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Differential effects of yolk testosterone and androstenedione in embryo development and nestling growth in the spotless starling (*Sturnus unicolor*)

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ABSTRACT

Yolk androgens in avian eggs play a significant role in embryo and nestling development. However, few studies have examined the differential effect of two of the main yolk androgens, testosterone (T) and androstenedione (A4). Here, we injected eggs of spotless starlings with physiological levels of either T, A4, the combination T + A4 or vehicle substance (control), to examine the differential ability of these steroids to influence nestling development. We found that the duration of the embryonic period was increased by T, and less so by A4, but not by the combination T + A4. Body condition was reduced in all experimental treatments where A4 was present, particularly so in the combination T + A4. Tarsus length was increased in males by A4, and in a lower degree by T, whereas the combination T + A4 inhibited growth. However, these differences in tarsus length between groups disappeared at the end of the nestling period. Cell-mediated immune responsiveness was marginally affected by the interaction between treatment and sex. These patterns suggest that in this species, T has a stronger influence during embryo development than A4, whereas during nestling development the capacities of both androgens to influence growth are similar. The combination T + A4 showed non-additive effects, suggesting either some kind of inhibition between the two androgens, or else an excessive effect due to a bell-shaped pattern of response. Our results suggest a complex picture of sex and age-dependent effects of T and A4, and underline the necessity of further research in the metabolism and action of egg androgens.

1. Introduction

Since the discovery of testosterone of maternal origin in the yolk of birds (Schwabl, 1993), many studies have studied the effects of these hormones on development, growth, behavior and adult phenotypes in the eggs of several bird species (Groothuis et al., 2005b; Gil, 2008). The initial excitement about the capacity of these hormones to boost growth (Winkler, 1993; Gil et al., 1999), has weaned after the publication of several studies that have shown that the effects are less spectacular than previously expected. For instance, several experiments have found weak or non-existing effects of experimental injections of androgens (Rubolini et al., 2006; Tobler et al., 2007), complex interactions with sex (Saino et al., 2006; von Engelhardt et al., 2006) or effects that are dependent on environmental factors (Lopez-Rull and Gil, 2009). Current opinion recognizes that the mechanisms that may mediate this phenomenon are extremely complex and that we are a long way from understanding them (von Engelhardt et al., 2009; Müller et al., 2012). However, numerous studies keep on finding exciting possibilities for this transfer of maternal hormones than vindicate their role in maternal adjustment to ecological demands (Tschirren et al., 2007; Poisbleau et al., 2012; Schwabl et al., 2012).

Three main androgens are present in avian egg yolks: testosterone (T), androstenedione (A4) and 5α-dihydrotestosterone (5α-DHT) (Schwabl, 1993; Hegyi and Schwabl, 2010). In the androgen synthesis pathway, A4 is converted to T by 17b-hydroxysteroid dehydrogenase (17b-HSD), but also these androgens are potentially converted to estrogens (estrone and estradiol), particularly by aromatase (CYP19). Additionally, 5α-reductase converts testosterone to 5α-DHT, an androgen which can act only via the androgen receptor and which cannot be converted to T or estrogens (Groothuis and Schwabl, 2008; Carere and Balthazart, 2007; Sanderson, 2006). Given that T was the androgen with the highest concentration in canaries, the first species in which it was studied, most research that followed used this hormone for manipulations and assays (e.g. Lipar and Ketterson, 2000). However, there are important species-specific differences in the abundance of these hormones, with contrasting patterns in whether T or A4 has the highest concentration. Thus, whereas canaries (*Serinus canaria*)

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have much higher levels of T than A4 (Schwabl, 1993), in the case of starlings (*Sturnus vulgaris*) (Pilz and Smith, 2004) or quails (Gil and Faure, 2007), the situation is reversed. Species-specific levels of A4 increase with body size, and shows particularly high levels in the Rallidae and Laridae families (Gil et al., 2007). On the other hand, 5α-DHT tends to be present in much smaller amounts in most species assayed so far (e.g. Schwabl, 1993; Pilz and Smith, 2004), even being undetectable in some species like the common starling (*S. vulgaris*) (Pilz and Smith, 2004).

