Context-dependent effects of yolk androgens on nestling growth and immune function in a multi-brooded 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 boost 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 multi-brooded 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 non-significant 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.

Key index words: Yolk androgens, testosterone, androstenedione, maternal effects, *Sturnus unicolor*, immune response, life history trade-offs, breeding conditions

61 **INTRODUCTION**

62

63 Female birds deposit variable amounts of physiologically active substances into egg
64 yolks (Ricklefs, 1984; Williams, 1994; Bernardo, 1996), which potentially affect
65 embryonic growth and development and can vary seasonally (Hargitai et al., 2009).
66 This flexible maternal mechanism may allow females to adjust the offspring phenotype
67 to specific requirements of the environment (Mousseau & Fox, 1998; Vergauwen *et al.*,
68 2012; Giordano *et al.*, 2014). Since the publication of the first study confirming the
69 presence of maternally derived hormones in the yolk (Schwabl, 1993), elucidating the
70 role of yolk androgens as modulators of maternal effects has been a subject of
71 intensive research during the last twenty years (Gil *et al.*, 1999; Schwabl, 1997;
72 Williams *et al.*, 2004; Räsänen & Kruuk, 2007). It is known that avian embryos actively
73 respond to variations in maternally derived androgens of the egg (Reed & Clarck,
74 2011), which may also affect a whole suite of nestling and adult traits including
75 growth, immunity, sexual development, dispersal or personality (reviewed in
76 Groothuis *et al.*, 2005a; Gil, 2008). Different androgens may have different biological
77 consequences (Hegyi *et al*., 2011; Muriel *et al*., 2013; Tschirren *et al*., 2014). Moreover,
78 a particular hormone can have different effects on a given trait, depending on the
79 species (reviewed in Groothuis *et al.*, 2005a; Gil, 2008) or the sex of the chick (Müller
80 *et al.*, 2005; von Engelhardt *et al.*, 2006; Saino *et al.*, 2006; Müller *et al.*, 2008; Müller
81 *et al.*, 2010; Ruuskanen & Laaksonen, 2010; but see Lipar & Ketterson, 2000). This
82 hormonal 'pleiotropy' could induce a number of life-history trade-offs (reviewed in
83 Williams, 2012), and studies that manipulate androgen levels are helpful to identify the
84 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 behavior in nestlings (Ketterson, 1992; Müller *et al.*, 2009; Müller *et al.*, 2012) or intensified begging behavior (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; Groothuis & Schwabl, 2008; but see Roberts *et al.*, 2004). 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; Navara *et al.*, 2005, Müller *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; Gil *et al.*, 2004; Uller *et al.*, 2005), parasite abundance (Tschirren *et al.*, 2004; Postma *et al.*, 2013) 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 (eg. Martínez-Padilla *et al.*, 2010, 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 defense, thermoregulation and parental care in general (Reed & Clark, 2011). In multi-brooded 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

132 that a given increase in androgen levels would result in contrasted effects on offspring
133 fitness at the beginning and at the end of the breeding season, when environmental
134 conditions become tougher.

135 We examined the effects of yolk androgens on embryo development, nestling
136 growth and chick's immune function in the spotless starling (*Sturnus unicolor*), taking
137 into account the different breeding attempts in the same breeding season. We
138 experimentally manipulated yolk androgen concentrations of whole clutches by in ovo
139 injection of a combination of testosterone (T) and androstenedione (A4) dissolved in
140 sesame oil or vehicle only (control). We measured hatching success, growth and
141 survival until nearly fledging (14 days age). We also studied gape width, which is a
142 temporary trait used by nestlings during begging displays to parents (Müller *et al.*,
143 2007b; Gil *et al.*, 2008). At that age, we also evaluated the immune function of
144 individuals using different indicators of both innate (number and proportion of
145 leukocytes, and level of IL-6) and adaptive immunity (lymphocyte proliferation and Ig-A
146 level), since several nestling immune function parameters are associated with survival
147 in the nest (Hõrak *et al.,* 1999; Merino *et al.*, 2000). In this way, we monitored a variety
148 of developmental and physiological parameters that may be affected by yolk
149 androgens, and that may allow us to track the variation in the trade-offs associated to
150 androgen allocation to eggs across the breeding season. We hypothesized that a
151 seasonal decline in yolk androgen allocation (López-Rull *et al.*, 2010; Vergauwen *et al.*,
152 2012) could be due to possible detrimental effects on the nestlings of the second
153 brood. The outcome of the androgen-mediated trade-off between offspring
154 development and immunocompetence is expected to depend on environmental
155 circumstances such as food availability (reviewed in Smiseth *et al.*, 2011; Royle *et al.*,

2001; Sockman *et al.*, 2006), ectoparasite load (Tschirren *et al.*, 2004, but see Müller *et al.*, 2007, 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.

MATERIAL 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 d 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 Salaberria *et al*., 2014; López-Rull *et al*., 2010). 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/m2 for the first brood, 18.95 ± 0.58 °C and 4.54 ± 0.46 l/m2 for replacement broods; and 25.14 ± 0.68 °C and 1.59 ± 0.63 l/m2 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 non-toxic waterproof marker as they were laid and measurements of length and width were taken with digital callipers (Mitutoyo Absolute, Japan, precision = 0.01 mm). Egg volume (mm^3) was calculated by the

