



Optimizing the trade-off between offspring number and quality in unpredictable environments: Testing the role of differential androgen transfer to collared flycatcher eggs

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ABSTRACT

According to the brood reduction hypothesis, parents adjust their brood size in response to current environmental conditions. When resources are abundant, parents can successfully raise all hatched offspring, but when resources are scarce, brood reduction, i.e., the sacrifice of some siblings to secure the quality of a subset of offspring, may maximize fitness. Differential transfer of maternal androgens is one potential proximate mechanism through which female birds may facilitate brood reduction because it may alter the relative competitive ability of sibling nestlings. We tested the hypothesis that female collared flycatchers (*Ficedula albicollis*) manipulate sibling competition by transferring less androgens to eggs late in the laying sequence. We experimentally elevated androgen levels in i) whole clutches and ii) only the two last laid eggs, and compared growth and begging behavior of offspring from these treatments with a control treatment. By using three treatments and video assessment of begging, we examined the effects of within-clutch patterns of yolk androgen transfer on levels of sibling competition in situ. When androgens were elevated in only the two last laid eggs, begging was more even among siblings compared to control nests. We also found that female nestlings receiving additional yolk androgens showed higher mass gain later in the breeding season, while their male counterparts did not. Our results suggest that females may improve reproductive success in unpredictable environments by altering within-clutch patterns of yolk androgen transfer. We discuss the possibility that life-history divergence between the co-occurring collared and pied flycatcher (*Ficedula hypoleuca*) is amplified by patterns of yolk androgen transfer.

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Introduction

In species with extensive parental care, such as many bird species, parents must resolve a trade-off between offspring number and the ability to provide sufficient resources to ensure offspring success and a high probability of recruitment into the breeding population (Lack, 1947). One way for parents to optimize the number of offspring that recruit into the breeding population is by producing more offspring than they can raise under normal resource conditions (Husby, 1986; Lack, 1947). If resources are abundant, parents can in this way raise more offspring than under normal conditions. However, when resources are scarce, some of the offspring may starve; the

parents should choose to provide resources to a subset of their offspring in order to raise some high quality offspring, and not merely many poor quality offspring. This strategy, in which parents adjust their brood size to current resource conditions, is known as the brood reduction hypothesis (e.g., Husby, 1986; Lack, 1947).

Mothers may optimize the trade-off between offspring number and quality in unpredictable environments by mediating levels of sibling competition (Marshall et al., 2008). If offspring are approximately equal in competitive ability, resources should be distributed evenly among siblings. If resources are scarce, however, equality in sibling competitive ability may not be optimal for parental fitness because none of the offspring may be of high enough quality to successfully recruit into the breeding population (Husby, 1986). By placing some offspring at a competitive disadvantage, and thereby reducing sibling competition among the other offspring, mothers may facilitate brood reduction.

One proximate mechanism by which female birds may mediate relative sibling competitive ability in the nest is through altering the

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level of androgen hormones, such as testosterone and androstenedione, experienced by their developing offspring (reviewed in Gil, 2008; Groothuis et al., 2005a). Testosterone in the yolk has been found to affect or correlate with a variety of offspring phenotypes, including growth, begging intensity, immune function, and survival (e.g., Groothuis et al., 2005b; Müller et al., 2010; Pilz et al., 2004; Schwabl, 1996; Sockman and Schwabl, 2000; von Engelhardt et al., 2006). Maternal transfer of androgens can vary both among females (Pilz et al., 2004; Tobler et al., 2007; Tschirren et al., 2009) and among eggs within a single clutch (Gil et al., 2006; Hegyi et al., 2010; Schmaltz et al., 2008; Schwabl, 1993; Sockman and Schwabl, 2000). The latter pattern, i.e. within-clutch variation in yolk androgens, may be an adaptive mechanism by which females can influence relative sibling competitive ability (reviewed in Gil, 2008; Groothuis et al., 2005a). Two general patterns of within-clutch variation in yolk androgens have been observed: an increase in yolk androgen levels over the laying sequence, and a decrease over the laying sequence. For species showing an increase over the laying sequence (e.g., pied flycatchers, Gil et al., 2006; red-winged blackbirds, Lipar et al., 1999; smooth-billed anis, Schmaltz et al., 2008; canaries, Schwabl, 1993; American kestrels, Sockman and Schwabl, 2000), enhanced amounts of androgens in later-laid eggs may offset any competitive disadvantage caused by hatching asynchrony (Lipar et al., 1999; Schwabl, 1993; but see Sockman and Schwabl, 2000) and lead to greater similarity in sibling competitive ability. By contrast, for species showing decreasing yolk androgen levels over the laying sequence (e.g., American coots, Reed and Vleck, 2001; cattle egrets, Schwabl et al., 1997), females may increase the competitive disparity among siblings, which may facilitate brood reduction when resources are scarce (Schwabl et al., 1997).

In this study, we tested whether differential transfer of maternally derived androgens to the eggs of collared flycatchers (*Ficedula albicollis*) may explain observed seasonal patterns of offspring survival. The survival probability of collared flycatcher nestlings declines over the breeding season (Qvarnström et al., 2009), as does food availability (Veen et al., 2010). Because parental strategies should adjust to offspring growth strategies (and vice versa; reviewed in Müller et al., 2007a; Tobler and Smith, 2010), we predicted that female collared flycatchers should respond to their offspring's poor ability to cope with unpredictable resource levels late in the breeding season. There are at least two possible ways by which female collared flycatchers could do this. First, they could lay a smaller clutch later in the season to reduce sibling competition for food. Second, they could manipulate the competitive ability of nestlings within the clutch. The steep decline in both clutch size and nestling survival as the breeding season progresses (Qvarnström et al., 2009) suggests that females have adopted a mixture of the two strategies. Thus, they appear to partly adjust their clutch size to the decline in predictability of resources, but not fully (a perfect adjustment would result in no increased mortality rate). Because the increased mortality rate over the breeding season is consistent with brood reduction, we hypothesized that female collared flycatchers may influence competitive disparity among siblings by transferring a lower amount of androgens to the last laid eggs in the clutch. To test this hypothesis, we used a manipulative field experiment in which we altered the yolk androgen content of natural clutches and subsequently measured morphological and behavioral responses in offspring raised in natural nests.

