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ORIGINAL PAPER

Long-term effects of yolk androgens on phenotype and parental feeding behavior in a wild passerine

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Abstract Early growth conditions, such as exposure to maternally derived androgens in bird eggs, have been shown to shape offspring in ways that may have important long-term consequences for phenotype and behavior. Using an experimental approach, we studied the long-term effects of yolk androgens on several phenotypic traits and parental behavior in adult and female collared flycatchers (*Ficedula albicollis*). We elevated yolk androgen levels and monitored the experimental recruits the following breeding seasons. Androgen treatment had a sex-dependent effect on adult body condition, yolk androgen-treated males being heavier than control males when controlling for size, a result which may be caused potentially by selective mortality, physiological differences, or different life-history strategies. Androgen treatment did not however affect the expression of sexually selected plumage ornaments (forehead and wing patch size), UV coloration, or parental feeding rate in either sex. Our results suggest that yolk androgens are unlikely to affect sexual selection via plumage characteristics or contribute to breeding success via altered parental care. Yolk androgens do not seem to act as a means for female collared flycatchers to enhance the attractiveness of their sons. The lower return rate previously observed for androgen-treated male offspring compared to controls may therefore not be due to lower mating or breeding success, but may rather reflect lower survival or higher dispersal propensity of yolk androgen-treated males.

Keywords Maternal effect · Testosterone · Bird · Plumage trait · Sexual selection

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Introduction

In addition to direct genetic effects, mothers influence the phenotype of their offspring via providing, for example, resources and care. These effects of maternal phenotype on offspring phenotype are referred to maternal effects (Mousseau and Fox 1998). Maternal effects seem to have long-lasting consequences on adult phenotype and fitness in several taxa, sometimes with adaptive consequences (reviewed by Bernardo 1996a; Bernardo 1996b; Mousseau and Fox 1998; Galloway 2005; Groothuis et al. 2005; Gil 2008; Hasselquist and Nilsson 2009). Especially transfer of maternal hormones to the eggs seems to be a major source of offspring phenotypic variation (from growth up to adult phenotype, behavior ,and reproductive physiology) and hormones play a potential role in mediating life-history trade-offs (Dufty et al. 2002; Groothuis et al. 2005; Uller et al. 2007; Gil 2008). Most previous experimental studies on egg yolk androgens have focused on their effects on early life stages (i.e., offspring growth, development, and behavior: Uller and Olsson 2003; Groothuis et al. 2005; Uller et al. 2007; Gil 2008; Navara and Mendonça 2008). However, because yolk androgens may have organizational effects and long-lasting activational effects (for example by modifying

receptor sensitivity or hormone production later in life), long-lasting consequences of early androgen exposure on offspring phenotype and behavior may be expected (reviewed in Carere and Balthazart 2007; Groothuis and Schwabl 2008; see also Müller et al. 2012).

Recently, a rapidly increasing number of studies in birds have indeed reported long-lasting effects of yolk androgens for example on adult plumage characteristics, behavior, and physiology (Strasser and Schwabl 2004; Daisley et al. 2005; Eising et al. 2006; Rubolini et al. 2006; Tschirren et al. 2007; Müller et al. 2008; Müller and Eens 2009). Plumage ornaments have been found to be larger in males originating from eggs with high yolk androgen levels in some species (Strasser and Schwabl 2004; Eising et al. 2006; reviewed in Müller and Eens 2009), but smaller/shorter in other species (e.g., spur length or wattle color in pheasants, Rubolini et al. 2006; Bonisoli-Alquati et al. 2011a). Long-lasting behavioral effects of yolk androgens have also been found, such as increased general activity, aggressiveness, boldness, and anti-predator behavior (Strasser and Schwabl 2004; Daisley et al. 2005; Eising et al. 2006; Tobler and Sandell 2007; Partecke and Schwabl 2008; Ruuskanen and Laaksonen 2010). However, not all studies detected such effects on adult phenotype and behavior (e.g., Müller and Eens 2009). The physiological mechanism for yolk androgens enhancing for example sexually selected plumage traits and behavior may relate in case of hormone-dependent traits, for example to increased sensitivity to androgen hormones as adults (e.g., Carere and Balthazart 2007). Elevated levels of androgens in eggs may also be aromatized to estradiol, which then may cause inhibition or decrease of male-like plumage traits (see e.g., Rubolini et al. 2006; Owens and Short 1995). Thus, the long-term effects of yolk androgens on adult phenotype and behavior are not completely clear, and studies from wild birds are rare.

