

Long-term fitness consequences of high yolk androgen levels: sons pay the costs

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Summary

1. Early growth conditions, for example hormonal environment during embryonic development, may have long-lasting effects on behaviour and phenotype, with subsequent fitness consequences. Yolk androgens have been shown to affect various offspring traits in the short-term, but fitness consequences for either offspring or parents, a prerequisite for studying the adaptiveness of this maternal effect, are poorly known in the wild.

2. We experimentally elevated yolk androgen levels of whole clutches in a wild population of collared flycatchers (*Ficedula albicollis*) and investigated the long-term effects of yolk androgens on offspring local recruitment, parental return rate, and timing and success of breeding in both parents and offspring in the breeding season(s) following the manipulation.

3. Yolk androgen elevation lowered the local recruitment rate of male, but not female offspring, but had no effect on any breeding parameters of offspring of either sex. Furthermore, yolk androgen elevation of the clutch had no effect on the return rate or any breeding parameters of the parents.

4. Our results indicate that high yolk androgen levels may impose a potential direct fitness cost for male offspring, but no long-lasting additional indirect fitness costs for parents (direct costs of transferring androgens to eggs not considered). Such a sex-specific cost on offspring could constrain yolk androgen deposition and select for sex-specific deposition mechanisms.

5. As yolk androgen levels in this population are heritable, our results on long-term fitness effect of yolk androgens support the idea that hormone-mediated maternal effects may evolve under selection and thereby affect evolutionary processes.

Key-words: bird, hormones, maternal effects, parent–offspring conflict, sexual conflict, testosterone

Introduction

Maternal effects are referred to when maternal phenotype affects offspring phenotype (Mousseau & Fox 1998a,b). Hormone-mediated maternal effects, such as exposure to androgens during early development have been shown to have both short- and long-term effects on various offspring traits, with potentially major fitness consequences in many vertebrate groups (e.g. Hews, Knapp & Moore 1994;

Lovern, McNabb & Jenssen 2001; Groothuis *et al.* 2005; Gil 2008; Love & Williams 2008). In egg-laying species, long-lasting effects of yolk androgens may be due to both carry-over effects and priming effects of early exposure to hormones on subsequent hormone production or sensitivity later in life (reviewed in Carere & Balthazart 2007; Groothuis & Schwabl 2008).

These long-lasting effects of egg yolk androgens may result in both positive and negative fitness consequences on either survival or reproductive success of offspring, potentially in a sex-specific way. Survival effects may be due for example to

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the suppressive effect of high androgen levels on the immune system (reviewed in Ketterson & Nolan 1992, 1999; Groothuis *et al.* 2005; Rutkowska, Wilk & Cichoń 2007; Gil 2008). Yolk androgens may also increase the predation risk via enhancing risk-taking behaviour (such as activity, exploration, aggressiveness, boldness and anti-predator behaviour; Strasser & Schwabl 2004; Daisley *et al.* 2005; Eising, Müller & Groothuis 2006; Tobler & Sandell 2007; Partecke & Schwabl 2008; Ruuskanen & Laaksonen 2010). Both effects may be sex-specific (e.g. Müller *et al.* 2005; Rutkowska, Wilk & Cichoń 2007; Ruuskanen & Laaksonen 2010). For males, a positive effect of yolk androgens on reproductive success may, for example, result from increased attractiveness and mating success of individuals from eggs with high yolk androgen levels (von Engelhardt 2004; Partecke & Schwabl 2008; but see Müller & Eens 2008 for a meta-analysis). On the contrary, yolk androgens may have direct negative effects on female reproductive physiology and performance, decreasing the number of copulations and the number and size of eggs laid (Uller, Eklöf & Andersson 2005; Rubolini *et al.* 2007; Müller, Vergauwen & Eens 2009; Bonisoli-Alquati *et al.* 2011a; but see Rutkowska, Wilk & Cichoń 2007). Thus, effects of yolk androgens on reproductive success may be opposite for females and males, causing a conflict over the optimal level of androgens *within* clutches. However, most long-term effects of yolk androgens on fitness-related traits have only been shown in domestic or captive species (e.g. Gil 2008). To our knowledge, only two studies have directly measured the consequences of variation in yolk androgen levels on a fitness component in wild birds: yolk androstenedione concentration has been found to positively correlate with recruitment rate in collared flycatchers (*Ficedula albicollis*) (Hegy *et al.* 2011) but an experimental elevation of yolk testosterone in great tits (*Parus major*) did not affect offspring recruitment rate in either sex (Tschirren, Fitze & Richner 2007). However, neither study investigated the effects of high yolk androgen levels on reproductive success of the recruited offspring.

Understanding the evolution of yolk androgen deposition and its potential adaptive value requires considering effects of yolk androgens on future survival and reproductive success of not only the offspring, but also the parents (e.g. Marshall & Uller 2007; Müller *et al.* 2007; Uller 2008). Large fitness costs of high yolk androgen levels for parents could select for depositing levels that are suboptimal for the offspring (i.e. parent-offspring conflict: Trivers 1974; Godfray 1995; Marshall & Uller 2007; Müller *et al.* 2007; Uller 2008). Direct costs for females could occur if depositing high hormone levels in the egg requires that the mother is exposed to high circulating hormone levels herself (Groothuis & Schwabl 2008), causing, for example, delayed reproduction, reduced clutch size or maternal immunosuppression (e.g. Clotfelter *et al.* 2004; Veiga *et al.* 2004; Rutkowska *et al.* 2005). Indirect costs for both parents could occur if rearing a brood from eggs with high androgen levels is highly energy demanding (for example due to high metabolic rate or activity of the chicks: Tobler, Nilsson & Nilsson 2007a), reducing the survival or breeding

success of the parents in the following breeding season. Furthermore, long-term fitness effects on parents could be sex-specific, for example if one parental sex is more responsive to the signals of nestlings from eggs with high yolk androgen levels (e.g. Müller *et al.* 2007; Tschirren & Richner 2008; Ruuskanen *et al.* 2009). More specifically, females have been suggested to potentially manipulate paternal investment via differential allocation of yolk androgens affecting offspring traits to which males respond (e.g. Müller *et al.* 2007, 2010; Tschirren & Richner 2008; Ruuskanen *et al.* 2009; Barnett *et al.* 2011; Laaksonen *et al.* 2011). This could generate long-term costs for males of raising a brood from eggs with high yolk androgen levels.