Methods

The collared flycatcher, *F. albicollis*, is a small, insectivorous passerine. It winters in southeastern Africa and migrates to summer breeding grounds in central and Eastern Europe and the Swedish islands of Öland and Gotland. This study was conducted during

May–June 2008 on collared flycatchers breeding in nestboxes on the Baltic island of Öland, Sweden (57°10' N, 16°58' E). The breeding season begins in late April, when male flycatchers begin to arrive and compete over breeding territories (Pärt and Qvarnström, 1997; Qvarnström, 1997). Females arrive approximately one week later, and after inspecting a number of males and their territories, select their mates and commence nest building. The breeding areas consist of deciduous and mixed coniferous–deciduous forest plots stretching across much of the island (see Qvarnström et al., 2009 and Figures 1 and 3 therein for more details on forest plot locations). Females lay one egg per day, with an average clutch size of about 6 eggs (Alatalo et al., 1990). After a 12-day incubation period, the eggs hatch, whereupon both parents feed insects to the nestlings. At 14–15 days post-hatching, the nestlings reach adult weight, and fledge from the nest. Fledglings in good condition, with relatively higher body mass for a given body size, have increased chances of recruitment into the breeding population (Lundberg and Alatalo, 1992).

To test our hypothesis that female collared flycatchers can affect levels of competitive disparity among siblings through within-clutch variation in androgen transfer, we manipulated egg androgen content in each clutch using three different injection treatments (described in greater detail below): 1) a control treatment, 2) a complete clutch androgen treatment in which all eggs in the clutch received additional androgens, and 3) a two last-laid eggs androgen treatment in which only the last two eggs laid in the clutch received additional androgens. We predicted that these androgen treatments would affect the level of competitive disparity in the nest by altering the begging intensity of those nestlings receiving additional androgens. A more intensely begging nestling should be more likely to beg first and receive food from a parent. Thus, if females transfer less androgens to last-laid eggs, and nestlings hatched from these eggs beg less intensely than their siblings, they may be less competitive than their siblings and receive less food. We therefore predicted that the intensity of the begging behavior of nestlings within clutches receiving the two last-laid eggs androgen treatment should be less variable than within nests receiving the other two treatments. We also predicted lower variance in nestling mass within clutches receiving the two last-laid eggs androgen treatment. Finally, if maternal androgen transfer influences begging intensity, we predicted that the complete clutch androgen treatment should lead to an overall increase in feeding rate compared to the control treatment. Most studies of the effects of yolk androgen transfer on offspring traits include only a control and a whole-clutch androgen treatment (e.g., Barnett et al., 2011; Müller et al., 2008; Pitala et al., 2009; von Engelhardt et al., 2006). By adding a third treatment (the two last-laid eggs treatment), we were able to investigate both the effects of yolk androgen transfer in general, and the effects of within-clutch patterns of yolk androgen transfer on levels of sibling competitive disparity.

All experimental procedures adhered to the standards established by the United States National Institutes of Health and were approved by the local Swedish Ethical Committee on Animal Research (Dnr 27-08) and the Bird Ringing Centre of the Swedish Museum of Natural History (Stockholm, Sweden; License number 605).

Egg handling

We began monitoring the nestbox plots for new collared flycatcher nests in late April. Upon location of a nest with three eggs or fewer, we marked each egg with a non-toxic marking pen. We then returned to the nest every day and marked each new egg with its order in the laying sequence. On the day it was laid, we collected the fourth-laid egg from every nest for a different experiment and replaced it with a dummy egg made from modeling clay. We continued daily visits to each nest, marking each new egg, until no new eggs were laid and incubation had started. On the fourth day of incubation, we collected the entire clutch of eggs and replaced them all with dummy eggs.

Collected eggs were placed in an incubator at a constant temperature (37 °C) and kept there for 1.65 ± 0.99 days (mean \pm SD) (range 1–6 days). Egg development was monitored by illuminating the eggs with a flashlight positioned under the egg. This was to ensure eggs were manipulated at the same developmental stage (see below) and to monitor embryo survival after egg manipulation. The day after injection, all manipulated eggs with live embryos were returned to their original nest, and the dummy eggs removed.

Hormone treatment injections

Each clutch was randomly assigned to one of the three androgen treatments: 1) a control treatment (N = 32 clutches), 2) androgen treatment of the complete clutch (N = 31 clutches), and 3) androgen treatment of only the two last-laid eggs (N = 30 clutches). For clutches assigned to the control treatment, each egg in the clutch was injected with 4 μ l of sterile sesame oil. For clutches assigned to the complete clutch androgen treatment (hereafter termed 'Androgen treatment'), each egg in the clutch was injected with 4 ng of testosterone (T) and 12 ng androstenedione (A4) dissolved in 4 μ l of sterile sesame oil. Finally, in the two last-laid eggs androgen treatment (hereafter termed 'Last Two treatment'), the final two eggs in the laying sequence received injections containing 4 ng T and 12 ng A4 dissolved in 4 μ l of sterile sesame oil, while the remaining eggs in the clutch received the control injection of 4 μ l of sterile sesame oil. The dose of injected androgens corresponds to 1 standard deviation from the mean concentrations of these hormones as measured in the yolks of a population of the closely related pied flycatcher from southern Sweden (Tobler et al., 2007). Yolk androgen analyses of collared flycatcher eggs from the nearby island of Gotland confirm that the dose is within the physiological range of the species (Tschirren et al., 2009). Notably, the dose used in the current study is considerably lower than the dose used by Pitala et al. (2009) in a yolk androgen manipulation experiment on collared flycatchers from the Gotland population. While the proportion of T in relation to A4 was similar in both studies, the dose injected by Pitala et al. (2009) was more than three times higher (14.4 ng of T and 50.8 ng of A4 in 4 μ l of oil) and aimed to increase average yolk androgen levels to the upper limit of the natural range.

We injected either the control or the T/A4 solution directly into the egg yolk using the protocol described by von Engelhardt et al. (2006). Androgens were injected at a stage when the embryonic disk was approximately 5 mm in diameter (approximately 3–4 days after the start of incubation; ca stage 15; Hamburger and Hamilton, 1951). This approach was chosen because the yolk sac has expanded at this stage and the yolk has started to dilute with albumin, which facilitates injection (M Tobler, personal observation). The injection site was cleaned by wiping with a small amount of 95% ethanol prior to the injection. Before the Hamilton syringe containing the injection solution was inserted into the egg, a small hole was made in the shell with a sterile, disposable 27 G needle (BD Microlance 3, 27 G \times 3/4"). After the injection, the hole in the shell was sealed with a small drop of superglue (Tobler and Sandell, 2007, 2009).