Despite these differences, only one experimental study to our knowledge has compared the differential effects of different yolk androgens on nestling phenotype (Hegyi and Schwabl, 2010). This study, performed in the Japanese quail (*Coturnix japonica*), species in which A4 is the predominant yolk androgen, showed effects of 5α-DHT or A4 in behavior, but not in growth, whereas T was shown to inhibit growth, implying that aromatization or other metabolic pathways may have interfered in the effects of androgens (Hegyi and Schwabl, 2010). In addition to this experiment, two comparative analyses have investigated the roles of different yolk androgens on development (Gil et al., 2007; Schwabl et al., 2007). The first of these two studies compared the relationship between species-specific androgen levels (both T and A4) and the duration of developmental periods in a large sample of species. The results showed that, across birds, neither androgen was related to species-specific differences in the overall duration of development. However, high levels of A4 (but not T) were correlated with relatively long incubation periods, and relatively short nestling periods (Gil et al., 2007). The second study (Schwabl et al., 2007), using a smaller sample of passerines, but a more precise androgen quantification protocol, including chromatographic column separation, found strong negative correlations between developmental periods and both T and 5α-DHT, as well as a positive relationship between any of these two androgens and growth rate. This relationship was not found for A4, suggesting that in passerines, the direct androgenic pathway (based in the androgen receptor) was the most important mechanism of yolk androgen action (Schwabl et al., 2007). Although it is difficult to reconcile the results of these studies, they all point to a differential role of the different androgens on embryo development.

In addition, prenatal androgen overexposure has been shown to negatively influence nestling immune capacity in several studies (Groothuis et al., 2005a; Müller et al., 2005; Navara et al., 2005, 2006), although some other studies have failed to find such an effect (e.g. Tschirren et al., 2005). Since negative effects of androgens in immunity may specifically depend on their pathway of action (Owen-Ashley et al., 2004), we decided to measure immune function. For this purpose, we used one of the most common and reliable immunological tests, cell-mediated immunity as measured by the swelling response to phytohaemagglutinin (PHA) (see e.g. Lochmiller and Deerenberg, 2000, López-Rull et al., 2011).

In this study we explore the effects of T and A4, separately and in combination, on embryo/nestling development in the spotless starling (*Sturnus unicolor*), a species whose eggs contain both androgens. In order to increase the power of our experimental design, we injected different eggs of the same clutch with either control, one of the two androgens, or the two androgens, always within physiological levels. We examined the possible effects of the treatment on incubation period, nestling growth (body mass and size) and immunity (cell-mediated immunity). We also studied the effects on gape width, which is a temporary trait used by nestlings to beg to parents that disappears at the end of the nestling period (Gil et al., 2008). Also, based on findings from previous studies (Saino et al., 2006; Müller et al., 2007), we explored interactions of the treatment effects with age and sex.

2. Material and methods

2.1. Study area and species

The experiment was conducted in April and May 2009, in central Spain (Soto del Real, Madrid), where spotless starlings breed in a nest-box colony monitored since 2001. The spotless starling (*S. unicolor*) is a facultatively polygynous passerine, that breeds in natural and artificial cavities, and shows sexually dimorphic characters (Cramp et al., 1982–1994; Moreno et al., 1999). Clutches are typically laid twice in the season, containing between 3 and 6 eggs. Incubation usually starts before the last egg is laid, and lasts for 12 days approximately. Parental care is provided by both pair members, although secondary females (i.e., the second female attracted by a bigamous male) often raise broods without male contribution (Moreno et al., 1999). The nestling period lasts about 21–22 days (Cramp et al., 1982–1994).

2.2. Field procedure and egg injections

The experiment was carried out in 84 nest-boxes, but due to predation events, we were only able to include data for 55 nest-boxes in the analysis (198 chicks: 102 males and 96 females). From the end of March onwards, nest-boxes were monitored each day to record laying date and laying order. Eggs were marked with a non-toxic waterproof marker as they were laid. Injections began when the third egg was found in the nest. Clutches were randomly assigned to one of the three experimental treatments. Within each clutch, eggs were injected with either control or experimental injections in alternation, following the laying order. Also, the order or 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. Treatments were alternated between clutches and consisted of either: (1) 6 ng T (T-86500, Testosterone, Fluka Analytical, Sigma–Aldrich Chemie, Steinheim, Germany) dissolved in 10 μl sesame oil (85067, Sesame Oil, Fluka Analytical, Sigma–Aldrich Chemie, Steinheim, Germany) (34 final chicks), (2) 17 ng A4 (A-9630, Sigma–Aldrich Chemie, Steinheim, Germany) dissolved in 10 μl sesame oil (38 final chicks), (3) 6 ng T + 17 ng A4 dissolved in 10 μl sesame oil (25 final chicks). The control treatment consisted of a 10 μl sesame oil injection (78 final chicks, higher number than any other treatment due to the necessary presence of control eggs in each nest). Androgen experimental modifications were performed with the aim of imposing variation levels within the physiological natural levels of each androgen. The injected doses were equivalent to 1 standard deviation of the population mean (testosterone: 9.79 pg/mg yolk [SD = 4.34], androstenedione: 36.27 pg/mg yolk [SD = 12.35], Gil D., unpublished data), adjusted for mean yolk mass (average yolk mass 1.4 g).