We investigated the long-term fitness consequences of yolk androgens on both offspring and their parents in a wild population of collared flycatchers (*Ficedula albicollis*) by experimentally elevating yolk androgen levels in whole clutches. Because yolk androgens were directly manipulated, this study however investigated only the potential indirect effects of high yolk androgen levels (via e.g. faster growth and higher food demand in nestlings) on parents, but not potential direct effects via yolk androgen production and deposition. In this species and in the pied flycatcher (*Ficedula hypoleuca*), a closely related species, among-clutch variation has been shown to correlate with both individual factors such as female condition (Tschirren *et al.* 2009; Tobler, Granbom & Sandell 2007b; but see Michl *et al.* 2005), male and female age (Michl *et al.* 2005; Laaksonen *et al.* 2011; B. Doligez & B. Tschirren, unpublished data), and environmental factors such as timing of breeding (Michl *et al.* 2005) and social stimulation (Hargitai *et al.* 2009). Furthermore, yolk androgen deposition has been shown to be repeatable and heritable (Tschirren *et al.* 2009). These results clearly call for investigating whether yolk androgen deposition may be subject to selection and thus exploring the fitness consequences of yolk androgen deposition.

We recorded offspring local recruitment, parental return rate and the timing and success of breeding of both the parents and the offspring of experimental nests in the subsequent breeding season(s). Yolk androgens may be expected to promote sexually selected secondary traits or behaviour (see examples above; review in Müller & Eens 2008); in that case, attractiveness, mating success and fecundity should be higher for androgen-treated males compared to controls. Conversely, high yolk androgen exposure may be expected to disrupt the female reproductive physiology (see examples above; Groothuis *et al.* 2005; Groothuis & Schwabl 2008); in that case, androgen-treated females should have lower reproductive success than controls. However, yolk androgen elevation has been reported to affect the male nestling growth negatively but have no (or weak opposite) effects on females at fledging in this population (Pitala *et al.* 2009). Because fledging condition may influence the future offspring fitness components (e.g. Tinbergen & Boerlijst 1990; Magrath 1991; Haywood & Perrins 1992; Lindén, Gustafsson & Pärt 1992), these short-term effects of yolk androgens may lead to the alternative predictions that male offspring from yolk

androgen-elevated eggs may have lower survival to the next year(s) and potentially lower breeding success compared to controls, whereas the opposite pattern (or no difference) may be expected for females. Furthermore, yolk androgen elevation of the clutch did not affect the parental feeding effort during the nestling period (Ruuskanen *et al.* 2009). Therefore, yolk androgen elevation may be expected not to affect the survival or future breeding success of the parents (via indirect cost), unless parental care after fledging is affected. Our study provides insight into whether yolk androgens may have fitness consequences through either indirect effects on parents and/or direct or indirect effects on offspring, and whether these consequences reflect the short-term effects observed during growth.

Materials and methods

STUDY SITE STUDY SPECIES AND POPULATION MONITORING

The experiment was conducted on the island of Gotland, Sweden (57°10'N, 18°20'E), in a nest box breeding population of collared flycatchers monitored since 1980 (Gustafsson 1989). The yolk hormone manipulations were conducted in 2007, and recruits were monitored during breeding seasons 2008–2009. The collared flycatcher is a small (*c.* 13 g), short-lived migratory passerine, which breeds in central and eastern Europe and on the islands of Gotland and Öland in Sweden. It readily breeds in artificial nest boxes, which provides easy access to detailed breeding data. The majority of breeding pairs in this population have six to seven eggs and three to four nestlings at fledging. Usually, both parents feed the young. The general population monitoring includes checking nest boxes every other day to determine the laying dates. Nests are monitored to record hatching date, number of hatchlings and fledging success, and nestlings are measured on day 12 after hatching. Females are caught in the nest box using a swing-door trap during incubation and both sexes when feeding 6–10-day-old nestlings. Therefore, male adult catching is biased towards successful individuals (see Doligez *et al.* 2011). Furthermore, this species is facultatively polygynous, thus approximately 10% of males succeed in attracting a secondary female and provide little parental care to the young of the secondary nest (Gustafsson 1989). As a consequence, some broods fail early and males cannot be identified. Adult age is determined in the field as 1-year old or older based on plumage characteristics. We chose to study the long-term fitness effects of yolk androgens in this particular population because local recruitment is high for such a short-lived species (*c.* 0.3–0.5 recruit per nest, Gustafsson 1986).