Circulating testosterone levels have often been found to decrease male parental care (Ketterson and Nolan 1992; Ketterson et al. 1992), an important determinant of breeding success in socially monogamous species, but we do not currently know whether yolk androgens may affect parental care as well via, e.g., an effect on circulating testosterone levels at adulthood (Carere and Balthazart 2007; Groothuis and Schwabl 2008). Finally, yolk androgens may have direct effects on female reproductive physiology and thus breeding performance via a decrease in copulation rate, egg number, and/or egg size (Uller et al. 2005; Rubolini et al. 2007; Müller et al. 2009; Bonisoli-Alquati et al. 2011b, but see Rutkowska et al. 2007). These changes in adult behavioral, morphological, or physiological traits in relation to yolk androgens experienced during growth could thus affect adult survival and reproductive success via, e.g., altered mating success, predation risk, parental care, or reproductive physiology (Partecke and Schwabl 2008; Müller and Eens 2009; Müller et al. 2009), potentially leading to fitness consequences (Ruuskanen et al. 2012).

If yolk androgens affect sexually selected male traits (e.g., ornaments or behavior) females could increase the attractiveness of their sons via differential yolk androgen deposition in their eggs (e.g., Groothuis and Von Engelhardt 2005). Depending on maternal strategies and costs of hormones transfer, females could be predicted to allocate high levels of hormones to (1) more valuable, high quality offspring (when pairing with high quality males) or, alternatively (2) low-quality offspring to compensate the lower expected quality/attractiveness of their sons (if heritable, when pairing with low quality males) (see, e.g., Gil et al. 1999; Michl et al. 2005; reviewed in Kingma et al. 2009). This maternal strategy could be favored especially when the variance in male mating success is high (e.g., in polygynous species). Yolk androgens seem to increase offspring quality or attractiveness (e.g., Strasser and Schwabl 2004; Eising et al. 2006; reviewed in Müller and Eens 2009), and there is some empirical evidence for both predictions. In some species, females allocate more androgens when paired with low quality/less attractive males (e.g., Michl et al. 2005; Navara et al. 2006; Laaksonen et al. 2011), whereas in other species, females mated with high quality males deposit high yolk androgens levels in their eggs (e.g., Gil et al. 1999; reviewed, e.g., in Groothuis and Von Engelhardt 2005; Gil 2008). However, yolk androgens do not always seem to affect sexually selected male traits (Rubolini et al. 2006; Müller and Eens 2009). Thus, it is important to continue assessing the potential role of yolk androgens as a maternal strategy to adjust their sons' quality or attractiveness.

Using an experimental approach, we studied the effects of yolk androgens on several adult phenotypic traits (morphological and plumage traits) and parental care behavior (feeding frequency) in a wild population of collared flycatchers (*Ficedula albicollis*). We elevated yolk androgen levels in eggs and measured phenotypic traits and parental feeding frequency of male and female experimental recruits during the three subsequent breeding seasons. Two mutually exclusive hypotheses on the effects of yolk androgens on especially male traits can be proposed: Firstly, if yolk androgens enhance sexually selected male traits, they could potentially function as a female strategy to increase the attractiveness of their sons (see above). To support this hypothesis, it has been found in our study species and a closely related species, the pied flycatcher (*Ficedula hypoleuca*), that females deposit higher yolk androgen levels when paired with young, potentially lower quality males (Michl et al. 2005; Laaksonen et al. 2011). If yolk androgens enhance sexually selected traits, depositing higher levels when pairing with low quality males could be a compensatory strategy. A second, alternative hypothesis is that yolk androgens may be expected to decrease sexually selected

male traits our study population. To support this latter hypothesis, we previously found that androgen-treated males had lower local recruitment rate but similar breeding success than control males, no difference among treatment groups being found in females (Ruuskanen et al. 2012). Because recapture rate is linked to breeding status and success, low recruitment of androgen-treated males could result from high mortality or dispersal, non-breeding or early breeding failure (i.e., non-capture). If androgen treated males would express shorter/smaller sexually selected traits, this could lead to lower attractiveness; consequently, a lower mating success for androgen-treated males could partly explain their low local recruitment rate. However, because the effects of yolk androgens on male traits at adulthood are still unclear (see references above), and because the effect of male quality on deposition of yolk androgens has not been previously described in our study population, either of the two mutually exclusive hypotheses may be possible. Furthermore, we also recorded parental feeding rates and hypothesized that if androgen-treated males would show lower feeding rate (due to, e.g., higher circulating androgen levels or sensitivity to androgens, Ketterson and Nolan 1992; Carere and Balthazart 2007; Groothuis and Schwabl 2008) higher rate of early breeding failure may partly explain their low local recruitment rate.