Measuring nestling growth and begging

The clutches were returned to their original nests the day following the injections. For a variety of reasons, including egg breakage during handling and development failure, the final sample sizes for each treatment were lower than the initial number of clutches injected (Control: N = 26 clutches; Androgen: N = 25 clutches; Last Two: N = 28 clutches). Starting twelve days after the last egg was laid, we made daily visits to each experimental nest to determine the day the eggs in each clutch hatched. Hatching asynchrony is slight in this species with sixth and seventh eggs hatching 10–30 h after the first egg (Rosivall et al., 2005), so we recorded hatching day as a

single date for the entire clutch. The injection procedure resulted in significantly lower hatching success for experimental versus non-experimental clutches (mean hatch rate \pm SD: 0.58 ± 0.27 and 0.80 ± 0.32 for experimental and non-experimental clutches respectively; Wilcoxon rank sum test, $W = 14141.5$, $N = 311$, $P < 0.0001$), but there were no differences in hatching success among the three experimental treatment groups (Kruskal–Wallis test, $H = 2.036$, $df = 2$, $P = 0.36$). Six days after hatching, we returned, ringed each nestling with individually numbered aluminum leg bands, measured nestling mass (within 0.1 g) using a Pesola spring scale, and collected a small blood sample using brachial vein puncture. Thirteen days after hatching (generally one to two days before fledging), we returned to the nests, measured mass again, and measured tarsus length (within 0.1 mm) using digital calipers.

Following Qvarnström et al. (2007), we measured offspring begging at ten days after hatching. To do this, we video-recorded begging behavior of nestlings within a subset of our experimental nests (N = 8, 10, and 8 for Control, Androgen, and Last Two treatments respectively). One day before the recording, a small IR-light camera (YOKO model YK-3045B, $f = 3.6$ mm broad lens) was placed in the top of each nestbox to allow both parents and nestlings to acclimate to its presence. These cameras were connected to digital video cameras (JVC GR-D30) placed near the base of the tree. Nestlings were individually marked on the top of the head with water-soluble correction fluid to allow identification on the video. We recorded begging and feeding behavior for 1 h, during either the morning (0600–1000) or the early evening (1600–1900). Video footage was analyzed by an observer blind to the experimental treatment using a digital videocassette recorder (Panasonic, DVCPRO model AJ-D230). By using video cameras, we were able to assess begging in a natural nest environment, where parents elicited the begging response and sibling competition was present. Most previous studies of androgen effects on begging have assessed begging behavior on individual nestlings removed from the nest environment (e.g., Goodship and Buchanan, 2007; Müller et al., 2010; von Engelhardt et al., 2006; but see Barnett et al., 2011).

For every feeding event, we recorded which parent brought food, the order in which the nestlings began to beg, and which nestling was fed. The nestling that began to beg first received a rank of 1, nestlings that started begging at the same time received the same rank, and nestlings that did not beg received the highest possible ranking. In total, 640 feeding events were scored. In 594 of these events only one nestling was fed, in 39 cases two nestlings were fed during the same feeding event, and in 7 events, three nestlings were fed.

Sexing the nestlings

To test whether the androgen injections had sex-specific effects, we used blood samples to determine the sex of each individual. DNA was extracted from each sample using a high-salt extraction procedure (Sambrook and Russell, 2001). The sex-linked CHD locus was then PCR-amplified and genotyped by gel electrophoresis to determine nestling sex, following Griffiths et al. (1998).

Statistical analyses

All statistical analyses were performed in R version 2.15.2 (R Core Team, 2012), using the *vegan* (Oksanen et al., 2011), *car* (Fox and Weisberg, 2011), *MASS* (Venables and Ripley, 2002), *effects* (Fox, 2003), *AICcmodavg* (Mazerolle, 2012), *lmtest* (Zeileis and Hothorn, 2002), and *lme4* (Bates et al., 2011) packages.

Evenness of begging ranks

We used Pielou's evenness index to assess variation among siblings in intensity of begging behavior within each of the videotaped experimental nests. Evenness is usually used as an indication of

species diversity in a community, taking into account both the number of species in the community and the number of individuals of each species. A community with one very common species and several rare species has a low evenness value. Pielou's evenness (J) is calculated using Eq. (1), with S equal to the number of species and p_i equal to the proportion of the total number of individuals in the community represented by species i .

$$J = \frac{-\sum_{i=1}^s p_i \log_e p_i}{\log S} \quad (1)$$

For our analysis, we likened the number of nestlings in each nest to the number of species in a community, and the number of times each nestling was the first to beg to the number of individuals of each species. We added 1 to the number of times each nestling was the first to beg in order to insure that the analysis took into account the presence of nestlings that never begged first. Thus, we calculated evenness in begging rank (B) within each nest using Eq. (2),

$$B = \frac{-\sum_{i=1}^c \frac{w_i + 1}{E} \log_e \frac{w_i + 1}{E}}{\log C} \quad (2)$$

where C is equal to the number of nestlings in each nest, w_i is equal to the number of times nestling i begged first, and E equals the total number of feeding events observed plus the number of nestlings. Nests with more equal sibling competitive abilities, in which all siblings are similarly likely to beg first, had higher evenness values.

If female collared flycatchers transfer less androgens to later laid eggs, we predicted that nests from the Last Two treatment should show higher evenness in begging than either the Control nests or the Androgen nests. The evenness values were negatively skewed and variances were heterogeneous across the three treatment groups. Thus, we transformed the data by first reflecting the evenness values (Quinn and Keough, 2002, p. 66) to create a positively skewed distribution, and then performing a Box–Cox power transformation ($\lambda = -6.5$). The transformed data satisfied parametric assumptions. To determine whether our androgen manipulations affected begging evenness, and to account for a variety of potential covariates, we fit a linear model with the following main effects and all two-way interactions: androgen treatment, laying date, number of nestlings in the nest, and the hour of day the begging video footage was recorded. Model simplification was carried out by stepwise removal of the least significant term starting with the highest order interactions. Likelihood ratio tests were then used to compare models with and without the focal term; if the simplified model explained significantly less variation in evenness based on the likelihood ratio test, the focal term was retained. We used post-hoc pairwise t -tests, with P -values adjusted for multiple testing following Holm (1979), to determine which treatment groups differed in begging evenness.