204 formula: 0.45 x length x width^2 (Worth 1940). For the analyses, we consider average
205 volume per clutch, because we could not assign individual chicks to the specific egg
206 they hatched from.
207
208 Although yolk A4 and yolk T may exert different biological effects (Hegyi *et al.*,
209 2011; Muriel *et al.,* 2013, Tschirren *et al.,* 2014), androgen-manipulation was done by
210 combining both hormones since they appear together in the yolk (Schwabl, 1993), and
211 are positively correlated among them (Groothuis & Schwabl, 2002; Gil *et al*., 2004;
212 Ruuskanen *et al*., 2009). Based on results obtained in a previous dose-response study
213 in the same study population (Muriel *et al.*, in press), we selected a dose of the
214 mixture of yolk androgens corresponding to 4 standard deviations of the mean amount
215 found in eggs in this population in an overall breeding season (testosterone: 14 ng/yolk
216 [SD = 6.0], androstenedione: 50 ng/yolk [SD = 17.1]; Gil D., unpublished data), adjusted
217 for mean yolk mass (average yolk mass 1.4 g). The maximum concentrations of yolk-T
218 and yolk-A4 that we have measured in this population are 25.9 and 141.76 pg/mg
219 yolk, respectively (Müller *et al*., 2007). According to mean yolk mass, this translates to
220 maxima of 36.3 ng T and 198.4 ng A4 per yolk, so that 4 SD injections result in total
221 androgen concentrations equal (for T) or below (for A4) the maximum levels found in
222 our population. We chose this concentration because this dose was found in a
223 previous study to induce maximum stimulatory effect on hatching nestling body mass
224 and skeletal growth (Muriel *et al.*, in press). Injections began when the fourth egg was
225 found in the nest, before embryonic development was triggered by the start of
226 parental incubation. Subsequently laid eggs were injected the same day they were laid.
227 Clutches were randomly injected with control or androgen injections. The mixture of

228 hormones (24 ng T (ref. 86500, Sigma-Aldrich, Steinheim, Germany) + 68 ng A4 (ref.
229 A9630, Sigma Aldrich) was dissolved in 10 µl of sesame oil (ref. 85067, Sigma-Aldrich).
230 Eggs in control clutches received 10 µl of sesame oil alone. In ovo injections were
231 performed in the field using a standard U-50 insulin syringe (Terumo Corporation,
232 Tokyo, Japan), following a standard protocol (Muriel *et al.*, 2013, Muriel *et al.*, in
233 press).

234 The experiment was carried out in 464 clutches, but 62 of them did not
235 produce any hatchlings because of predation (6.25%), clutch sabotage by conspecifics
236 (62.5%) or abandonments (31.25%). The reason for this unusually large sample size is
237 that this experimental setup is part of large scale study where we will explore the long
238 term effects of our manipulation at the adult stage. The final number of
239 control/androgen clutches per breeding attempt was 90/99 in first, 38/36 in
240 replacement and 62/78 in second broods. We recorded the hatching success of 33
241 uninjected clutches in order to compare the effect of our injection protocol per se on
242 egg hatchability with the natural levels in our population. As found in previous studies
243 (Pilz *et al.*, 2004 (35%); Müller *et al.*, 2007b (30%); Pitala *et al.*, 2009 (32.85%); see
244 results), egg injections led to a certain level of hatching failure, whereby brood size
245 was reduced in some nests. In order to reach the modal brood size in our population
246 (mean ± SD = 4.72 ± 0.57) and to avoid an unusually low level of sibling competition,
247 we performed a post-hatch brood amalgamation of those broods in which only one to
248 three chicks had hatched (163 C and 167 treated out of 977 chicks were moved from
249 their original nests). This was conducted at the age of 3 days. Amalgamated broods
250 were performed trying to minimize the genetic variation of the final brood, pooling
251 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 C and 286 treated in first, 85 C and 76 treated in replacement and finally 114 C 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 in order to check hatching time. We recorded hatching success and computed incubation time or embryonic development period (EDP) as the elapsed time (days ± 4 hours) 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, Japan, 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 aluminum 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 lab in cooled containers (approx. 4°C) to conduct immune measurements (see below and Supporting Information). 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 experimental chicks from first brood and 11 control plus 23 experimental chicks from second brood). On arrival to the lab, blood samples were gently but thoroughly mixed to obtain a uniform distribution of blood cells. We obtained blood smears that were fixed by 3 minutes immersion in methanol, air-dried and stained with commercial Giemsa diluted with PBS pH 6,8 (1:2). Slides were examined under microscope (1,000x magnification with oil immersion) to estimate the proportion of different types of leukocytes (Campbell & Ellis, 2007). Examination continued until 100-120 leukocytes had been found per slide (Salaberria *et al.*, 2013). We measured the number and proportion of leukocytes since these are part of the primary line of defense 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), since increasing H/L ratios are associated with a higher physiological stress in birds (Gross & Siegel, 1983; Maxwell & Robertson, 1998).

Lymphocyte proliferation

298 Our lymphocyte proliferation assay measured the ability of lymphocytes placed in
299 short-term tissue culture to undergo a clonal proliferation when stimulated in vitro by
300 phytohemagglutinin (PHA). Higher levels of proliferation are associated with a better
301 acquired T-cell mediated immune response. This allowed an evaluation of the
302 functional capabilities of T cells (Talebi *et al.*, 1995), whose proliferation and
303 differentiation also involves IL-6 levels (Holsti & Raulet, 1989; Croft & Swain, 1991;
304 Zhang *et al.*, 2000). For the analysis of this parameter, blood was kept on ice and taken
305 to the lab for differential separation of white blood cells and measurement of
306 lymphocyte T proliferation of cells exposed to PHA by means of the AlamarBlue®
307 technique. Plates were incubated at 38ºC for 72 hours, measuring absorbance at 0, 24,
308 48 and 72 hours. The intra-assay variation coefficient was 4.80% (see Supporting
309 Information for details of the technique).
310
311 *Plasma IL-6 concentration*
312 This pro-inflammatory cytokine exhibits a wide range of functions in the regulation of
313 innate immunity and the inflammatory response, directing leukocyte movement and
314 stimulating haematopoiesis (reviewed in Zimmerman *et al.*, 2014; Heinrich *et al.*, 2003;
315 Kishimoto, 2005). A high IL-6 level can be associated with increased susceptibility to
316 infections. We developed an indirect ELISA for chicken IL-6, using rabbit IgG anti-
317 chicken IL-6 as primary antibody and goat IgG anti-rabbit IgG conjugated with
318 horseradish peroxidase as secondary antibody. The intra-assay variation coefficient
319 was 6.79% and the inter-assay was 11.56% (see Supporting Information for details of
320 the technique).
321

