Females can influence the phenotype of their offspring through genes and somatic investments. Mousseau and Fox (1998) define maternal effects as epigenetic modifications of offspring phenotype caused by the environment provided by the mother during development. These mechanisms of phenotypic plasticity can cause evolutionary changes in some traits because they affect the expression of traits under selective pressure from heterogeneous environmental conditions (Mousseau and Fox, 1998; Price, 1998; Räsänen and Kruuk, 2007; Wolf and Wade, 2009).

Avian models are ideal systems for studying maternal effects in an evolutionary framework, because their embryos develop outside the mother's body in independent structures (i.e. eggs). Egg production represents a substantial maternal investment for birds, with a strong influence on offspring development and survival (Williams, 1994; Christians, 2002). Hormone concentrations in the yolk of oviparous vertebrates are considered a clear case of maternal effects (Gil, 2008) as they are transferred from the mother (Williams et al., 2004) and may adjust offspring phenotype to environmental pressures (Adkins-Regan et al., 1995; Mousseau and Fox, 1998; Groothuis and Schwabl, 2008). For example, studies across a variety of passerine species have confirmed links between egg yolk androgen levels and growth, maternal parasite exposure, breeding densities, the timing of breeding or food abundance (reviewed in Gil, 2003, 2008; Tschirren et al., 2009). Avian yolks contain three different androgens: androstenedione (A4), testosterone and 5α-dihydrotestosterone (DHT) (Schwabl, 1993). All these androgens share a common synthesis pathway, in which A4 can be directly converted into oestradiol (E2) or testosterone. Testosterone, in turn, can be directly converted into E2 or DHT (Groothuis et al., 2005). The concentrations of these three androgens vary greatly both within and among species (Groothuis et al., 2005; Schwabl et al., 2007; Gil et al., 2007) and their effects on several offspring traits can be detected at different stages of offspring development (Mousseau and Fox, 1998; Griffith and Buchanan, 2010).

Since the seminal paper of Schwabl (1993) showing systematic intra-clutch variation of testosterone levels in avian eggs, the field of hormone-mediated maternal effects in birds has developed rapidly, focusing on the effects of yolk testosterone on postnatal growth and behaviour within an adaptive framework. The differential deposition of yolk hormones may not only modulate the level of within-brood competition but also prepare offspring for certain environmental conditions (Schwabl, 1997). Variations in yolk androgen levels may result in a wide array of effects on offspring traits. Thus, an increase in yolk androgen levels may cause faster embryonic development (Eising et al., 2001; Eising and Groothuis, 2003; but see Sockman and Schwabl, 2000; Muriel et al., 2013), greater development of the hatching muscle (Lipar and Ketterson, 2000; Lipar, 2001), higher aggressiveness (Müller et al., 2009, 2012), intensified begging behaviour (Schwabl, 1996; Eising and Groothuis, 2003), enhanced nestling growth (Eising et al., 2001; Pilz et al., 2004; but see Sockman and Schwabl, 2000; Muriel et al., 2013) and higher metabolic rates (Tobler et al., 2007), as well as higher plasma testosterone levels in nestlings (Müller et al., 2007). Although most studies report a significant effect of the focal steroid hormone on early growth or survival (Smiseth et al., 2011), this effect often has associated negative consequences on offspring fitness (Uller et al., 2005), such as decreased immune responsiveness (Groothuis et al., 2005; Sheldon and Verhulst, 1996; Duffy et al., 2000; Demas, 2004; Müller et al., 2005; Navara et al., 2005) and increased oxidative stress probably resulting from faster growth (Alonso-Alvarez et al., 2007). Also, sex differences in the effects of maternal androgens on offspring growth or survival

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have been reported (Smiseth et al., 2011), suggesting that androgens may have different costs and benefits for males and females (von Engelhardt et al., 2006; Saino et al., 2006; Müller et al., 2008, 2010; Ruuskanen and Laaksonen, 2010). Although it is clear that maternal effects mediated by yolk androgens may alter offspring phenotype (see above), there is a likely confounding effect due to large differences between studies in the dose and type of androgens injected (Gil, 2008; Groothuis et al., 2005). Furthermore, several androgens appear together in yolk, but it has been shown that the effects of testosterone and A4 are neither equivalent nor additive (Muriel et al., 2013; Hegyi and Schwabl, 2010; Tschirren et al., 2014). Non-linear, dose–response effects of steroids may shift the balance from benefits to costs (Navara et al., 2005). Non-linear responses are common in the steroid literature. For example, Norton and Wira (1977) showed that *in ovo* injections of a low testosterone dose stimulated growth of the bursa of Fabricius, while a high dose had the opposite effect. In humans, research shows that different androgen-dependent processes have different testosterone dose–response relationships (Bhasin et al., 2001). Thus, dose–response studies manipulating the specific pool of hormones present in the yolk are essential to fully understand the effects of these molecules on offspring phenotype.

Here, we explored the dose-dependent response to maternal yolk hormones and how several offspring traits are affected by them, by experimentally injecting three different doses of testosterone+A4 into the egg yolks of spotless starlings (*Sturnus unicolor* Temminck 1820). We chose this combination of hormones as they appear together in the yolk (Schwabl, 1993) and are positively correlated (Groothuis and Schwabl, 2002; Gil et al., 2004; Ruuskanen et al., 2009). Androgen doses were calculated considering the population mean±s.d. of both hormones, and were defined as 'low' (2 s.d.), 'intermediate' (4 s.d.) and 'high' (8 s.d.) doses. Control eggs received injections of the vehicle only. Because hormone effects can strongly depend on environmental conditions (reviewed in Smiseth et al., 2011), we performed our experiment in a single breeding season. In order to increase the power of our experimental design, we injected different eggs of the same clutch with either control or one of the three androgen doses. We studied the effects of our treatments on the length of the embryonic development period (EDP), hatching success, nestling survival and growth (tarsus length, body mass and body condition). We also studied gape width, which is a temporary trait used by nestlings during begging displays to parents (Müller et al., 2007; Kilner, 2006). On the basis of previous studies (Müller et al., 2007; Saino et al., 2006), we explored how these effects varied between sexes and throughout the nestling period. We hypothesized that an elevation of yolk androgen levels should improve offspring development and growth, although we took into account the possibility of finding inverted U-shaped effects due to non-linear dose-dependent responses (Groothuis and Schwabl, 2008).