Feeding rate

To test whether feeding rate was higher at nests receiving the Androgen treatment versus the Control treatment, we fit a linear model with the number of feedings during the 1-hour recording time as the response, and androgen treatment (Control or Androgen) as a main effect. We included three covariates in the model—hatching date, number of nestlings in the nest, and the hour of the video recording—and all two-way interactions between androgen treatment and each covariate. We removed non-significant terms from the model one by one, and validated each removal using likelihood ratio tests as described above. All parametric assumptions were satisfied.

Variance in nestling mass

If females can manipulate levels of sibling competitive disparity through differential transfer of androgens, then our androgen treatments should affect variance in nestling mass at fledging. Because begging is more even in nests receiving the Last Two treatment (see Results section), and begging rank predicts the number of times a nestling is fed (Qvarnström et al., 2007), we predicted that nestlings would be fed more evenly and thus, variance in mass would be lower in the Last Two treatment compared to the Control and Androgen treatments. Variance in mass was positively skewed, so we transformed this response using a Box–Cox power transformation ($\lambda = -0.02$). Transformed variance in mass was normally distributed and variance was homogeneous across our treatment groups. To determine whether our androgen treatments affected variance in mass, we fit a linear model with androgen treatment as a fixed effect. We included hatching date and the number of nestlings in the nest in the model as potential covariates, along with all two-way interactions with androgen treatment. Because previous work indicated that timing of breeding affects nestling mass and survival (Qvarnström et al., 2005, 2009), we predicted that hatching date might impact any relationship between begging and nestling mass. We simplified the model as described above. If androgen treatment affected variance in mass, the best-fit model should include the androgen treatment effect and should explain significantly more variation than a reduced model without the androgen treatment effect.

We also tested for a linear relationship between nest evenness in begging rank and variance in nestling mass at fledging. We predicted that nests with greater evenness in begging rank would show lower variance in nestling mass at fledging. We used untransformed Pielou's evenness in begging rank as a fixed effect. We included hatching date and number of nestlings in the nest in the model as potential covariates, as well as all interactions. Before fitting the linear model, we transformed evenness in nestling mass at fledging to achieve normality using a Box–Cox power transformation ($\lambda = 0.14$).

General effects of androgens

To test for general effects of androgen treatment on growth, we fit linear mixed effect models using maximum likelihood to our data with nest ID as a random effect. Full models included fixed effects of treatment, offspring sex, number of nestlings in the nest, hatching date, and their interactions. We used mass gain (from day 6 to day 13 post-hatching) and mass at fledging as response variables. Mass at fledging was negatively skewed, so we first reflected it and then performed a Box–Cox power transformation ($\lambda = 0.14$) to achieve a normally-distributed response. Stepwise model simplification was carried out as described above, using likelihood ratio tests to validate the removal of each term. If treatment with androgens affected nestling growth or fitness, then the best-fit models should include androgen treatment. At the same time, if treatment with androgens had differential effects on the two sexes, the best-fit models should include the androgen treatment by sex interaction. Because we were unable to determine after hatching which nestlings from the Last Two nests had been injected with androgens and which with

Table 1

Analysis of variance (type II tests) in transformed and reflected Pielou's evenness in begging ranks in relation to androgen treatment, laying date, video recording time, and number of nestlings in the nest (Adjusted $R^2 = 0.45$, $F_{8,16} = 3.44$, $P = 0.017$).

| | SS | df | F-value | P-value |
|--|--------|----|---------|---------|
| Androgen treatment | 0.0143 | 2 | 7.238 | 0.006 |
| Laying date | 0.0032 | 1 | 3.256 | 0.090 |
| Recording time | 0.0007 | 1 | 0.704 | 0.414 |
| Number of nestlings in nest | 0.0008 | 1 | 0.774 | 0.392 |
| Laying date \times recording time | 0.0077 | 1 | 7.819 | 0.013 |
| Treatment \times number of nestlings in nest | 0.0047 | 2 | 2.384 | 0.124 |
| Residual | 0.0158 | 16 | | |

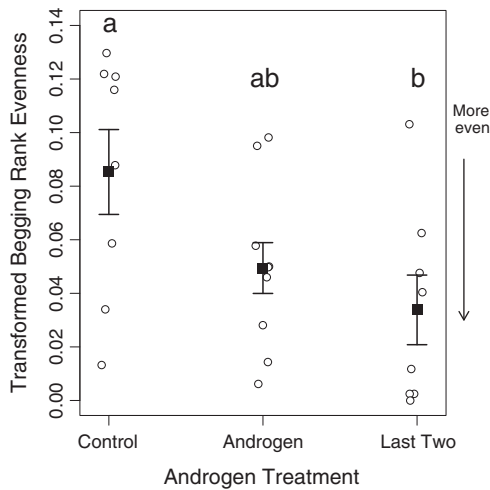


Fig. 1. Mean transformed begging rank evenness for nests receiving the three androgen treatments. Black squares are means, and the error bars show standard errors around the mean. Treatment groups labeled with different letters are significantly different in transformed begging evenness based on pairwise t-tests with *P*-values corrected for multiple testing following Holm (1979). Control vs. Androgen, *P* = 0.11; Androgen vs. Last Two, *P* = 0.38; Control vs. Last Two, *P* = 0.03.

the control solution, we included only nestlings from the Androgen and Control treatments in these analyses (*N* = 102 and *N* = 107 nestlings for mass gain and mass at fledging respectively). We included data from all nests that were treated and for which no data were

missing (*N* = 38 and *N* = 41 nests for mass gain and mass at fledging respectively), not only the subset of nests for which begging behavior was recorded.

We also tested for an effect of androgen treatment on hatching time, again including only the nestlings receiving the Androgen and Control treatments. Maternal androgens may act to accelerate or delay hatching, which could influence level of sibling competitive disparity in our Last Two nests. We fit a linear mixed effect model using maximum likelihood with nest ID as a random effect. We used hatching time (the difference between hatching date and laying date) as our response variable (*N* = 162). Because hatching time was positively skewed, we performed a Box-Cox power transformation ($\lambda = -1.64$) to achieve a normally-distributed response. Our full model included fixed effects of androgen treatment and offspring sex, as well as their interaction. We carried out stepwise model simplification as described above.

