

Experimentally altering pre-breeding sex steroids reduces extra-pair paternity in female tree swallows

Lisha L. Berzins  | J. Mark Shrimpton | Russell D. Dawson

Ecosystem Science and Management Program, University of Northern British Columbia, Prince George, BC, Canada

Correspondence

Lisha L. Berzins, Department of Biology, University of Saskatchewan, Saskatoon, SK, Canada.
Email: lisha.berzins@usask.ca

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Abstract

Extra-pair paternity commonly occurs in many socially monogamous animal species, yet the reasons why females mate with males outside the social pair bond remain poorly understood. Because sex steroids mediate aggressive and sexual behaviors that peak prior to egg laying in female birds, we tested whether they also influence the rate of extra-pair paternity in nests of female tree swallows (*Tachycineta bicolor*). In one treatment, we experimentally elevated testosterone (T) using implants containing exogenous T, and in another treatment, we blocked the estrogenic and androgenic actions of T using implants containing 1,4,6-androstatrien-3,17-dione in combination with flutamide (ATD+F). Females with empty implants served as controls. Nests of females treated with T and those with ATD+F were less likely to contain extra-pair offspring and had a lower proportion of extra-pair offspring, compared to control females. Treating females with T also delayed clutch initiation dates and disrupted incubation behavior, as found in previous studies, but clutch sizes of T females did not differ from controls. Females treated with ATD+F tended to lay larger clutches than control and T-treated females, which may be due to their clutch sizes not declining with later initiation dates as found in the T and control treatments; however, after controlling for brood size, ATD+F females produced nestlings that were lighter at day 16 than control females. Although the effect of T on extra-pair paternity that we observed in tree swallows is consistent with previous studies in female birds, our results demonstrate that blocking the estrogenic and/or androgenic actions of T during pre-breeding also lowers extra-pair paternity. Overall, our study suggests that extra-pair paternity in tree swallows is mediated by sex steroids of females.

KEYWORDS

1,4,6-androstatrien-3,17-dione, extra-pair paternity, flutamide, promiscuity, social monogamy, *Tachycineta bicolor*, testosterone

1 | INTRODUCTION

In many socially monogamous animal species, females commonly engage in extra-pair copulations, which may result in offspring being sired by males other than a female's social mate (Griffith, Owens, & Thuman, 2002). As females may incur costs by copulating with

males outside of the social pair bond, many hypotheses have suggested that females obtain fitness benefits from extra-pair mating (reviewed in Petrie & Kempenaers, 1998). Recently, alternative hypotheses also have proposed that extra-pair copulation behavior of females evolved as a correlated response to either selection on extra-pair copulation behavior of males, or selection on fecundity

or other behaviors expressed by females (i.e., genetic constraints hypothesis, Forstmeier, Nakagawa, Griffith, & Kempenaers, 2014). In line with the latter hypothesis, studies have suggested that variation among females in rates of extra-pair paternity may be due to intrinsic differences in behavioral traits, such as aggression (While, Sinn, & Wapstra, 2009) or sexual behavior (Forstmeier, 2007). Such relationships may exist if behavioral traits and extra-pair copulation behavior of females are mediated by the same genes or hormones, and consequently correlated in their expression (i.e., pleiotropic effects, Forstmeier et al., 2014; Ketterson & Nolan, 1999).

In female birds, concentrations of circulating sex steroids, such as testosterone (T) and 17β -estradiol (E_2), peak prior to egg laying (Ketterson, Nolan, & Sandell, 2005; Williams, Kitaysky, & Vézina, 2004), which coincides with an increase in aggressive (Cristol & Johnsen, 1994) and copulatory behavior (Barber & Robertson, 2007; Crowe et al., 2009). Aggressive behavior may influence extra-pair paternity in species where females search for extra-pair mates by invading neighboring territories defended by resident females (see Kempenaers et al., 1992). Experimentally elevating T concentrations increases aggressive behavior in female birds, such as dark-eyed juncos (*Junco hyemalis*; Zysling et al., 2006), European starlings (*Sturnus vulgaris*; Sandell, 2007), and tree swallows (*Tachycineta bicolor*; Rosvall, 2013), but studies investigating whether elevated T mediates extra-pair paternity in female birds have produced mixed results. T-treated female dark-eyed juncos did not differ from control females in the proportion of extra-pair offspring in their nests (Gerlach & Ketterson, 2013), whereas extra-pair paternity was lower for T-treated female spotless starlings (*Sturnus unicolor*) and blue tits (*Cyanistes caeruleus*) when compared to control females (García-Vigón, Cordero, & Veiga, 2008; de Jong et al., 2017; respectively). Furthermore, de Jong et al. (2017) treated a third group of female blue tits with flutamide (F), which blocks androgen receptors, but reported no difference between F-treated and control females in the number of nests containing extra-pair young. Collectively, while these experimental studies suggest that sex steroids in females may not play a role in mediating extra-pair paternity, none of these investigations manipulated sex steroids by inhibiting the conversion of T to its estrogenic metabolites. Experimental studies have demonstrated that decreasing levels of E_2 lowers sexual behavior of female birds, such as solicitation (reviewed in Ritters & Alger, 2011), and blocking the estrogenic actions of T, in addition to androgen receptors, is necessary to fully reduce aggressive behavior (Archawaranon & Wiley, 1988). Therefore, the use of an aromatase inhibitor to suppress the conversion of T to E_2 in combination with F is necessary to fully understand the effects of sex steroids on the mating behavior of females.

In this study, our aim was to examine whether sex steroids influence extra-pair paternity and reproductive success, using female tree swallows as a study species. Female tree swallows produce T throughout the breeding season, but levels in females are lower than in males (Staley, Vleck, & Vleck, 2011). In general, T in female birds is produced primarily by the gonad (i.e., ovaries; Rosvall, Bergeon Burns, Hahn, & Ketterson, 2013), but also by the

adrenal gland (Staub & De Beer, 1997), and females have lower aromatase, but not androgen receptors, in the brain than males (Rosvall et al., 2013). Female tree swallows are highly aggressive, especially during pre-breeding, when competing with conspecifics for, and in defense of, a nest site (Leffelaar & Robertson, 1985; Rosvall, 2008), and those that are more aggressive are more likely to obtain nest sites when their availability is experimentally reduced (Rosvall, 2008). Tree swallows also exhibit high rates of extra-pair paternity, with up to 85% of nests containing at least one extra-pair young (O'Brien & Dawson, 2007). Females in this species engage in extra-pair copulations with males from neighboring territories (Venier, Dunn, Lijfeld, & Robertson, 1993), and presumably at roost sites away from the nest (Dunn & Whittingham, 2005; Stapleton & Robertson, 2006). Both males and females initiate extra-pair copulations, but those solicited by the female are more likely to result in fertilization (Venier et al., 1993). Forced extra-pair copulations from males are rare (see Venier et al., 1993), and male tree swallows do not guard their social mate (Leffelaar & Robertson, 1984), but copulate with them frequently to protect their paternity within the brood (Crowe et al., 2009). Therefore, as female tree swallows exhibit aggressive behavior to secure and defend nest sites, and this behavior coincides with social and extra-pair mating, sex steroids that mediate aggressive and sexual behavior of females may influence extra-pair paternity.