Methods

Study site, study species, and general 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. The yolk hormone manipulations were conducted in 2007 and experimental recruits were monitored during breeding seasons 2008–2010. The collared flycatcher is a small (ca. 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 accepts to breed in artificial nest boxes, which provides easy access to detailed breeding data. We chose to study long-term effects of yolk androgens on adult phenotypic and behavioral traits in this particular population because local recruitment is relatively high for such a short-lived species (0.3–0.5 local recruits per nest; Gustafsson 1986). Average clutch size in this population is 6–7 eggs, and average brood size 3–4 nestlings at fledging. The general population monitoring includes checking nest boxes every other day to determine laying dates. All nests are further monitored to record final clutch size, hatching date, number of hatchlings and fledging success, and nestling body mass and tarsus length are measured at 12 days of age. Females are caught for identification, measurement and aging in the nest box using a swing-door trap during incubation and both parents when feeding 6- to 12-day-old nestlings. Therefore, when broods fail early, males cannot be identified and male adult catching is biased towards successful individuals (see Doligez et al. 2011). Furthermore, the species is facultatively polygynous, with approx. 10 % of males successful in attracting a secondary female. Polygynous males provide little parental care to the young of the secondary nest and consequently have lower chances to be identified.

Yolk androgen manipulation experiment

In spring 2007, on the estimated day of clutch completion (i.e., the sixth egg laying day), each experimental clutch was randomly assigned to either the control or the androgen-manipulation group. All the eggs in a clutch were treated the same way. The eggs were replaced by dummy eggs during the time of the injections (ca 30–60 min) and the procedure did not generate desertion in the experimental clutches (see Pitala et al. 2009; Ruuskanen 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 μl sesame oil. In the control group (N=120 nests), eggs were injected with 4 μl 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, 14.2 ng/yolk, SD 4.7 ng/yolk, maximum, 28.8 ng/yolk; A4 mean, 60.3 ng/yolk, SD 16.2 ng/yolk, maximum 111.1 ng/yolk). The injected amount of androgens thus corresponded to 3.1 times the SD for both T 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 (1,460 eggs in total), we aimed at obtaining about 70 recruits.

Hatching success of experimental nests (mean±SD, excluding deserted and predated nests, where no egg hatched)

was 70.2±24.3 % for androgen-treated nests and 74.1± 23.0 % for control-treated nests and did not differ between treatments (logistic regression: χ_1^2=1.32, p=0.25, N=210 nests). 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 hatching success. 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, to investigate the short-term effects of yolk androgens on feeding rates of the parents of experimental nests (Ruuskanen et al. 2009). In the other half of the nests, nestlings were not cross-fostered, but were blood sampled for molecular sexing and challenged with a subcutaneous injection of a non-pathogenic antigen, the phytohemagglutinin (PHA, Sigma, code L8754), to investigate the short-term effects of yolk androgens on nestling 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 is unlikely to affect our results on the long-term effects of yolk androgens. We checked this by including the PHA injection treatment (treated or not) as a fixed factor in all statistical analyses. As expected, the PHA injection treatment was not significant for any of the measured traits (results not detailed), and was therefore removed from final models.

Measurements from fledglings

Fledgling body mass (to the nearest 0.1 g), tarsus (to the nearest 0.1 mm) at 12 days of age were recorded as a part of the general population monitoring. Tarsus length is an important structural size trait that should be fixed at the end of the growth period (here at 12 days of age, Alatalo et al. 1990; Merilä 1997), and thus direct effects of androgens should be apparent then, while other morphological and plumage traits considered for recruits (see below) vary along adult's lifetime. Thus, in contrast to other traits measured at adulthood (see below), we analyzed tarsus length and body condition both at fledging (year 2007) and adulthood (years 2008–2010). Note that a sex difference on fledgling body size in response to our manipulation of yolk androgens has previously been reported for ca. half of the broods (Pitala et al. 2009). However, that analysis used an index combining body mass and tarsus length obtained from a Principal Component Analysis, and thus not directly tarsus length.

Measurements of experimental recruits

In the following three breeding seasons (2008–2010), reproductive attempts of experimental recruits were monitored from the end of April as part of the general population monitoring to record basic breeding parameters and phenotypic data (N=70 captures of 47 different recruits in total). In addition to the general monitoring of nests, males were caught in nest boxes before pair formation in most parts of the study area in 2008. We included experimental recruits caught in the 3 years after the manipulation in the analyses, because about 30 % of offspring are caught as recruits for the first time when 2 years old and 7 % when 3 years old (Doligez et al. 2004). Weather conditions were also particularly poor in 2009, which may have led to lower capture rate and/or higher rate of postponed breeding in that year.