YOLK ANDROGEN-MANIPULATION EXPERIMENT

In spring 2007, nest boxes were checked every other day to determine the laying dates. On the estimated day of clutch completion (*i.e.* the sixth egg-laying day), each clutch was randomly assigned to either the control or the androgen-manipulation group. The eggs were replaced by dummy eggs during the time of the injections (*c.* 30–60 min), and the procedure did not generate desertion in the experimental clutches. See the studies by Ruuskanen *et al.* (2009) and Pitala *et al.* (2009) for more details on the injection protocol. In the androgen-manipulation group ($n = 120$ nests), eggs were injected with 14.4 ng of testosterone (T, Fluka) and 50.8 ng of androstenedione (A4, Fluka) dissolved in

4 μ L of sesame oil. In the control group ($N = 120$ nests), eggs were injected with 4 μ L of sesame oil only. The amount of injected androgens corresponded to the difference between mean and maximum values of androgens per yolk calculated using previous data on natural yolk androgen levels in the same population ($n = 120$ eggs; T mean \pm SD, 14.2 \pm 4.7 ng per yolk; maximum, 28.8 ng per yolk; A4 mean \pm SD, 60.3 \pm 16.2 ng per yolk; maximum, 111.1 ng per yolk). The injected amount of androgens corresponded to 3.1 times the SD for testosterone and A4. Eggs with very high natural androgen levels thus probably ended up with levels above the previously observed maximum value; however, the distribution of natural yolk androgen levels is highly right-skewed in this population (B. Doligez and B. Tschirren, unpublished data), and thus the final androgen levels should be above the observed maximum value only in a small number of eggs. Furthermore, in this species, yolk androgen levels are not correlated with egg or yolk size (Tschirren *et al.* 2009) and there seems to be no within-clutch pattern in either yolk androgen levels, egg size or yolk size (Michl *et al.* 2005). Variation in egg size or initial yolk androgen levels along the laying sequence is therefore unlikely to bias our results or lead to pharmacological concentrations. Nests were checked on the following day and the seventh egg was injected if present, according to the clutch treatment. Only one experimental nest had eight eggs (this additional egg was not injected). By manipulating 240 nests (and a total of 1460 eggs), we aimed at obtaining about 70 recruits (see general recruitment rate above).

Nests were monitored to record hatching date, number of hatchlings and fledging success as part of the population monitoring. Hatching success (*i.e.* proportion of hatched eggs within a nest, mean \pm SD, excluding deserted and predated nests, where no egg hatched) was 70.2 \pm 24.3% for androgen-treated nests and 74.1 \pm 23.0% for control-treated nests (with a similar proportion of nests where all or no eggs hatched for both treatments) and did not differ between treatments (logistic regression: $n = 210$ nests, $\chi^2 = 1.32$, $p = 0.25$). The natural hatching success of non-injected clutches (also excluding nests where no egg hatched) in this population was 93.8% in 2007 (unmanipulated nests, $n = 326$); thus, the injection protocol lowered the hatching success, as observed in previous studies using the same protocol. Two days after hatching, complete broods were cross-fostered between manipulated nests (matched for hatching date and brood size) in *ca.* half of the nests used in the experiment, to investigate the short-term effects of yolk androgens on parental feeding rates (see details in Ruuskanen *et al.* 2009). In the other half of the nests, nestlings were blood sampled for molecular sexing and challenged (subcutaneous injection) with a non-pathogenic antigen, phytohaemagglutinin (PHA; Sigma, St. Louis, MO, USA, code L8754), to investigate the short-term effects of yolk androgens on cellular immune response at 11 days of age (see details in Pitala *et al.* 2009). Because there was no difference in nestling immune response to PHA between treatments (Pitala *et al.* 2009), we consider that immunization does not affect our results on the long-term effects of yolk androgens. In total, 305 and 301 young fledged from the androgen-manipulated and control nests, respectively (from 80 and 81 nests, respectively). All experiments were conducted under licences from the Swedish National Board for Laboratory Animals and the Bird Ringing Centre of the Swedish Museum of Natural History (Stockholm, Sweden).

BREEDING DATA OF THE RECRUITS AND PARENTS OF EXPERIMENTAL NESTS

In the following two breeding seasons (2008 and 2009), reproductive attempts of experimental recruits were monitored from the end of April as part of the general population monitoring to record basic

breeding parameters (laying date, final clutch size, hatching date, number of hatchlings and fledging success). Males were caught before pair formation in most parts of the study area (only in 2008) or during the chick feeding period. Females were caught during incubation. Nestlings were also measured when 12 days old (tarsus and body mass), and averages of each brood were analysed. Because about 30% of offspring are caught as recruits for the first time when 2 years old (Doligez, Gustafsson & Pärt 2009), we collected breeding data from all recruits during 2 years. For parents, we decided to limit the data to the following year (2008) only to avoid cumulative effects on breeding success of an experimental and a non-experimental year. For the purpose of another experiment, part of the nestlings of experimental recruits and parents were cross-fostered on day 2 after hatching (always retaining the original brood size), and we always used the fostered nestling condition, because nestling condition has been shown to be largely dependent on growth environment compared to genetics (e.g. Merilä, Kruuk & Sheldon 2001). Part of the nests of recruits and experimental parents were also included in brood size manipulations; this was controlled for in the statistical analyses.

Environmental conditions seemed to drastically differ during the two breeding seasons after the manipulation (i.e. 2008 and 2009). To quantify this difference, we obtained weather data from a meteorological station close to the study population (Table S1, Supporting Information). Temperatures were colder and rain was more abundant, reflecting harsher weather conditions, in 2009 than in 2008 (Table S1, Supporting Information). Thus, the potential effect of different environmental conditions on breeding parameters was accounted for in the statistical analyses by including year as a fixed factor. Local habitat quality can also be an important factor determining the offspring quality. In a subset of experimental nests, fledging condition strongly differed between two of the patches where the experiment was conducted (Pitala *et al.* 2009). However, (i) the experimental manipulations were conducted in a total of 12 different areas overall, with both treatments homogeneously distributed in space, (ii) recruits and parents may disperse to different patches in the study area (Pärt & Gustafsson 1989) and (iii) local habitat quality varies in time and space in this population (Doncaster *et al.* 1997). It is therefore unlikely that local return rates and breeding variables of recruits and parents show systematic biases with respect to treatment due to local habitat quality.