Yolk injections were performed in the field using a 0.5-ml insulin syringe (Terumo Corporation, Tokyo, Japan), following a standard protocol. We calculated the mean and standard deviation of injection volumes by performing mock injections within 0.2 ml eppendorf tubes and measuring the weight with a precision balance (A-2005, Sartorius Analytical Balance, Goettingen, Germany, accuracy = 0.0001 g) in the lab. The volume injected into the eggs was 10.15 ± 1.05 (mean ± SD) μl. For injections, eggs were placed with the pointed end facing up in a custom-made foam egg-candling device, so that we could monitor whether the needle penetrated the yolk before performing the injection. After some seconds, the yolk migrates up into the pointed end, and then we cleaned the injection point with a 95% ethanol swab, allowed it to dry, and inserted the needle into the yolk, where the substances were slowly injected. Holes were sealed with a tiny layer of cyanoacrylate glue, allowed to dry, and eggs were returned to the

nest-box within 15–20 min. In a pilot study conducted in 2010, we checked whether the injections done with this method were indeed delivered into the yolk. For this, we injected eggs with sesame oil stained with neutral red, froze the eggs and dissected them to locate the red spot. In all cases ($N = 10$), the oil-vehicle was found within the yolk.

2.3. Nestling growth

Each chick was assigned to the egg it hatched from by daily nest monitoring around hatching time. In order to ensure the correct identification and labelling of chicks (by differential down cuttings), broods were frequently visited from the tenth days after the last egg was laid. Nestlings hatching from manipulated eggs were measured on days 4, 7, 10, and 14 post-hatching. All measurements were performed by the same individual (JM), blind with respect to treatment. We also recorded hatching success and computed incubation time by counting the number of days between hatching date and the day after the last egg in a clutch was laid. 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.

Morphological measurements were taken on each of these ages and included body mass (Ohaus Scout II SC2020 balance, China, accuracy = 0.1 g), gape width (recorded as the maximum width comprising the beak flanges) and tarsus length (digital calliper: MITUTOYO, Japan, accuracy = 0.01 mm). At day 1, only body weight and gape width were recorded. 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).

2.4. Cell-mediated immunity and sex determination

We used the phytohaemagglutinin skin test to measure nestling immune function (Smits et al., 1999). This test has been found useful in linking immunity to the evolution of life-history strategies (Møller et al., 2001) and post-fledging recruitment (Lopez-Rull et al., 2011) in a variety of bird species. On the 14th day of age, nestlings were injected subcutaneously with 0.05 ml of a 5 mg/ml solution of phytohaemagglutinin (L-8754, Sigma–Aldrich Chemie, Steinheim, Germany) in a previously plucked and marked area of the wing web. We measured the thickness of the nestlings' wing web at the marked site immediately prior to injection and 24 ± 1.3 h after injection with a thickness gauge (Mitutoyo Co., Tokyo, Japan) to the nearest 0.01 mm. The swelling response was estimated by subtracting the pre-injection measurement from the 24-h measurement. All measurements were done by the same individual (LPR), blind with respect to treatment. After measuring the PHA-response, we took a blood sample of each nestling for molecular sexing by brachial vein puncture. Blood samples were preserved in 96% ethanol in small cryogenic vials and frozen at −20 °C. Later on, total DNA was extracted from samples using ammonium acetate techniques (Bensch and Åkesson, 2003), and diluted to a working DNA concentration of 25 ng/μl. This solution was used in a polymerase chain reaction (using the primers P2 and P8) to amplify a part of the CHD-W gene in females and the CHD-Z gene in both sexes (see Griffiths et al., 1998). Amplified products were visualized in 1.5% agarose gels stained with SYBR safe (Invitrogen, Carlsbad, CA).

2.5. Statistical analysis

Descriptive and bivariate analyses were performed using chi-square test with the software STATISTICA v7.0 (StatSoft Inc., Tulsa, OK, 214 USA) using a significance level of 0.05. The rest of statistical analyses were conducted with SAS 9.2 (SAS Institute Inc., Cary, NC, USA). Incubation period, wing swelling and size and growth measures were analysed using mixed models followed by planned comparison post hoc tests (Proc Mixed, normal distribution). All morphometric variables and body condition were analyzed separately using mixed models for repeated measures (PROC MIXED, normal distribution). Experimental treatment, sex and age were fixed factors (predictor variables). Nest was defined as random effect. Information criteria (AICC values) were used to select a simple covariance structure (Burnham and Anderson, 2002) for repeated measures analysis. Initial models controlled for egg volume, laying order, brood size, body size (except when this was the dependent variable), treatment and sex. All biologically meaningful double and triple interactions were also included. In particular, since we expected treatment effect to change along age and differ between sexes, the interaction treatment * sex * age was included in all models. Values represented are means ± SE. We present final models in the text, and initial rejected models can be found in the supplementary data (Table S1). To inspect differences between androgen treatments on body condition, tarsus length and gape width, we performed post hoc pairwise comparisons from the final models (Table S2).