Results

Evenness of begging ranks

Compared to nests receiving the Control treatment, nests receiving the Last Two treatment, in which only the two last-laid eggs received additional androgens, showed greater evenness in begging rank (Table 1, Fig. 1). We also found a significant interaction effect of laying date and video recording time on begging rank evenness (Table 1, Fig. 2). Begging rank evenness in the early morning is constant across the breeding season. Evenness in the afternoon, however, decreases as the breeding season progresses (Fig. 2).

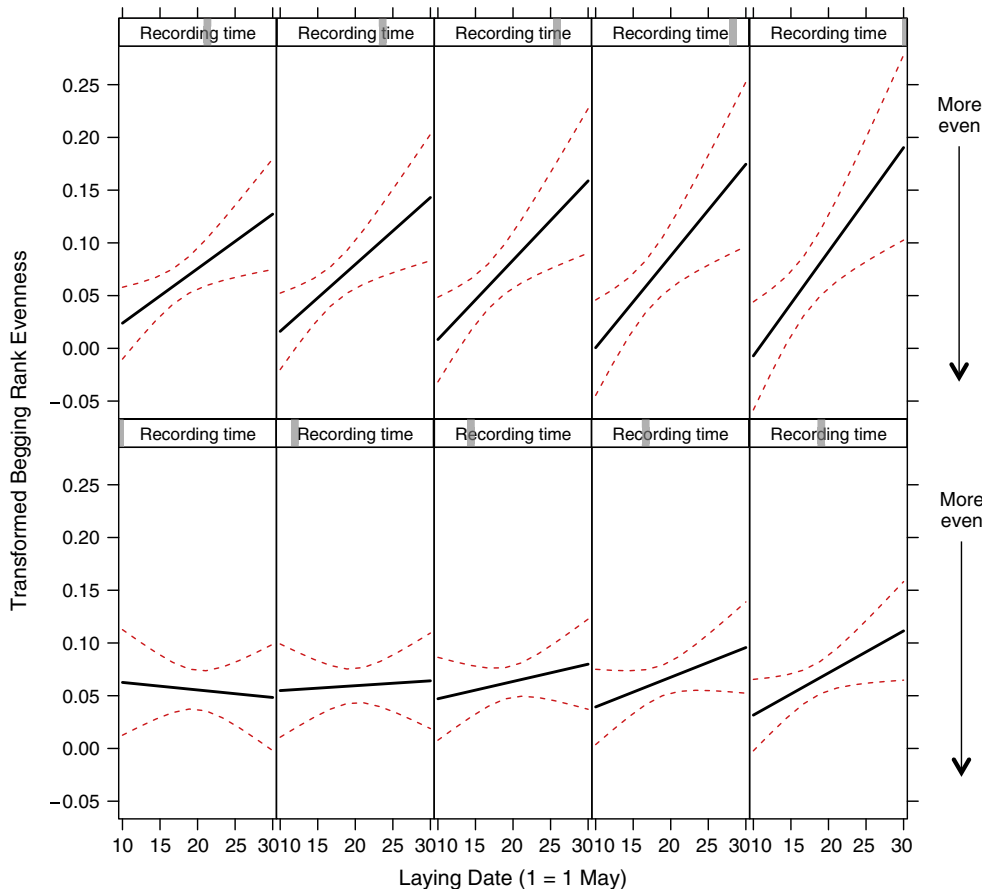


Fig. 2. Effect plot of the laying date by video recording time interaction. Each panel represents the relationship between laying date and begging rank evenness at a different recording time (dashed lines represent 95% confidence bands), increasing in hour from the lower left to the top right panel. Small values on the y-axis indicate higher evenness in begging. Evenness in begging in the early morning is constant across the breeding season (bottom), while evenness later in the day decreases across the breeding season (top).

Table 2
Tests for general effects of androgens on nestling growth. Full models include androgen treatment, sex, number of nestlings in the nest, hatching date, and their interactions as fixed effects. Reduced models include only the specified fixed effects. Corrected Akaike information criterion (AICc) values are listed for each model. Chi-square and *P*-values correspond to likelihood ratio tests comparing the fit of the given model to the model listed directly above (except where noted; $P > 0.05$ indicates that the given model does not explain significantly less variation than the model above, validating the model simplification). Boldface signifies the best-fit model for each response (i.e., lowest AICc).

| Response | Model | AICc | Chi-square | <i>P</i> -value |
|------------------|--|--------------|-------------------------|-------------------------|
| Mass gain | Full | 372.4 | | |
| | Treatment × Sex × Hatch date (including all interactions) | 358.6 | 8.10 | 0.42 |
| | Treatment + Sex + Hatch date + all 2-way interactions | 360.7 | 4.49 | 0.03 |
| Mass at fledging | Full | 94.1 | | |
| | Treatment × Sex | 70.6 | 7.42 | 0.83 |
| | Treatment + Sex | 70.2 | 1.82 | 0.18 |
| | Treatment | 99.0 | 31.16 | 2.4×10^{-8} |
| | Sex | 68.3 | 0.27^a | 0.61^a |

^a Corresponds to likelihood ratio test comparing this model to the model including Treatment + Sex.

Feeding rate

We found no differences between the Control and Androgen treatments in feeding rate. The simplified model was non-significant overall (ANOVA, type II tests: $F_{6,10} = 2.155$, $P = 0.136$), and included androgen treatment, hour of the video recording, hatching date, number of nestlings in the nest, the treatment by hour of video interaction, and the treatment by hatching date interaction.

Variance in nestling mass

We found no effect of androgen treatment on nest variance in mass. The best-fit model included only hatching date, and was non-significant overall (ANOVA: $F_{1,67} = 1.598$, $P = 0.211$). Including androgen treatment in the model did not explain significantly more variation in nest variance in mass than this best-fit model (Likelihood ratio test: $\chi^2 = 0.32$, $P = 0.85$). Similarly, we found no significant relationship between evenness in begging rank and variance in nestling mass at fledging (Linear regression, $R^2 = 0.03$, $F_{1,20} = 0.514$, $P = 0.482$).

General effects of androgens

We found no evidence of an effect of androgen treatment on mass at fledging. The best-fit model did not include the treatment effect or the treatment by sex interaction (Table 2).