RESULTS

Embryonic development and hatching success

In agreement with previous studies, the overall hatching success was around 67% (details in supplementary material Table S1). Control eggs had a significantly lower hatching success than non-injected eggs from a random sample of nests in the same colony (χ^2=8.64, d.f.=1, P=0.003), but hatching success was not influenced by treatment (χ^2=1.58, d.f.=3, P=0.662), suggesting that the levels of testosterone+A4 that we used did not have a negative effect on embryo survival. Chick mortality during the nestling period did not differ between treatments (χ^2=0.471, d.f.=3, P=0.925).

EDP was evaluated considering only recently hatched nestlings, those for which hatching hour could be precisely recorded. EDP was significantly affected by treatment ($F_{1,31.2}$=9.65, P=0.004, estimate±s.e.=−0.057±0.0186; Fig. 1), after controlling for laying order ($F_{1,27.8}$=9.84, P=0.004, estimate±s.e.=0.121±0.0387), without a significant effect of hatchling sex ($F_{1,30.3}$=0.01, P=0.92). EDP linearly decreased with increasing androgen levels in egg yolks, so that time to hatch was shorter for those nestlings that had been treated with the highest dose (Fig. 1). We analysed in unhatched eggs whether there were differences in the phase in which chicks had stopped their development, but did not find significant differences between treatments in early (χ^2=0.69, d.f.=3, P=0.873) or late stages of development (χ^2=1.89, d.f.=3, P=0.594).

Hatchling body mass was affected by experimental treatment with a negative quadratic effect ($F_{1,143}$=13.68, P=0.0003, estimate±s.e.=−0.0231±0.0061) after controlling for hatchling wetness level [$F_{2,136}$=52.53, P<0.0001, estimate±s.e.=(level 1) 5.59±0.078, (2) 6.01±0.073 and (3) 6.66±0.077]. This inverted U-shaped effect of androgen treatment remained when potential outliers (i.e. points deviating more than 2 s.d. from the mean of each group) were removed from the analysis ($F_{1,119}$=8.20, P=0.0049, estimate±s.e.=−0.0168±0.0058). Thus, we found higher masses when the androgen dose was increased, but only from the control to the intermediate concentrations, as the highest dose reduced body mass to the level of controls (Fig. 2). Also, hatchling mass was positively affected by egg volume ($F_{1,78.5}$=92.74, P<0.0001, estimate±s.e.=0.0009±0.0001) and clutch laying date ($F_{1,72.3}$=9.05, P=0.0036, estimate±s.e.=0.0132±0.0044), but did not vary between sexes ($F_{1,131}$=0.52, P=0.471).

Nestling growth

Regarding skeletal growth, we detected a negative quadratic (inverted U-shaped) effect of androgen levels in the interaction with age on tarsus length (Table 1), the effect of the treatment being more intense in the early days of development (supplementary material Table S3; Fig. 3A). This effect was independent of sex (supplementary material Table S2). Although throughout all ages, males showed longer tarsi than females (repeated-measures ANOVA: $F_{1,575}$=10.99, P=0.0010), males and females had the

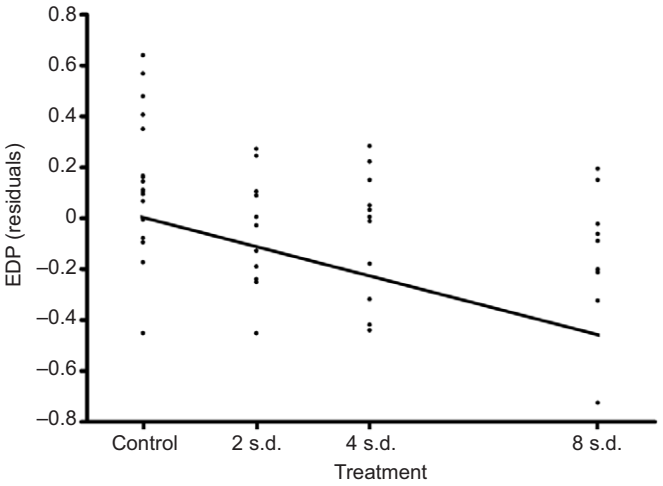

Fig. 1. Linear negative effects of yolk androgen doses on embryonic development period (EDP). Data plotted are residuals from the final model excluding treatment. Similarly, regression lines were generated by the graphics program from those data, and are only for illustration purposes. For the real estimates of the final models, please refer to Results.

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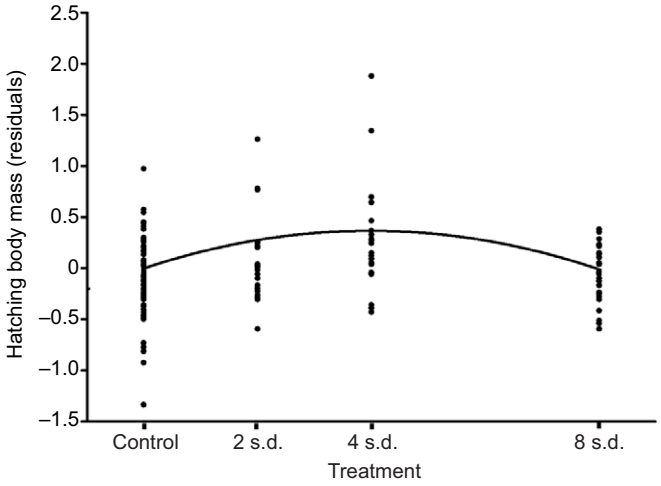