3. Results

Hatching success did not differ between eggs from the four different treatments ($\chi^2 = 0.93$, $P = 0.82$). However, the injection itself led to a statistically non-significant reduction in hatching success with respect to non-injected eggs: chicks hatched from control-injected eggs had 75.38% hatching success, whereas those hatched from non-injected eggs had a 91.32% success ($\chi^2 = 1.52$, $P = 0.22$). Nestling mortality did not differ between the four treatments ($\chi^2 = 2.18$, $P = 0.53$).

Embryonic development period (EDP) was significantly affected by treatment ($F_{3,127} = 2.67$, $P = 0.05$; Fig. 1), after controlling for laying order ($F_{1,120} = 105.47$, $P < 0.001$) and sex ($F_{1,121} = 0.12$, $P = 0.72$). *Post-hoc* contrasts indicated that nestlings hatching from the T treatment showed longer embryonic development periods than nestlings hatching from control ($F_{3,128} = 2.48$, $P = 0.014$) or from the T + A4 treatment ($F_{3,132} = 2.31$, $P = 0.022$). The effect of A4 treatment on EDP was intermediate between control and testosterone (although not statistically different from either of them), and the combination T + A4 was undistinguishable from the control treatment (Fig. 1).

Hatchling body condition was mainly affected by egg volume ($F_{1,104} = 83.84$, $P < 0.0001$, estimate (SE) = 0.0009 ± 0.00009), laying order ($F_{1,181} = 8.69$, $P = 0.0036$, estimate (SE) = −0.112 ± 0.03805), incubation period ($F_{1,91.7} = 8.13$, $P = 0.005$, estimate (SE) = 0.250 ± 0.08793) and nest laying date ($F_{1,51.7} = 25.09$, $P < 0.0001$, estimate (SE) = 0.182 ± 0.03649); thus hatchlings in better condition were produced from large eggs, from eggs laid in early laying positions and dates, and from clutches that were incubated for longer. However, hatchling condition was not affected by experimental treatment ($F_{3,151} = 0.89$, $P = 0.445$).

During nestling development, treatment significantly affected body condition similarly for both sexes (Table 1). Inspection of the residuals from the model (Fig. 2) suggests that during most of their development, nestlings which had received any treatment where A4 was present, were in a lower condition than controls, and this effect was stronger in the group which received the combination T + A4 ($F_{1,150} = 6.32$, $P = 0.013$; post hoc comparisons in Table S2). As shown in Fig. 2, T nestlings had a superior condition compared to those in both A4 and T + A4 groups ($F_{1,34.3} = 4.7$, $P = 0.037$ and $F_{1,30.5} = 5.85$, $P = 0.021$, respectively; Suppl. Table 2), although no differences were found with control nestlings.

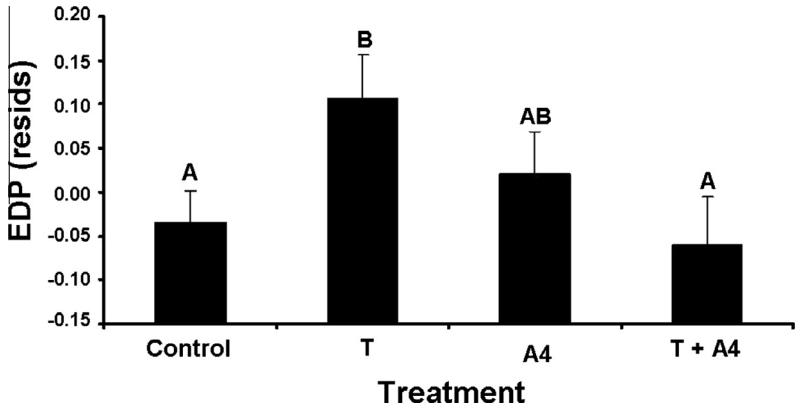

Fig. 1. Differences in embryonic development period (EDP), shown as residuals from the model, according to treatment. Different letters above bars indicate significant ($P \leqslant 0.05$) differences between treatment groups based on post hoc average comparisons.