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 inter-assay were 3.69% and 1.85%, respectively (see Supporting Information 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 posthatch/ hatchings) between experimental groups were analysed using chi-square tests (χ^2) with the software STATISTICA v7.0 (StatSoft Inc., Tulsa, OK, 214 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

346 breeding attempt (First, replacement, second brood) were considered as categorical
347 variables. In the analysis of gape width, we controlled for nestling size by including
348 tarsus length as a covariate. We also included the person who took the morphometric
349 measurements and the day on which immunological assays were performed as factors
350 in the models for these response variables. Arcsine square-root and logarithmic
351 transformations were applied to leukocyte proportions and H/L ratios, respectively. All
352 biologically meaningful double and triple interactions were also included in the main
353 models. Values represented are means ± SE. Starting from the saturated model, a
354 backward stepwise procedure was used to remove terms with $P > 0.05$. The normality
355 assumption was confirmed by checking the residuals of the models. To inspect
356 differences between androgen treatment and breeding attempts on the biological
357 variables commented above, we performed Fisher's least significant difference (LSD)
358 post hoc test from the final models (see Table S1 and Table S2 in the Supporting
359 Information).

360

361 **RESULTS**

362

363 **Embryonic development and offspring survival**

364 The overall hatching failure in first, replacement and second broods was 31.66%, 43.50%
365 and 47.41% respectively, based on 1950 eggs. We found no significant differences in
366 hatching success between control and androgen-injected eggs across the different
367 reproductive attempts (1^{st}: $\chi^2 = 0.15$, d.f. = 1, $P = 0.695$; Replacement: $\chi^2 = 0.07$, d.f. = 1, $P =$
368 0.784; 2^{nd} brood: $\chi^2 = 0.01$, d.f. = 1, $P = 0.896$). However, overall hatching success of

control eggs was significantly lower than that of non-injected clutches (χ^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 (χ^2 = 1.78, d.f. = 1, P = 0.181). However, there was a marginal effect of treatment in replacement broods (χ^2 = 3.44, d.f. = 1, P = 0.063), that turned significant in second broods (χ^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 posthatch 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 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 Supporting Table S1).

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 Supporting 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

392 ($F_{2,511}$ = 1.49, *P* = 0.226). Overall condition was better in males than in females (Table 1;
393 estimate ± SE (males) = 0.097 ± 0.047), and it was worse as brood size increased (Table
394 1; estimate ± SE = -0.163 ± 0.042).

395 Structural body size, as measured by tarsus length, also showed an interaction
396 effect between treatment and breeding attempt (Table1): experimental and control
397 chicks had similar tarsus lengths regardless of attempt and treatment, but controls
398 from replacement broods had shorter tarsi than the rest (Fig. 2b and Supporting Table
399 S1). Consistently with the sexual dimorphism of this species, males had longer tarsi
400 than females (Table 1).

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

410

411 **Nestling Immunity**

412 *Differential WBC Counts*

413 Neither percentages of the different leukocyte types (heterophils, eosinophils,
414 basophils, lymphocytes or monocytes) nor H/L ratio were affected by androgen
415 treatment, breeding attempt or the interaction between these two variables (all $P >$
416 0.143). Percentage of basophils covaried positively with the body weight of the chick
417 ($F_{1,67} = 4.27$, $P = 0.042$, estimate ± SE = 0.0008 ± 0.0004).

418

419 *Interleukin-6 (IL-6) and lymphocyte proliferation*

420 IL-6 plasma concentration in chicks hatching from androgen injected eggs was
421 marginally higher than that from control chicks ($F_{1,33.9} = 3.93$, $P = 0.056$, estimate ± SE
422 (control) = -1.011 ± 0.510), irrespective of breeding attempt ($F_{1,39.5} = 0.29$, $P = 0.59$). IL-
423 6 levels were negatively related to body weight ($F_{1,56.7} = 4.15$, $P = 0.046$, estimate ± SE =
424 -0.072 ± 0.035).

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

433

434 *Immunoglobulin A (IgA)*

435 Faeces produced by nestlings hatching from androgen-injected eggs showed higher IgA
436 levels than controls, although this effect was only marginally significant ($F_{1,25.9}$ = 4.10, P
437 = 0.053, estimate ± SE (control) = -0.248 ± 0.123). However, IgA levels did not vary with
438 breeding attempt ($F_{1,38.3}$ = 0.37, P = 0.548) or with the interaction with treatment
439 ($F_{1,24.4}$ = 0.38, P = 0.542).