The following morphological measurements were taken on recruits: body mass (to the nearest 0.1 g), tarsus (to the nearest 0.1 mm), wing and tail length (to the nearest 0.5 mm), white wing patch size (on primaries; in mm), UV coloration measured from the white part of the middle tertial feather and, for males, white forehead patch area (in square millimeter). Forehead and wing patch size are highly variable breeding plumage features in collared flycatchers: forehead patch size is an important sexually selected trait in males, related in particular to male–male competition (Pärt and Qvarnström 1997), female choice (Qvarnström et al. 2000) and parental care (Qvarnström 1997) in this population. Wing patch size (on the primaries) is also, but to a lesser extent, sexually selected in males (Sheldon and Ellegren 1999; de Heij et al. 2011). We estimated forehead patch area and wing patch size as described in Pärt and Qvarnström (1997). Since the white forehead patch is roughly rectangular in shape, its area was estimated by multiplying width and height (measured to the nearest millimeter). The size of the wing patch was measured as the sum of the length of white on the inner parts of the outer web of the first seven primaries (measured from the tips of primary coverts—see Pärt and Qvarnström 1997 for more details). The UV coloration is also a sexually selected male trait in pied flycatchers (Siitari et al. 2002) although its role in sexual selection in collared flycatchers, as well as its variation in females, have not been investigated so far. UV coloration was measured on the white part of a tertial feather because white feathers typically have higher UV reflectance than dark feathers (Eaton and Lanyon 2003; Sirkiä and Laaksonen 2009). We used the UV chroma, defined as the proportion of reflectance in the UV spectrum, i.e. (reflectance over the 320-400 nm interval)/(reflectance over the 320-700 nm interval) (Andersson and Prager 2006), measured in the laboratory with a spectrophotometer (Avantes 2048, Avantes DH-S light source; white standard WS-2). UV chroma was computed as the mean of three measurements from each feather. The repeatability of the UV chroma (sensu Lessells and Boag 1987), calculated on a random sample of 15 individuals, was high (r=0.89, p<0.0001).

We used feeding rate (shown to correlate positively with parental energy expenditure in this population: Pärt et al.

1992), as a measure of parental investment. Feeding rates were recorded at the nests of experimental recruits ca. 9 days post-hatching (mean nestling age±SD, 9.25±0.72 days) using a camouflaged video recorder placed at a minimum distance of 6–8 m from the nest. Feeding rates were all recorded during the morning (mean starting time±SD, 9.27±1.06 h). Nestling age and starting time of recordings were added as covariates in the analyses of feeding rate but were not significant (results not detailed) and were thus removed from final models. Ca. 2 h of feeding time was recorded and the first 15 min were excluded for habituation of the parents (mean duration of the recording analyzed± SD, 1 h 45 min±25 min). Feeding rate was measured for each parent as the number of feeding visits by the parent per hour. Feeding rate could not be measured for all recruits because of (1) early nest failure (N=13), (2) early caught recruits not observed breeding later on (N=6, in 2008), or (3) missing recording (N=5). Nests where no male was seen feeding during the recording were also excluded from the analyses (N=4). All measurements in the field and in the laboratory were done blindly with respect to the treatment received by the recruit.

Statistical analyses

All analyses were conducted with SAS 9.2. For experimental fledglings, we first analyzed tarsus length of fledglings of known sex, to check for a potential sex effect (ca half of the broods, see above; N=238). This was done using mixed models (proc MIXED, normal distribution) with sex, treatment and their interaction as explanatory variables. Since there was no strong effect of sex (see results), we further analyzed the full dataset (N=617 fledglings) using a mixed model to test for a general treatment effect. Similar model (sex, treatment and interaction as fixed factors) was run for adult tarsus. Similarly, we also compared the treatment effect on body mass in adults and fledglings and thus analysed fledging body condition (1) for fledglings of known sex and (2) for the whole dataset. We always included adult tarsus length as a covariate in the analyses of body mass, and therefore refer to them as analyses of body condition. Brood identity was included as a random effect to control for the non-independence of sibling measurements. The Kenward–Roger method was used to calculate the degrees of freedom of fixed effects (Littell et al. 2006).

Adult morphological and plumage traits (body mass, tarsus, wing and tail length, forehead and wing patch size, UV-chroma and parental feeding rate) were analyzed with linear mixed models (proc MIXED, normal distribution) including sex (when applicable), yolk androgen treatment and age (1-year-old vs. older, when applicable), and all their two-level interactions as explanatory variables (see details below). When analyzing adult body condition, only individuals caught during the same reproductive phase (i.e., the chick feeding period) were included (N=52), to avoid biases due to body mass changes during the breeding season. Bird identity was included as a random effect in all models, because 15 out of a total of 47 recruits were caught and measured two or three times in the years following the manipulation. Restricting analyses to measurements from the first observation of each experimental recruit however yielded similar results (N=47 for morphological and plumage traits and N=32 for feeding rates, results not detailed). Similarly, restricting analyses to individuals caught only during the chick feeding period (i.e., excluding males caught early, see "Methods" section) to compare individuals that reached the same reproductive phase yielded similar results (N=60, results not detailed). Parental feeding rate (i.e., number of feeding visits/h) was analyzed using a model similar to the morphological traits in which fledgling number and partner feeding rate were added as covariates, since they both have been shown to affect feeding rate in this population (Doligez et al. 2004; Ruuskanen et al. 2009).