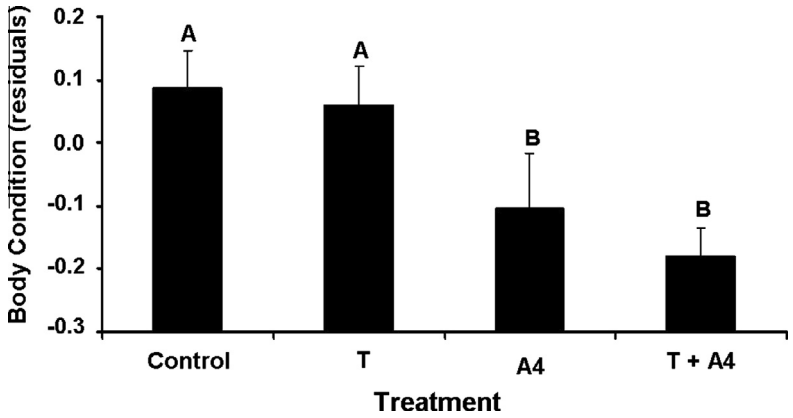

Fig. 2. Differences in nestling body condition, shown as residuals from statistical models, according to treatment. Different letters above bars indicate significant ($P \leqslant 0.05$) differences between treatment groups based on post hoc average comparisons.

Table 1
Summary of final repeated-measures mixed models showing the effect of yolk androgen treatment on nestling biometry, taking into account age, sex, laying order, egg volume and brood size. Models were run using Proc Mixed (SAS) with Satterthwaite correction to adjust the degrees of freedom.

Trait	Independent variable	df	*F*	*P*
Body condition	Androgen treatment	3240	3.81	0.0107
	Laying order	1228	35.17	<0.0001
	Egg volume	1148	6.51	0.0117
	Brood size	166.9	4.36	0.0406
Tarsus length	Androgen treatment	3226	1.62	0.1849
	Age	3533	11264.8	<0.0001
	Androgen treatment × Age	9533	1.96	0.0415
	Sex	1218	9.88	0.0019
	Sex × Age	3533	3.09	0.0267
	Laying order	1214	12.91	0.0004
	Egg volume	1174	8.78	0.0035
	Nest laying date	150.4	5.04	0.0292
Gape width	Androgen treatment	3206	0.04	0.989
	Age	3531	1068.08	<0.0001
	Androgen treatment × Age	9528	2.65	0.0051
	Sex	1209	22.71	<0.0001
	Tarsus length	1768	12.97	0.0003
	Brood size	174.1	3.00	0.0876

Body size, as measured by tarsus length, showed interactions between treatment and age and between sex and age (Table 1; Fig. 3). We divided the data set to explore this effect further and found a lack of effect of age * treatment in females (repeated-measures ANOVA: $F_{9,246} = 1.04$, $P = 0.406$), but a significant effect in males (repeated-measures ANOVA: $F_{9,257} = 2.47$, $P = 0.0102$). An examination of the residuals (Fig. 3a) shows that during the first half of the nestling period, male nestlings tended to grow longer tarsus than controls when they have received an A4 egg injection ($F_{3,164} = 2.40$, $P = 0.069$; Table S2), whereas the combination T + A4 affected tarsus length negatively (Fig. 3a) in relation to the other experimental groups (Table S2). During the second part of the nestling period, differences evened up between all groups.

Gape width was larger for males than females and also showed a treatment * age effect (Table 1, Fig. 4). Post-hoc pairwise comparisons showed that the main difference driving this effect is due to the T + A4 group (Table S2) presenting narrower gapes early on development in comparison with other groups (Fig. 4), but showing wider gapes than any other group at the end of the nestling period.

Nestling body condition and tarsus length were positively related with egg volume, and negatively with laying order (Table 1). Moreover, chicks that shared their nests with a higher number of siblings showed poorer body condition and smaller gapes width than chicks raised in smaller brood sizes.

Mass gain between ages 4 and 10 after hatching (at the time when growth rate is highest), was much higher for males than for females ($F_{1,175} = 21.76$, $P < 0.0001$), but treatment did not have a significant effect in either sex ($F_{3,168} = 0.52$, $P = 0.668$). Chicks hatching from eggs that experiencing longer embryonic development period had a lower mass gain, irrespectively of treatment ($F_{1,153} = 25.29$;