440

441 **DISCUSSION**

442

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

450

451 **Offspring development and survival**

452 In first broods, yolk androgen injections significantly affected the EDP, accelerating
453 embryonic development and reducing hatching time (Eising *et al.*, 2001; Eising &
454 Groothuis, 2003; Muriel *et al.*, in press), while no effects on nestling body size (Tobler
455 *et al.*, 2007a) or survival on day 14 posthatch were found (Pilz *et al.*, 2004; von
456 Engelhardt *et al.*, 2006; Pitala *et al.*, 2009; Muriel *et al.*, in press). This reduction in
457 hatching time could be a consequence of a stimulatory effect of androgens on the

458 hatching muscle (*musculus complexus*) (Lipar & Ketterson, 2000; but see Lipar, 2001),
459 which could help the chick break the eggshell during hatching.
460
461 By contrast, in second broods, androgen treatment led to an increase in
462 nestling mortality (Sockman & Schwabl, 2000; but see Schwabl *et al.*, 2011), with no
463 effects on embryo or nestling development (Sockman & Schwabl, 2000 and Tobler *et*
464 *al.*, 2007a; respectively). This increase in mortality until fledging contrasts with
465 previous studies showing that yolk androgens often lead to higher survival (Eising &
466 Groothuis, 2003; Pilz *et al.*, 2004; von Engelhardt *et al.*, 2006; Müller *et al.*, 2007b).
467 Therefore, our results suggest that, in a context in which late breeding conditions are
468 harsher than early conditions (Ilyina *et al.*, 2013) and chicks are in low body condition
469 (Hõrak *et al.*, 1999; Serra *et al.*, 2012; but see Pilz *et al.*, 2004), decreased survival of
470 experimental chicks may be explained by a greater susceptibility of these nestlings to
471 disease (Folstad & Karter, 1992; Buchanan *et al.*, 2003; Roberts *et al.*, 2004; Navara *et*
472 *al.*, 2005; but see Evans *et al.*, 2000; Navara *et al.*, 2006).
473
474 In replacement broods, androgen treatment triggered an accelerated
475 embryonic development, which also resulted in chicks from androgen-treated eggs
476 attaining larger body sizes than controls, but with no significant effects on survival.
477 This stimulating effect of androgens on growth rate or body size was consistent with
478 many previous studies (Eising *et al.*, 2001; Navara *et al.*, 2006; Schwabl, 1996a;
479 Tschirren *et al.*, 2005; Eising & Groothuis, 2003; Müller *et al.*, 2007b) . This particularly
480 strong effect of androgen on the embryonic period in this breeding attempt may have
481 also conferred these nestlings a competitive advantage, allowing them to reach a
482 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 experimental clutches.

In general, hatching success decreased as the breeding season progressed, without differences between experimental groups as reported by other authors (Schwabl, 1996a; Hegyi & Schwabl, 2010; Müller *et al.*, 2010; Muriel *et al.*, in press; but see Navara *et al.*, 2005). 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 non-significantly different. This is possibly

507 due to the low functionality of gapes at day 14, when this trait was measured, as gapes
508 play a major role during begging at earlier ages (Gil *et al.*, 2008; Wiebe & Slagsvold,
509 2012).
510
511 **Nestling Immunity**
512 According to life-history theory, since reproduction and body maintenance are costly
513 activities, there is an optimal allocation of limited resources among the different
514 organism functions (Stearns, 1992). Since androgens can increase nestling growth
515 (Schwabl, 1996a; Eising *et al.*, 2001), one might expect androgen injections to entail an
516 imbalance of the trade-off between growth and the immune response (reviewed in
517 Sheldon & Verhulst, 1996; Demas, 2004; Saino *et al.*, 1998; Soler *et al.*, 2003), where
518 major nutritional and energetic demands could be associated with a higher growth at
519 the expense of immunocompetence (Brzęk & Konarzewski, 2007).
520
521 Even though IL-6 and Ig-A levels did not change between breeding attempts,
522 they were marginally increased by the androgen treatment. Recently it has been
523 shown that taking the parasite community into account is essential for the proper
524 interpretation of immune indices (Biard *et al.*, 2015). Bearing this in mind, a likely
525 explanation for this result is that the suppression of the first line of defences by
526 androgens could increase susceptibility to pathogens or parasites, leading to a
527 subsequent activation of these immunological variables. Il-6 is a protein required for
528 the activation of the immune system (Rose-John, 2012), and is considered a main
529 inflammatory marker (Kishimoto, 2005; Raman *et al.*, 2013). It is assumed that
530 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), since 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 (Sockman *et al.*, 2006; Verboven *et al.*, 2003), 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

555 second broods (López-Rull *et al*., 2010) could explain the contrasted effects on cell-
556 mediated immunity detected (de Lope *et al.*, 1998; Biard *et al.,* 2015; López-Arrabé *et*
557 *al.*, 2015). Finally, this context dependent effect of androgens on immunity would also
558 help to explain the controversial results obtained when addressing the
559 immunocompetence handicap hypothesis (Owen-Ashley *et al.*, 2004, Roberts *et al.*
560 2004, Navara *et al.*, 2006, Alonso-Alvarez *et al.*, 2009). Although in our study
561 differences in growth were only significant in the replacement brood on day 14 post-
562 hatch, we have shown before that these effects are stronger at earlier developmental
563 stages, and it is therefore possible that we may have missed it in first and second
564 broods at an earlier age (Muriel *et al.*, in press).
565
566 In summary, we found evidence that the effect of yolk androgens both on pre- and
567 post-hatching development and immune function is context-dependent. Our results also
568 showed a negative effect of increased androgen levels on the nestling survival in second
569 clutches, but not in first or replacement clutches. Taken together, our findings could
570 explain, from an adaptive perspective, how prenatal environmental factors, such as food
571 availability or ectoparasite load may act as maternal cues to adjust the yolk androgen
572 levels to each breeding context (Gil *et al.*, 2006; Tobler *et al.*, 2007b; López-Rull *et al.*,
573 2010) in order to maximize offspring fitness (Mousseau & Fox, 1998). Considering this
574 context-dependent effect of androgens on nestling development could improve our
575 understanding of how mothers cope with variable environments when seeking for optimal
576 hormone-mediated maternal effects.
577

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958

959 **FIGURE LEGENDS**

960 **Figure 1.** Differences in Embryonic Development Period (EDP), shown as residuals from
961 the final model, according to treatment and breeding attempt (white bars: control and
962 black bars: androgen treated). Different letters above bars indicate significant ($P \leq$
963 0.05) differences between treatment groups based on Fisher's post-hoc comparisons.