Fig. 2. Inverted U-shaped effect of yolk androgen dose on hatchling body mass. Data plotted are residuals from the final model excluding treatment. Regression lines were generated by the graphics program from those data, and are only for illustration purposes. This quadratic effect remains when potential outliers (i.e. points deviating >2 s.d. from the mean of each treatment) are removed from the analysis. For the real estimates of the final models, please refer to Results.

same response to treatment. Nestlings hatching from eggs treated with androgens showed progressively longer tarsus lengths when the testosterone+A4 concentration was increased, reaching a maximum at intermediate doses but not increasing when the concentration was even higher. Tarsus length was also positively affected by egg volume and nest laying date, but negatively by laying order, incubation period and brood size (supplementary material Table S3); thus, nestlings with larger tarsi hatched from larger eggs, occupied earlier positions in the laying sequence, belonged to clutches that had been laid later in the season and incubated for a shorter time, and had been raised with a lower number of siblings.

Body mass showed a significant positive effect of treatment in interaction with age (Table 1), but the interaction between treatment and sex was not significant (supplementary material Table S2). Inspecting the residuals from the model (Fig. 3B), we found that the pattern conformed to a threshold effect, strongly contrasting with that observed for tarsus length: heavier chicks were those who hatched from eggs receiving the highest androgen dose, with virtually no effect of the lower doses of hormone (Fig. 3B), and this effect was particularly strong during the early nestling period. We also found a positive effect of egg size, and negative effects of laying order, EDP and brood size (supplementary material Table S3).

Nestling body condition (residuals from the regression of body mass on tarsus length) showed a marginally significant quadratic effect of androgen dose (Table 1), and did not differ between males and females or ages (supplementary material Table S2). If we examine the residuals from the model (Fig. 3C), we find a similar trend to that observed in nestling body mass, although no interaction with age was found here. We also found a positive effect of egg size, and negative effects of clutch laying date and laying order (supplementary material Table S3).

Gape width was positively and linearly affected by androgen dose (supplementary material Table S3), with nestlings showing wider gapes as androgen dose increased (Fig. 3D). This effect was independent of sex and age (supplementary material Table S2), although males showed wider gapes than females (supplementary material Table S3). Although there was no significant interaction between treatment and age, the largest differences in gape width between control and 8 s.d. nestlings were detected on day 3. There was also a negative effect of clutch laying date on gape width and a positive effect of incubation period and brood size (supplementary material Table S3). In absolute terms, males had wider gapes than females (supplementary material Table S3), after controlling for body size as measured by tarsus length ($F_{1,588}$=71.74, P<0.001, estimate±s.e.=0.258±0.030).

DISCUSSION

Despite many experimental studies on maternal effects mediated by yolk androgens in birds, the large variability in the dose and type of androgens injected makes it difficult to perform comparisons between studies (see Table 2), which are essential to understand certain inconsistent patterns in the literature (Gil, 2008; Groothuis et al., 2005; Groothuis and Schwabl, 2008). Because complex dose–response patterns may shift the balance from benefits to costs (Navara et al., 2005; Bhasin et al., 2001), we experimentally tested in a single breeding season the differential effect of physiological

Table 1. Summary of final repeated-measures mixed models of the effect of yolk androgen treatment

	Tarsus length			Body mass			Body condition			Gape width		
Independent variable	d.f.	*F*	*P*	d.f.	*F*	*P*	d.f.	*F*	*P*	d.f.	*F*	*P*
Age	3,538	3257.4	<0.001	3,539	1662.52	<0.001	–	–	–	3,548	924.72	<0.001
Sex	1,575	10.99	0.001	1,577	15.74	<0.001	–	–	–	1,596	9.98	0.002
Sex×age	3,538	5.68	<0.001	3,539	7.46	<0.001	–	–	–	–	–	–
Treatment×age	3,538	2.30	0.076	3,539	2.08	0.102	–	–	–	–	–	–
Treatment^2	1,585	0.69	0.406	1,584	0.73	0.395	1,565	3.76	0.0530	–	–	–
Treatment^2×age	3,538	4.94	0.002	3,539	4.17	0.006	–	–	–	–	–	–
Treatment	1,560	0.88	0.350	1,566	0.02	0.899	1,589	0.34	0.5623	1,591	10.83	0.001
Clutch laying date	1,130	26.65	<0.001	–	–	–	1,89.6	13.96	0.0003	1,88.4	15.55	<0.001
Egg volume	1,261	5.41	0.021	1,233	9.26	0.003	1,140	9.77	0.0022	–	–	–
Laying order	1,571	95.01	<0.001	1,574	90.77	<0.001	1,600	18.40	<0.001	–	–	–
EDP	1,422	20.84	<0.001	1,418	13.11	<0.001	–	–	–	1,250	3.97	0.047
Brood size	1,582	3.65	0.057	1,583	10.87	0.001	–	–	–	1,590	9.86	0.002
Tarsus length	–	–	–	–	–	–	–	–	–	1,588	71.74	<0.001

Data show the effect of yolk androgen treatment on tarsus length, body mass, body condition and gape width of nestlings, taking into account age, sex, laying date, egg volume, laying order, embryonic development period (EDP) and brood size.
Models were run using Proc Mixed with Satterthwaite correction to adjust the degrees of freedom. Treatment and Treatment^2 show the effects of the linear and quadratic terms of hormone treatment, respectively. Age was measured in days.

