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Context-dependent effects of yolk androgens on nestling growth and immune function in a multibrooded passerine

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Abstract

Female birds may adjust their offspring phenotype to the specific requirements of the environment by differential allocation of physiologically active substances into yolks, such as androgens. Yolk androgens have been shown to accelerate embryonic development, growth rate and competitive ability of nestlings, but they can also entail immunological costs. The balance between costs and benefits of androgen allocation is expected to depend on nestling environment. We tested this hypothesis in a multibrooded passerine, the spotless starling, *Sturnus unicolor*. We experimentally manipulated yolk androgen levels using a between-brood design and evaluated its effects on nestling development, survival and immune function. Both in first and replacement broods, the embryonic development period was shorter for androgen-treated chicks than controls, but there were no differences in second broods. In replacement broods, androgen-treated chicks were heavier and larger than those hatched from control eggs, but this effect was not observed in the other breeding attempts. Androgen exposure reduced survival with respect to controls only in second broods. Regarding immune function, we detected nonsignificant trends for androgen treatment to activate two important components of innate and adaptive immunity (IL-6 and Ig-A levels, respectively). Similarly, androgen-treated chicks showed greater lymphocyte proliferation than controls in the first brood and an opposite trend in the second brood. Our results indicate that yolk androgen effects on nestling development and immunity depend on the environmental conditions of each breeding attempt. Variation in maternal androgen allocation to eggs could be explained as the result of context-dependent optimal strategies to maximize offspring fitness.

Introduction

Female birds deposit variable amounts of physiologically active substances into egg yolks (Ricklefs, 1984; Williams, 1994; Bernardo, 1996), which potentially affect embryonic growth and development and can vary seasonally (Hargitai *et al.*, 2009). This flexible maternal mechanism may allow females to adjust the offspring phenotype to specific requirements of the environment (Mousseau & Fox, 1998; Vergauwen *et al.*, 2012; Giordano *et al.*, 2014). Since the publication of the first study confirming the presence of maternally derived hormones in the yolk (Schwabl, 1993), elucidating the role of yolk androgens as modulators of maternal effects has been a subject of intensive research during the last 20 years (Schwabl, 1997; Gil *et al.*, 1999; Williams *et al.*, 2004; Räsänen & Kruuk, 2007). It is known that avian embryos actively respond to variations in

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maternally derived egg androgens (Reed & Clark, 2011), which may also affect a whole suite of nestling and adult traits including growth, immunity, sexual development, dispersal or personality (reviewed in Groothuis *et al.*, 2005a; Gil, 2008). Different androgens may have different biological consequences (Hegyi *et al.*, 2011; Muriel *et al.*, 2013; Tschirren *et al.*, 2014). Moreover, a particular hormone can have different effects on a given trait, depending on the species (reviewed in Groothuis *et al.*, 2005a; Gil, 2008) or the sex of the chick (Müller *et al.*, 2005; von Engelhardt *et al.*, 2006; Saino *et al.*, 2006; Müller *et al.*, 2008, 2010; Ruuskanen & Laaksonen, 2010; but see Lipar & Ketterson, 2000). This hormonal 'pleiotropy' could induce a number of life-history trade-offs (reviewed in Williams, 2012), and studies that manipulate androgen levels are helpful to identify the mechanisms underlying these processes (Andersson *et al.*, 2004; Groothuis *et al.*, 2005b). Androgen injection studies have shown that small changes in yolk hormone levels induce a wide range of effects (reviewed in Groothuis *et al.*, 2005a; Gil, 2008). Some of these effects, such as accelerated embryonic development (Eising *et al.*, 2001; Eising & Groothuis, 2003; Muriel *et al.*, in press), increased growth rate (Eising *et al.*, 2001; Pilz *et al.*, 2004; Muriel *et al.*, in press), improved competitive behaviour in nestlings (Müller *et al.*, 2009, 2012) or intensified begging behaviour (Schwabl, 1996a; Eising & Groothuis, 2003), suggest that maternal yolk androgens are generally beneficial to offspring.

However, androgens can also entail some negative side effects. For instance, the immunocompetence handicap hypothesis (Folstad & Karter, 1992) proposes that androgens could be beneficial for some traits such as the production of male secondary sexual traits, but also harmful due to their immunosuppressive effects (reviewed in Owen-Ashley *et al.*, 2004; but see Roberts *et al.*, 2004; Groothuis & Schwabl, 2008). It has indeed been found that prenatal androgen overexposure may decrease cellular and humoral immune responsiveness elicited by standard *in vivo* challenges by lipopolysaccharides, phytohemagglutinin or sheep red blood cells (Saino *et al.*, 1995; Verhulst *et al.*, 1999; Duffy *et al.*, 2000; Groothuis *et al.*, 2005b; Müller *et al.*, 2005; Navara *et al.*, 2005; Sandell *et al.*, 2009). However, the effects of yolk androgens on other components of the immune system remain understudied.