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

Figure 3. Differences in nestling lymphocyte proliferation shown as residuals from final statistical models, according to 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.

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 posthatch. 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	–	–	–	–	–	–

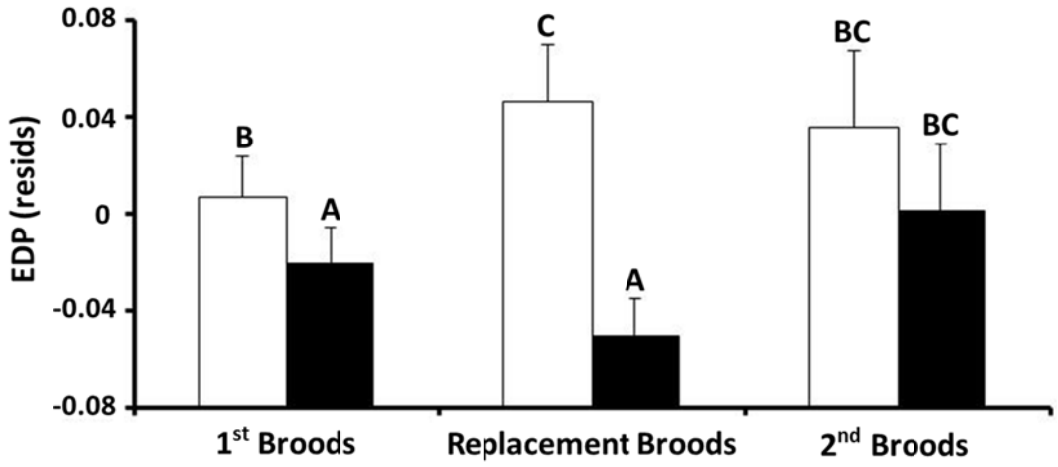
0.08
0.04
0
-0.04
-0.08
EDP (resids)
B
A
C
A
BC
BC
1st Broods
Replacement Broods
2nd Broods

Fig. 1

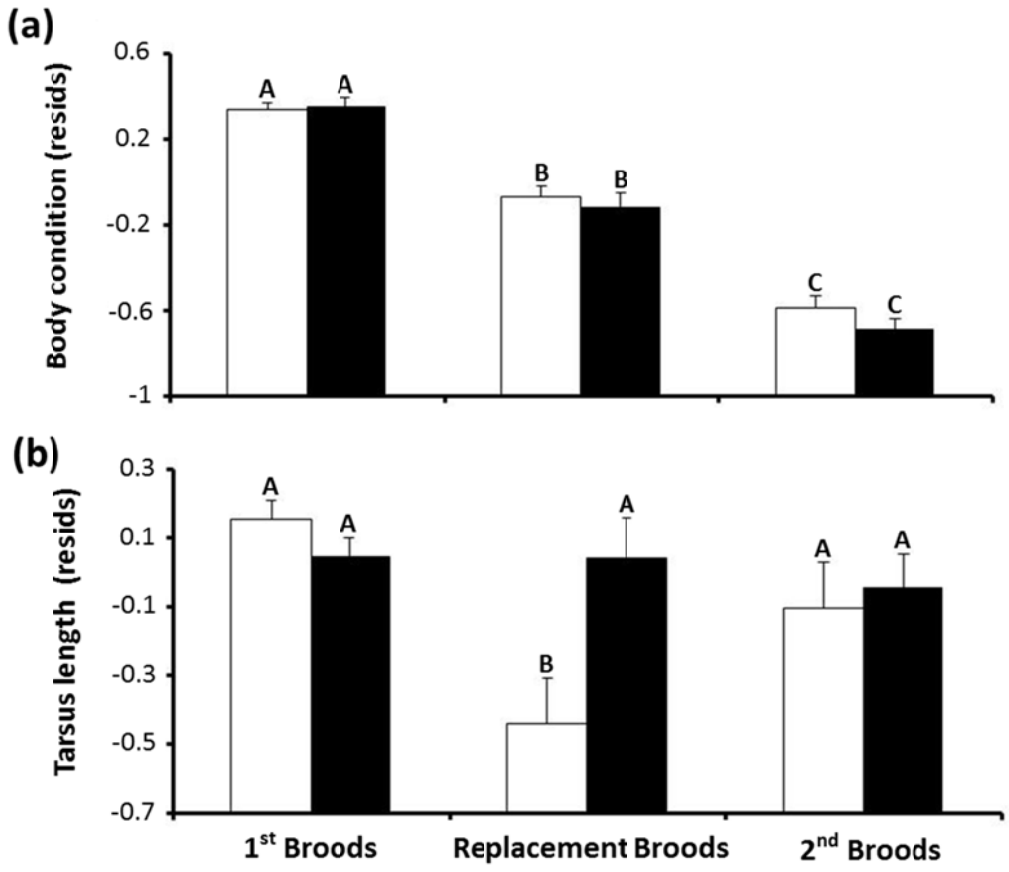
(a)
Body condition (resids)
0.6
0.2
-0.2
-0.6
-1
A
A
B
B
C
C
(b)
Tarsus length (resids)
0.3
0.1
-0.1
-0.3
-0.5
-0.7
A
A
B
A
A
A
1st Broods
Replacement Broods
2nd Broods