The normality and homoscedasticity of residuals was checked for all analyses. 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, removing non-significant interactions, covariates and main effects except sex, androgen treatment and their interaction, starting with the least significant. To confirm the non-significance of the removed interactions and main effects, each term was added to the final model separately.

Results

The morphological and plumage traits as well as feeding rates of experimental recruits according to sex and yolk androgen treatment are summarized in Table 1. Depending on the trait considered, we obtained up to 8 and 21 measures on androgen-treated and control males, respectively, and up to 22 and 19 measures on androgen-treated and control females, respectively.

General morphological traits in fledglings and adults

Among fledglings of known sex, tarsus length only tended to depend on the interaction between sex and treatment (sex×treatment interaction: $F_{1,\ 194}$=3.3; p=0.07), androgen-treated males having shorter tarsi than control males, but no difference among females (post-hoc test androgen-treated vs. control males: $t_{1,\ 83.4}$=1.99, p=0.05, other post-hoc tests among groups p>0.05). There was no main effect of sex or treatment (sex: $F_{1,\ 194}$=0.85, p=0.36; treatment: $F_{1,\ 65.3}$= 1.68, p=0.20, in a model excluding the interaction). Thus, the yolk androgen treatment did not strongly differently affect

Table 1 Least square means (±SE) of morphological and plumage traits and parental feeding behavior of experimental recruits in relation to yolk androgen treatment and sex

	Male		Female	
	AT	CO	AT	CO
Tarsus (mm)				
LSmean ± SE	19.1±0.2	19.2±0.1	19.5±0.1	19.7±0.2
N	7	20	21	18
Body mass (g)				
LSmean ± SE	13.4±0.3	12.7±0.2	13.3±0.2	13.5±0.2
N	6	15	17	14
Wing (mm)				
LSmean ± SE	84.0±0.7	82.7±0.5	81.5±0.5	81.8±0.5
N	7	20	21	18
Tail (mm)				
LSmean ± SE	52.8±0.7	51.6±0.5	51.0±0.5	51.3±0.5
N	7	20	21	18
Forehead patch (mm^2)				
LSmean ± SE	60.1±6.2	71.1±4.1		
N	7	20		
Wing patch (mm)				
LSmean ± SE	36.3±3.2	34.7±2.1	18.9±2.0	20.0±2.1
N	7	20	21	18
UV chroma				
LSmean ± SE	0.192±0.003	0.188±0.002	0.172±0.002	0.170±0.002
N	8	13	21	17
Feeding rate/h				
LSmean ± SE	22.6±2.3	17.7±1.8	18.1±1.8	18.6±2.0
N	8	11	11	12

LSmeans are from models presented in Table 2. For body mass, only individuals measured during the chick feeding stage are included (see text). Forehead patch size is given for males only

AT=androgen-treated, *CO*=control, *N*=sample size per group

male and female fledging structural size. When analyzing all broods without a sex effect, tarsus length at fledging was significantly shorter in androgen-treated young compared to controls (Table 2; marginal means±SE (millimeter): androgen-treated: 19.4±0.07; controls 19.6±0.07, controlling for brood identity). However, among experimental recruits, adult tarsus length did not differ between treatments (Table 2); but females showed longer tarsi than males (Table 2; marginal means±SE (mm) males: 19.1±0.1, females 19.6±0.1).

Body condition at fledging, accounting for tarsus length as a covariate, did not differ between treatments among fledglings of known sex (sex×treatment interaction: $F_{1,182}=1.57$, $p=0.21$; treatment: $F_{1,\ 68}=0.00$, $p=0.95$), although males were in better body condition than females (sex: $F_{1,\ 183}=5.01$, $p=0.03$; marginal means±SE (grams): males: 14.1±0.02; females 13.9±0.02, controlling for tarsus length). There was no difference among treatments when all fledglings were included in the analysis (Table 2). However, body condition at adulthood was affected by yolk androgen treatment in males only (significant treatment×sex interaction; Table 2, controlling for tarsus length). Androgen-treated males had better body condition than control males, while no difference was observed among females (post-hoc test: control vs. androgen-treated males: $t_{1,\ 24.4}=-2.06$, $p=0.05$; control vs. androgen-treated females: $t_{1,\ 21.3}=1.1$, $p=0.28$; Fig 1).

Wing and tail length at adulthood did not differ between androgen-treated and control individuals in either sex (Tables 1 and 2). As previously found in this species, females and 1-year-old individuals had shorter wings than males and older individuals, respectively (marginal means± SE (millimeter): females, 81.7±0.3, males, 83.3±0.4; 1-year-old, 81.6±0.3, older, 83.4±0.4; Table 2). No other interaction between treatment, sex and age, and no other main effect were significant (all p values>0.4).