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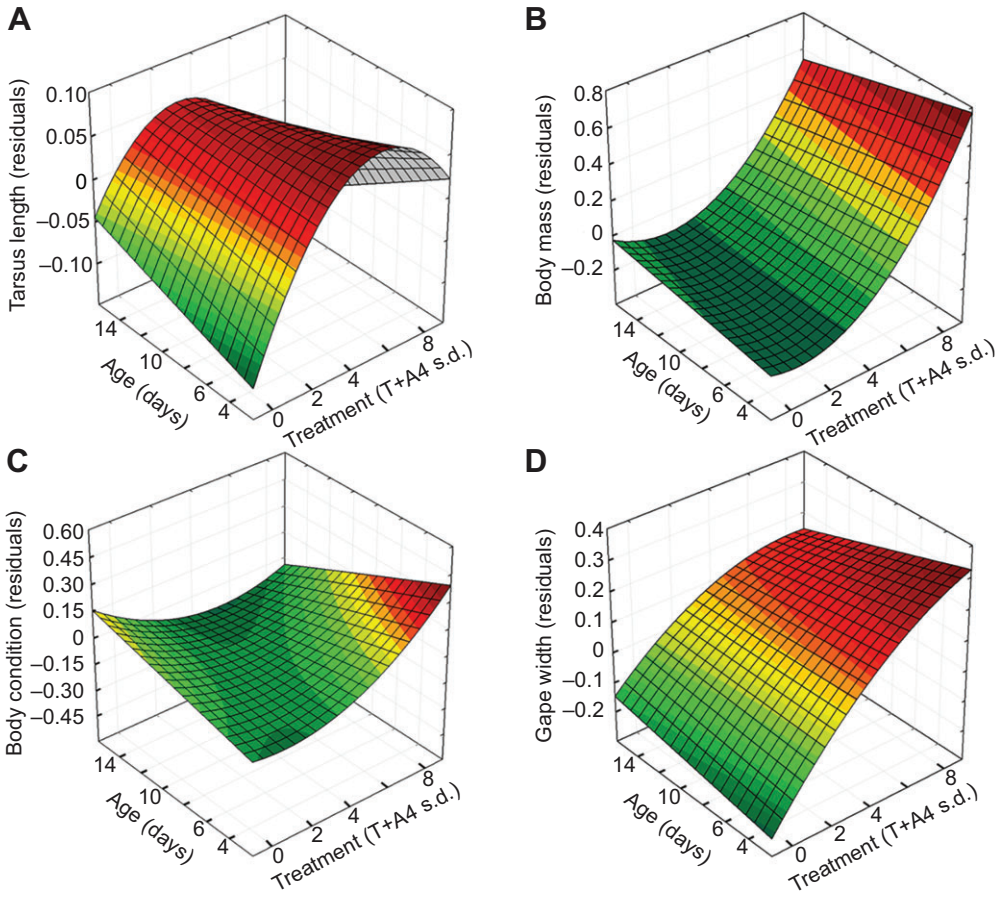

Fig. 3. Effect of yolk androgen dose on growth parameters during the nestling period. (A) Tarsus length, (B) body mass, (C) body condition and (D) gape width. Data plotted are residuals from the final model excluding treatment and age. Similarly, regression splines were generated by the graphics program from those data, and are only for illustration purposes. For the real estimates of the final models, please refer to supplementary material Table S3. T, testosterone; A4, androstenedione.

in ovo injections of three different androgen doses of the naturally occurring mixture of yolk testosterone and A4 on offspring development. We found that in wild spotless starlings, yolk androgens showed different dose–response patterns depending on the observed trait (for a similar situation with men and testosterone, see Bhasin et al., 2001). For some traits we found non-linear dose–response patterns with both negative quadratic effects (on hatchling body mass and nestling skeletal growth) and positive quadratic or threshold effects (on nestling body mass). In addition, yolk androgen exerted a linear effect for other traits (i.e. embryonic development period and nestling gape width). Our results suggest differential tissue responsiveness to yolk androgens, which may select for compromises in maternal allocation to produce adapted phenotypes (Gil, 2008).

In contrast to a similar study in which different testosterone doses were injected (Navara et al., 2005), we found no differential embryo or nestling mortality among groups due to increases in androgen levels (Pitala et al., 2009; Müller et al., 2010). Based on previous studies (Eising et al., 2001; Eising and Groothuis, 2003; Schwabl et al., 2007; Müller and Eens, 2009), we predicted a negative effect of yolk androgens (testosterone+A4) on the duration of the EDP. Our results confirm this prediction, showing a consistent linear reduction of EDP with increasing yolk androgen levels, at least within the range that we studied. Such an effect could be particularly relevant in situations of strong competition in broods composed of offspring of variable size (Smith and Bruun, 1998; Pilz and Smith, 2004; Hadfield et al., 2013). The difference in EDP that we found could be a consequence of the effect of yolk androgens on the development of the musculus complexus (hatching muscle), as has been showed in a previous study (Lipar and Ketterson, 2000; but see Lipar, 2001), which may reduce the competitive disadvantage of last-hatched chicks. So, our results support the hypothesis that yolk androgens may function as a compensatory mechanism for delayed hatching (Gil, 2008; Schwabl, 1993; Müller et al., 2005) and thus play a role during the earliest life stages (Groothuis et al., 2005). In a previous study in the spotless starling (Muriel et al., 2013), no differences were observed in EDP from eggs injected with testosterone+A4, but in that case the injected dose was smaller (only 1 s.d. of the population mean). In contrast, an opposite effect (i.e. longer EDPs for androgen-injected eggs) was found in two different studies performed in American kestrels (*Falco sparverius*) and zebra finches (*Taeniopygia guttata*) (testosterone+A4: Sockman and Schwabl, 2000; testosterone: Boncoraglio et al., 2011). This suggests strong species-specific differences in the effects of yolk androgens. In addition, this discrepancy of effects might be due to dose–response patterns, which probably differ between species.