We experimentally elevated concentrations of T in female tree swallows using implants containing exogenous T in one treatment, and blocking the estrogenic and androgenic actions of T using implants containing 1,4,6-androstatrien-3,17-dione (ATD) and flutamide (F) in combination (ATD+F; i.e., decreasing the effects of T and E_2) in another treatment; control females received empty implants. As far as we are aware, the combined use of ATD+F has not been employed previously to examine how T-mediated behavior influences extra-pair paternity in female birds. As female tree swallows are highly aggressive during the pre-breeding period to secure and defend a nest site (Leffelaar & Robertson, 1985; Rosvall, 2008), and rates of extra-pair paternity are high in this species (O'Brien & Dawson, 2007), we predicted based on the biology of the species that nests of females treated with T would contain a greater number of extra-pair offspring than controls. Alternatively, if T-treated female tree swallows are aggressive (Rosvall, 2013) and exhibit increased territorial behavior and/or lowered attractiveness to potential mates (Ketterson et al., 2005), then the alternative outcome of elevating T is that nests of T-treated females would contain fewer extra-pair offspring than controls, as previously demonstrated in other species of birds (García-Vigón et al., 2008; de Jong et al., 2017). For ATD+F females, if they are less aggressive or had lower sexual interest toward extra-pair mates as a result of blocking the androgenic and estrogenic actions of T, then we predicted nests of these females should contain fewer extra-pair offspring than controls. Following the experimental alteration of T and its estrogenic metabolites, we subsequently used microsatellite markers to assign parentage to offspring and determined the proportion of extra-pair paternity in nests. We also monitored the nests of females during

the breeding season to examine how the treatments influenced reproductive success.

2 | MATERIALS AND METHODS

2.1 | Study area and species

We studied tree swallows breeding in nest boxes west of Prince George, BC, Canada (53°N, 123°W), from May to August 2012. The study area consisted of open agricultural areas intermixed with small wetlands and patches of coniferous and deciduous trees (see Dawson, Lawrie, & O'Brien, 2005 for more details). Tree swallows are small, migratory aerial insectivores that are socially monogamous and exhibit high rates of extra-pair paternity (e.g., O'Brien & Dawson, 2007). Clutch size is typically 4–7 eggs, which are incubated only by the female, and both sexes contribute to provisioning offspring (Winkler et al., 2011).

2.2 | General field methods and hormone manipulation

During early May, we visited nest boxes daily to document nest building and captured females in boxes once construction of the nest cup was complete, but prior to eggs being laid. We collected blood samples (approx. 50–80 μ l) from a subset of females ($N = 22$) to measure pre-laying levels of T and E_2 ; logistical constraints prevented additional sampling. Blood samples were collected in heparinized capillary tubes and stored on ice until they were centrifuged for 5 min in the laboratory. Plasma was transferred to microcentrifuge tubes and stored at -80°C until hormone analysis (see below).

Age of females was determined according to plumage color as being in either their second year (SY) of life or older (after-second year, ASY; Hussell, 1983); only ASY females were used in this study because SY females often breed later than ASY females (Stutchbury & Robertson, 1988), and their broods may be less likely to contain extra-pair offspring than ASY females (Kempnaers, Congdon, Boag, & Robertson, 1999). ASY females were sequentially allocated by capture order to the T, ATD+F, or control treatment after determining the treatment order randomly. Females received two 6-mm-long implants constructed from Silastic[®] laboratory tubing (1.47 mm I.D., 1.96 mm O.D.; Dow Corning #508-006) sealed at both ends with Silastic glue. T implants were packed with 3.5 mm of testosterone (approx. 0.0048 g; Sigma-Aldrich # T1500); a dose of 3.5 mm of T

was used in our study as Rosvall (2013) previously reported that 5 mm of T in female tree swallows lowered incubation temperature and resulted in hatching failure of eggs. ATD+F implants were packed with 3.5 mm of ATD (approx. 0.0052 g; Steraloids, Inc., #A4100-000) and 3.5 mm of F (approx. 0.0035 g; Sigma-Aldrich #F9397), a dosage similar to previous studies in female birds (Brown, Johnson, & Bottjer, 1993). Control implants were empty. Implants from all treatments were cleaned with ethanol and incubated at 37°C overnight in individual tubes containing 0.9% saline (Sigma-Aldrich #S8776) to begin the diffusion process of hormones across the tubing and avoid the surge of hormone release that can occur after implantation (Smith, Damassa, & Davidson, 1977). In the field, we treated all females with a topical anesthetic and then made a small incision using surgical scissors above the left flank. Two implants (T and empty, ATD and F, or two empty) were inserted subcutaneously under the skin along the flank, and the incision site was sealed with cyanoacrylate tissue adhesive (Vetbond[™] #1469SB). We implanted a total of 12 T, 15 ATD+F, and 13 control females for this study (see Table 1). As two crews implanted birds and the order in which each crew allocated birds to treatments differed, samples sizes differ among treatments. All females were implanted from May 19 to May 30, 2012, and the date females received implants did not differ by treatment ($F_{2,37} = 0.02, p = .99$).

After the hatching of eggs in June, we captured adult males while they were feeding offspring and collected a blood sample for paternity analysis. Control- and ATD+F-treated females were recaptured on days 12–16 of the nestling period to remove implants ($N = 19$), and when possible, we collected blood samples to measure post-implant levels of T and E_2 ($N = 15$). We also recaptured T-treated females and their social mates when possible ($N = 7$) before collecting the clutch (see below); however, we were unable to capture the social mate at five nests where the female had been implanted with T.

2.3 | Reproductive success

To examine whether experimentally altering T and its estrogenic metabolites influenced reproductive success in females, all nests boxes were monitored daily to record the date of clutch initiation (where January 1 = 1) and clutch size. Freshly laid eggs were numbered with a non-toxic marker for identification and were weighed with a digital scale (nearest 0.01 g). Once a clutch was complete, we checked nests daily to determine the onset of incubation, at which time nests were left undisturbed. Nests for T-treated females

TABLE 1 Number of female tree swallows that received implants containing 1,4,6-androstatrien-3,17-dione and flutamide (ATD+F), testosterone (T), or empty implants (control). “Initiated clutch” refers to the number of females that laid at least one egg. “Hatched eggs” refers to the number of females that hatched at least one egg. “Disappeared” refers to females who were usurped or abandoned their nesting attempt, or whose eggs were depredated

Treatment	Manipulated	Initiated clutch	Hatched eggs	Provisioned nestlings	Disappeared
ATD+F	15	12	11	10	5
Control	13	11	9	8	4
T	12	12	0	—	0

were monitored for 1 week after clutch completion to determine the onset of incubation, but as none of the T-treated females incubated their clutches, the eggs were collected (approx. 7–9 days after clutch completion) and stored at -20°C until DNA extraction. Females whose clutches were collected were observed at their nests during later stages of the breeding period (Berzins, personal observation), and so despite not incubating their eggs, none of the T-treated females abandoned their nest sites after receiving implants (Table 1). Nests in the ATD+F and control treatments were checked daily beginning 12 days after clutch completion to record the day of hatch, designated as day 0 of the brood-rearing period. Any eggs that failed to hatch from these nests were collected and stored as described above.