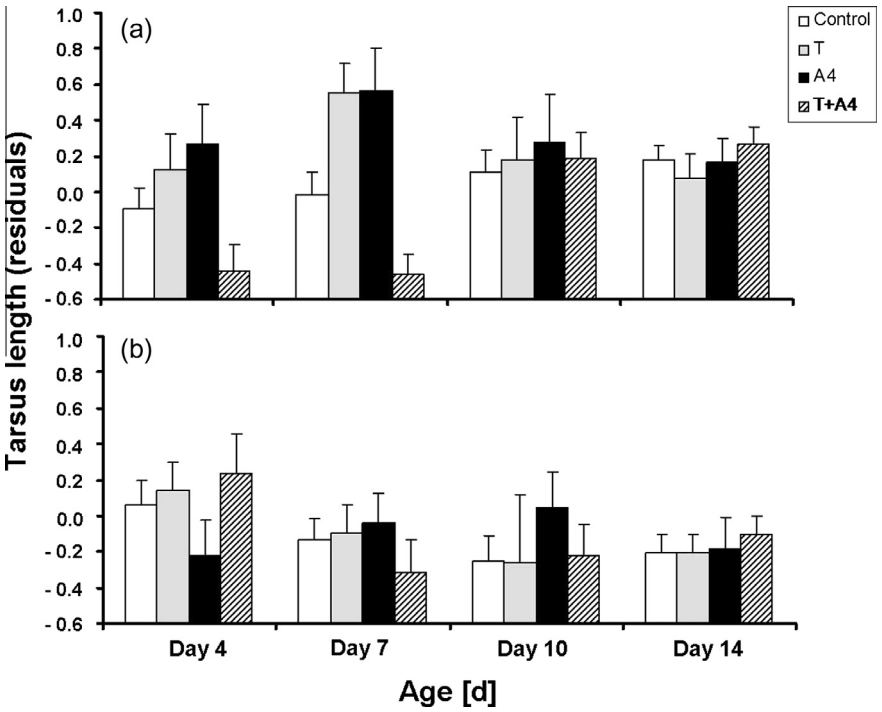

Fig. 3. Differences in nestling tarsus length for males (a) and females (b) shown as residuals from statistical models, according to treatment and age (white bars: control, grey bars: testosterone, black bars: androstenedione and striped bars: testosterone + androstenedione).

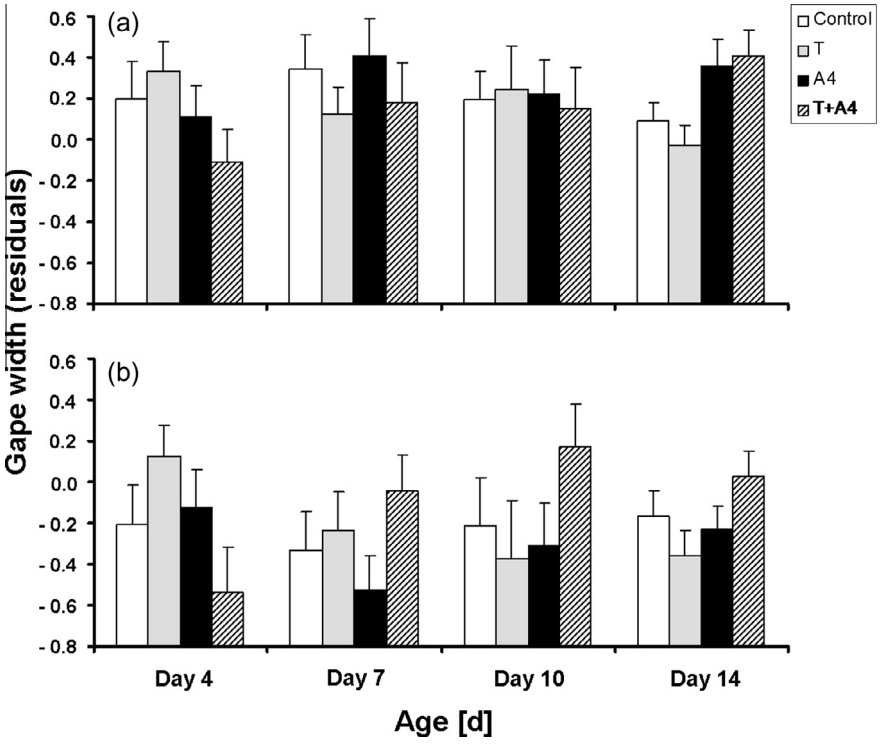

Fig. 4. Differences in nestling gape width for males (a) and females (b) shown as residuals from statistical models, according to treatment and age (white bars: control, grey bars: testosterone, black bars: androstenedione and striped bars: testosterone + androstenedione).

$P < 0.0001$; estimate (SE) = −0.016 ± 0.00330). Chicks who shared the nest with a higher number of siblings also had a higher mass gain ($F_{1,56.2} = 4.54$; $P = 0.037$; estimate (SE) = 0.005 ± 0.00237).