Beyond parent–offspring and sexual conflict over parental investment (Trivers, 1974; Godfray, 1995; Müller *et al.*, 2007a), maternal deposition of yolk hormones may also influence trade-offs experienced by the offspring (e.g. balance between growth and immunocompetence; Saino *et al.*, 1998; Soler *et al.*, 2003) whose optimal resolution is context dependent. In fact, although androgen levels may covary positively with female quality or with egg position in the laying sequence (Schwabl, 1993; Lipar *et al.*, 1999; Pilz *et al.*, 2003; Tanvez *et al.*, 2007), several studies have shown that this variation may also depend largely on the environmental features that are affecting the breeding female, such as nutritional conditions (Verboven *et al.*, 2003; Gasparini *et al.*, 2007; Benowitz-Fredericks *et al.*, 2013), photoperiod (Schwabl, 1996b), aggressive interactions (Whittingham & Schwabl, 2002), the attractiveness of their mates (Gil *et al.*, 1999, 2004; Uller *et al.*, 2005), parasite abundance (Tschirren *et al.*, 2004; Postma *et al.*, 2014) or breeding density (Schwabl, 1997; Groothuis & Schwabl, 2002; Pilz & Smith, 2004). Such maternal modulation of yolk androgens as a function of the environmental conditions could be an adaptive strategy to handle the context- and dose-dependent effect of androgens (e.g. Martinez-Padilla *et al.*, 2010; Martínez-Padilla *et al.*, 2014). However, such hypothetical context-dependent effect of yolk androgens on offspring physiology has scarcely been explored (Verboven *et al.*, 2003; Gasparini *et al.*, 2007; Benowitz-Fredericks *et al.*, 2013).

Seasonal variation in environmental conditions is one of the main factors that impacts on the breeding context, as it may affect the resources available for foraging, antiparasite defence, thermoregulation and parental care in general (Reed & Clark, 2011). In multibrooded bird species, females are expected to adjust the allocation of egg resources –including yolk androgens – in different broods to maximize reproductive success and offspring fitness (Tinbergen, 1987; Stouffer, 1991; Verhulst *et al.*, 1997; Styrsky *et al.*, 1999; Robinson *et al.*, 2010; Giordano *et al.*, 2014). If the reason for such seasonal variation in androgen allocation to yolks is an adjustment to balance the costs and benefits of hormones according to environmental conditions, we would expect that a given increase in androgen levels would result in contrasted effects on offspring fitness at the beginning and at the end of the breeding season, when environmental conditions become tougher.

We examined the effects of yolk androgens on embryo development, nestling growth and chick's immune function in the spotless starling (*Sturnus unicolor*), taking into account the different breeding attempts in the same breeding season. We experimentally manipulated yolk androgen concentrations of whole clutches by in ovo injection of a combination of testosterone (T) and androstenedione (A4) dissolved in sesame oil or vehicle only (control). We measured hatching success, growth and survival until nearly fledging (14 days age). We also studied gape width, which is a temporary trait used by nestlings during begging displays to parents (Müller *et al.*, 2007b; Gil *et al.*, 2008). At that age, we also evaluated the immune function of individuals using different indicators of both innate (number and proportion of leucocytes, and level of IL-6) and adaptive immunity (lymphocyte proliferation and Ig-A level), as several nestling immune function parameters are associated with survival in the nest

(Hõrak *et al.*, 1999; Merino *et al.*, 2000). In this way, we monitored a variety of developmental and physiological parameters that may be affected by yolk androgens and that may allow us to track the variation in the trade-offs associated with androgen allocation to eggs across the breeding season. We hypothesized that a seasonal decline in yolk androgen allocation (López-Rull *et al.*, 2010; Vergauwen *et al.*, 2012) could be due to possible detrimental effects on the nestlings of the second brood. The outcome of the androgen-mediated trade-off between offspring development and immunocompetence is expected to depend on environmental circumstances such as food availability (reviewed in Royle *et al.*, 2001; Sockman *et al.*, 2006; Smiseth *et al.*, 2011), ectoparasite load (Tschirren *et al.*, 2004; but see Müller *et al.*, 2007a,b; López-Rull *et al.*, 2010) and perhaps climatic conditions during breeding (Wingfield, 2003). Based on the context dependence of early maternal effects (Krist *et al.*, 2015), we predicted that androgen treatment (compared to control treatment) would have a positive effect on chick growth and less immunosuppressive side effects during the first brood, because of more suitable breeding conditions that would balance energy requirements (Monaghan, 2008; Ilyina *et al.*, 2013). In contrast, during the second brood, characterized in our study site by low precipitations that dramatically reduce prey abundance (Turner, 1983), increased nest ectoparasite abundance (López-Rull *et al.*, 2010) and high thermal stress for nestlings (Salaberria *et al.*, 2014), we would expect that the costs of increased yolk androgens would overcome their benefits for nestlings.