2.4 | Offspring performance and fledging success

On day 4 after hatching, we uniquely marked the legs of each nestling in a brood with a non-toxic marker for individual identification. We measured mass with a spring balance (nearest 0.125 g) and length of the combined head and bill (hereafter, head-bill) with digital calipers (nearest 0.1 mm) every 2 days from day 4 to 16, and length of the ninth primary feather with a ruler (nearest 0.5 mm) from day 8 to 16. On day 12, a small blood sample (20 μl) was collected for paternity analysis from the brachial vein of nestlings and stored in Queen's lysis buffer at 4°C (Seutin, White, & Boag, 1991) until DNA extraction (see below). From nestlings found dead in the nest prior to day 12, we collected brain tissue and stored it in the same manner as blood. On day 22, we visited nests to determine the number of nestlings that successfully fledged.

2.5 | Hormone measurements

We quantified concentrations of T in plasma samples of control- and T-treated females using a T enzyme immunoassay (EIA) kit (Expanded Range Salivary Testosterone EIA kit, Salimetrics #1-2402). As ATD has a high cross-reactivity with the T assay kit (Vandermeer, 2013), we instead quantified E_2 concentrations in plasma samples of females in the control and ATD+F treatment using an E_2 EIA kit (High Sensitivity Salivary 17β -estradiol EIA, Salimetrics #1-3702). Both assay kits have been used previously to quantify T and E_2 directly from avian plasma without extraction (Hall & MacDougall-Shackleton, 2012; Washburn, Millsbaugh, Morris, Schulz, & Faaborg, 2007) and were validated for use in tree swallows by demonstrating a displacement curve from a serial dilution of pooled plasma samples that was parallel to the standard curve for each assay. For most control females, we had sufficient plasma to perform both assays; plasma was limited for three females, and their plasma was randomly allocated to either the T or E_2 assay. As the T assay kit has a high cross-reactivity with other androgens (see Appendix S1), we report our results from the T assay as "androgen concentrations" as opposed to T concentrations. Samples were run in a single assay for each kit, and additional details are provided in Appendix S1. The intra-assay coefficient of variation calculated from high and low

controls of known concentration provided with each kit was 8.2% and 1.4% for T, and 0.7% and 1.2% for E_2 .

2.6 | Paternity analysis

Genomic DNA was extracted using Qiagen kits (Qiagen #69506 and #56304) and amplified by polymerase chain reaction (PCR) at either five or six microsatellite loci developed for *Tachycineta swallows* (Tle19, TaBi34, TaBi6, Tal8, TaBi10, and/or TaBi8; Makarewich, Stenzler, Ferretti, Winkler, & Lovette, 2009). PCR products were run on an ABI 3130xl automated sequencer (Applied Biosystems) to obtain individual genotypes and assign paternity.

We classified offspring as within-pair if they matched the resident male at five loci and extra-pair offspring if they mismatched the resident male at a minimum of one locus. For this experiment, only a single nestling mismatched the resident male at one locus; this mismatch was a heterozygous mismatch at the TaBi10 locus, and this mismatching allele was shared with another sibling that mismatched the resident male at a total of four loci (Tle19, TaBi6, TaBi10, and TaBi8). All other mismatches among nestlings and resident males occurred at two or more loci. We were able to assign parentage using five loci with no apparent null alleles to all offspring except for four offspring genotyped at only four loci either due to null alleles or poor amplification. For these four offspring, we calculated the probability of chance inclusion following O'Brien and Dawson (2007). All four offspring were within-pair, and the probability that a randomly chosen male would match these offspring at all four loci was low ($\bar{x} \pm \text{SE}$, 0.0028 ± 0.0025 ; range: 0.0010–0.0064). Further details outlining the paternity analysis are described in Appendix S1.

2.7 | Statistical analysis

To test whether treating females with implants altered concentrations of androgens and E_2 , we used a repeated measures analysis of variance (ANOVA), with timing of blood sample (pre-breeding or nestling rearing) as a within-subject repeated measure, treatment as a between-subject factor, and an interaction between timing of blood sampling and treatment. As only a few females from each treatment were blood-sampled both before and after receiving an implant ($N = 3$ –5 females sampled at both times per treatment), we also used a one-way ANOVA to test for differences in T and E_2 levels among treatment groups after females were implanted.

To test whether implanting females with T, ATD+F, or empty implants influenced extra-pair paternity, we first tested whether the presence of extra-pair offspring (yes or no) within a female's nest differed by treatment using a likelihood ratio test. In this analysis, we were able to include nests from the T treatment where the social mate of the female was captured ($N = 7$) as well as the nests where the female's social mate was not captured ($N = 5$, see Methods above). For these five clutches, we assessed the number of paternal alleles (i.e., alleles for each offspring that did not match the genetic mother), and clutches containing three or more different paternal alleles at a minimum of two loci were considered to be sired by multiple

TABLE 2 The number of female tree swallows whose nests contained extra-pair offspring after receiving implants containing 1,4,6-androstatrien-3,17-dione and flutamide (ATD+F), testosterone (T), or empty implants (control)

Nest contained extra-pair offspring	ATD+F	Control	T ^a
No	6	1	7
Yes	5	8	4

^aOne nest was excluded from this analysis because the clutch contained only a single offspring (see Methods for more details) and so sample sizes for this table differ for the T treatment from those presented in Table 1.

males (Borkowska, Borowski, & Krysiuk, 2009). For two nests, only two paternal alleles were detected at all loci (no extra-pair offspring), but in two other nests, we detected three paternal alleles at three to five loci (contained extra-pair offspring); one nest contained a single egg, so we were unable to determine its paternity status and excluded this clutch from the analysis (leaving $N = 11$ nests total for this analysis; Table 2). Four females initiated clutches the day after receiving implants (three T and one ATD+F); all three T females then delayed egg laying for 2–4 days after laying their first egg, and we included these T females in the analysis, but only considered eggs laid after the delay in laying for the analysis as the effects of implants can be detected in plasma or on behavior within a day (see De Ridder, Pinxten, & Eens, 2000; Smith et al., 1977). The ATD+F female continued to lay eggs after initiating the clutch and so we performed the analysis with and without the inclusion of this female.

We also tested whether the proportion of extra-pair offspring in the nests of females differed by treatment using a generalized linear model (GLM) fitted with binomial errors and logit link function for proportion data (Crawley, 2013). The response variable was the number of within-pair offspring, and the binomial denominator was the number of offspring in the nest. Treatment was included as a fixed factor, and the number of days a female was implanted prior to initiating a clutch ($\bar{x} \pm SE$, 9.63 ± 1.35 days, range: 1–30) was included as a covariate. We also included the interaction between treatment and the number of days a female was implanted. Clutch initiation date and number of days implanted were correlated ($r = .93$, $p < .0001$), so we did not include clutch initiation date in the analysis. To account for overdispersion, we refitted the model with a quasi-binomial error structure (Crawley, 2013). Following Crawley (2013), we tested the significance of the interaction and covariate by assessing the analysis of deviance between models. Of the four females that initiated clutches the day following the manipulation (see above), we had paternity data for two (one T and one ATD+F), and as described above, we included the T-treated female in the analysis (but only considered the eggs after the delay in breeding) and performed the analysis with and without the inclusion of the ATD+F female that continued to lay eggs; all other females initiated clutches at least 4 days after being implanted.