A marginally significant sex * treatment interaction was observed for cell-mediated immune response ($F_{3,162} = 2.40$, $P = 0.069$), in which control males showed better immune capacity than control females. Females hatching from eggs treated with androgens produced greater inflammatory responses, but just the opposite trend was observed in males. Nestlings' swelling response was principally influenced by offspring body condition at 14 days, the age when the test was performed ($F_{1,181} = 9.69$, $P = 0.002$, estimate (SE) = 0.016 ± 0.00538).

4. Discussion

We tested the differential effect of physiological in ovo injections of two different androgens (T and A4) and their combination in embryo and nestling growth in a species in which these two androgens are present, with A4 in a much larger concentration than T (ratio 3:1; see Section 2). We found different effects in embryonic and nestling development of the two androgens, suggesting that androgen effects are specific to the particular stage when they are measured and to specific androgenic pathway which they are subject to (Groothuis et al., 2005b; Navara et al., 2005; Rubolini et al., 2006; von Engelhardt et al., 2009).

In the case of embryonic growth, we found that the duration of the embryonic period (EDP) was increased in T-injected eggs with respect to controls, whereas the combination of T + A4 showed no difference with respect to controls, and A4 was intermediate. A similar increase in the EDP of T-injected eggs has been found in other studies conducted with zebra finches (*Taeniopygia guttata*) and American kestrels (*Falco sparverius*) (Sockman and Schwabl, 2000; Boncoraglio et al., 2011). However, an opposite effect, with shorter incubation times for androgen-injected eggs was found for the black-headed gull (*Larus ridibundus*) (Eising et al., 2001), whereas in the case of the Japanese quail *C. japonica* (Hegyi and Schwabl, 2010) no effects were found. Differences between species may be due to differential androgen metabolism by the embryo, but also to different methodologies used in each study (doses, androgen combination, etc.). The fact that A4 was intermediate between control and T could be explained not only by the lower androgenic capacity of A4 (Sonneveld et al., 2006), but also by the fact that A4 is a prohormone that renders T depending on the intracellular presence of 17beta-hydroxysteroid dehydrogenases (17beta-HSD) (Labrie et al., 2000; Adamski and Jakob, 2001). So an increase of A4 levels in the developing embryo may lead to an enhancement of T synthesis in those cellular types with 17beta-HSD activity (Bruggeman et al., 2002). However, it is not straightforward to understand why the T + A4 combination did not lead to an increase in EDP comparable or larger than that found with T, since both androgens, although differing in androgenic capacity, are not expected to inhibit each other. One possibility is that if the effect of the two androgens is additive, the summation of the two may have led to a large androgenic effect in the form of a bell-shaped response, as found in other androgen-mediated systems (Lee et al., 1995). Another possibility is that the resultant enhanced T levels after T + A4 administration could activate the oxidizing form of 17beta-HSD thus reducing T available for critical processes on embryo development. Different forms of 17beta-HSD differ in tissue distribution, catalytic preferences, substrate specificity, subcellular localization, and mechanisms of regulation (Luu-The, 2001) and are present in chick embryo as soon as in the second day of incubation (Bruggeman et al., 2002). Additionally, a possible pathway is that of aromatase (CYP19) and 5α-reductase in the conversion of T/A4 to estrogens and T to 5α-DHT respectively. Unfortunately, the mechanisms of regulation of CYP19 and other steroidogenic enzymes in birds, particularly in the embryo and nestling phase, are still poorly known (Sanderson, 2006). Although it is essential for chick development (Rantakari et al., 2010), its organ- and time-pattern of activity has not been precisely determined yet. Slower embryonic development can produce higher quality offspring under the physiological trade-off hypothesis (Ricklefs, 1992; Martin and Schwabl, 2008). Thus, it is unclear what a longer EDP may mean in terms of development, since we did not find differences between experimental groups in hatchling body condition (Eising and Groothuis, 2003). The possibility that the immune system has benefited from extra development time (Ricklefs, 1992) could not be ruled out completely because marginally sex differences were found between experimental groups in immune response. Females from androgen-injected eggs benefited from an increase in androgen concentration, while the males may suffer a slight immunosuppressive effect (for additional examples of sex-dependent effects on immunity see: Groothuis et al., 2005a; Navara et al., 2006). However, it could be argued that early developmental differences in immunity may not be detected by this test, which was performed at 14 days of age.

In the case of nestling body condition we found no effect of T treatment, but there was a general reduction of body condition in all experimental treatments where A4 was present, particularly so in the T + A4 combination. In contrast to what found in EDP, the combination T + A4 showed a pattern of summation of effects, leading to a larger decrease in body condition. In agreement with these data, gape width followed the completely opposite pattern, as expected by previous data which has shown that this trait does not shrink with age in nestlings in bad condition (Gil et al., 2008). Thus, it makes sense that the group that showed worse body condition through development T + A4 also kept wider gapes during that time, since an increased gape could attract more feedings from parents and thus compensate the difference in growth (Wiebe and Slagsvold, 2009). In a similar study, spotless starling chicks whose eggs had been injected with T + A4 injections were found to develop higher body mass gain and wider gapes than control-chicks (Müller et al., 2007). However, other experimental study which added T + A4 to the yolk had very little effect on nestling development (Pitala et al., 2009). The high variation in androgen effects between studies suggests that the costs and benefits of these hormones may depend on environmental contexts.