One of the most remarkable results of this study is the inverted U-shaped effect of treatment on hatchling body mass. Many studies have found effects of androgens on body condition or growth in chicks hatching from androgen-injected eggs during post-embryonic development (Groothuis et al., 2005; Eising et al., 2001; Sockman and Schwabl, 2000; Pilz et al., 2004; Navara et al., 2005; Cucco et al., 2008), but our study is the first to explore quadratic effects right from the hatching stage. Studies that have measured body mass at hatching (Eising et al., 2001; Eising and Groothuis, 2003; Schwabl, 1996; Pilz et al., 2004) or in early post-embryonic development (Navara et al., 2005) found no effects of androgens on this trait (but see Schwabl, 1996). The inverted U-shaped effect that we found may result from two different effects

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Table 2. Overview of experimental studies in which yolk levels of androgens in avian eggs were experimentally increased

Species	Androgen	Oil (µl)	T (ng yolk^{-1})	T (ng)	%T	A4 (ng yolk^{-1})	A4 (ng)	%A4	Effect	Ref.
Falco sparverius	T+A4	50	44.85	100	223.0	1513.68	4000	264.3	[+EDP/–G/+M], [–G]	1, 2
Ficedula albicollis	T+A4	4	14.2	14.4	101.4	60.3	50.8	84.2	[G*], [M*]	3, 4
Hirundo rustica	T+A4	4	1.15	1	87.0	4.12	3.5	85.0	[G*]	5
Larus ridibundus	T+A4	50	111.26	90	80.9	6162.36	7500	121.7	[+G]	6
	T+A4	50	111.26	120	107.9	6162.36	10,000	162.3	[–EDP/+G], [–EDP/+BI], [–I]	6, 7, 8
Rissa tridactila	T +A4	50	423.13	153	36.2	5684.8	2695	47.4	[+A/+D]	9
Sturnus unicolor	T+A4	10	10.29	6	58.3	38.13	17	44.6	[G*/+GW/+AP], [–BC/G*/GW*]	10, 11
Sturnus vulgaris	T+A4	10	0.96	1	104.2	43.6	50	114.7	[–EDP]	12
Sturnus unicolor	A4	10	–	–	–	38.13	17	44.6	[–BC/G*]	11
Coturnix japonica	A4	20	–	–	–	372.32	200	53.7	[+RB]	13
	T	20	103.83	50	48.2	–	–	–	[–G]	13
Agelaius phoeniceus	T	5	36.93	110	297.9	–	–	–	[+CM]	14
Coturnix chinensis	T	5	289	300	103.8	–	–	–	[I*], [–TS/–ES]	15, 16
Faisianus cochicus	T	20	73.4	40	54.5	–	–	–	[DR*]	17
Parus major	T	5	8.87	30	338.2	–	–	–	[+DD], [+G]	18, 19
Passer domesticus	T	5	47.85	200	418.0	–	–	–	[SO*/+FC], [M*]	20, 21
Perdix perdix	T	20	20.85	20,000	95,923.3	–	–	–	[–G/–I]	22
	T	20	20.85	200	959.2	–	–	–	No effect	22
	T	20	20.85	20	95.9	–	–	–	[+G/+I]	22
Serinus canaria	T	5	56.55	100	176.8	–	–	–	[+G/BI], [G*/SD*/RS*]	23, 24
	T	5	56.55	50	88.4	–	–	–	[G*/+A], [G*]	25, 26
Sialia sialis	T	5	121.45	3000	2470.2	–	–	–	[–HS/+BC/–I]	27
	T	5	121.45	300	247.0	–	–	–	[–HS/+G]	27
Sturnus vulgaris	T	5	10	100	1000.0	–	–	–	[G*/BI*/–M]	28
Sturnus unicolor	T	10	10.29	6	58.3	–	–	–	[+EDP]	11
Taeniopygia guttata	T	5	1.125	0.5	44.4	–	–	–	[+EDP/BI*/G*/–M], [+RMR]	29, 30, 31
Troglodytes aedon	T	5	1.225	2	163.3	–	–	–	[+BI/+FS]	32

Androgens injected into eggs are reported here as a percentage of the mean rather than in s.d. units because s.d. data were not available for most studies. However, the comparison is still relevant in showing the very large range of doses that different studies have used.

'Androgen' refers to hormone of interest (T, testosterone; A4, androstenedione; T+A4, mixture of both androgens); 'Oil' indicates the injected volume of oil as vehicle; 'T' and 'A4' refer to the natural androgen concentration (here, yolk mass was adjusted for each species); 'Effect' indicates the reported effects per reference (+, increase; –, decrease; *, androgen interaction with other variable).

A, aggression; AP, endogenous plasma levels of androgens; BC, body condition; BI, begging intensity; CM, muscle complexus mass; D, dominance; DD, dispersal distance; DR, digit ratio; EDP, embryonic development period; ES, egg size; FC, food competitiveness; FS, fledging success; G, growth; GW, gape width; HS, hatching success; I, immunity; M, mortality; RMR, resting metabolic rate; RS, reproductive success; SD, song development; SO, sexual ornaments; TS, testis size.

[1]Sockman and Schwabl, 2000, [2]Sockman et al., 2008, [3]Pitala et al., 2009, [4]Ruuskanen et al., 2012, [5]Saino et al., 2006, [6]Eising et al., 2001, [7]Eising and Groothuis, 2003, [8]Müller et al., 2005, [9]Müller et al., 2012, [10]Müller et al., 2007, [11]Muriel et al., 2013, [12]Müller and Eens, 2009, [13]Hegyi and Schwabl, 2010, [14]Lipar and Ketterson, 2000, [15]Andersson et al., 2004, [16]Uller et al., 2005, [17]Romano et al., 2005, [18]Tschirren et al., 2007, [19]Tschirren et al., 2005, [20]Strasser and Schwabl, 2004, [21]Schwabl et al., 2012, [22]Cucco et al., 2008, [23]Schwabl, 1996, [24]Müller et al., 2008, [25]Müller et al., 2010, [26]Vergauwen et al., 2011, [27]Navara et al., 2005, [28]Pilz et al., 2004, [29]von Engelhardt et al., 2006, [30]Tobler et al., 2007, [31]Nilsson et al., 2011, [32]Barnett et al., 2011.