To test whether clutch initiation dates differed among females after receiving implants, we used a general least-squares model from the R package nlme (Pinheiro, Bates, DebRoy, Sarkar, & R Core

Team, 2016) to account for heteroscedasticity in variances, following Cleasby and Nakagawa (2011). From this analysis, we excluded the three T and one ATD+F female that initiated a clutch the morning after receiving implants because the physiological processes associated with egg formation would have been initiated before these females were implanted (Williams, 2012), and so it is unlikely the hormones influenced initiation dates in these cases. Because the effects of silastic implants on T and aromatase activity can be detected in plasma within a day of being implanted (see Balthazart, Foidart, & Hendrick, 1990; Smith et al., 1977), all four females were included in analyses for clutch size and egg mass. We tested whether clutch size and average egg mass differed among females in each treatment using analysis of covariance (ANCOVA) for each variable separately, which included clutch initiation date as a covariate to control for the seasonal decline in clutch size that occurs in tree swallows (Winkler et al., 2014). We z-transformed initiation date so that the effect of treatment on clutch size or average egg mass could be interpreted in the presence of a treatment by initiation date interaction (Schielzeth, 2010). The eggs of one T female were depredated after clutch initiation and so sample sizes for clutch size and egg mass differ for T-treated birds from that listed in Table 1.

To determine whether offspring performance differed among the nests of females in the ATD+F and control treatments, we examined mass and size (length of ninth primary feather and head-bill) at day 16, and the growth rates of nestlings. Growth rate constants were calculated for each nestling using a logistic model for mass, Gompertz model for head-bill, and linear model for ninth primary following Dawson et al. (2005). All nests had complete growth data, but one nest was measured on day 17 and was not included in the analysis of mass or size at day 16. Moreover, as we were interested in the effects of female treatment on nestling growth and size, we excluded from these analyses two nests (one ATD+F and one control) where the female, although observed during egg laying and/or incubation, was not observed after the hatching of eggs and a second (helper) female was provisioning the nestlings. We used random intercept linear mixed models from the R package nlme (Pinheiro et al., 2016) with mass or size at day 16, or growth rate constants, as dependent variables, treatment as the fixed factor, and brood identity as a random factor. Brood size at day 16 and time of day measurements were both z-transformed and included as covariates in models analyzing mass and size at day 16; only brood size was used in models analyzing growth rate constants. Models also included the interaction between treatment and brood size at day 16.

We tested whether the fledging success of nestlings, that is, number fledged in relation to clutch size, differed between the ATD+F and control treatments for broods that hatched at least one nestling using a GLM fitted with quasi-binomial errors and logit link function as described above. Treatment was included as a fixed factor, and brood size at day 16 and hatch date as covariates.

For all analyses, we removed interactions and covariates that did not approach significance ($p \geq .10$) in a backward, stepwise fashion (Montgomery, Peck, & Vining, 2012; see, e.g., García-de Blas, Mateo, & Alonso-Alvarez, 2015). We retained variables in models that

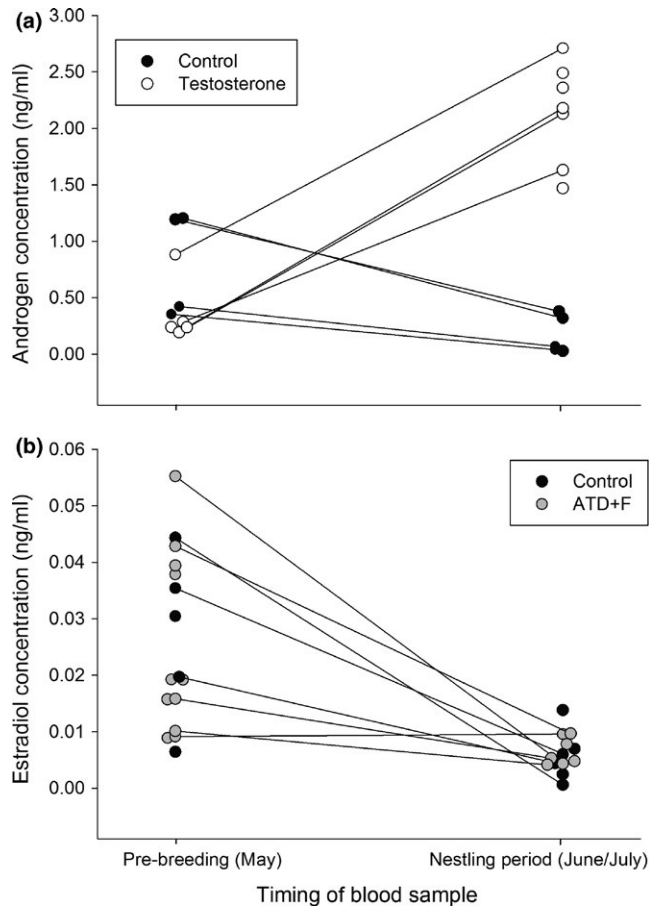


FIGURE 1 Concentrations of (a) androgens and (b) estradiol in female tree swallows, measured prior to receiving implants during pre-breeding in May and after receiving implants containing testosterone (T) or 1,4,6 androstatrien-3,17-dione and flutamide (ATD+F), and sham implants during the nestling period in June/July. Black circles represent control females, white circles represent T-treated females, and gray circles represent females treated with ATD+F. Repeated measures for individuals are connected by a line, whereas individual circles indicate single measurements where repeated measurements were not available. Data points were jittered for easier interpretation

approached significance ($p \leq .10$) to ensure that biologically meaningful, but not statistically significant, patterns in the data were not overlooked (i.e., reduce the possibility of type II errors, Golberg & Cho, 2004; Moreira et al., 2005) because our sample sizes are small. Nevertheless, results were considered significant at $p \leq .05$. Least square means from models ± 1 standard error (SE; lsmeans; Lenth, 2016) are reported, unless otherwise stated. The overall significance of omnibus tests was examined using post hoc tests with a false discovery rate adjustment for multiple comparisons (Verhoeven, Simonsen, & McIntyre, 2005). Effect sizes are calculated as general eta-squared (η_G^2) for the repeated measures analysis following Lakens (2013) and as the correlation coefficient (r) for post hoc comparisons following Field, Miles, & Field (2012). All statistical analyses were performed using SPSS (IBM Corp., 2011) and R (R Development Core Team, 2015) statistical software.