Plumage traits

Neither white forehead patch area in males nor white wing patch sizes in either sex were affected by androgen treatment (Tables 1and 2). As previously found in this species, wing patch size depended on the interaction between the individual's age and sex (Table 2); the wing patch was larger in old males than in other groups, which did not differ from each other (marginal means±SE: old males, 48.5±2.5, old females, 21.4±2.0; 1-year old males, 22.5±2.3,: 1-year old

Table 2 Effect of yolk androgen treatment and sex on morphological and plumage traits and parental feeding behavior of experimental birds

Trait	ndf, ddf	*F*	*p*
A. Fledgling measurements			
Tarsus at fledging, *N*=617			
Treatment	1, 220	4.51	0.03
Body mass at fledging, *N*=617			
Treatment	1, 246	1.14	0.28
Tarsus length	1,613	303.4	<0.001
B. Adult measurements			
Tarsus, *N*=66			
Treatment	1, 41.6	0.36	0.55
Sex	1, 41.6	6.89	0.01
Treatment*sex	1, 41.6	0.16	0.69
Body mass, *N*=52			
Treatment	1, 24.3	0.87	0.36
Sex	1, 22.6	3.18	0.09
Treatment*sex	1, 23.3	5.43	0.03
Tarsus length	1, 37.2	8.54	0.006
Wing length, *N*=66			
Treatment	1, 37.4	0.65	0.43
Sex	1, 37.1	9.27	0.0043
Treatment*sex	1, 37.2	2.08	0.16
Age	1, 37.2	17.97	0.0001
Tail length, *N*=66			
Treatment	1, 42.2	0.64	0.43
Sex	1, 42.2	3.77	0.06
Treatment*sex	1, 42.2	1.96	0.17
Forehead patch size, *N*=27			
Treatment	1, 18.8	2.14	0.16
Wing patch size, *N*=66			
Treatment	1, 45	0.01	0.91
Sex	1, 44.8	45.80	<0.0001
Treatment*sex	1, 45	0.31	0.58
Age	1, 50.4	63.42	<0.0001
Age*sex	1, 50.4	33.62	<0.0001
UV chroma, *N*=59			
Treatment	1, 33.6	2.19	0.15
Sex	1, 34	71.75	<0.0001
Treatment*sex	1, 34	0.21	0.65
Age	1, 44.3	15.05	0.0003
Parental feeding rate, *N*=42			
Treatment	1, 27.5	1.21	0.28
Sex	1, 26.9	0.83	0.37
Treatment*sex	1, 26.9	1.97	0.17
Number of fledglings	1, 25	18.51	0.0002
Partner feeding rate	1, 36	4.07	0.05

Non-significant factors were removed from the final models (except treatment, sex and their interaction, when applicable)

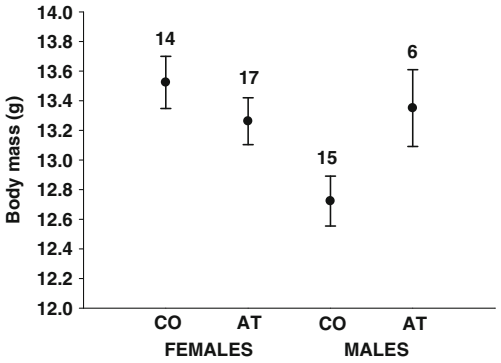

Fig. 1 Adult body mass (marginal means ± SE, adjusted for tarsus length) of experimental recruits according to yolk androgen treatment and sex. Bird identity was included as a random effect to control for several measurements from same individual. *CO*=control, *AT*=androgen-treated. *Numbers above the bars* indicate sample size

females, 17.4±1.8, post-hoc tests: old males vs. other groups $t<-8.55$, $p<0.0001$, other comparisons $p>0.09$). UV chroma of the white wing patch was not affected by androgen treatment in either sex (Tables 1 and 2). UV coloration was brighter (i.e., UV chroma was higher) in males than females (marginal means±SE, males, 0.190±0.002; females, 0.171±0.001, Table 2) and in older than in 1-year old individuals (marginal means±SE, 1-year-old, 0.177±0.001; older, 0.184±0.001, Table 2). No other interaction between treatment, sex and age, and no other main effects were significant (all *p* values>0.11).

Feeding rates

Yolk androgen treatment did not affect parental feeding rate of either sex (Tables 1 and 2). As previously found in this species, feeding rate was positively correlated with partner feeding rate (partial regression coefficient±SE, 0.31±0.15, Table 2) and number of fledglings (partial regression coefficient±SE, 2.00±0.47, Table 2). No interaction between age, sex, and treatment, and no other main effects were significant (all *p* values>0.66).

Discussion

We studied the long-term effects of early androgen exposure on different adult phenotypic traits (morphological and sexually selected plumage traits) and parental care behavior in a wild population of collared flycatchers. We found that androgen treatment affected a structural morphological trait, tarsus length (fixed at fledging), and had a sex-dependent effect on adult body condition. Yolk androgen treatment was not associated with expression of sexually selected plumage ornaments or UV coloration and it did not affect parental feeding rate in either sex.