(benefits and costs) balancing out in a dose–response manner, with intermediate dosages having greater positive net effects than either lower or higher doses (Groothuis and Schwabl, 2008). In birds, prenatal exposure to androgens can have positive effects on growth (Eising et al., 2001; Schwabl, 1996; Pilz et al., 2004; Cucco et al., 2008; Pitala et al., 2009; Bentz et al., 2013), probably mediated by an increase in metabolic rate (Tobler et al., 2007; Nilsson et al., 2011). However, androgen levels that are too high might have higher associated costs to fuel metabolism, like excessive energy expenditure or increased susceptibility to oxidative damage as a consequence of free radical production (Alonso-Alvarez et al., 2007), and this could ultimately result in a loss of body mass at hatching. Furthermore, the effects of elevated yolk androgens on growth may be dependent on egg quality, relating to a likely prenatal context dependency (Williams, 1994; Vergauwen et al., 2011). For a better understanding of the effects of maternal yolk steroids, we need a broader and more specific knowledge of the mechanisms of androgen actions in the embryo. Previous studies have given rise to suggestions for a number of phenomena that need to be considered, including differences in yolk steroid metabolism [e.g. via the oestrogen receptor pathway after its aromatization to oestrogens (Hegyi and Schwabl, 2010), alterations in hormone secretion, saturation of steroid receptors, androgen sensitivity of the embryo and, more generally, gene expression (Gil, 2008)].

Regarding skeletal growth, we found an inverted U-shaped effect of treatment on tarsus length, as well as an age-dependent effect of yolk androgens on growth. Several previous studies have found a positive effect of androgens on chick growth (reviewed in Gil, 2008; Groothuis et al., 2005; Groothuis and Schwabl, 2008; Bentz et al., 2013), but only a few have showed an interaction between treatment and age (e.g. Schwabl, 1996; Pilz et al., 2004; Müller et al., 2010; Hegyi and Schwabl, 2010). As discussed in the case of hatchling body mass, yolk androgens had a stimulatory effect on growth, where an intermediate dose (4 s.d.) led to the maximum tarsus length. In this case, skeletal growth of treated chicks could be benefited either by a possible increase in competitive ability or begging levels for obtaining food or simply because of the benefits of early hatching (see above). However, as happened with hatchling body mass, excessively high doses of androgens may involve costs (Cucco et al., 2008) that may cause a decline or a plateau in growth. This effect was detectable throughout nestling growth, but was greater in the early days of development, as found in previous

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studies (Schwabl, 1996; Pilz et al., 2004; Cucco et al., 2008). Our finding is consistent with a similar trend described by Navara and co-authors (2005) who, at 2 days post-hatch, found that moderate levels of yolk testosterone, but not the high dose, tended to have a stimulatory effect on skeletal size of the resulting offspring in the eastern bluebird (*Sialia sialis*). Our finding that chicks grew faster than controls, particularly at the beginning of the nestling period, together with our finding of effects on embryo development suggest that androgen sensitivity may be particularly high during early development (Pilz et al., 2004; Cucco et al., 2008). This could be mediated through an increase in bone growth factors (Kasperk et al., 1990), or may simply arise because of the higher number of androgen receptors in bone and cartilage (Corvol et al., 1992). Perhaps for this reason, an increase in size would not necessarily be linked to a gain in nestling body condition (see below). Similarly, it has been suggested that although egg components are important early in ontogeny, their effects quickly dissipate and genetic and other environmental influences have a stronger role after this (Smith and Bruun, 1998). These findings are consistent with comparative data by Schwabl and co-authors (2007) who found that the relationship between androgen levels and growth was stronger for the embryonic than for the nestling developmental period.

Contrary to our findings for hatchling body mass and nestling tarsus length, we found that low doses of androgen exerted virtually no effect on nestling body mass, but high doses resulted in much higher nestling body mass than controls. Such a non-linear pattern can be interpreted as a threshold effect of yolk androgens on mass. These results are consistent with those of Schwabl (1996), who found that high-yolk androgen levels resulted in heavier offspring of better body condition (but see Cucco et al., 2008). Low and intermediate doses may derive a weight advantage from fast hatching, but this difference may not be enough to offset the costs and increase mass accordingly. This pattern may be explained if body mass and skeletal growth trade-off against each other: if we compare the shape of the dose–response effects of treatment on tarsus length and body mass we see that at low and intermediate doses, androgens enhanced skeletal growth at the expense of a parallel increase in body mass. Thus, we may speculate that above a certain threshold androgen level, the benefits of the hormones (shortest EDP, enhanced competitive ability) would offset the potential costs associated with the accelerated growth mentioned above. The fact that gape width increased linearly with androgen dose (particularly in the early stages of post-hatching development, discussed below) would support this hypothesis as wider gapes may attract a greater number of feedings (Gil et al., 2008), allowing the chick to fulfil the energetic requirements of accelerated growth. Taking into account the differential effect of androgens on body size and mass, we can understand why an increase in growth was not mirrored by an improved body condition. According to our results, androgens would be generating larger but relatively lighter chicks, leading to a reduced body condition. Similarly, in a previous study (Muriel et al., 2013) we found a reduction in body condition of a testosterone+A4 nestling group (1 s.d.). Nevertheless, in the present study, hatchlings from 8 s.d. injected eggs were those with the best body condition, as these chicks had greater tarsus length and body mass.

Our finding of a positive, linear effect of yolk androgens on gape width is in agreement with that of a previous study in the same species (Müller et al., 2007). Although the interaction between treatment and age was not significant, there appears to be a trend towards a greater effect of treatment at earlier stages of the nestling period (Fig. 3D). During this initial period of nestling development, chick survival strongly depends on attracting parental feedings (Gil et al., 2008; O'Connor, 1978; Wiebe and Slagsvold, 2012), so it makes sense that gape growth should be particularly labile during this time. As expected from a trait directly involved in sibling competition for parental feedings (Gil et al., 2008), we also found that nestlings from larger broods, where sibling competition is more intense, developed wider gapes. These results support previous evidence showing that birds can use adaptive developmental plasticity responses derived from maternal androgens (Gil, 2008; Groothuis et al., 2005).