Fig. 2

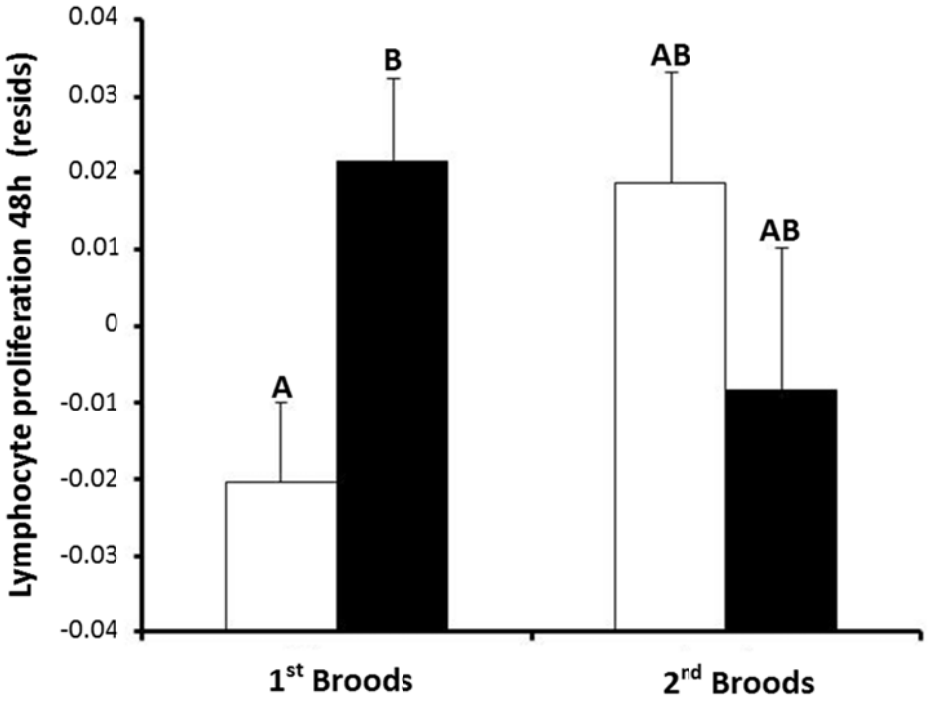
Lymphocyte proliferation 48h (resids)
0.04
0.03
0.02
0.01
0
-0.01
-0.02
-0.03
-0.04
A
B
AB
AB
1st Broods
2rd Broods

Fig. 3

SUPPLEMENTARY METHODS

Blood cell isolation and immune tests

White blood cell isolation

Blood was centrifuged at 3000 rpm and 4ºC for 5 minutes, just enough to allow partial plasma recovery without forming a compact pellet. Plasma was stored at -20ºC until IL-6 analysis and cells were immediately processed for WBC isolation. The isolation procedure was based in procedures already described (Strain & Matsumoto, 1991; Finkelstein *et al*., 2003; Gil & Culver, 2011). Briefly, blood was diluted (1:1) in Roswell Park Memorial Institute 1640 medium with hepes (RPMI, Sigma, St. Louis, MO) containing 1% bovine serum albumin (BSA, Sigma, St. Louis, MO), and penicillin-streptomycin −neomycin (200 U – 0.2 mg - 0.4 mg/ml, respectively, Sigma, St. Louis, MO) and mixed gently (this mixture will be referred to as RPMI+). This mixture was set above an equal volume of a double layer of Histopaque gradient (Sigma, St. Louis, MO): HP 1.119:HP 1.077, and centrifuged at 700g during 30 minutes. The layer above HP 1.077 containing the lymphocytes was collected and transferred to a clean tube with 400 µl RPMI+ and centrifuged at 250g for 12 min. The supernatant was aspirated and the cells resuspended in 400 µl RPMI+, and thoroughly mixed to avoid cell aggregates. The final pellet was gently resuspended in 200 µl RPMI+. We counted the number of alive lymphocytes in a 15 µl aliquot mixed with 5 µl tripan blue in a counting chamber. We used this figure to finally dilute the homogenate to a final concentration of 10^5 lymphocytes per 100 µl .

Lymphocyte T proliferation

Lymphocyte T proliferation was measured in flat-bottom 96-well plates (Nunc, Roskilde, Denmark). Two duplicates per sample of the 200 µl of lymphocyte suspension (containing 10^5 lymphocytes per 100µl) were incubated with 20 µl of AlamarBlue® (AbD Serotec). This dye indicates the oxidation-reduction state of the medium, measuring both the intensity and velocity of the proliferation process. One of the two duplicates (experimental) received 20 µl of a PBS solution containing 50 µg of phytohemaglutinin (PHA) (Sigma, L8902), and the other well (control) received a

similar volume of PBS. Plates were incubated at 38ºC during 72 hours, and we read absorbances at 0, 24, 48 and 72h of the process. Readings were done in a plate reader at both 570 and 600 nm and calculations performed following the instructions from the commercial kit after adjusting to our species.

To adjust the AlamarBlue procedure to our species, calculations were done considering the molar extinction coefficients of the reduced and oxidized form of AlamarBlue at the two wavelengths recommended in the in the commercial kit insert. Lymphocyte proliferation was calculated in two ways, as proliferation *per se,* this is, the reduction obtained once the reduction of the respective negative controls had been discounted, and as velocity of proliferation, this is, the percentage of reduction of the experimental wells compared with the respective positive controls (see commercial insert for formulae). Since results were very similar between these two types of measurement, we chose to use the more commonly used proliferation estimate. The above conditions were decided after assaying different plating densities (ranging from 10000 to 1000 cells per 100 µl) at different incubation times (24, 48 and 72h) with spotless starling blood. Proliferation increased with incubation time (Fig. S1), although between 48 and 72 hours the increase was smaller, with some cells starting to decline at 72, and thus we chose to use 48 hours as our standard point (correlation between 48 and 72 hour scores: $r = 0.930$, $N = 96$, $P < 0.001$).