STATISTICAL ANALYSES

All statistical analyses were conducted with SAS 9.2 program (SAS Institute Inc., Cary, NC, USA). Because nestlings were sexed in half of the experimental nests only, we could not test the sex-specific effect of treatment on the probability of local recruitment of each offspring in the following breeding seasons using generalized linear mixed models. We therefore used two different approaches. First, we calculated the expected number of recruits for each sex and treatment and compared observed and expected recruit numbers using a 2 by 2 (two sexes and two treatments) chi-square test. Expected numbers of recruits were calculated based on the total number of fledged young, the total number of recruits and the proportion of males in the sexed broods. Importantly, we assume that fledglings were equally distributed between treatments, which is supported by the absence of sex-specific mortality in the sexed broods (see Pitala *et al.* 2009). For details on the calculations of the expected numbers of recruits, see Appendix S1 (Supporting Information). Second, for each sex separately (as a *post hoc* test), we directly compared the number of observed recruits in each treatment using a chi-square test, to test the assumption of equal numbers of recruits in both treatment groups per

sex. We included in the analysis all recruits captured either in 2008 or in 2009 (caught early in the season and during breeding, see above), but including only recruits captured in year 2008 yielded qualitatively similar results.

Age at first breeding of experimental offspring, that is first breeding at age 1 (in 2008) or 2 (in 2009), was analysed using a generalized linear model (proc GENMOD, binomial distribution and logit link). It is possible that part of the recruits first caught breeding in 2009 were breeding outside our study area already in 2008. However, we aimed at comparing the observed age at first breeding between treatment groups and assume here that the proportion of missing individuals should be similar in both groups, that is, temporary dispersal is not affected by our treatment (see Discussion). Timing of breeding (laying date of the first egg) and clutch size of the experimental offspring were analysed using a mixed model (proc MIXED, normal distribution). Hatching success, that is number of hatched eggs/clutch size, was analysed using a generalized linear mixed model (proc GLIMMIX, binomial distribution of events/trials, logit link). Fledging success, that is the number of fledged young, was analysed with a generalized linear model (proc GLIMMIX, negative binomial distribution). Tarsus and body mass at fledging were analysed using a mixed model (proc MIXED, normal distribution). In the analysis of body mass, we included tarsus length as a covariate to estimate the body condition.

In all analyses of offspring breeding parameters, sex, androgen treatment and their interaction were included as explanatory variables. We also included year and its interactions with sex and treatment (i.e. testing whether breeding success of recruits from different treatments is differently affected by environmental conditions). Nests used in large brood size manipulation experiments (i.e. ≥ 2 chicks added or removed, $n = 3$) were excluded from the analysis of fledging success and nestling size and condition, but nests where brood size was manipulated by only one chick ($+1$ or -1 , $n = 11$) were included and manipulation was controlled for in the analysis. We included bird identity as a random intercept effect in all analyses of offspring breeding parameters, because some birds were caught in both years following the manipulation. Thus, only one random factor was included in each model.

The probability for parents of experimental nests to return in the year following the yolk androgen manipulation (2008) was analysed using generalized linear mixed model (proc GLIMMIX, binomial distribution and logit link) with parental sex, androgen treatment of the nest and their interaction as explanatory variables. Nest (in year 2007) was included both as a random intercept and as a random coefficient on sex (Schielzeth & Forstmeier 2009). Timing of breeding, clutch size, hatching and fledging success, parental condition at the chick feeding stage, nestling size and condition for experimental parents in the year following the manipulation were analysed with similar models as for offspring (using proc GLIMMIX), except that bird identity and year were excluded. The parents of experimental nests where none of the eggs hatched were excluded from the analyses, because no effect of androgen manipulation on subsequent survival or reproductive success of parents was expected in this case. Nests used in large brood size manipulation experiments were again excluded from the analysis of fledging success and nestling condition ($n = 14$, see above). In addition, in the analyses of both offspring and parental breeding data, we controlled for covariates that are known to affect the breeding parameters in the study population (see Appendix S2, Supporting Information for details). Including these covariates did not qualitatively change the results; thus, they were removed from final models and are not presented here.

For all analyses, the normality of model residuals was checked. The Kenward–Roger method was used to calculate the degrees of

freedom of fixed effects (Littell *et al.* 2006). We used a backward model selection procedure starting with the least significant terms, always retaining treatment, sex and their interaction in the models. To confirm the non-significance of the removed covariates, each term was added to the final model separately. To further assess the biological significance of the differences tested, we also calculated effect sizes (Cohen's *d*, Cohen 1988) for breeding parameters of offspring and parents, separately for each sex.

Results

LONG-TERM EFFECTS OF YOLK ANDROGENS ON RECRUITS

We caught in total 45 experimental recruits in the two study years (37 recruits in 2008 and 8 new recruits in 2009) and recorded the breeding parameters of 52 breeding events (31 in 2008 and 21 in 2009; six male recruits caught early in 2008 were not caught as breeders later on; see above). The total number of experimental recruits caught was lower than expected (see Materials and methods and Doligez, Gustafsson & Pärt 2009), which was probably due to low breeding success, thus low capture rate of new breeding recruits in 2009. Overall, the observed number of recruits differed between sexes and treatment groups ($\chi^2_1 = 6.18$, $P = 0.009$, Fig 1a, see Appendix S1, Supporting Information): local recruitment rate was lower than expected in androgen-treated males compared to other groups. Sex-specific tests confirmed that recruitment was lower for male offspring from the androgen than control treatment, whereas there was no such difference in females (control vs. androgen-treated, males: $\chi^2_1 = 4.26$, $P = 0.039$; females: $\chi^2_1 = 0.15$, $P = 0.69$, Fig 1a). The same result was obtained when restricting to local recruitment in one breeding season (2008) after the androgen manipulation only (general test including both sexes: $\chi^2_1 = 7.13$, $P = 0.007$; sex-specific tests: control vs. androgen-treated, males: $\chi^2_1 = 6.25$, $P = 0.012$; females: $\chi^2_1 = 0.05$, $P = 0.83$).