Materials and methods

Study area and species

This study was conducted between April and June 2011 in a nest-box population of spotless starlings (*Sturnus unicolor*) located in central Spain (Soto del Real, Madrid). The study area is covered by a woodland of oak (*Quercus pyrenaica*) and ash (*Fraxinus angustifolius*) with abundant open areas used by grazing cattle. It exhibits a continental Mediterranean climate [Köppen–Geiger climate classification: Csb category (reviewed in Peel *et al.*, 2007)] with hot and dry summers. The spotless starling is a facultative polygynous passerine that breeds in tree holes and artificial cavities (Moreno *et al.*, 1999; Veiga, 2002), showing high breeding synchrony. Modal clutch size is five eggs (López-Rull *et al.*, 2007), and fledglings leave the nest around 22 days of age (Cramp, 1998). Generally, females invest more than males in rearing the brood (Jimeno *et al.*, 2014), although paternal care varies widely (Moreno *et al.*, 1999). In our study area, most spotless starling pairs rear two broods. The first one between mid-April and the beginning of May, and the second one at the end of May (Salaberria *et al.*, 2014), investing more resources in early than in late clutches (López-Rull *et al.*, 2010). When the first breeding attempt is truncated due to sabotage by conspecifics or predation, they lay a replacement clutch (Müller *et al.*, 2007b). In our study area, food availability and offspring quality decreases as the season advances (i.e. from first to second broods, see López-Rull *et al.*, 2010; Salaberria *et al.*, 2014). The daily average maximum temperature and precipitation (mean ± SE) recorded per each breeding attempt for the year of study were 18.71 ± 0.63 °C and 3.32 ± 0.48 L m^{-2} for the first brood, 18.95 ± 0.58 °C and 4.54 ± 0.46 L m^{-2} for replacement broods; and 25.14 ± 0.68 °C and 1.59 ± 0.63 L m^{-2} for the second brood [Data provided by the Spanish Meteorological Agency (AEMET)].

Field procedure and egg injections

From early April onwards, nest boxes were inspected daily to determine laying date and laying order. Eggs were marked with a nontoxic waterproof marker as they were laid and measurements of length and width were taken with digital callipers (Mitutoyo Absolute, Kawasaki, Japan, precision = 0.01 mm). Egg volume (mm^3) was calculated by the formula: 0.45 × length × $width^2$ (Worth, 1940). For the analyses, we consider average volume per clutch, because we could not assign individual chicks to the specific egg they hatched from.

Although yolk-A4 and yolk-T may exert different biological effects (Hegyi *et al.*, 2011; Muriel *et al.*, 2013; Tschirren *et al.*, 2014), androgen manipulation was performed by combining both hormones as they appear together in the yolk (Schwabl, 1993) and are positively correlated among them (Groothuis & Schwabl, 2002; Gil *et al.*, 2004; Ruuskanen *et al.*, 2009). Based on results obtained in a previous dose–response study in the same study population (Muriel *et al.*, in press), we selected a dose of the mixture of yolk androgens corresponding to four standard deviations of the mean amount found in eggs in this population in an overall breeding season (testosterone: 14 ng per yolk [SD = 6.0], androstenedione: 50 ng per yolk [SD = 17.1]; D. Gil, unpublished), adjusted for mean yolk mass (average yolk mass 1.4 g). The maximum concentrations of yolk-T and yolk-A4 that we have measured in this population are 25.9 and 141.76 pg mg^{-1} yolk, respectively (Müller *et al.*, 2007b). According to mean yolk mass, this translates to maxima of 36.3 ng T and 198.4 ng A4 per yolk, so that 4 SD injections result in total androgen concentrations equal (for T) or below (for A4) the maximum levels found in our population. This concentration induced stimulatory effect on hatching nestling body mass and skeletal growth (Muriel *et al.*, in press). Injections began when the fourth egg was found in the nest, before embryonic development was triggered by the start of

parental incubation. Subsequently, laid eggs were injected the same day they were laid. Clutches were randomly injected with control or androgen injections. The mixture of hormones 24 ng T (ref. 86500; Sigma-Aldrich, Steinheim, Germany) + 68 ng A4 (ref. A9630, Sigma-Aldrich) was dissolved in 10 μL of sesame oil (ref. 85067; Sigma-Aldrich). Eggs in control clutches received 10 μL of sesame oil alone. In ovo injections were performed in the field using a standard U-50 insulin syringe (Terumo Corporation, Tokyo, Japan), following a standard protocol (Muriel *et al.*, 2013, in press).

The experiment was carried out in 464 clutches, but 62 of them did not produce any hatchlings because of predation (6.25%), clutch sabotage by conspecifics (62.5%) or abandonments (31.25%). The reason for this unusually large sample size is that this experimental set-up is part of large-scale study where we will explore the long-term effects of our manipulation at the adult stage. The final number of control/androgen clutches per breeding attempt was 90/99 in first, 38/36 in replacement and 62/78 in second broods. We recorded the hatching success of 33 uninjected clutches to compare the effect of our injection protocol per se on egg hatchability with the natural levels in our population. As found in previous studies (Pilz *et al.*, 2004 (35%); Müller *et al.*, 2007b (30%); Pitala *et al.*, 2009 (32.85%); see Results), egg injections led to a certain level of hatching failure, whereby brood size was reduced in some nests. In order to reach the modal brood size in our population (mean $\pm$ SD = 4.72 $\pm$ 0.57) and to avoid an unusually low level of sibling competition, we performed a post-hatch brood amalgamation of those broods in which only one to three chicks had hatched (163 control and 167 treated of 977 chicks were moved from their original nests). This was conducted at the age of 3 days. Amalgamated broods were created trying to minimize the genetic variation of the final brood, pooling broods of the same treatment and age and composed by nestlings of similar size (Muriel *et al.*, in press). Finally, we were able to include in the development analysis data from 977 chicks (259 control and 286 treated in first, 85 control and 76 treated in replacement and finally 114 control and 157 treated chicks in second broods).