3 | RESULTS

3.1 | Androgen and E₂ concentrations

Results of the repeated measures ANOVA showed that within individual females, androgen concentrations differed between the two sampling periods, that is, before females received implants during pre-breeding in May and after receiving implants when measured during the nestling period in June/early July ($F_{1,6} = 31.95$, $p = .001$, $\eta_G^2 = 0.59$; Figure 1a), and between the T and control treatments ($F_{1,6} = 10.68$, $p = .02$, $\eta_G^2 = 0.56$; Figure 1a). The interaction between treatment and sampling time was also significant ($F_{1,6} = 129.50$, $p < .0001$, $\eta_G^2 = 0.85$), and when we analyzed the data separately by treatment, the results showed that androgen concentrations decreased in control females after receiving empty implants ($F_{1,3} = 15.39$, $p = .03$, $\eta_G^2 = 0.48$; Figure 1a), but increased in females after being implanted with T ($F_{1,3} = 155.31$, $p = .001$, $\eta_G^2 = 0.87$; Figure 1a). As the number of females blood-sampled both before and after receiving implants was small ($N = 4$ for each treatment), we also compared circulating levels of androgens for all control- and T-treated females that were captured, either pre-breeding in May or during the nestling period. There was no difference in androgen concentration between treatments prior to females receiving implants ($F_{1,7} = 12.84$, $p = .14$, $\eta_G^2 = 0.29$; Figure 1a), but T-treated females had a higher concentration of androgens compared to control females during the nestling period ($F_{1,11} = 86.56$, $p < .0001$, $\eta_G^2 = 0.90$; Figure 1a).

The concentration of E₂ decreased from pre-breeding in May to day 12 of the nestling period among control females and those treated with ATD+F implants ($F_{1,6} = 12.95$, $p = .01$, $\eta_G^2 = 0.52$; Figure 1b); however, there was no effect of treatment on E₂ concentration ($F_{1,6} = 0.07$, $p = .80$, $\eta_G^2 = 0.006$) and the interaction between time and treatment also was not significant ($F_{1,6} = 0.44$, $p = .53$, $\eta_G^2 = 0.04$). As only three control females were blood-sampled both before and after receiving implants, we also compared E₂ concentrations between treatment groups post-implant. E₂ concentration did not differ between ATD+F-treated and control females for blood samples collected on day 12 of the nestling period ($F_{1,11} = 0.14$, $p = .71$, $\eta_G^2 = 0.01$; Figure 1b).

3.2 | Extra-pair paternity

Treatment of female tree swallows with ATD+F, T, or empty implants, influenced whether nests contained extra-pair offspring ($G_2 = 6.83$, $N = 31$, $p = .03$; Table 2). To further examine these results, we performed subanalyses between pairs of treatment groups, which showed that fewer broods in the ATD+F treatment contained extra-pair offspring compared to control broods ($G_1 = 4.61$, $N = 20$, $p = .04$; Table 2). Excluding the data for the female that initiated a clutch the day after being implanted with ATD+F resulted in a similar trend ($p = .059$). Fewer nests in the T treatment contained extra-pair offspring compared to controls ($G_2 = 6.22$, $N = 20$, $p = .01$; Table 2). In this analysis, we considered

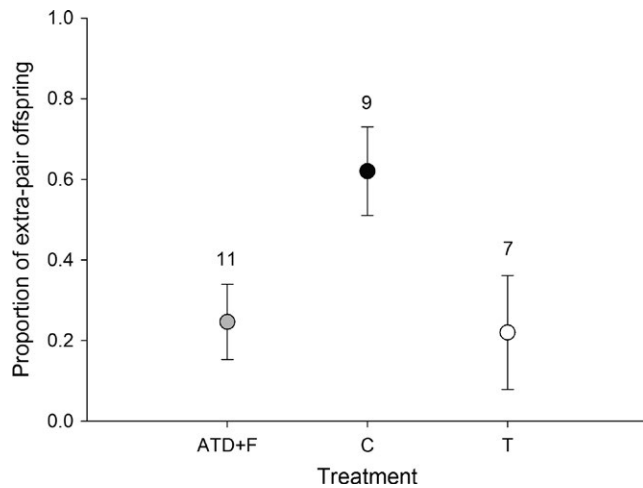


FIGURE 2 The proportion (\pm SE) of extra-pair offspring in nests of tree swallows where females were either implanted with 1,4,6 androstatrien-3,17-dione and flutamide (ATD+F) or testosterone (T), and for controls (C; sham implanted). Sample sizes are given above error bars

all nests with available paternity data; however, paternity data were incomplete for five nests due to the disappearance or breakage of a single egg in each of the nests (see above). Performing the analysis with the exclusion of these data did not alter the conclusion of the results (p -values $<.05$).

The proportion of extra-pair paternity also differed significantly among treatment groups ($F_{2,24} = 4.12$, $p = .03$; Figure 2). The number of days a female was implanted prior to initiating a clutch, as well as the interaction between treatment and the number of days a female was implanted, were not significant predictors of extra-pair paternity (p -values $>.20$) and were removed from the model. Post hoc tests showed that females treated with ATD+F had a lower proportion of extra-pair offspring in their nests compared to controls (estimate = -1.74 ± 0.73 , $t_{24} = -2.37$, $p = .04$, $r = -.43$; Figure 2). Similarly, when compared to control females, females treated with T also produced a lower proportion of extra-pair offspring (estimate = -2.16 ± 0.99 , $t_{24} = -2.18$, $p = .04$, $r = -.41$; Figure 2). There was no difference in the proportion of extra-pair offspring between nests where females were treated with ATD+F and T (estimate = 0.43 ± 0.98 , $t_{24} = 0.44$, $p = .66$, $r = .09$). Excluding the ATD+F female that initiated the day after being implanted (see Methods) from this analysis did not change our conclusions (treatment: $p = .04$; ATD+F versus control: $p = .04$; T vs. control: $p = .04$). Additionally, performing the analysis with the exclusion of nests with incomplete paternity data (see above) did not alter the conclusion of the results (p -values $<.05$).

3.3 | Reproductive success

Clutch initiation date differed by treatment ($F_{2,28} = 3.52$, $p = .04$). Post hoc tests showed that T-treated females tended to initiate clutches later than control females (estimate = -7.90 ± 3.32 , $t_{28} = -2.38$, $p = .07$, $r = -.44$), but not ATD+F females (estimate = -5.25 ± 3.61 ,

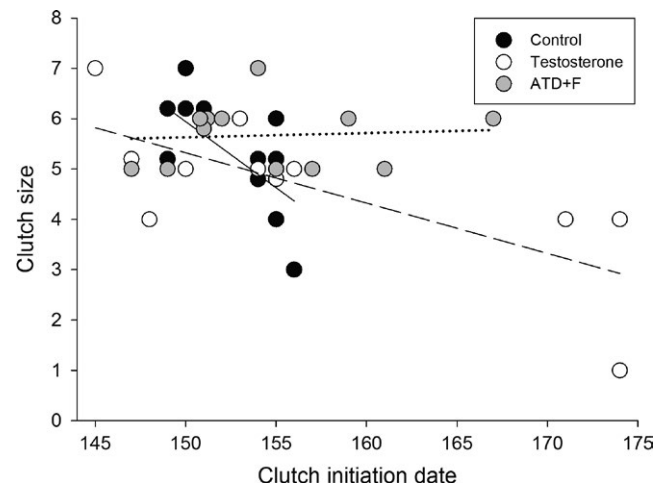


FIGURE 3 Relationship between clutch size and initiation date (1 January = 1) for nests where female tree swallows were implanted with 1,4,6 androstatrien-3,17-dione and flutamide (ATD+F; dotted line), testosterone (dashed line), or received sham implants (solid line). Data points for controls were jittered for easier interpretation