Androgens led to an increase in skeletal growth in the first half of development, but only in males, with A4 showing a larger effect than T. Similarly, offspring from eggs with relatively higher A4 concentrations within a clutch were relatively large after hatching in the Japanese quail (Hegyi et al., 2011). The combination T + A4 showed a strong reduction in growth (Pitala et al., 2009), also limited to that early period, since differences disappeared at the end of nestling period. Sex-specific effects in growth have been previously reported for yolk androgens, although the sex that is favored depends on the species: females in zebra finches and American kestrels (von Engelhardt et al., 2006; Sockman et al., 2008), and males in swallows *Hirundo rustica* (Saino et al., 2006). Our study shows time-dependent growth differences, but not definite growth differences in developmental end-points, suggesting that sexual mediators of androgen action may affect within-nest competition patterns (Müller and Groothuis, 2013).

We found a stronger effect of T in the embryo period than in the nestling period, whereas A4 followed the opposite trend. A similar pattern was found in a comparative analysis (Schwabl et al., 2007), in which species-specific differences in T were more strongly correlated with embryo than with nestling developmental periods. These differences may be due to several factors, most importantly the differential presence of metabolizing enzymes in embryo and the chick (Bruggeman et al., 2002). To date, no study has examined in detail the differences of the metabolizing pathways of androgens in these two systems, although data suggest extensive metabolization of maternal androgens from day 1 of incubation (Fivizzani et al., 1986; von Engelhardt et al., 2009). The most comprehensive study so far (von Engelhardt et al., 2009) has detected a far ranging modification of original androgens to conjugated and unconjugated steroids, half way through embryo development. Further work is required to specifically identify which metabolites are produces, and whether these products are active or not.

Leaving aside the specific effects that androgen metabolites may have by non-genomic means (Foradori et al., 2008), androgen receptors (AR) exist in the embryo from very early on (day 7 in the zebra finch) (Godsave et al., 2002), and thus there is scope for a

direct androgenic effect of maternal steroids. Evidence to this comes from a study in which eggs treated with the AR blocker Flutamide failed to show an anabolic effect of yolk androgen detected in control eggs (Lipar and Ketterson, 2000). Furthermore, embryos exposed to high levels of steroids during early development may also present altered hormone synthesis pathways during subsequent development (Badyaev, 2002).

In principle, the androgenic effect of A4 is much lower than that of T, because of different affinities for the AR (Sonneveld et al., 2006). However, a whole series of metabolizing pathways have been inferred during embryo development by means of thin-layer chromatography (von Engelhardt et al., 2009), suggesting the action of numerous enzymes that can dramatically alter the androgenic capacity of yolk androgens. These enzymes can either transform androgens into androgen metabolites, more or less active, and also conjugate them with proteins that may increase transport and availability (Payne and Hales, 2004; von Engelhardt et al., 2009). For instance, as discussed above, the conversion of A4 or T into 5α-DHT depends on 17beta-HSD or 5α-reductase, respectively, but its availability may differ between species or across different tissues or developmental stages (Payne and Hales, 2004). Embryo and nestling development rates could also be directly induced by 5α-DHT (Schwabl et al., 2007), so its manipulation would have been useful to understand completely the mechanisms of androgen action. However, since Pilz and Smith (2004) did not find detectable levels of 5α-DHT in the European starling, we did not consider this hormone in our experimental setup in the closely related Spotless starling. Additionally, this would have led to a high number of different combinations, unfeasible in a field study.

Strong positive effects of large egg size (Smith and Bruun, 1998) and early laying order (Clotfelter et al., 2000) were found throughout development. We were surprised to see that differences in egg size could be detected even at the end of the developmental period, suggesting that this maternal effect may provide an important survival boost in this species (Cordero et al., 2001; Christians, 2002). In the case of laying order, since we allowed natural incubation to take place, we could not distinguish between the effect of early incubation that typically leads to hatching asynchrony (Ellis et al., 2001), and differences in egg composition that may covary with laying order (Boncoraglio et al., 2011; Müller and Groothuis, 2013).

In summary, our results suggest a complex picture of sex and age-dependent effects of T and A4 administration, and underline the necessity of further research in the metabolism and action of egg androgens.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2013.09.013.

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