Recruit breeding parameters according to sex and yolk androgen treatment are described in Table 1a. Age at first breeding was not affected by the androgen treatment ($\chi^2_1 = 0.05$, $P = 0.82$), sex ($\chi^2_1 = 1.71$, $P = 0.19$) or their interaction ($\chi^2_1 = 0.12$, $P = 0.73$). Timing of breeding, clutch size, hatching and fledging success, nestling size and condition were also not affected by the androgen manipulation in either sex (Tables 1a and 2a–f). The effect sizes for breeding parameters of recruits also provide little support for differences between treatment groups for any of the parameters in either sex, except nestling size and condition (Table 3). Nestling measurements appeared higher for androgen-treated compared to control males, but the sex-by-treatment interaction was far from approaching significance (Table 2e,f), probably due to small sample sizes (Table 1a). These effects thus remain speculative, and we therefore do not further comment on them. Timing of breeding was earlier in 2009 than in 2008 (marginal means \pm SE, 21.04 ± 1.10 and 17.20 ± 1.36 for years 2008 and 2009, respectively; 1 = 1st May, Table 2a),

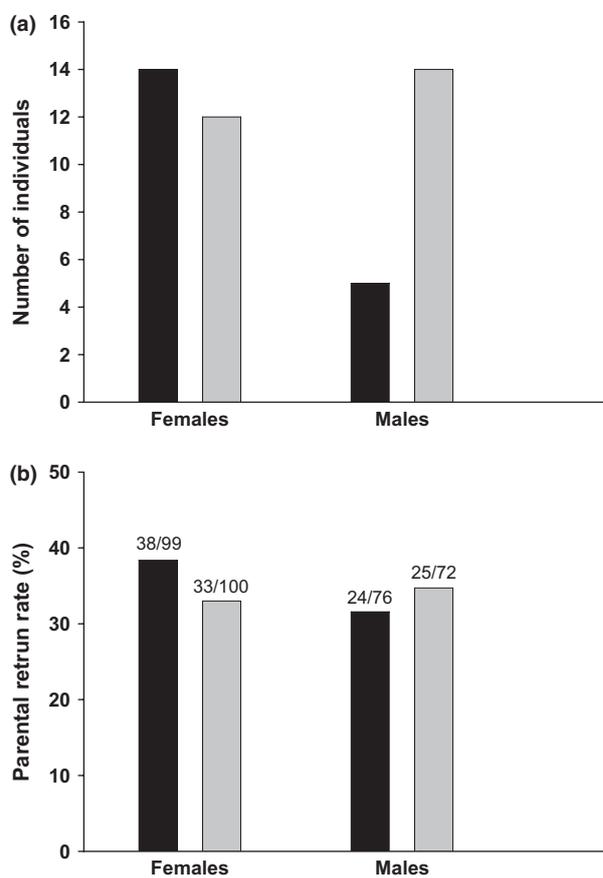


Fig. 1. (a) Number of experimental offspring locally recruited and (b) parental return rate for the androgen-manipulated (black bars) and control (grey bars) nests during the subsequent breeding season(s), according to sex. Graphs show (a) total number of offspring that returned (2008–2009) and (b) percentage of returning individuals for parents. Figures indicate the number of parents that returned to breed in 2008/the number of experimental parents caught in 2007.

which likely reflects a recruit age effect because year and breeding experience were highly correlated (see Appendix S2, Supporting Information). Overall, male recruits had more fledglings than females [Table 2d, back-transformed marginal means with asymmetrical SE, males: 3.68 (upper and lower SE, 0.8, 0.65); females: 2.01 (0.40, 0.34)], but this difference most likely reflects the fact that males were caught at a later step (during chick feeding) than females (during incubation), which biases male breeding success towards successful nests (males of unsuccessful nests have an overall lower probability of being caught and thus identified; Doligez *et al.* 2011). The breeding parameters of androgen-treated and control recruits of either sex were not differently affected by the environmental conditions (i.e. year) (all P -values for main effect of year and all interactions with year > 0.11).

LONG-TERM EFFECTS OF YOLK ANDROGENS ON PARENTS

We caught in total 347 parents of experimental nests (199 females and 148 males) in the year of yolk androgen manipulation (2007); 118 of them were recaptured in 2008 (70 females

Table 1. Breeding parameters for (a) recruits (years 2008–2009) and (b) parents (year 2008) of the experimental nests in the following breeding season(s)

Sex	Treatment	Timing of breeding		Clutch size		No. of hatchlings		No. of fledglings		Nestling body mass		Nestling tarsus	
		Mean (SD)	<i>n</i>	Mean (SD)	<i>n</i>	Mean (SD)	<i>n</i>	Mean (SD)	<i>n</i>	Mean (SD)	<i>n</i>	Mean (SD)	<i>n</i>
(a) Recruits													
Female	CO	20.2 (5.8)	16	6.2 (1.1)	16	5.4 (1.5)	16	2.1 (1.9)	15	12.4 (2.8)	10	18.6 (1.5)	10
Female	AT	20.1 (6.1)	19	5.9 (0.5)	19	5.4 (0.9)	19	2.1 (2.2)	18	13.7 (1.5)	8	19.2 (0.5)	8
Male	CO	18.1 (4.6)	10	6.2 (1.0)	10	4.9 (1.6)	10	3.6 (2.4)	8	13.2 (1.9)	8	19.2 (0.9)	8
Male	AT	19.5 (6.7)	6	6.3 (0.5)	6	5.8 (1.2)	6	3.7 (3.2)	6	14.5 (1.6)	2	20.0 (0.1)	2
(b) Parents													
Female	CO	19.6 (5.4)	30	6.4 (0.7)	30	5.6 (1.4)	30	2.8 (2.1)	24	12.1 (2.0)	20	18.5 (1.2)	20
Female	AT	19.1 (7.0)	34	6.5 (1.1)	34	5.4 (2.0)	35	3.3 (2.8)	31	12.6 (1.9)	25	18.5 (1.0)	25
Male	CO	17.2 (5.7)	18	6.5 (0.7)	18	5.9 (0.8)	18	4.2 (2.4)	15	12.7 (1.9)	16	19.0 (1.2)	16
Male	AT	18.5 (1.1)	19	6.0 (1.3)	19	5.2 (1.8)	19	3.7 (2.5)	17	13.6 (1.2)	14	19.3 (0.4)	14

AT, androgen treatment; CO, control.