$t_{28} = -1.46$, $p = .16$, $r = -.27$), and no difference in clutch initiation date was observed between ATD+F-treated and control females (estimate = -2.63 ± 1.83 , $t_{28} = -1.44$, $p = .16$, $r = -.26$). However, the trend for later breeding in the T treatment was due to the influence of three females that initiated their clutch approximately 25–34 days later than the other females in this treatment group (see Figure 3). As these females did not receive implants later than the other females in this treatment group, we suspect that later initiation dates by these females likely were due to elevated T. Removing these three females from the analysis showed that clutch initiation date no longer differed by treatment ($F_{2,25} = 1.41$, $p = .26$).

After controlling for clutch initiation date (estimate = -1.90 ± 0.75 , $F_{1,28} = 6.45$, $p = .02$), clutch sizes differed among treatment groups ($F_{2,28} = 3.31$, $p = .05$; mean clutch size: ATD+F = 5.67 ± 0.26 ; control = 4.63 ± 0.37 ; T = 4.84 ± 0.27). Post hoc tests suggested that females treated with ATD+F tended to lay larger clutches than females in the T (estimate = 0.80 ± 0.39 , $t_{28} = 2.20$, $p = .065$, $r = .38$) and control treatments (estimate = 0.96 ± 0.43 , $t_{28} = 2.20$, $p = .065$, $r = .38$). Clutch size of control and T-treated females did not differ by treatment (estimate = 0.16 ± 0.44 , $t_{28} = 0.35$, $p = .73$, $r = .07$). There also was a significant interaction between treatment and initiation date ($F_{2,28} = 3.58$, $p = .04$; Figure 3). Analyzing data by treatment revealed a negative relationship between clutch size and initiation date in control (estimate = -1.87 ± 0.73 , $F_{1,9} = 6.61$, $p = .03$, $r = -.63$) and T treatment groups (estimate = -1.09 ± 0.34 , $F_{1,9} = 9.92$, $p = .01$, $r = -.72$), but no relationship was detected in the ATD+F treatment group (estimate = 0.06 ± 0.26 , $F_{1,10} = 0.05$, $p = .82$, $r = .07$). As noted above, three T-treated females initiated their clutches later than the other T-treated females. Removal of these females from the analysis changed the results so that no effect of treatment on clutch size was detected ($F_{2,25} = 1.21$,

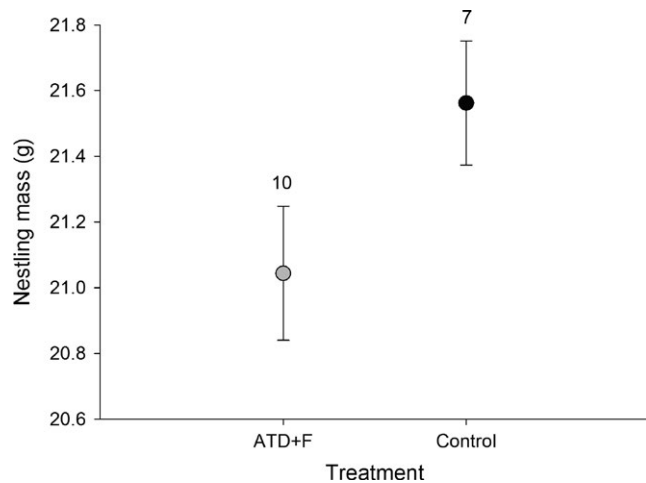


FIGURE 4 Mean (\pm SE) body mass at day 16, after controlling for brood size, of nestling tree swallows raised in broods where females were treated with 1,4,6 androstatrien-3,17-dione and flutamide (ATD+F) and sham implants. Sample sizes indicate the number of broods within each treatment and are given above error bars

$p = .31$), even after controlling for clutch initiation date (estimate = -1.18 ± 0.43 , $F_{1,25} = 7.70$, $p = .01$); however, there was still a significant interaction between treatment and clutch initiation date ($F_{2,25} = 3.42$, $p = .048$). This interaction was due to the decline in clutch size with later laying dates in control nests only (see above) as clutch sizes no longer declined with later clutch initiation dates in the T treatment once the three late-breeding birds were removed from the analysis (estimate = -0.24 ± 0.35 , $F_{1,6} = 0.50$, $p = .51$). Average egg mass did not differ among treatment groups ($F_{2,30} = 0.67$, $p = .52$; ATD+F = 1.80 ± 0.04 ; control = 1.78 ± 0.04 ; T = 1.85 ± 0.05).

3.4 | Offspring performance and fledging success

Nestlings were lighter at day 16 in the ATD+F than control treatments (estimate = -0.52 ± 0.29 , $F_{1,13} = 6.05$, $p = .02$; Figure 4), after controlling for brood size (estimate = -0.63 ± 0.18 , $F_{1,13} = 3.73$, $p = .08$). There also was a significant interaction between treatment and brood size (estimate = 0.80 ± 0.27 , $F_{1,13} = 8.71$, $p = .01$). Analyzing data by treatment indicated that there was a negative relationship between brood size and mass at day 16 for nestlings reared in control broods (estimate = -0.62 ± 0.15 , $F_{1,5} = 16.12$, $p = .01$), but no relationship was observed in ATD+F broods (estimate = 0.18 ± 0.18 , $F_{1,8} = 1.00$, $p = .35$). Nestlings reared in ATD+F and control broods did not differ in length of ninth primary ($F_{1,14} = 0.14$, $p = .72$) after controlling for time of measurement ($F_{1,14} = 3.89$, $p = .07$) or length of head-bill ($F_{1,15} = 2.02$, $p = .18$). Nestlings grew at similar rates regardless of the treatment of the female (ninth primary, head-bill, and mass, all p -values $>.16$). Fledging success of nestlings did not differ by treatment ($F_{1,18} = 0.90$, $p = .35$); $86.2 \pm 0.06\%$ of nestlings in the ATD+F and $94.0 \pm 0.04\%$ of nestling in the control treatments fledged successfully.

4 | DISCUSSION

In our study, we treated female tree swallows with T, ATD+F, or empty implants to examine whether sex steroids influence rates of extra-pair paternity. Female tree swallows are highly aggressive when securing and defending nest sites (Leffelaar & Robertson, 1985; Rosvall, 2008), which coincides with the timing of social and extra-pair mating. As this aggressive behavior may facilitate their ability to intrude on neighboring territories to pursue extra-pair copulations, we predicted that females treated with T would have a higher proportion of extra-pair offspring in their nests. Although we based this prediction on the biology of the species, our results instead showed that T-treated females, which had elevated levels of androgens after being treated with T (Figure 1a), produced fewer extra-pair offspring than control females (Table 2; Figure 2), a finding consistent with previous studies in other species of female birds (García-Vigón et al., 2008; de Jong et al., 2017).