We found evidence that treatment with androgens had sex-specific effects on mass gain. The best-fit model included a significant three-way interaction between androgen treatment, sex, and hatching date (Tables 2 and 3). Mass gain in female nestlings receiving additional androgens increased across the breeding season more than did mass gain in control females (Fig. 3A). In contrast, mass gain in male nestlings receiving additional androgens was relatively constant across the breeding season, while mass gain in males receiving the control treatment increased across the breeding season (Fig. 3B). The

Table 3

Results from a linear mixed effects model (Analysis of deviance, type II Wald chi-square tests) testing for the effects of androgen treatment, sex, and hatching date on nestling mass gain. Only nestlings receiving either the control or whole-clutch Androgen treatment were included in this model.

| | Wald chi-square | df | <i>P</i> -value |
|----------------|-----------------|----|-----------------|
| Treatment (A) | 4.035 | 1 | 0.045 |
| Sex (B) | 0.050 | 1 | 0.823 |
| Hatch date (C) | 0.719 | 1 | 0.397 |
| A × B | 0.018 | 1 | 0.893 |
| A × C | 0.046 | 1 | 0.831 |
| B × C | 0.107 | 1 | 0.743 |
| A × B × C | 4.751 | 1 | 0.029 |

androgen-treated females thus showed a similar relationship between hatching date and mass gain to the control-treated males (Fig. 3).

Treatment with androgens did not accelerate or delay hatching. The best-fit model included the treatment effect (Table 4), but an analysis of deviance indicated that there was no significant difference in hatching time for nestlings receiving the Control versus the Androgen treatment (Wald chi-square = 1.49, $df = 1$, $P = 0.22$).

Discussion

We tested the hypothesis that within-clutch patterns of maternal androgen transfer to collared flycatcher eggs cause competitive disparity among siblings. In collared flycatchers (*F. albicollis*), nestling growth and survival decline over the breeding season (Qvarnström et al., 2005, 2009). The decline in growth was not present when brood size was experimentally reduced, however (Qvarnström et al., 2009). This suggests that female collared flycatchers may produce more offspring than they can successfully raise when food availability is low. We hypothesized that females may facilitate brood reduction during unfavorable conditions by manipulating levels of sibling competitive disparity through within-clutch variation in yolk androgen levels. We tested this hypothesis by experimentally manipulating yolk androgen levels in collared flycatcher clutches and subsequently measuring begging behavior, mass, and fledging success in nestlings from the experimental nests.

Evenness of begging ranks

Nests receiving the Last Two treatment, in which we elevated the androgens in only the two last laid eggs, showed greater evenness in begging rank (Table 1, Fig. 1). This effect was not due to an acceleration or delay of hatching due to elevated androgens, because nestlings receiving the Control and the Androgen treatments did not differ in hatching time. These results were consistent with our prediction, and suggest that female collared flycatchers may increase competitive disparity among siblings by reducing levels of androgens transferred to eggs late in the laying sequence. Prior evidence has been mixed for a decrease in yolk androgen transfer with laying order as a proximate mechanism for increasing competitive disparity among siblings (Smiseth et al., 2011). Females should be under selection to influence offspring phenotypes in a way that maximizes their own fitness, but if this conflicts with the fitness interests of individual offspring, then offspring themselves should be under selection to circumvent potentially harmful maternal manipulations (Müller et al., 2007a; Tobler and Smith, 2010). Our results suggest that in collared flycatchers, offspring remain susceptible to maternal manipulation of sibling competitive disparity levels through within-clutch patterns of yolk-androgen transfer. One possible reason for this susceptibility is that nestlings may also benefit from information provided by the mother through yolk androgen transfer (Tobler and Smith, 2010),

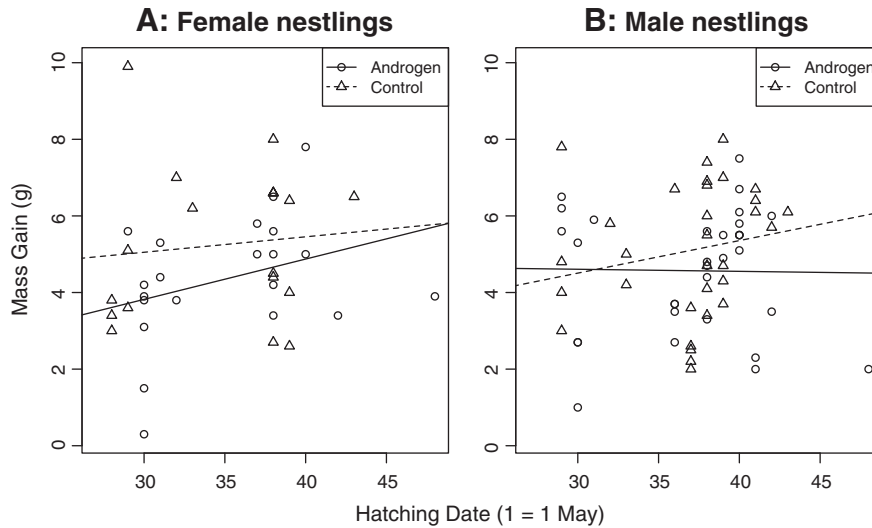


Fig. 3. Interaction among androgen treatment, sex, and hatching date on mass gain. A, female nestlings. B, male nestlings. Circles represent nestlings hatched from eggs receiving the whole-clutch Androgen treatment and triangles represent nestlings hatched from eggs receiving the Control treatment. Regression lines are provided for illustration purposes only. Mass gain in female nestlings receiving additional yolk androgens increased across the breeding season, similar to male nestlings receiving the control treatment. In contrast, mass gain in male nestlings receiving additional yolk androgens was relatively constant across the breeding season.

such as information about maternal (Hegyi et al., 2010) or paternal (Michl, 2004) quality, or the surrounding social environment (Hargitai et al., 2009).

Interestingly, von Engelhardt et al. (2006) found that treatment of zebra finch eggs with testosterone led to effects on begging only at younger ages. By day 10 post-hatching, when we measured begging in the present study, there were no differences in begging between control and testosterone-treated zebra finches (von Engelhardt et al., 2006). von Engelhardt and colleagues measured the begging behavior of each nestling in isolation from its nestmates, which precluded the possibility of observing differences among siblings in begging. Nestlings are likely to behave differently when in the presence of their sibling competitors than they would in isolation (Marques et al., 2011). Barnett et al. (2011) measured the effect of elevated yolk androgen levels on begging in house wrens in a natural nest environment, with parents soliciting the begging response. Yet, similar to von Engelhardt et al. (2006), they found that elevated yolk androgens only led to an increase in begging early in the nestling period. Because they used audio recordings to measure begging responses, however, Barnett et al. (2011) were unable to assess the level of variation among individual nestlings in begging behavior. Our experimental design, with three treatments and video assessment of begging responses in situ, allowed us to evaluate the importance

of within-clutch variation in yolk androgen levels for determining relative competitive ability of nestlings in a natural nest environment. Our results suggest that yolk androgens may have longer-lasting effects on individual begging behavior and on relative competitive ability in the natural nest environment than previously recognized.