Mean (SD) values are shown according to sex and yolk androgen treatment. For timing of breeding, 1 = 1st May. For fledgling number, size and condition, nests used in large brood size manipulation experiments were excluded (see text), explaining the lower sample sizes. Average nestling mass and tarsus length of 12 day nestlings per nest is presented.

Table 2. Effects of yolk androgen treatment on the breeding parameters of recruits. Breeding parameters in two subsequent breeding seasons after the androgen-manipulation experiment (2008 and 2009) have been analysed. Environmental conditions (year) and brood size manipulation (see Materials and methods) were included in the models and are shown only when significant. In the analysis of fledgling number, size and condition, nests used in large brood size manipulation experiments were excluded (see text). We included bird identity as a random effect in all models, because some birds were caught in both years following the manipulation. Nd.f. and dd.f. indicate the numerator and denominator degrees of freedom

	Nd.f., dd.f.	<i>F</i>	<i>P</i>
(a) Timing of breeding, <i>N</i> = 52			
Androgen treatment	1, 36	0.25	0.47
Sex	1, 35.8	0.37	0.46
Androgen treatment × sex	1, 36	0.12	0.63
Year	1, 31.1	4.37	0.04
(b) Clutch size, <i>N</i> = 52			
Androgen treatment	1, 48	0.00	0.99
Sex	1, 48	0.30	0.58
Androgen treatment × sex	1, 48	0.84	0.36
(c) Hatching success, <i>N</i> = 52			
Androgen treatment	1, 43.82	1.62	0.21
Sex	1, 43.82	0.42	0.52
Androgen treatment × sex	1, 42.32	0.47	0.49
(d) Fledging success, <i>N</i> = 48			
Androgen treatment	1, 17.18	0.04	0.84
Sex	1, 17.18	4.88	0.04
Androgen treatment × sex	1, 17.18	0.06	0.81
(e) Nestling tarsus, <i>N</i> = 28			
Androgen treatment	1, 24	2.29	0.14
Sex	1, 24	1.98	0.17
Androgen treatment × sex	1, 24	0.06	0.81
(f) Nestling condition, <i>N</i> = 28			
Androgen treatment	1, 23	0.00	0.98
Sex	1, 23	0.39	0.54
Androgen treatment × sex	1, 23	0.08	0.78
Tarsus length	1, 23	43.44	<0.0001

and 48 males) and 106 were breeders (67 females and 39 males; the remaining 12 were caught early only). The probability for parents to return in the year following the manipulation was affected neither by their sex nor by the androgen treatment of their clutch (treatment, $F_{1,195.1} = 0.26$, $P = 0.61$; sex, $F_{1,343} = 0.28$, $P = 0.60$; treatment × sex interaction, $F_{1,343} = 0.50$, $P = 0.48$, Fig. 1b). Timing of breeding, clutch size, hatching and fledgling success, nestling size and condition in the breeding season following the androgen manipulation were not affected by the androgen treatment of the clutch for parents of either sex (Tables 1b and 4a–g). The effect sizes for breeding parameters of parents also provide little support for differences between treatment groups in any of the parameters in either parental sex (Table 3).

Discussion

We examined the long-term fitness consequences of elevated yolk androgens in both parents and offspring in a wild bird population. We found that high early androgen exposure lowered the local recruitment rate of male, but not female offspring in subsequent breeding seasons. We however detected no differences in age at first breeding or breeding parameters (timing of breeding, clutch size, hatching or fledgling success, nestling body size and condition) between recruits from the androgen and control treatments in either sex. Furthermore, raising a brood hatched from eggs with elevated yolk androgen levels did not affect local return rate or breeding parameters of the parents in the following season. If local return rate reflects survival or breeding prospects, these results suggest that potential trade-offs related to yolk hormone deposition may arise mainly through sex-specific direct effects of yolk androgens on offspring fitness rather than indirect costs on parents' fitness (note again that direct costs on mothers have not been investigated here).

Table 3. Effect sizes (Cohen's *d*) of yolk androgen treatment on breeding parameters of both recruits and parents of experimental nests. Effects sizes have been calculated separately for each sex, comparing androgen-treated vs. control individuals (a positive value indicating that the parameter is higher for androgen-treated than control individuals). An effect size below 0.2 is considered to indicate a small difference, around 0.5 a moderate difference and above 0.8 a large difference (Cohen 1988)

	Effect sizes (Cohen's <i>d</i>)				Nestling tarsus	Nestling body mass
	Timing of breeding	Clutch size	No. of hatchlings	No. of fledglings		
(a) Recruits						
Females (AT vs. CO)	-0.02	-0.28	-0.01	0.04	0.54	0.58
Males (AT vs. CO)	0.18	0.29	0.6	0.04	1.25	0.74
(b) Parents						
Females (AT vs. CO)	-0.08	0.11	-0.11	0.2	0	0.26
Males (AT vs. CO)	0.31	-0.48	-0.12	0.2	0.34	0.57