García-Vigón et al. (2008) hypothesized that T-treated female spotless starlings may have produced fewer extra-pair offspring because T reduced the attractiveness of females, T-treated females invested more time in aggressive encounters and territory defense, or they rejected copulations from extra-pair males because they were more aggressive. T has been shown to have masculinizing effects on female traits in a variety of taxa (Ketterson et al., 2005), and given that exogenous T increases aggressive behavior in female tree swallows (Rosvall, 2013), females in our study, if they behaved aggressively, may have appeared unattractive or were aggressive to extra-pair mates. Moreover, T-treated females also may have pursued or solicited extra-pair copulations less if they spent more time involved in aggressive interactions or territory defense, as previously suggested (García-Vigón et al., 2008; de Jong et al., 2017). As such, female tree swallows treated with T may have copulated less with potential extra-pair mates than their social mate, leading to a greater proportion of within-pair offspring in their nests.

We predicted that broods produced by female tree swallows treated with ATD+F would have a lower proportion of extra-pair offspring, and although ATD+F-treated females did not have lower E_2 concentrations than control females (Figure 1b), our results were consistent with this prediction (Table 2; Figure 2). We suspect that we did not detect a difference in plasma E_2 levels between ATD+F and control females because we collected post-implant blood samples during the nestling period (approx. 42.93 ± 2.56 days after receiving implants) when E_2 levels would have been naturally low in control females (e.g., Schwabl, Lindsay, Barron, & Webster, 2014; Williams et al., 2004; Figure 1b). Nevertheless, low E_2 levels late in the breeding season for females treated with ATD+F do not indicate that ATD treatment was unsuccessful at altering female behavior; for instance, male European stonechats (*Saxicola torquata rubicola*) exhibited reduced aggressive behavior toward decoy intruders following treatment with ATD+F despite having naturally low plasma E_2 levels both before and after receiving implants (Canoine & Gwinner, 2002). In line with this, studies in songbirds that have altered aggressive or song behavior of males using ATD+F implants have reported

unchanged or elevated levels of E_2 (Archawaranon & Wiley, 1988; Soma, Sullivan, & Wingfield, 1999), and/or elevated levels of T, luteinizing hormone, or 5α -dihydrotestosterone after treatment (Hau, Wikelski, Soma, & Wingfield, 2000; Moore, Walker, & Wingfield, 2004; Soma et al., 1999; Van Duyse, Pinxten, Snoeijs, & Eens, 2005). It has been previously suggested that ATD suppresses E_2 levels in the brain rather than in plasma (Soma et al., 1999), but we have no data on aromatase activity for ATD+F-treated female swallows, and unfortunately, ATD has a high cross-reactivity with the T assay kit that we used (Vandermeer, 2013) so we were not able to measure T levels for these females. Nevertheless, that fewer extra-pair offspring were observed in broods produced by ATD+F females compared to controls (Table 2; Figure 2) demonstrates that the ATD+F treatment successfully altered rates of extra-pair paternity.

Although we were unable to directly observe how our experimental manipulation altered behavior related to extra-pair paternity, due to the rarity of witnessing extra-pair copulations in wild birds (e.g., Venier et al., 1993), we propose two mechanisms to explain the lower proportion of extra-pair offspring in broods of females treated with ATD+F (Figure 2). First, extra-pair paternity in tree swallows often results when females visit neighboring territories to copulate with the resident males (Venier et al., 1993). As resident females vigorously defend their territories from intruding females seeking extra-pair copulations (e.g., blue tits, Kempenaers et al., 1992), females treated with ATD+F, if they were less aggressive as a result of blocking the actions of T and its estrogenic metabolites, may not have pursued extra-pair copulations from males on neighboring territories. Consequently, females in the ATD+F treatment may have experienced limited opportunities for encountering extra-pair males in territories other than their own and copulated more frequently with their social mate, resulting in more within-pair offspring produced (Crowe et al., 2009).

Second, solicitation of extra-pair copulations by female tree swallows treated with ATD+F may have been reduced compared to controls as a result of blocking the estrogenic and androgenic actions of T. For example, T can mediate sexual behavior of females by being aromatized to E_2 in the brain and acting on estrogen receptors (Riters & Alger, 2011), and experimental studies in birds and mammals have demonstrated that these behaviors can be delayed or suppressed using an aromatase inhibitor (Belle, Sharp, & Lea, 2005; Rissman, Clendenon, & Krohmer, 1990; but see Tomaszycski, Banerjee, & Adkins-Regan, 2006). For instance, treating female canaries (*Serinus canaria*) with an aromatase inhibitor delayed copulation solicitation displays (Leboucher, Béguin, Mauget, & Kreutzer, 1998). Alternatively, the lower rate of extra-pair paternity in broods of ATD+F females may have been due to F preventing T from binding directly to the androgen receptors and altering the sexual behavior of females toward extra-pair males. For example, female goats (*Capra aegagrus hircus*) treated with F were less receptive toward males than females treated with sex steroids (Imwalle & Katz, 2004), although a recent study in blue tits that treated females with F alone reported no difference in the rate of extra-pair paternity in their broods compared to control

females (de Jong et al., 2017). Although our study cannot distinguish whether the effect of the ATD+F on extra-pair paternity in tree swallows is due to inhibiting aromatase activity, androgen receptor binding, or both, the lower rate of extra-pair paternity in broods produced by ATD+F females may be due to these females soliciting fewer extra-pair copulations from extra-pair males and copulating more often with their social mates. Future studies could use implants containing E_2 and other aromatase inhibitors, such as fadrozole, to examine the proximate effects of female sex steroids on extra-pair paternity.

Although it may be difficult to reconcile how rates of extra-pair paternity were lower when we elevated T (T treatment) and also blocked the androgenic and estrogenic actions of T (ATD+F treatment) in female tree swallows (Table 2; Figure 2), the similar outcomes we observed for both treatment groups are likely the result of sex steroids altering the attractiveness of females to potential mates or extra-pair copulation behavior of females, albeit through different mechanisms as described above. It is unlikely that the lower rates of extra-pair paternity were the result of implants themselves as the broods of control females did not exhibit lower rates of extra-pair paternity compared to those previously reported at our study area and others (see O'Brien & Dawson, 2007 and references therein). While it is possible that the lower rates of extra-pair paternity were due to chance given our small sample sizes (Table 1), our sample sizes are similar to other studies demonstrating altered behavior following treatment with ATD+F and/or T (e.g., Canoine & Gwinner, 2002; Hau et al., 2000; Moore et al., 2004). Moreover, we tested the effect of ATD+F and T treatments on extra-pair paternity using two different analyses, number of nests containing extra-pair offspring and the proportion of extra-pair offspring in nests, and both led to the same conclusion; sex steroids of females influenced extra-pair paternity.