Nestling measurements and sampling

Broods were visited daily from the 10th day after the last egg was laid to check hatching time. We recorded hatching success and computed incubation time or embryonic development period (EDP) as the elapsed time (days $\pm$ 4 h) from start of incubation (fourth egg laid) until hatching. Nestlings were measured on day 14 post-hatching. At this age, we recorded body mass with a digital balance (Ohaus Scout II SC2020, China, precision= 0.1 g), gape width (recorded as the maximum width comprising the beak flanges) and tarsus length with digital callipers (Mitutoyo Absolute, accuracy = 0.01 mm). 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 this time, all chicks were ringed with numbered aluminium bands and a blood sample was collected by puncture of the brachial vein for molecular sexing (Griffiths *et al.*, 1998). In a random sample of 53 and 41 chicks from first and second broods, respectively, 600 μL of blood was collected from the jugular vein with heparinized syringes for immunological tests. Also, in a subset of those chicks (21 from first and 32 from the second brood), a faecal sample was collected for Ig-A analyses. Blood and faecal samples were transported immediately to the laboratory in cooled containers (approx. 4 °C) to conduct immune measurements (Data S1). No additional biometric measures were taken from day 14 onwards because of the high risk of premature fledging that would result from handling the birds.

Immunological tests

Blood differential counts

This assay was performed with 82 blood smears (28 control plus 20 treated chicks from first brood and 11 control plus 23 treated chicks from second brood). On arrival to the laboratory, blood samples were gently but thoroughly mixed to obtain a uniform distribution of blood cells. We obtained blood smears that were fixed by 3 min immersion in methanol, air-dried and stained with commercial Giemsa diluted with PBS pH 6.8 (1:2). Slides were examined under microscope (1000× magnification with oil immersion) to estimate the proportion of different types of leucocytes (Campbell & Ellis, 2007). Examination continued until 100–120 leucocytes had been found per slide (Salaberria *et al.*, 2013). We measured the proportion of leucocytes as this is part of the primary line of defence of the innate immune system (Dhabhar *et al.*, 1995; Müller *et al.*, 2011), whose deviation from a normal range could indicate infectious processes. We also calculated the heterophil: lymphocyte ratio (H/L), as increasing H/L ratios are associated with a higher physiological stress in birds (Gross & Siegel, 1983; Maxwell & Robertson, 1998).

Lymphocyte proliferation

Our lymphocyte proliferation assay measured the ability of lymphocytes placed in short-term tissue culture to undergo a clonal proliferation when stimulated *in vitro* by phytohemagglutinin (PHA). Higher levels of proliferation are associated with a better acquired T-cell-mediated immune response. This allowed an evaluation of the functional capabilities of T cells (Talebi *et al.*, 1995), whose proliferation and differentiation also involves IL-6 levels (Holsti & Raulet, 1989; Croft & Swain, 1991; Zhang *et al.*, 2000). For the analysis of

this parameter, blood was kept on ice and taken to the laboratory for differential separation of white blood cells and measurement of lymphocyte T proliferation of cells exposed to PHA by means of the AlamarBlue® (AbD Serotec, Oxford, UK) technique. Plates were incubated at 38 °C for 72 h, measuring absorbance at 0, 24, 48 and 72 h. The intra-assay variation coefficient was 4.80% (see Data S1 for details of the technique).

Plasma IL-6 concentration

This pro-inflammatory cytokine exhibits a wide range of functions in the regulation of innate immunity and the inflammatory response, directing leucocyte movement and stimulating haematopoiesis (reviewed in Heinrich *et al.*, 2003; Kishimoto, 2005; Zimmerman *et al.*, 2014). A high IL-6 level can be associated with increased susceptibility to infections. We developed an indirect ELISA for chicken IL-6, using rabbit IgG anti-chicken IL-6 as primary antibody and goat IgG anti-rabbit IgG conjugated with horseradish peroxidase as secondary antibody. The intra-assay variation coefficient was 6.79%, and the inter-assay was 11.56% (see Data S1 for details of the technique).

Faecal sampling and immunological test

Secretory immunoglobulin-A (Ig-A) plays an important role in protecting against infection in the intestinal immune system (Davis *et al.*, 1978), where high Ig-A levels could be correlated with a primary or secondary infection. Thus, we measured Ig-A levels in faeces to obtain a measure of humoral immune condition (Snoeck *et al.*, 2006). The method used for extraction and depuration of faecal immunoglobulin was adapted from that used by Peters *et al.* (2004). Subsequently, Ig-A level was quantified with an ELISA kit developed for chicken Ig-A (Bethyl Lab). Coefficients of intra- and interassay were 3.69% and 1.85%, respectively (see Data S1 for details of the technique).