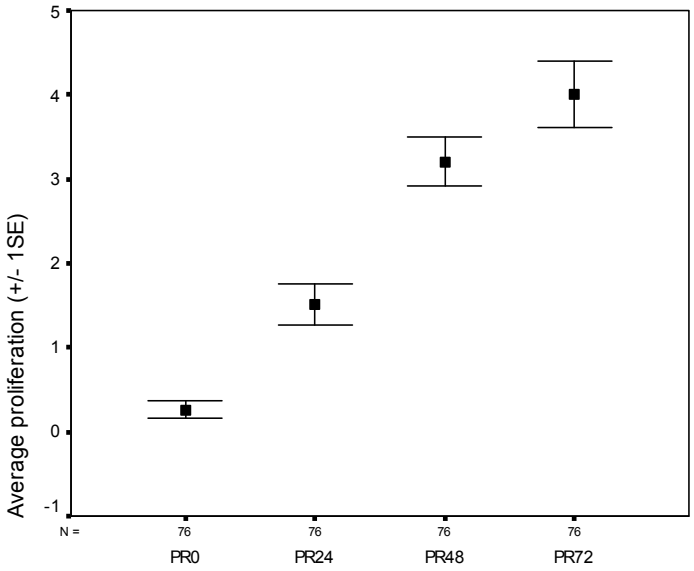

Fig. S1. Mean (±1 SE) lymphocyte proliferation as measured by the AlamarBlue technique at different time intervals since incubation: 0, 24, 48 and 72 hours.

Plasma IL-6 concentration and validation procedure

We developed an indirect ELISA using chicken IL-6 as antigen (Mybiosource, MBS 232222), a rabbit IgG anti-chicken IL-6 as primary antibody (MybioSource, MBS 220073) and goat IgG anti-rabbit IgG conjugated with horseradish peroxidase as secondary antibody (MybioSource, MBS 235191). Wells in a 96-well plate were covered with 100 µl of either standard solutions or plasma samples and left 2 hours at room temperature. Wells contents were aspirated and washed 5 times with 200 µl of Tris-buffer-saline containing 0.05% Tween 20, (TBST) pH 8.O (Sigma C3041). Inespecific binding sites were blocked with 200 µl ELISA SYMBLOCK (AbD Serotec BUF034A) for 1 h at room temperature. Wells contents were then aspirated and washed 5 times with 200 µl of TBST. Both standards and samples received 100 µl of a 5 µg/ml solution of primary antibody, left overnight at 4ºC and washed 5 times with 200 µl of TBST. Identical procedure was followed for the secondary antibody which was diluted 1:10000 in TBST with 1% albumin. After washing, 100 µl de TMB (Sigma Aldrich, Sigma T0440), were added to every well, and the plate was then incubated in darkness for 15-30 min and the reaction stopped by adding 100 µl 2M H_2SO_4 to every well. Absorbance at 450 nm was read in the next 30 min in a plate reader. Eight solutions containing 0.125-0.250-0.5-1-2-4-6-8 in HISPEC assay diluent (AbD Serotec BUF049A) were used as standard solutions. These conditions were chosen after tritiation experiments using 0.125-10 µg/ml as antigen solutions, 1-5 µg/ml as primary antibody solutions and 1.10000-1:50000 solution of the secondary antibody. The intra-assay variation coefficient was 6.79% and the inter-assay was 11.56%.

Fecal sampling and immunological test

IgA extraction from feces

Chicks usually defecate when manipulated for morphometric measurements and blood collection. We collected the whole fecal pellet in cold containers that were transported to the lab in 2-4 hours after delivery and conserved at -20ºC until analysis. After been taken from the freezer, they were cleaned with filter paper (the main part of the pellet of uric acid was removed) and exposed to 30-35ºC until weight was constant. The

following steps used for extraction of fecal immunoglobulin were adapted from that used by Peters *et al.* 2004. Samples were weighed and grounded with a mortar, and TBST (Tween buffer saline with 0.5% Tween 20) pH 7.4 was added in proportion 0.5 g feces/2ml TBST. They were kept under agitation for 60 min and centrifuged at 1600 g during 15 min at 4ºC. The supernatant was transferred to a sterile Eppendorf tube and mixed with a protease inhibitor cocktail (Sigma-Aldrich), in proportion 2ml extract to 20 µl cocktail and centrifuged at 10000 × g for 10 min at 4ºC for optimal removal of solid material. The supernatant was preserved at -20ºC until analysis.

IgA quantification

An ELISA quantification set developed for chicken IgA by Bethyl Lab (E30-103) was used to measure IgA in feces extracts. We followed the commercial procedure. In brief, 96-well immunoplates (Maxisorb; Nunc, Roskilde, Denmark) were covered with the first antibody, incubated at room temperature during 1 hour and washed with TBST (Tween buffer saline with 0.05% Tween 20 pH 8.0, Sigma T9039). TBS with 1% BSA (Bovine serum albumin) was used as blocking solution for a further 30 min period and washed again. A known amount of antigen (recombinant chicken IgA) or samples were added and incubated for 1 hour, the wells being washed again. The second antibody which was conjugated with HRP (horseradish peroxidase) was added and incubated for 1 hour. After washing, TMB (Sigma T0440) was added, maintained in darkness for 15 min, and the reaction stopped with H_2SO_4 0.18 M. The absorbance was read in a plate reader at 450nm, immediately. Both standards and samples were run in duplicate. Washing procedures were repeated 5 times throughout.