In summary, we have documented that yolk androgens show complex dose–response effects during early development, including both linear and non-linear responses for different traits (Rubolini et al., 2006). Thus, our results highlight the importance of considering dose-dependent effects when studying the effects of yolk androgens in the future. For some traits, yolk androgen effects were mostly detected at earlier phases of the nestling period, whereas for others the effect was stronger in later stages, thus illustrating the variability in responsiveness to the hormone across traits. Also, we show for the first time that although androgens accelerate embryonic development, this does not necessarily lead to a disadvantage in terms of body mass at hatching. Besides their implications for a better understanding of the effects of yolk androgens on offspring development, our results beg the question of why females vary in their allocation of these hormones (Gil et al., 2004). It is possible that the answer lies not only in the differential effects that we found here for different traits but also in the potential costs for offspring in the long term, or just how costly the mobilization and allocation of yolk androgens is for females.

MATERIALS AND METHODS

Study species and site

The experiment was conducted in central Spain (Soto del Real, Madrid, ca. 40°45′N, 3°48′W, 920–940 m above sea level), in a large nest-box colony of spotless starlings (*S. unicolor*). The yolk hormone manipulations were conducted in April and May 2010 and nestlings were monitored until June. The study area is a Dehesa ecosystem used by cattle towards mid-May, and covered by a deciduous woodland of oak (*Quercus pyrenaica*) and ash (*Fraxinus angustifolius*). The spotless starling is a relatively long-lived, colonial and sedentary passerine species that exhibits a facultative polygynous breeding system (Moreno et al., 1999; Veiga, 2002). Females can lay up to two clutches per season; the first is started in early April and the second around the end of May in our study area. Incubation usually starts before the last egg is laid (3–6 eggs per clutch), and it is done mainly by females (lasting for approximately 12 days). Although parental care is provided by both pair members (Moreno et al., 1999), females invest more than males in rearing the brood (Jimeno et al., 2014). The nestling period lasts about 21–22 days (Cramp, 1998).

Egg injections

From the end of March onward, nest-boxes were inspected each day to determine laying date and laying order. Eggs were marked with a non-toxic waterproof marker as they were laid. To minimize nest disturbance, injections began when the third egg was found in the nest, before embryonic development was triggered by the start of parental incubation. All subsequent eggs were injected as they were laid. Clutches were randomly assigned to one of the three androgen doses, and eggs within each clutch received either control or experimental injections, alternating between the two, following the laying order. Also, the order of the injections across the laying sequence was inverted for each nest. With this balanced schedule we controlled for a possible confounding effect of laying sequence (for instance, naturally deposited testosterone increases with laying order, while A4 decreases; López-Rull and Gil, 2009). Treatments were alternated between clutches and consisted of: (1) low dose: 12 ng testosterone (ref.

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86500, Sigma-Aldrich, Steinheim, Germany)+34 ng A4 (ref. A9630, Sigma-Aldrich); (2) intermediate dose: 24 ng testosterone+68 ng A4; or (3) high dose: 48 ng testosterone+136 ng A4. In all cases, the mixture of hormones was dissolved in 10 µl of sesame oil (ref. 85067, Sigma-Aldrich), which is generally used as a solvent (Love et al., 2005) as steroid hormones are cholesterol derivatives and are lipid soluble. Within each clutch, all control eggs received 10 µl of sesame oil alone. The low, intermediate and high doses were equivalent to, respectively, 2, 4 and 8 s.d. of the population mean (testosterone: 14±6.0 ng yolk^{-1}; androstenedione: 50±17.1 ng yolk^{-1}, means±s.d.; D.G., unpublished data), adjusted for mean yolk mass (1.4 g). The maximum levels of yolk testosterone and yolk A4 that we have measured in this population are 36.3 and 198.4 ng per yolk, respectively (Müller et al., 2007). The high dose injections that we used (8 s.d.) are slightly above and below, respectively, the maximum levels of testosterone and A4 found in our population. Note, however, that previous studies indicate that injections *in ovo* might not mimic the natural distribution of hormones in the yolk, leading to variable exposure of the developing embryo depending on the position of the blastodisc (Von Engelhardt et al., 2009). Therefore, the potential degradation or incomplete incorporation of the injected androgens into the yolk must be considered (Navara et al., 2005), as not all the hormone injected is finally assimilated by the developing embryo. Furthermore, the choice of dose was based on a literature comparison (Table 2), which revealed a huge range of variation in previous experiments. A previous study in this species (Muriel et al., 2013) in which 1 s.d. of the mean of the combination of testosterone+A4 was injected found a slight growth inhibition, whereas many previous studies have used values that amount to much higher hormone dosages. We opted for levels that would cover, to some extent, the range of injections of previous studies.

In ovo injections were performed in the field using a standard U-50 insulin syringe (Terumo Corporation, Tokyo, Japan), following a standard protocol described elsewhere (Muriel et al., 2013). An injection volume of 10.15±1.05 µl (mean±s.d.) was used for each egg, based on mock injections performed within 0.2 ml Eppendorf tubes, with mass measured using a precision balance (accuracy 0.0001 g; A-2005, Sartorius Analytical Balance, Goettingen, Germany). The accuracy of injections and the diffusion of the oil in the egg yolks were assessed in a pilot study, consisting of injection of 10 µl of sesame oil stained with Neutral Red (a eurhodin dye used in histology; Winckler, 1974) into seven eggs from three non-experimental nest boxes, followed by retrieval 5 days later. Yolks of all injected eggs contained a homogeneous amount of Neutral Red throughout (albumin contained none), suggesting that the treatments diffuse uniformly into the yolk within a few days.