Statistical analysis

For each breeding attempt, differences in hatching success (number of hatchings/clutch size) and nestling survival (number of chicks on day 14 post-hatch/hatchings) between experimental groups were analysed using chi-square tests (χ^2) with the software STATISTICA v7.0 (StatSoft Inc., Tulsa, OK, USA). Data from 33 uninjected clutches were not included in statistical analyses, except to compare the natural hatching success. The remaining analyses were conducted with SAS 9.2 (SAS Institute Inc., Cary, NC, USA). Morphometric variables, body condition, EDP and immunological parameters were analysed using mixed models (SAS, PROC MIXED, normal distribution), in which nest of origin was defined as random effect affecting model intercept. The following variables were included in the main model: treatment, sex, breeding attempt, egg volume, laying order, brood size and EDP (except when EDP was the dependent variable). Treatment (Control vs. treated), sex (male vs. female) and breeding attempt (first, replacement, second brood) were considered as categorical variables. In the analysis of gape width, we controlled for nestling size by including tarsus length as a covariate. We also included the person who took the morphometric measurements and the day on which immunological assays were performed as factors in the models for these response variables. Arcsine square root and logarithmic transformations were applied to leucocyte proportions and H/L ratios, respectively. All biologically meaningful double and triple interactions were also included in the main models. Values represented are means ± SE. Starting from the saturated model, a backward stepwise procedure was used to remove terms with $P > 0.05$. The normality assumption was confirmed by checking the residuals of the models. To inspect differences between androgen treatment and breeding attempts on the biological variables commented above, we performed Fisher's least significant difference (LSD) *post hoc* test from the final models (see Tables S1 and S2).

Results

Embryonic development and offspring survival

The overall hatching failure in first, replacement and second broods was 31.66%, 43.50% and 47.41% respectively, based on 1950 eggs. We found no significant differences in hatching success between control and androgen-injected eggs across the different reproductive attempts (1st: $\chi^2 = 0.15$, d.f. = 1, $P = 0.695$; Replacement: $\chi^2 = 0.07$, d.f. = 1, $P = 0.784$; 2nd brood: $\chi^2 = 0.01$, d.f. = 1, $P = 0.896$). However, overall hatching success of control eggs was significantly lower than that of noninjected clutches ($\chi^2 = 11.92$, d.f. = 1, $P < 0.001$). This suggests that increased hatching failure of injected eggs is the result of eggshell drilling, rather than yolk androgen manipulation. Overall nestling survival in first, replacement and second broods was 91.49%, 81.90% and 80.94%, respectively. In first broods, nestling survival was not affected by treatment ($\chi^2 = 1.78$, d.f. = 1, $P = 0.181$). However, there was a marginal effect of treatment in replacement broods ($\chi^2 = 3.44$, d.f. = 1, $P = 0.063$), which turned significant in second broods ($\chi^2 = 6.57$, d.f. = 1, $P = 0.010$). In both cases, chicks hatched from androgen-injected eggs had a higher mortality during the first 14 days post-hatch than controls.

EDP was negatively affected by both average egg volume (Table 1, estimate ± SE = −0.009 ± 0.001) and clutch size (Table 1, estimate ± SE = −0.145 ± 0.023), so that EDP was shorter for chicks hatched from larger eggs and from those laid in larger clutches. EDP was also significantly affected by treatment, but this effect

was different for each breeding attempt (Table 1, treatment × breeding attempt interaction): nestlings hatching from the androgen-treated eggs showed shorter EDPs than controls in first and replacement clutches, but no difference was found in second broods (see Fig. 1 and Table S1).

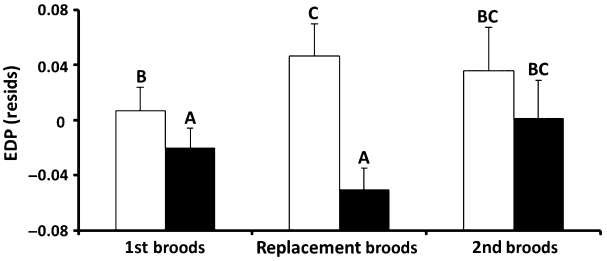

Fig. 1 Differences in Embryonic Development Period (EDP) shown as residuals from the final model, according to the treatment and breeding attempt (white bars: control and black bars: androgen treated). Different letters above bars indicate significant ($P \leq 0.05$) differences between treatment groups based on Fisher's *post hoc* comparisons.

Nestling development

Nestling body condition at day 14 was dramatically affected by the breeding attempt ($F_{2,817} = 245.00$, $P < 0.001$), as it decreased as the breeding season advanced (Fig. 2a and Table S1). We did not detect an effect of androgen treatment on condition, either alone ($F_{1,664} = 0.32$, $P = 0.573$) or in interaction with breeding attempt ($F_{2,511} = 1.49$, $P = 0.226$). Overall, condition was better in males than in females (Table 1; estimate ± SE (males) = 0.097 ± 0.047), and it was worse as brood size increased (Table 1; estimate ± SE = −0.163 ± 0.042).

Structural body size, as measured by tarsus length, also showed an interaction effect between treatment and breeding attempt (Table 1): treated and control chicks had similar tarsus lengths regardless of attempt and treatment, but controls from replacement broods had shorter tarsi than the rest (Fig. 2b and Table S1). Consistently with the sexual dimorphism of this species, males had longer tarsi than females (Table 1).

Gape width was marginally influenced by treatment (Table 1; estimate ± SE (control) = −0.114 ± 0.070) and significantly affected by breeding attempt (Table 1, estimate ± SE (1st) = 0.303 ± 0.072, estimate ± SE (replacement) = 0.178 ± 0.121). Chicks hatched from androgen-treated eggs showed a trend to exhibit wider gapes than controls, and this trait was reduced as breeding season progressed. On average, and controlling for sexual dimorphism in body size, males had wider gapes than females (Table 1, estimate ± SE (males) = 0.507 ± 0.059). Interestingly, even though gape width was measured fourteen days after hatching, we observed a positive effect of egg volume on the development of this trait (Table 1; estimate ± SE = 0.012 ± 0.003).