Long-term effects of yolk androgen on morphology

In a previous study based on the same yolk androgen manipulation experiment, we investigated the short-term effects of yolk androgen treatment on offspring, and found that yolk androgen-treated male nestlings suffered from reduced growth during the nestling period compared to controls, whereas the pattern was weak but opposite in female nestlings (Pitala et al. 2009). When analyzing the full data focusing only on a structural size trait, tarsus length, which is fixed at fledging, we found that elevated yolk androgen levels lead to shorter tarsus at fledging compared to control treatment, with only a tendency for a sex difference. Smaller structural size in androgen-treated individuals compared to controls was also reported in the pied flycatcher (Ruuskanen and Laaksonen 2010) and the American kestrel (using a PCA for size measurements; Sockman and Schwabl 2000). In contrast, many other studies showed that elevated yolk androgen levels increase growth (either body mass and/or skeletal growth; Schwabl 1996; Eising et al. 2001; Groothuis et al. 2005; Navara et al. 2005; Gil 2008; Navara and Mendonça 2008), but most of these studies did not report effects on final structural size.

Contrary to fledging tarsus length, adult tarsus length did not differ among treatments and we found no effect of yolk androgens on wing and tail length at adulthood either. However, adult body condition (i.e., body mass controlled for tarsus length) was lower in control than androgen-treated males. Previous studies on captive birds reported lack of difference in morphological traits between androgen-treated and control individuals (Strasser and Schwabl 2004; Uller et al. 2005; Rubolini et al. 2006; Ruuskanen and Laaksonen 2010). The difference in the effect of the treatment on a fixed trait (tarsus length) measured at fledging and adulthood may arise through selection on this trait before recruitment, leading to differential survival (natural selection) or breeding probability (sexual selection) depending of tarsus length: larger fledgling size has been suggested to increase first-year survival in many species, including collared flycatchers (e.g., (Gustafsson and Sutherland 1988; Lindén et al. 1992; Potti et al. 2002). Thus, recruited adults represent a non-random sample from fledglings with respect to size measurements. Therefore, the higher body condition of androgen-treated males compared to controls and the absence of difference in tarsus length at adulthood despite smaller tarsus length at fledging, may potentially be explained by selection among androgen-treated males: only those in best condition and the largest ones (if reflecting high individual quality) would be able to survive and breed successfully. This may in particular result from potentially detrimental effects of high levels yolk androgens on males (such as immunosuppression, see "Introduction" section) such that only the highest quality males can successfully overcome these effects. Alternatively, differences in body condition may potentially be explained by other physiological changes or different life history-strategies (investment in self vs. offspring) between androgen-treated and control males, but these remain to be studied. No differences in adult body condition among androgen treatment and controls have been reported in previous studies (Uller et al. 2005; Rubolini et al. 2006; Müller et al. 2008), but because these results have all been obtained from captive populations, they may not reflect the effects of selection that could act in a wild population such as here.

No long-term effects of yolk androgens on sexually selected plumage traits and parental care

Yolk androgen treatment affected none of the measured secondary sexual male traits (forehead and wing patch size, UV chroma) in recruits. These results suggest that yolk androgens are unlikely to enhance sexually selected traits and thus affect sexual selection via male plumage characteristics in collared flycatchers. Therefore, differential allocation of yolk androgens into eggs would not represent a female strategy to increase their sons' attractiveness. The alternative hypothesis that yolk androgens could decrease sexually selected traits, thus explaining the lower recruitment of androgen-treated males, seem to not be supported either. We cannot exclude that the absence of effect was due to low sample size, i.e., a low statistical power to detect effects. However, we were able to detect previously described effects of age and sex on these variables, and we observed a difference in adult body mass between androgen-treated and control males. This suggests that sample size was sufficient to detect the effects of major biological factors, thus any effect of yolk androgen treatment on sexually selected traits must be small compared to these effects. Importantly, as mentioned above, recruited adults have been under sexual selection before successfully mating and being caught. If yolk androgens negatively affected sexually selected ornaments (especially in males), individuals with small sexually selected traits may not have accessed breeding, and this bias towards larger ornaments may mask the difference among treatment groups. Thus, we can conclude with confidence that yolk androgens did not enhance male sexually selected traits, but we cannot say for sure that they did not negatively affect these traits.