Standard solutions for the calibration curve were obtained from an initial antigen solution in TBST of 1000 ng/ml. After trying a long dilution series, we chose the central lineal part of the curve, between 400 and 6.25 ng/ml. A parallelism test was run to asses that the chicken antibodies discriminate different concentrations of IgA from *Sturnus unicolor.* To that end, we extracted the IgA from a pool of several fecal extract with magnetic beads so that a solution with an absorbance 2400 was obtained. Several dilutions allowed as to run a curve that paralleled to the standard curve run at the

same time. R^2 for different standard curves was > 98.7. The coefficients intra and inter-assay were 3.69% and 0.46%, respectively.

IgA extraction with magnetic beads for parallelism curve

Dynabeads M-270 Epoxy from Life Technologies were used to extract IgA from a pool of fecal extract. The commercial procedure was followed with minor modifications. In short, 2 x 108 beads were covered with 80 µg of the primary chicken antibody used in the previous ELISA procedure. After adding 20 µl of $(NH4)_2SO4$ 5M they were incubated for 20 hours with a vortex so that beads were not allowed to settle down. Beads were recovered under a magnetic field (Dynamag, Life technologies) washed and mix with 8 ml pool of fecal extracts. After 4 hours under agitation with vortex, they were allowed to sediment with the magnetic field, washed with PBS and resuspended in 200 ml TBST pH 8. IgA were released to the medium from the beads by adding 300 µl of citric acid 3.1 M (three sequential additions of 100 µl). Beads were settled down with the magnetic field and the supernatant was transferred to a clean eppendorf, immediately neutralized with NaOH to pH 7.4 and this solution was used to raise serial dilutions with TBST pH 8.

SUPPLEMENTARY REFERENCES

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Supplementary 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). Fixed factors are coded as Treat (Treatment; control: 0, androgen treated: 1) and Attempt (Breeding Attempt; first brood: 1st, replacement: R, second brood: 2nd).

Diff. between groups				EDP				Tarsus length				Body condition			
Treat	**Attempt**	**Treat**	**Attempt**	**Estimate ± SE**	**d.f.**	**t**	***P***	**Estimate ± SE**	**d.f.**	**t**	***P***	**Estimate ± SE**	**d.f.**	**t**	***P***
0	1st	0	R	-0.145±0.072	915	-2.00	0.045	0.949±0.172	428	5.51	<0.001	0.483±0.121	548	3.97	<0.001
0	1st	0	2nd	0.054±0.062	922	0.87	0.383	0.368±0.157	566	2.34	0.019	1.102±0.103	704	10.71	<0.001
0	1st	1	1st	0.115±0.053	851	2.17	0.030	0.171±0.119	386	1.44	0.151	-0.099±0.084	518	-1.18	0.240
0	1st	1	R	0.282±0.083	885	3.42	<0.001	0.133±0.189	390	0.71	0.481	0.534±0.129	503	4.11	<0.001
0	1st	1	2nd	0.059±0.039	793	1.52	0.128	0.241±0.129	929	1.87	0.062	1.263±0.078	939	16.19	<0.001
0	R	0	2nd	0.199±0.085	894	2.35	0.019	-0.581±0.202	440	-2.88	0.004	0.619±0.138	559	4.47	<0.001
0	R	1	1st	0.260±0.078	842	3.34	<0.001	-0.778±0.172	368	-4.52	<0.001	-0.582±0.124	466	-4.71	<0.001
0	R	1	R	0.427±0.100	872	4.26	<0.001	-0.816±0.227	358	-3.59	<0.001	0.051±0.160	464	0.32	0.749
0	R	1	2nd	0.204±0.076	923	2.67	0.007	-0.708±0.187	453	-3.78	<0.001	0.780±0.129	592	6.04	<0.001
0	2nd	1	1st	0.061±0.046	818	1.32	0.189	-0.196±0.145	878	-1.36	0.175	-1.201±0.088	965	-13.60	<0.001
0	2nd	1	R	0.228±0.084	931	2.70	0.007	-0.235±0.212	487	-1.11	0.269	-0.568±0.141	634	-4.01	<0.001
0	2nd	1	2nd	0.005±0.063	931	0.07	0.941	-0.127±0.171	634	-0.74	0.457	0.161±0.110	824	1.47	0.142
1	1st	1	R	0.167±0.078	917	2.15	0.032	-0.038±0.184	424	-0.21	0.836	0.633±0.126	555	5.03	<0.001
1	1st	1	2nd	-0.057±0.053	915	-1.05	0.292	0.069±0.135	600	0.51	0.608	1.362±0.089	748	15.25	<0.001
1	R	1	2nd	-0.223±0.084	907	-2.66	0.008	0.108±0.202	439	0.53	0.594	0.729±0.136	571	5.34	<0.001

Supplementary Table S2. Fisher's LSD post hoc test of androgen treatment effects on lymphocyte proliferation at 48 hours on day 14 posthatch between first and second brood (summary statistics of final models in Table 1). Fixed factors were coded as Treatment (Treat; control: 0, androgen treated: 1) and Attempt (Breeding Attempt; first brood: 1st, second brood: 2nd).

Diff. between groups				Lymphocyte proliferation			
Treat	**Attempt**	**Treat**	**Attempt**	**Estimate ± SE**	**d.f.**	**t**	***P***
0	**1st**	**0**	**2nd**	-0.06191±0.05353	67	-1.16	0.2516
0	**1st**	**1**	**1st**	-0.04543±0.02011	67	-2.26	0.0272
0	**1st**	**1**	**2nd**	-0.03317±0.06115	67	-0.54	0.5893
0	**2nd**	**1**	**1st**	0.01648±0.05395	67	0.31	0.7609
0	**2nd**	**1**	**2nd**	0.02874±0.02955	67	0.97	0.3341
1	**1st**	**1**	**2nd**	0.01226±0.06151	67	0.20	0.8426