Nestling immunity

Differential WBC counts

Neither percentages of the different leucocyte types (heterophils, eosinophils, basophils, lymphocytes or monocytes) nor H/L ratio were affected by androgen treatment, breeding attempt or the interaction between these two variables (all $P > 0.143$). Percentage of basophils covaried positively with the body weight of the chick ($F_{1,67} = 4.27$, $P = 0.042$, estimate ± SE = 0.0008 ± 0.0004).

Interleukin-6 (IL-6) and lymphocyte proliferation

IL-6 plasma concentration in chicks hatching from androgen-injected eggs was marginally higher than that from control chicks ($F_{1,33.9} = 3.93$, $P = 0.056$, estimate ± SE (control) = −1.011 ± 0.510), irrespective of breeding attempt ($F_{1,39.5} = 0.29$, $P = 0.59$). IL-6 levels were negatively related to body weight ($F_{1,56.7} = 4.15$, $P = 0.046$, estimate ± SE = −0.072 ± 0.035).

Table 1 Summary of final repeated-measures mixed models showing the effect of yolk androgen treatment on embryo development period (EDP) and nestling development (tarsus length, body condition and gape width) on day 14 post-hatch. Models were run using Proc Mixed (SAS) with Satterthwaite correction to adjust the degrees of freedom.

	EDP			Tarsus length			Body condition			Gape width		
Independent variable	d.f.	*F*	*P*	d.f.	*F*	*P*	d.f.	*F*	*P*	d.f.	*F*	*P*
Treat	1,922	21.20	<0.001	1,538	6.61	0.010	–	–	–	1,889	2.72	0.089
Breeding attempt	2,897	0.02	0.977	2,914	7.81	<0.001	2,817	245.00	<0.001	2,812	8.95	<0.001
Treat × Breeding attempt	2,876	6.29	0.002	2,377	7.65	<0.001	–	–	–	–	–	–
Sex	–	–	–	1,914	9.01	0.003	1,890	4.21	0.040	1,870	74.64	<0.001
Egg volume	1,810	34.09	<0.001	1,281	3.71	0.055	–	–	–	1,408	18.96	<0.001
Clutch size	1,929	39.29	<0.001	–	–	–	–	–	–	–	–	–
Brood size	–	–	–	–	–	–	1,778	14.97	<0.001	–	–	–
EDP	.	.	.	1,459	22.17	<.0001	–	–	–	–	–	–
Measurer	–	–	–	1,248	6.86	0.009	–	–	–	–	–	–

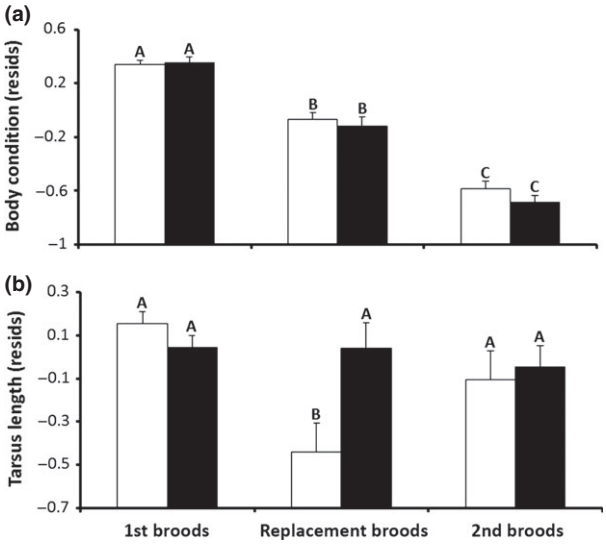

Fig. 2 Differences in nestling body condition (a) and tarsus length (b) shown as residuals from final statistical models, according to the treatment and breeding attempt (white bars: control and black bars: androgen treated). Different letters above bars indicate significant ($P \leq 0.05$) differences between treatment groups based on Fisher's *post hoc* comparisons.

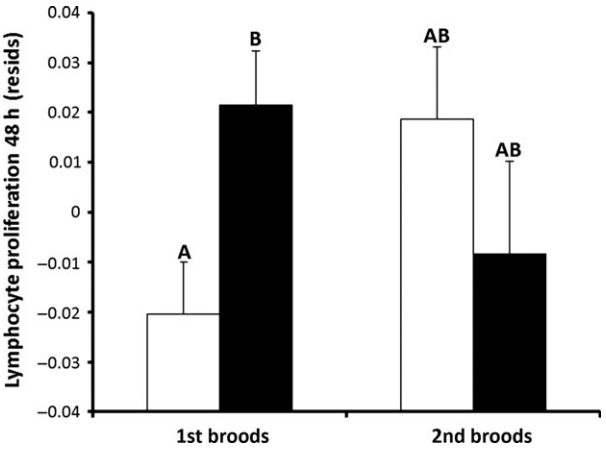

Fig. 3 Differences in nestling lymphocyte proliferation shown as residuals from final statistical models, according to the treatment between the first and the second brood (white bars: control and black bars: androgen treated). Different letters above bars indicate significant ($P \leq 0.05$) differences between treatment groups based on Fisher's *post hoc* comparisons.