Our results show that female collared flycatchers have the potential to increase competitive disparity among their nestlings by transferring less androgens to later laid eggs. However, females, even from the same population, may vary in the strategy they employ for coping with unpredictable environments. Recently, Hegyi et al. (2010) measured yolk androgen levels in Hungarian collared flycatchers and found no trend in yolk testosterone levels over the laying sequence, but an increase in yolk androstenedione over the laying sequence for small or low-condition females (Hegyi et al., 2010). Given our results, this suggests that low-condition females may adopt a strategy leading to reduced sibling competitive disparity, perhaps precluding brood reduction. Possible adaptive explanations could be that low-condition females produce smaller clutches to start with (i.e. leaving little scope for brood reduction) or that they are willing to pay a higher cost of current reproduction if they have lower survival chances. However, we also need to keep in mind that Hegyi et al. (2010) studied a different population of collared flycatchers. Swedish collared flycatcher nestling survival declines over the breeding season (Qvarnström et al., 2009), which may result from greater resource unpredictability late in the season (Veen et al., 2010). Given that the Swedish and the Hungarian populations occur at quite different latitudes (57°10' N and 47°43' N respectively), over-all levels of resources as well as the predictability of those resources likely differ between these populations. If female collared flycatchers respond to the resource environment by manipulating yolk androgen levels, then we may expect within-clutch trends in yolk androgen levels to vary between these populations. Populations of the closely related pied flycatcher exhibit geographic variation in egg androgen levels (Ruuskanen et al., 2011). Whether they also exhibit variation in within-clutch patterns of yolk androgens remains unclear. A systematic analysis of geographic variation in within-clutch yolk androgen transfer has not yet been done, although studies on both Swedish (Tobler et al., 2007) and Spanish populations (Gil et al., 2006) suggest pied flycatchers increase androgen transfer with laying order. However, both studies also found that within-clutch patterns potentially vary with environment quality. The relationship between

Table 4

Tests for general effects of androgen treatment on hatching time. The full model includes androgen treatment, sex, and their interaction as fixed effects. Reduced models include only the specified fixed effects. Corrected Akaike information criterion (AICc) values are listed for each model. Chi-square and *P*-values correspond to likelihood ratio tests comparing the fit of the given model to the model listed directly above (except where noted; *P* > 0.05 indicates that the given model does not explain significantly less variation than the model above, validating the model simplification). Boldface signifies the best-fit model (i.e., lowest AICc).

| Response | Model | AICc | Chi-square | <i>P</i> -value |
|---------------|------------------|----------------|---------------------|--------------------------------------|
| Hatching time | Full | −2262.5 | | |
| | Treatment + Sex | −2417.5 | 0 | 1 |
| | Treatment | −2937.7 | 0 | 1 |
| | Sex | −2202.4 | 217.27 ^a | 2.2 × 10 ^{−16} ^a |

^a Corresponds to likelihood ratio test comparing this model to the model including Treatment + Sex.

androgen transfer and laying order differed with female expression of a condition-dependent ornament (Gil et al., 2006), and between first and replacement clutches (Tobler et al., 2007). These results are consistent with the idea that adjusting within-clutch patterns of androgen transfer may allow females to improve their reproductive success in unpredictable environments.

Across all treatment groups, competitive disparity among siblings increases as the breeding season progresses and food resources are depleted, and this effect is especially strong in the afternoon (Table 1, Fig. 2). Diurnal variation in begging behavior has not been directly studied to our knowledge, and it may result from either exogenous (e.g., level of food deprivation; Leonard and Horn, 2006; Marques et al., 2011) or endogenous sources (e.g., circadian rhythms; Konturek et al., 2011). That we observed greater diurnal variation late in the breeding season, when food supplies are more unpredictable, suggests that exogenous forces may play a larger role in this system. Early in the breeding season when food resources are more abundant, parents may be better able to feed nestlings evenly; even feeding in the morning may result in even begging in the afternoon. In contrast, late in the breeding season when food resources are depleted, nestlings may not be fed evenly. Those who received more food in the morning may beg less intensely in the afternoon, leading to heightened competitive disparity among siblings and greater diurnal variation in begging.

Feeding rate

We predicted that the parental feeding rate should be higher for nests in the Androgen treatment than for the Control treatment if additional yolk androgens affected begging behavior and begging is an honest signal of need (Ottosson et al., 1997; Qvarnström et al., 2007; Tschirren and Richner, 2008). However, we found no differences between the Control and Androgen treatments in feeding rate (ANOVA, type II tests: $F_{6,10} = 2.155, P = 0.136$). Experimental elevation of yolk androgens in the nearby Gotland population of collared flycatchers also failed to result in increased parental feeding rates (Ruuskanen et al., 2009). These results suggest either that levels of yolk androgens do not influence begging behavior at all, that yolk androgens influence nestling competitive ability but not frequency of begging, or that parents do not adjust their feeding behavior to match nestling begging behavior. Our results indicate that treatment with androgens affected relative competitive ability and evenness in begging ranks (Table 1, Fig. 1), so we suggest that some combination of the latter two non-mutually exclusive explanations is more likely. In nests showing increased begging evenness, the overall frequency of begging may not change; thus, parents may not increase their overall feeding rates, but may instead distribute the food more evenly among the nestlings. Also, parents may fail to adjust their feeding behavior for at least two reasons: 1) They may already be investing maximum effort in feeding the nestlings, and are physically unable to further increase their feeding rate; or 2) They face a trade-off between current parental investment and future reproductive output (Gustafsson and Sutherland, 1988; Williams, 1966), and further increasing the feeding rate will result in an overall decrease in lifetime reproductive success.