Table 4. Effects of the yolk androgen treatment of the clutch on the breeding parameters of the parents in the year following the androgen-manipulation experiment (2008). In the analysis of fledgling number, size and condition, nests used in large brood size manipulation experiments were excluded (see text). Nd.f. and dd.f. indicate the numerator and denominator degrees of freedom

	Nd.f., dd.f.	<i>F</i>	<i>P</i>
(a) Parental condition, <i>N</i> = 99			
Androgen treatment	1, 94	0.48	0.49
Sex	1, 94	10.97	0.003
Androgen treatment × sex	1, 94	0.25	0.61
Tarsus length	1, 94	8.59	0.004
(b) Timing of breeding, <i>N</i> = 101			
Androgen treatment	1, 97	0.07	0.79
Sex	1, 97	1.05	0.30
Androgen treatment × sex	1, 97	0.36	0.54
(c) Clutch size, <i>N</i> = 105			
Androgen treatment	1, 101	1.62	0.21
Sex	1, 101	0.49	0.49
Androgen treatment × sex	1, 101	1.30	0.26
(d) Hatching success, <i>N</i> = 102			
Androgen treatment	1, 98	1.66	0.20
Sex	1, 98	1.13	0.29
Androgen treatment × sex	1, 98	0.29	0.59
(e) Fledgling success, <i>N</i> = 87			
Androgen treatment	1, 34	0.43	0.51
Sex	1, 34	2.37	0.13
Androgen treatment × sex	1, 34	0.61	0.41
(f) Nestling tarsus, <i>N</i> = 76			
Androgen treatment	1, 72	1.66	0.20
Sex	1, 72	3.37	0.07
Androgen treatment × sex	1, 72	0.06	0.89
(g) Nestling condition, <i>N</i> = 76			
Androgen treatment	1, 71	1.25	0.26
Sex	1, 71	0.72	0.39
Androgen treatment × sex	1, 71	0.44	0.55
Tarsus length	1, 71	85.14	< 0.0001

Sex-specific costs of yolk androgens on offspring may therefore constrain optimal deposition of androgens and may thus select for sex-specific deposition mechanisms. Alternative mechanisms are also discussed later.

CONSEQUENCES OF HIGH YOLK ANDROGEN LEVELS FOR OFFSPRING

Several non-mutually exclusive explanations can be proposed for the lower local recruitment rate of male offspring from the androgen compared to control treatment: (i) lower survival, (ii) higher non-breeding or early breeding failure probability and/or (iii) higher dispersal probability out of the study area. The effects of yolk androgens on early development provide support for the lower survival alternative: males from androgen-treated eggs reached smaller body size 12 days post-hatching than controls (Pitala *et al.* 2009), and offspring size at fledging has been suggested to positively affect first-year survival in many species, including collared flycatchers (e.g. Gustafsson & Sutherland 1988; Lindén, Gustafsson & Pärt 1992; Potti *et al.* 2002). This is probably the most likely explanation for our results. However, higher mortality of male offspring from androgen-treated eggs may also result from the immune suppressive effect of high androgen levels or higher predation risk via androgens affecting behavioural traits, both of which have been reported to be sex-specific (see Introduction). Long-term effects of yolk androgens on survival in the wild are poorly known; the only existing experimental study found no effect on recruitment in either sex in great tits (Tschirren, Fitze & Richner 2007). However, correlative data from a Hungarian collared flycatcher population indeed suggested that yolk androstenedione concentration may be positively correlated with recruitment rate, although sex-dependent effects were not reported (Hegyí *et al.* 2011; see also Groothuis *et al.* 2005; Rutkowska & Cichoń 2006 for sex-specific effects on survival until fledging).

Alternatively, the lower return rate of male offspring from the androgen compared to control treatment could reflect lower breeding probability or higher early breeding failure, thus lower capture probability, rather than lower survival. Because capture probability is strongly linked to breeding status and success in this population, an effect of yolk androgens on capture probability should still reflect a fitness effect. The effects of yolk androgens on male behaviour or phenotype (e.g. plumage characteristics, aggressiveness or song quality; reviewed in Groothuis *et al.* 2005; Gil 2008) have often been predicted to increase male attractiveness or ability to occupy

high-quality territories (Groothuis *et al.* 2005; Müller & Eens 2008), but the empirical evidence is conflicting (e.g. Rubolini *et al.* 2006; Bonisoli-Alquati *et al.* 2011b). Here, yolk androgens could have decreased male attractiveness or territory quality, leading to low local recruitment. Yolk androgens may also increase the breeding failure probability, for example by decreasing the male parental care (Ketterson & Nolan 1992, 1999), and thus such effects need to be further studied. Finally, high yolk androgen levels may also increase the male natal dispersal probability or distance (see Tschirren, Fitze & Richner 2007), thereby decreasing the male capture rate within the study population (see Doligez & Pärt 2008). Such a sex-specific effect of yolk androgens on natal dispersal has not been previously documented and therefore should be further explored. Identifying the proximate mechanisms underlying the effect of early androgen exposure on local recruitment of male offspring thus needs further investigation.

Whatever the mechanism, the lower local recruitment of males from androgen-treated eggs potentially reflects a fitness cost for males (and thus for parents as well) because this low local recruitment was not compensated by higher breeding performance. The fitness consequences of a lower local recruitment explained by higher dispersal out of the study area are not entirely clear, but dispersal costs have been suggested in many species (reviewed in e.g. Béchon, Clobert & Massot 1996; Doligez & Pärt 2008; Bonte *et al.* 2011), including the collared flycatcher (Pärt 1991, 1994). Furthermore, long-term costs and benefits of early androgen exposure are expected to be context dependent (Groothuis *et al.* 2005). In line with this expectation, environmental and social conditions, such as breeding density, parasite load, food supply, mate attractiveness or female condition, have been shown to correlate with variation in yolk androgen levels (reviewed in Groothuis *et al.* 2005; Gil 2008). We found that the different environmental conditions in the two study years did not differently affect the breeding parameters in relation to androgen treatment. However, other factors such as breeding density and the level of competition for access to breeding may promote such context dependence. Experimental manipulations would be needed to specifically test the impact of environmental and social factors on the fitness consequences of variation in yolk androgen levels. So far, only a few experimental studies have tested the long-term adaptive significance of this variation, especially on birds (Tschirren, Fitze & Richner 2007).