Nevertheless, our results confirm previous results on quails, pheasants, and starlings, in which none of the measured plumage traits (throat feather characteristics, wattle characteristics, iridescence, badge size) were affected by androgen treatment (e.g., Uller et al. 2005; Rubolini et al. 2006; Müller and Eens 2009; but see Strasser and Schwabl 2004; Eising et al. 2006, Bonisoli-Alquati et al. 2011a). A recent meta-analysis concluded that the effects of yolk

androgens on male phenotypic traits and sexual selection are low (Müller and Eens 2009), suggesting that yolk androgens are likely to cause only minor variation in androgen- and non-androgen dependent sexually selected traits. This may be explained by the many other environmental and/or genetic factors influencing the expression of sexually selected male characters (such as condition-dependence, developmental stress, trade-offs with reproductive effort, and parasitism; Hamilton and Zuk 1982; Andersson 1986; Gustafsson et al. 1995; Qvarnström 1999). However, the direct effect of yolk androgens on male attractiveness should be investigated using, e.g., mate choice tests. To our knowledge, only two studies have conducted such tests: in pheasants, yolk androgen-treated males obtained more copulations than control males (Bonisoli-Alquati et al. 2011b), but in zebra finches, yolk androgen treatment did not affect mate choice of untreated females (Rutkowska et al. 2007). In captive canaries, females mated with males from androgen-treated eggs increased their investment in eggs as measured by clutch size and mass (Müller et al. 2008). This may indicate that females either assessed androgen-treated males as more attractive or compensated for lower mate quality or future offspring viability (Müller et al. 2008). Overall, the possibility for female to increase the attractiveness of their sons through differential allocation of yolk androgens via their effects on secondary sexual traits remains unclear.

Yolk androgens did not induce male-like plumage ornaments (e.g., large forehead and wing patches) in females, and thus did not interfere with sexual differentiation. This could be expected as the default plumage is the male plumage (males being the homogametic sex) and as female plumage results from exposure to estrogens (Owens and Short 1995; Carere and Balthazart 2007). Also, the hormone dosages used in experiments resulting in interference in sexual differentiation are much higher than used in maternal effect studies (as suggested by Groothuis and Schwabl 2008). The result is also in accordance with previous studies, where yolk androgen manipulations within the physiological range did not induce the development of ornaments typical of one sex in the other (e.g., badge size in quails; spurs, wattles and ear tufts in pheasants: Strasser and Schwabl 2004; Rubolini et al. 2006).

High circulating testosterone levels have often been found to decrease both male and female parental care behavior (e.g., Ketterson and Nolan 1992; Ketterson et al. 1992; Stoehr and Hill 2000; Peters et al. 2002; Van Roo 2004; Lynn 2008; Veiga and Polo 2008), but potential effects of yolk androgens on subsequent adult parental care remained unexplored. We found here that feeding rate of either male or female parents was not affected by the yolk androgen treatment of eggs they originated from. This suggests that early exposure to androgens during the development has no such organizing effect on physiology that would affect future parental care. Again, we cannot exclude that the lack of effect results from a low statistical power due to a low number of recruits. However, we were able to detect the previously described major effects of fledgling number and parental age on feeding rate (e.g., Doligez et al. 2004). This suggests that any biological effect of yolk androgen treatment on feeding behavior must be small.

We previously found that androgen-treated males had lower local recruitment than control males, while no difference between treatment groups was found in females. Breeding success was however not affected by androgen treatment in either sex (Ruuskanen et al. 2012). Similar breeding success of recruits from different treatments likely results from the absence of difference in breeding investment and parental care among individuals from different treatments, as found here. Because parental care was not affected by yolk androgen treatment, the lower return rate of androgen-treated males compared to control males is unlikely to result from early breeding failure. Furthermore, because sexually selected male traits affecting mating success did not seem to be affected by yolk androgen treatment either (except if recruits are not a representative sample of experimental fledglings with respect to these traits due to a sexual selection process), the lower return rate of androgen-treated males is also unlikely to result from lower mating success. Thus, this lower local recruitment rate of androgen-treated males should be due to lower local survival or higher dispersal. We found here and previously (Pitala et al. 2009) that androgen-treated individuals, especially males, are smaller at fledging, which could lead to lower first-year survival (Lindén et al. 1992; Potti et al. 2002) and explain the observed low local recruitment. Lower recruitment may also be due to effects of yolk androgens on physiology, immune function or dispersal behavior, androgen-treated males dispersing out of our study area out of the study area (Tschirren et al. 2007). Especially the effects of yolk androgens on dispersal should be further investigated to understand causes of low recruitment better.

Ultimately, if yolk androgens have generally few effects on sexually selected traits and they do not increase the reproductive success of the offspring, but may even decrease male local recruitment in the long-term (Ruuskanen et al. 2012), what may be the benefits of allocating more (potentially costly) hormones in eggs? One hypothesis may be that the main role of yolk androgens is in modulating development, growth, and begging behavior during early life, for which there is quite strong evidence (reviewed in, e.g., Groothuis et al 2005; Gil 2008). Some of the effects observed in adults (see references in "Introduction") may then be carry-over effects of the early conditions (e.g., Metcalfe and Monaghan 2001; Groothuis and Schwabl 2008).

Ethical standards

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). All experiments comply with the current laws of Sweden.

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