Lymphocyte proliferation, expressed as proliferation *per se* (see Supplemental Information), at 48 h of incubation was affected by the interaction between treatment and the breeding attempt ($F_{1,72} = 4.54$, $P = 0.036$), after controlling for day of the assay ($F_{7,72} = 3.97$, $P = 0.001$): nestlings hatching from androgen-treated eggs in first broods showed higher lymphocyte proliferation than controls, whereas no significant differences were found in second broods (see in Fig. 3 and Table S2). Lymphocyte proliferation at 72 h of incubation showed very similar patterns (data not shown).

Immunoglobulin A (IgA)

Faeces produced by nestlings hatching from androgen-injected eggs showed higher IgA levels than controls, although this effect was only marginally significant ($F_{1,25.9} = 4.10$, $P = 0.053$, estimate ± SE (control) = −0.248 ± 0.123). However, IgA levels did not vary with breeding attempt ($F_{1,38.3} = 0.37$, $P = 0.548$) or with the interaction with treatment ($F_{1,24.4} = 0.38$, $P = 0.542$).

Discussion

We investigated how the effects of yolk androgens on developmental and immunological traits in spotless starling chicks changed depending on the breeding attempt, as the environmental conditions become harsher (Salaberria *et al.*, 2014) and parental energetic reserves are gradually reduced (Stouffer, 1991; Verhulst & Tinbergen, 1991; Wiggins *et al.*, 1994; Styrsky *et al.*, 1999; Reed & Clark, 2011). Our results supported context-dependent effects of yolk androgens on early development, survival and cell-mediated adaptive immunity.

Offspring development and survival

In first broods, yolk androgen injections significantly affected EDP, accelerating embryonic development and reducing hatching time (Eising *et al.*, 2001; Eising & Groothuis, 2003; Muriel *et al.*, in press), while no effects on nestling body size (Tobler *et al.*, 2007a) or survival on day 14 post-hatch were found (Pilz *et al.*, 2004; von Engelhardt *et al.*, 2006; Pitala *et al.*, 2009; Muriel *et al.*, in press). This reduction in hatching time could be a consequence of a stimulatory effect of androgens on the hatching muscle (*musculus complexus*) (Lipar & Ketterson, 2000; but see Lipar, 2001), which could help the chick break the eggshell during hatching.

By contrast, in second broods, androgen treatment led to an increase in nestling mortality (Sockman & Schwab, 2000; but see Schwabl *et al.*, 2011), with no effects on embryo or nestling development (Sockman & Schwab, 2000 and Tobler *et al.*, 2007a; respectively). This increase in mortality until fledging contrasts with previous studies showing that yolk androgens often lead to higher survival (Eising & Groothuis, 2003; Pilz *et al.*, 2004; von Engelhardt *et al.*, 2006; Müller *et al.*, 2007b). Therefore, our results suggest that, in a context in which late breeding conditions are harsher than early conditions (Ilyina *et al.*, 2013) and chicks are in

low body condition (Hõrak *et al.*, 1999; Serra *et al.*, 2012; but see Pilz *et al.*, 2004), decreased survival of treated chicks may be explained by a greater susceptibility of these nestlings to disease (Folstad & Karter, 1992; but see Evans *et al.*, 2000; Buchanan *et al.*, 2003; Roberts *et al.*, 2004; Navara *et al.*, 2005; Navara *et al.*, 2006).

In replacement broods, androgen treatment triggered an accelerated embryonic development, which also resulted in chicks from androgen-treated eggs attaining larger body sizes than controls, but with no significant effects on survival. This stimulating effect of androgens on growth rate or body size was consistent with many previous studies (Schwabl, 1996a; Eising *et al.*, 2001; Eising & Groothuis, 2003; Tschirren *et al.*, 2005; Navara *et al.*, 2006; Müller *et al.*, 2007b). This particularly strong effect of androgen on the embryonic period in this breeding attempt may have also conferred these nestlings a competitive advantage, allowing them to reach a larger size than controls by the end of the nestling phase (Fig. 2b). This is consistent with a previous study (Muriel *et al.*, in press) showing that chicks hatched from eggs injected with the same androgen dose as here had greater size than controls. Females laying a replacement clutch may have suffered resource limitations from their double laying effort (Bolton *et al.*, 1992; Hipfner *et al.*, 1999; Gasparini *et al.*, 2006; but see Gasparini *et al.*, 2007), so it is possible that yolk androgen injection may have compensated to some extent this constraint, bolstering nestling development of treated clutches.

In general, hatching success decreased as the breeding season progressed, without differences between experimental groups as reported by other authors (Schwabl, 1996a; but see Navara *et al.*, 2005; Hegyi & Schwabl, 2010; Müller *et al.*, 2010; Muriel *et al.*, in press). Similarly, nestling survival on day 14 was lower in late than in early broods, suggesting that environmental conditions experienced during late clutches may be detrimental for fledglings. Despite the effect found in body size, nestling body condition was not affected by the treatment or its interaction with breeding attempt, although it decreased over the breeding season. As expected, and regardless of the breeding attempt, chicks that shared their nests with more siblings showed poorer body condition, likely because of increased nestling competition for the limited resources provided by the parents. Also, gapes were significantly wider in chicks hatching from first clutches, perhaps because natural androgen concentration are higher in these first clutches (López-Rull *et al.*, 2010), and androgens exert a positive effect on this trait (Müller *et al.*, 2007b; Muriel *et al.*, in press). Consequently, we found that androgen-treated chicks had a tendency to show wider gapes than controls, although these differences were nonsignificantly different. This is possibly due to the low functionality of gapes at day 14, when this trait was measured, as gapes play a major role during begging at earlier ages (Gil *et al.*, 2008; Wiebe & Slagsvold, 2012).