EVOLUTION OF HORMONE-MEDIATED MATERNAL EFFECTS: COSTS, CONSTRAINTS AND FAMILY CONFLICTS

Understanding the evolution of hormone-mediated maternal effects requires assessing their fitness consequences and constraints from the perspective of all family members. Both parent-offspring conflict and sexual conflict over parental investment have been suggested to shape the evolution of yolk hormone deposition (see Introduction). High yolk androgen

levels have been shown to potentially entail high direct costs for mothers (reviewed in Groothuis *et al.* 2005), and our results suggest potential fitness costs of high yolk androgen levels for male offspring without clear fitness benefits for female offspring. Therefore, overall, low yolk androgen levels could be beneficial for both offspring and parents, thus implying a lack of parent-offspring conflict. This further suggests that the original yolk androgen levels in experimental clutches may have been optimal here. Furthermore, the lack of sex-specific indirect effects on parents both in the short term (Ruuskanen *et al.* 2009) and in the long term (this study) in our study species suggests that females and males do not respond differently to characteristics of nestlings from eggs with different androgen levels. Thus, yolk androgens are unlikely to mediate sexual conflict over parental care in this species.

Sex-specific effects of yolk androgens may further constrain the evolution of yolk androgen deposition (e.g. Müller *et al.* 2005; von Engelhardt *et al.* 2006; Rubolini *et al.* 2006; Rutkowska & Cichoń 2006; Saino *et al.* 2006; Rutkowska, Wilk & Cichoń 2007; Partecke & Schwabl 2008; Sockman *et al.* 2008; Pitala *et al.* 2009; Ruuskanen & Laaksonen 2010). The possible fitness cost found here for male offspring should select for low levels of yolk androgens in male eggs. Such sex-specific long-term fitness effects on offspring may therefore select for differential yolk androgen deposition by mothers according to offspring sex (e.g. Müller *et al.* 2002; Gilbert *et al.* 2005; Groothuis *et al.* 2005; Gil 2008), for example via sex-specific temporal clustering of growing oocytes (e.g. Young & Badyaev 2004; Badyaev *et al.* 2005, 2006b; Badyaev, Oh & Mui 2006a). However, it is not clear whether such a mechanism is possible, because sex determination (meiotic division) occurs after yolk deposition (see Badyaev *et al.* 2005, 2006b; Badyaev, Oh & Mui 2006a; Groothuis & Schwabl 2008; Rutkowska & Badyaev 2008). Alternatively, selection could act on offspring sensitivity to yolk androgens in a sex-specific way, thus decreasing male sensitivity to androgens (for example, through different receptor locations and densities; see e.g. Müller *et al.* 2007; Groothuis & Schwabl 2008). However, this may also incur costs because the same receptors are involved in sexual differentiation (Carere & Balthazart 2007). Finally, because females did not appear to benefit from elevated yolk androgen levels in the long-term (a weak positive effect on female nestling growth was previously reported, Pitala *et al.* 2009), it appears that there is no evidence for selection towards higher yolk androgen levels in this population. It needs to be remembered that such selection could however occur as a by-product of selection on female physiology during egg-laying or over the whole life span of an individual, which we could not study here. Whether the current yolk androgen levels are individually optimized or whether selection could occur towards lower yolk androgen levels still remains to be investigated.

It has been recently shown that yolk androgen deposition in our study population is heritable (Tschirren *et al.* 2009). The possible long-term fitness effect of yolk androgens revealed in this study (given that low local return rate reflects

low survival) thus opens up the possibility for hormone-mediated maternal effects to respond to selection and thereby affect evolutionary processes. The potential for maternal effects, including yolk androgens, to mediate evolutionary change is indeed gaining increasing interest in empirical studies (e.g. Badyaev *et al.* 2002, 2003; Reinhold 2002; Räsänen & Kruuk 2007; Badyaev 2008; Uller 2008; Badyaev & Uller 2009). Such mediating effects could occur for example via androgens affecting the evolution of behavioural syndromes (e.g. Tobler & Sandell 2007; Groothuis *et al.* 2008; Ruuskanen & Laaksonen 2010) or the colonization of new environments and the dynamics of spatial distribution of populations (e.g. Kokko & López-Sepulcre 2006; Duckworth & Badyaev 2007; Tschirren, Fitze & Richner 2007). These processes clearly deserve further investigation.

Acknowledgements

This study was financially supported by Turku University foundation, Finnish Cultural foundation (grants to S.R.), Emil Aaltonen Foundation (a grant to T.L.), the ANR (Agence Nationale de la Recherche – grant ANR-06-JCJC0082 to B.D.) and the CNRS (Centre National de la Recherche Scientifique – PICS) (grants to B.D.). We thank Maaike de Heij, Tuomo Jaakkonen, Melissa Lemoine, Christoph Meier and all the field assistants, especially Lise Duconte, Laure Cauchard and Laurent Brucy, for help in collecting the breeding data.

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Received 22 July 2011; accepted 16 March 2012

Handling Editor: Keith Sockman

Supporting Information

Additional supporting information may be found in the online version of this article.

Appendix S1. Calculations of the expected number of recruits for each sex and treatment.

Appendix S2. Effect of covariates in the analyses of breeding data of experimental recruits and parents.

Table S1. Comparison of environmental conditions between the two study years.

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