The experiment was carried out in 88 nests from the first clutch, but 41 of them could not be included in the analysis because of predation or sabotage by other females, or the impossibility of assigning hatchlings to their experimental group (see below). From the remaining successful nests, we included in the analysis data from 153 chicks (75 males and 78 females). This rate of nest failure is not unusual in this population (see, for instance, Müller et al., 2007).

Nestling growth

Broods were visited several times a day from the 10th day after the last egg was laid to check hatching time and ensure the correct assignment and labelling of chicks originating from the different experimental groups (chicks were labelled by distinct down cutting). We also recorded hatching success and computed incubation time or EDP, as the number of days from the last egg in a clutch being laid to the hatching date of a given egg. This procedure does not take into account incubation time before the completion of the clutch, but this variable was controlled by taking laying order into account in the analysis. Three days after the nest hatching date, we opened un-hatched eggs in order to check the phase at which the chick had stopped its development. We set up two categories based on the development stages established by Hamburger and Hamilton (1951): early (stages 0–28) and late (stages 29–38).

Nestlings were measured on days 3, 6, 10 and 14 post-hatching. At these ages, we recorded body mass with a digital balance (accuracy=0.1 g; Ohaus Scout II SC2020, China), gape width (recorded as the maximum width comprising the beak flanges) and tarsus length with digital callipers (accuracy=0.01 mm; Mitutoyo Absolute, Japan). An index of body condition was estimated using the residuals from a regression of body mass on tarsus length (Schulte-Hostedde et al., 2005). At the same time as yolk manipulation, measurements of egg length and width were taken with the digital callipers and egg volume (mm^3) was calculated by the formula: 0.45×length×width^2 (Worth, 1940). At day 1, we recorded body mass and categorized hatchling stage by their level of wetness (1: just hatched, down still moist and stuck to the skin; 2: 1–2 h after hatching, down almost dry but still not fully erect; and 3: >2 h after hatching, down totally dry and erect). All measurements were performed by the same individual (J.M.), blind to individual treatment whenever possible. At 15 days of age, a blood sample was extracted from all birds for molecular sexing. DNA was extracted from these samples (Bensch and Åkesson, 2003), and diluted to a working concentration of 25 ng µl^{-1} for PCR of the *CHD-W* gene in females and the *CHD-Z* gene in both sexes (Griffiths et al., 1998). The procedure was run twice on a subsample of 32 nestlings on several occasions to check error rate – in all cases the sex determination was identical. No additional biometric measures were taken from day 14 onwards because of the high risk of premature fledging that would result from handling nestlings at that age.

Given that egg injections typically lead to a certain level of hatching failure (Pilz et al., 2004, 35%; Müller et al., 2007, 30%; Pitala et al., 2009, 32.85%), the number of siblings was reduced in some nests in our study. This might result in unusually low levels of sibling competition in some of the experimental nests, which could affect the traits considered (Muriel et al., 2013). Thus, in order to reach the modal brood size in our population (mean±s.d.=4.72±0.57 nestlings per brood), we performed post-hatch brood amalgamation of broods in which only 1–3 chicks had hatched (37 out of 152 chicks were moved from their original nests). This procedure involved broods of the same age and androgen treatment and was performed when chicks were 3 days old. In all cases, broods were matched by average nestling size and the larger brood acted as the host nest. In those cases where broods to be pooled presented the same number of nestlings, the host nest was chosen at random.

Statistical analysis

Hatching success and nestling mortality were analysed using the chi-square test with the software STATISTICA v7.0 (StatSoft Inc., Tulsa, OK, USA) adopting a significance level of 0.05. Other statistical analyses were conducted with SAS 9.2 (SAS Institute Inc., Cary, NC, USA). In all cases the treatment was considered as a continuous variable because dose increased in a linear way (0, 2, 4 and 8 s.d. of the mean natural values), and we had *a priori* expectations of quadratic effects. Incubation period and hatchling body mass (controlled by the level of wetness) were analysed using mixed models. All morphometric variables and body condition were analysed separately using mixed models for repeated measures (SAS, Proc Mixed, normal distribution), with Satterthwaite correction to adjust the degrees of freedom. To control for non-independence of individuals from the same nest, which share strong genetic and early maternal effects over traits studied, the nest of origin was defined as a random effect affecting the model intercept. Using nest of adoption, instead of nest of origin, as random effect yielded the same results, but given that nest of origin explained much more variance, we kept this factor for all the mixed models presented here. Also, nestlings that remained in their original nests and translocated nestlings did not differ in any of the studied traits (all P>0.292). The identity of the individual was entered as a repeated factor in the models. The following variables were included in the main model: treatment, treatment^2, sex, age, egg volume, laying order, laying date, EDP, brood size, body size (except when this was the dependent variable), and all possible interactions. Sex (male: 1, female: 2) and age (3, 6, 10, 14 days) were considered as categorical variables. Input variables (raw parameters measured) were standardized to a mean of 0 and a s.d. of 0.5 before model analysis (reviewed in Grueber et al., 2011). Non-significant (P>0.05) terms were sequentially removed from the initial models, starting with interactions, following a backward stepwise procedure, until only the significant explanatory variables or interactions were retained in the models. When a significant interaction occurred, main effects of each factor involved in the interaction were also kept in the final model. As an exception, when brood size and treatment^2 (as explanatory variables of tarsus length and body condition

respectively, Table 1) showed marginal significance levels ($0.05<P<0.06$), they were retained in the final models to explore these trends. We included EDP as a covariate in these analyses because it may affect nestling development in addition to androgen effects per se. All biologically meaningful double and triple interactions were also included. In particular, as we expected the treatment effect to change along with age and to differ between sexes, the interaction treatment×sex×age was included in all initial models. Values represented are means±s.e. We present final models in the text, and initial rejected models can be found in supplementary material Table S2.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
D.G. conceived and designed the project. M.P. developed methodology and commented on the manuscript. J.M., D.G. and L.P.-R. performed the experiments, analysed the data and wrote the manuscript.

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Supplementary material
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