Variance in nestling mass

Contrary to our predictions, androgen treatment did not affect nest variance in mass, and nest evenness in begging rank did not predict variance in nestling mass at fledging. Reduced levels of sibling competition in our nests due both to the removal of the fourth-laid egg and the reduced hatching success from our experimental manipulation (see Methods) may explain this. If androgens affect nestling growth by altering begging or another aspect of sibling competition, then the reduced sibling competition in our study might have allowed

all nestlings to obtain sufficient food to achieve maximal growth, regardless of sibling disparity in begging behavior. The relationships among yolk androgen levels, begging, and growth are currently unclear (Smiseth et al., 2011). However, the effects of yolk androgens may vary with food availability (reviewed in Smiseth et al., 2011), which was altered when we removed the fourth-laid egg. A previous experiment on collared flycatchers, in which clutch size was not manipulated, demonstrated that begging intensity predicts the number of times a nestling is fed, and that the number of times a nestling is fed predicts mass at fledging (Qvarnström et al., 2007). It is therefore possible that the observed effects of the androgen treatment on begging evenness would have larger effects on mass at fledging, if levels of sibling competition were not reduced (but also see below).

General effects of androgens

Elevated levels of yolk androgens led to sex-specific effects on mass gain (Tables 2 and 3, Fig. 3), with androgen-treated females showing increased mass gain as the breeding season progressed. Control males, but not androgen-treated males, exhibited a similar increase in mass gain over the breeding season. Pitala et al. (2009) found sex-specific effects of experimental manipulation of yolk T and A4 in a different Swedish population of collared flycatchers: Additional androgens led to larger body size in female nestlings but reduced body size in male nestlings. In the present study, nestling females and males did not differ in their mean responses to androgen treatment (Table 3, Treatment \times Sex interaction), but rather differed in their seasonal patterns of response to androgen treatment (Table 3, Fig. 3).

This result provides an alternative explanation to the absence of a straightforward relationship between evenness in begging rank and variance in mass. Higher levels of yolk androgens may increase the competitive ability of nestlings (i.e. in terms of their ability to gain a favorable begging rank) but the effect on their mass gain seems to differ depending on their sex and on external conditions (i.e. their parents' timing of breeding). Early in the season, male nestlings receiving extra androgens grew just as well as control males (Fig. 3B), while female nestlings receiving extra androgens grew less well than control females (Fig. 3A). Late in the season when the amount of food is declining (Veen et al., 2010), this relationship swaps: Female nestlings receiving extra androgens grew just as well as control females (Fig. 3A), while male nestlings receiving extra androgens grew less well than control males (Fig. 3B). Female collared flycatchers may therefore be able to achieve adaptive brood reduction either by adjusting patterns of within-clutch yolk androgen transfer and/or by adjusting the ovulation order of sons and daughters. It is also important to consider that the position of sons and daughters across the laying sequence affects oocyte exposure to maternal steroids. Adjustment of the laying sequence of sons and daughters is therefore assumed to be an important mechanism enabling sex-specific maternal allocation of steroids and antioxidants (e.g. Badyaev et al., 2006a,b; Bowden et al., 2000). Our results suggest that adaptive brood reduction through variation in yolk androgen transfer needs to be considered in relation to adaptive adjustments of the laying sequence of sons and daughters.

We can only speculate about the underlying mechanisms through which yolk androgens affect begging and growth in our study species. As reviewed by Groothuis and Schwabl (2008), yolk androgen manipulation may alter endogenous production of as well as sensitivity towards specific steroid hormones. With respect to offspring begging, Godsave et al. (2002) demonstrated the presence of androgen receptors in the syrinx of embryo and hatchling zebra finches (*Taeniopygia guttata*). If differences in yolk androgen levels result in differences in syrinx androgen receptor expression, this may directly translate into differences in begging rate. There is also evidence that endogenous androgen levels in nestlings of passerine birds positively affect

begging behavior. Experimentally elevated circulating levels of testosterone resulted in more intense begging behavior of nestling pied flycatchers (Goodship and Buchanan, 2007). Likely, the two mechanisms (production and sensitivity) are not mutually exclusive.

Interestingly, yolk androgens have also been found to affect body structures relevant to begging (and growth). Lipar and Ketterson (2000) found that yolk testosterone enhanced the growth of the neck muscle (musculus complexus) that is assumed to play an important role in the frequency and persistence of begging behavior. Moreover, Müller et al. (2007b) showed that embryonic exposure to elevated androgen levels resulted in larger beak flanges (i.e. larger gape area) in ca. two week old spotless starlings (*Sturnus unicolor*). Larger gape area is likely to affect begging success and, hence, its frequency and persistence. How exactly yolk testosterone affects muscle or beak flange growth, whether through increased hormone production or enhanced receptor expression in specific tissues, is unknown, however. Finally, sex differences of yolk androgen effects such as those reported in this study may result from sex differences in the distribution of hormone receptors and the timing of hormone receptor expression (sensu Groothuis and Schwabl, 2008). They may come about either through direct action of androgens or indirectly through sex-specific effects of yolk androgens on other physiological components (e.g. immune system) or behavior.

Implications for life-history divergence

Our results show that female collared flycatchers could decrease competitive disparity among their offspring by transferring more androgens to the last eggs in their laying sequence (as in our Last Two treatment). However, it may often be in the mother's interest to maintain competitive disparity among her offspring, in order to improve reproductive success when resources are unpredictable. When resources are scarce, adaptive brood reduction may result, which could in part explain the decline in offspring survival probability across the breeding season (Qvarnström et al., 2009). Offspring of the closely related pied flycatcher (*F. hypoleuca*) are not as sensitive to harsh environmental conditions and they do not show a decline in survival probability across the breeding season, even where the species co-occur on the Baltic island of Öland, Sweden (Qvarnström et al., 2009). Moreover, Tobler et al. (2007) found that yolk androgen concentration usually increased or remained constant across the laying sequence in pied flycatchers breeding on the Swedish mainland. Disentangling the relative roles of tightly co-evolved maternal and offspring strategies in causing population divergence in life history traits may prove to be difficult. However, the flexible nature of maternal effects means that they may have a strong impact on the initial direction of evolution when populations experience new environmental conditions.

Conclusions

Female collared flycatchers have the potential to manipulate levels of sibling competition through within-clutch patterns of yolk androgen transfer. This may allow females to improve their reproductive success in unpredictable environments. Maternal effects likely play an important role in adaptation to unpredictable environments in this species. Additional work will be necessary to determine the importance of maternal androgen transfer and other underlying mechanisms for mediating interspecific life-history divergence and coexistence.

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