Nestling immunity

According to life-history theory, as reproduction and body maintenance are costly activities, there is an optimal allocation of limited resources among the different organism functions (Stearns, 1992). As androgens can increase nestling growth (Schwabl, 1996a; Eising *et al.*, 2001), one might expect androgen injections to entail an imbalance of the trade-off between growth and the immune response (reviewed in Sheldon & Verhulst, 1996; Saino *et al.*, 1998; Soler *et al.*, 2003; Demas, 2004), where major nutritional and energetic demands could be associated with a higher growth at the expense of immunocompetence (Brzęk & Konarzewski, 2007).

Even though IL-6 and Ig-A levels did not change between breeding attempts, they were marginally increased by the androgen treatment. Recently, it has been shown that taking the parasite community into account is essential for the proper interpretation of immune indices (Biard *et al.*, 2015). Bearing this in mind, a likely explanation for this result is that the suppression of the first line of defences by androgens could increase susceptibility to pathogens or parasites, leading to a subsequent activation of these immunological variables. Il-6 is a protein required for the activation of the immune system (Rose-John, 2012) and is considered a main inflammatory marker (Kishimoto, 2005; Raman *et al.*, 2013). It is assumed that mounting an immune response has energetic and/or nutrient costs which may interfere with metabolic processes (Demas *et al.*, 1997), resulting in a possible loss of body weight. In this scenario, it makes sense that heavier chicks presented lower levels of IL-6 as observed in our study. On the other hand, the similar tendency for increased IgA levels observed in the faeces of androgen chicks could be due to increased levels of IL-6 (Beagley *et al.*, 1989; Ramsay *et al.*, 1994), as this pro-inflammatory cytokine could induce a higher IgA production by B cell from Peyer's patches (Beagley *et al.*, 1989). Accordingly, our data would suggest that an inflammatory process is taking place in chicks hatched from androgen-treated eggs, with both innate and adaptive processes working at higher rates than in control chicks.

Regarding cell-mediated adaptive immunity, we found higher lymphocyte proliferation in androgen chicks than in controls in first broods, but an opposite trend in second broods. This pattern could be responsible, in part, for the lower nestling survival observed in this breeding attempt. This contrasted pattern of first vs. second broods could be attributed to differences in food availability, as it is known that nutrient availability may mediate the costs of immune defence (Norris &

Evans, 2000; Zuk & Stoehr, 2002). The fact that proliferation was higher in first clutches (but see Merino *et al.*, 2000), when breeding conditions were the most suitable (reviewed in Lindén & Møller, 1989; Styrsky *et al.*, 1999; Serra *et al.*, 2012; Salaberria *et al.*, 2014), is in agreement with this context-dependent effect of androgens (Verboven *et al.*, 2003; Sockman *et al.*, 2006), which could be beneficial when plenty nutritional resources were available, but detrimental when food was scarce (reviewed in Smiseth *et al.*, 2011). Not only differences in overall food quantity and quality across the breeding season, but also the differential exposure to parasites and pathogens of first and second broods (López-Rull *et al.*, 2010) could explain the contrasted effects on cell-mediated immunity detected (de Lope *et al.*, 1998; Biard *et al.*, 2015; López-Arrabé *et al.*, 2015). Finally, this context-dependent effect of androgens on immunity would also help to explain the controversial results obtained when addressing the immunocompetence handicap hypothesis (Owen-Ashley *et al.*, 2004; Roberts *et al.*, 2004; Navara *et al.*, 2006; Alonso-Alvarez *et al.*, 2009). Although in our study differences in growth were only significant in the replacement brood on day 14 post-hatch, we have shown before that these effects are stronger at earlier developmental stages, and it is therefore possible that we may have missed it in first and second broods at an earlier age (Muriel *et al.*, in press).

In summary, we found evidence that the effect of yolk androgens on both pre- and post-hatching development and immune function is context dependent. Our results also showed a negative effect of increased androgen levels on the nestling survival in second clutches, but not in first or replacement clutches. Taken together, our findings could explain, from an adaptive perspective, how prenatal environmental factors, such as food availability or ectoparasite load may act as maternal cues to adjust the yolk androgen levels to each breeding context (Gil *et al.*, 2006; Tobler *et al.*, 2007b; López-Rull *et al.*, 2010) in order to maximize offspring fitness (Mousseau & Fox, 1998). Considering this context-dependent effect of androgens on nestling development could improve our understanding of how mothers cope with variable environments when seeking for optimal hormone-mediated maternal effects.

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Conflict of interest

The authors declare no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Data S1 Methods.

Table S1 Fisher's LSD *post hoc* test of androgen treatment effects on EDP and nestling development (tarsus length and body condition) on day 14 posthatch across breeding attempts (summary statistics of final models in Table 1).

Table S2 Fisher's LSD *post hoc* test of androgen treatment effects on lymphocyte proliferation at 48 h on day 14 posthatch between first and second brood (summary statistics of final models in Table 1).

Data deposited at Dryad: doi: 10.5061/dryad.d1t81

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