In species where females initiate extra-pair copulations, studies have demonstrated the importance of females pursuing and soliciting extra-pair copulations. For instance, broods produced by females that foray into neighboring territories often contain extra-pair offspring (Chiver, Stutchbury, & Morton, 2008; Double & Cockburn, 2000; but see Celis-Murillo, Schelsky, Benson, Louder, & Ward, 2017). Likewise, solicitation of extra-pair copulations also may be an important mechanism by which females obtain extra-pair copulations. Sheldon (1994) hypothesized that female chaffinch (*Fringilla coelebs*) performed solicitation displays to signal their fertility to neighboring extra-pair males, and female reed warblers (*Acrocephalus scirpaceus*) that exhibited solicitation behavior toward extra-pair males were more likely to have extra-pair offspring in their broods (Hoi, Krištofik, & Darolová, 2013). Although our results are consistent with sex steroids mediating rates of extra-pair paternity by influencing the extra-pair copulation behavior of females, further studies that combine observations of female behavior in aviaries (Drevon & Slagsvold, 2005) with new technologies in the field to track extra-territorial forays (Celis-Murillo et al., 2017; Schlicht, Valcu, & Kempenaers, 2015) would greatly improve our understanding of the behavioral mechanisms that underlie variation in extra-pair paternity among females.

Reproductive success of females treated with T was negatively affected as these females did not incubate their eggs, even though they developed brood patches and continued to defend, enter, and perch on their nest box during the incubation and nestling periods of the breeding season (Berzins, personal observation). These results are similar to a previous study in tree swallows that experimentally elevated T levels of females during incubation and reported cooler nest temperatures and complete hatching failure (Rosvall, 2013). Female great tits (*Parus major*) implanted with T during nest building also had lower incubation temperatures and hatching success (de Jong et al., 2016), but such negative effects of T on incubation behavior were not observed in female dark-eyed juncos implanted prior to breeding (Clotfelter et al., 2004). The results of our study and others suggest that, at least in some species, elevated T levels disrupt incubation behavior. In contrast, ATD+F females tended to lay larger clutches than females in the T and control treatments, but this result is likely driven by clutch size not declining with initiation date as observed in the control and T treatments (Figure 3), and was no longer detected when the three late-breeding T-treated females were removed from the analysis. Female zebra finches treated with ATD+F had lower egg production compared to control females (Tomaszycki et al., 2006); however, we suspect that we did not detect such an effect of ATD+F treatment on female tree swallows because we used a much lower dose of ATD+F in our study (approximately 0.17 mm/g of body weight in our study compared to 0.82 mm/g of body weight in Tomaszycki et al., 2006), as our intention was to reduce, but not completely impair, aromatase activity. Females treated with ATD+F did have lighter nestlings in their brood on day 16, even after controlling for brood size, compared to control females (Figure 4). This may be the consequence of ATD+F being transmitted to the egg yolk and either blocking embryonic exposure to maternal sex steroids or production of sex steroids by the embryo (Groothuis, Müller, von Engelhardt, Carere, & Eising, 2005), or ATD+F-treated females or their mates provisioning nestlings less than in the control group. Nonetheless, despite nestlings reared in the ATD+F treatment being lighter on day 16, nestlings were of similar structural size, grew at similar rates, and were as likely to fledge as those raised by control females.

In conclusion, our study demonstrates that experimentally altering T and/or its estrogenic metabolites prior to breeding in female tree swallows reduces the rate of extra-paternity in their nests. Our results are similar for both treatments because T and ATD+F likely influenced either the attractiveness of females or whether females pursued, solicited, or were receptive to extra-pair copulations from potential mates; both mechanisms would ultimately lead to social mates of treated females securing a higher proportion of within-pair offspring. Overall, the results of our study suggest that extra-pair paternity in tree swallows is mediated by sex steroids of females.

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ORCID

Lisha L. Berzins  <http://orcid.org/0000-0001-8277-9828>

REFERENCES

- Archawaranon, M., & Wiley, R. H. (1988). Control of aggression and dominance in white-throated sparrows by testosterone and its metabolites. *Hormones and Behavior*, 22, 497–517. [https://doi.org/10.1016/0018-506X\(88\)90054-2](https://doi.org/10.1016/0018-506X(88)90054-2)
- Balthazart, J., Foidart, A., & Hendrick, J. C. (1990). The induction by testosterone of aromatase activity in the preoptic area and activation of copulatory behavior. *Physiology and Behavior*, 47, 83–94. [https://doi.org/10.1016/0031-9384\(90\)90045-6](https://doi.org/10.1016/0031-9384(90)90045-6)
- Barber, C. A., & Robertson, R. J. (2007). Timing of copulations and the pattern of paternity in relation to laying order in tree swallows *Tachycineta bicolor*. *Journal of Avian Biology*, 38, 249–254. <https://doi.org/10.1111/j.0908-8857.2007.04042.x>
- Belle, M. D. C., Sharp, P. J., & Lea, R. W. (2005). Aromatase inhibition abolishes courtship behaviours in the ring dove (*Streptopelia risoria*) and reduces androgen and progesterone receptors in the hypothalamus and anterior pituitary gland. *Molecular and Cellular Biochemistry*, 276, 193–204. <https://doi.org/10.1007/s11010-005-4060-6>
- Borkowska, A., Borowski, Z., & Krysiuk, K. (2009). Multiple paternity in free-living root voles (*Microtus oeconomus*). *Behavioral Processes*, 82, 211–213. <https://doi.org/10.1016/j.beproc.2009.05.003>
- Brown, S. D., Johnson, F., & Bottjer, S. W. (1993). Neurogenesis in adult canary telencephalon is independent of gonadal hormone levels. *Journal of Neuroscience*, 13, 2024–2032. <https://doi.org/10.1523/JNEUROSCI.13-05-02024.1993>
- Canoine, V., & Gwinner, E. (2002). Seasonal differences in the hormonal control of territorial aggression in free-living European stonechats. *Hormones and Behavior*, 41, 1–8. <https://doi.org/10.1006/hbeh.2001.1720>
- Celis-Murillo, A., Schelsky, W., Benson, T. J., Louder, M. I. M., & Ward, M. P. (2017). Patterns, correlates, and paternity consequences of extraterritorial foray behavior in the field sparrow (*Spizella pusilla*): An automated telemetry approach. *Behavioral Ecology and Sociobiology*, 71, 45. <https://doi.org/10.1007/s00265-017-2273-9>

- Chiver, I., Stutchbury, B. J. M., & Morton, E. S. (2008). Do male plumage and song characteristics influence female off-territory forays and paternity in the hooded warbler? *Behavioral Ecology and Sociobiology*, *62*, 1981–1990. <https://doi.org/10.1007/s00265-008-0629-x>
- Cleasby, I. R., & Nakagawa, S. (2011). Neglected biological patterns in the residuals. *Behavioral Ecology and Sociobiology*, *65*, 2361–2372. <https://doi.org/10.1007/s00265-011-1254-7>
- Clotfelter, E. D., O'Neal, D. M., Gaudio, J. M., Casto, J. M., Parker-Renga, I. M., Snajdr, E. A., ... Ketterson, E. D. (2004). Consequences of elevating plasma testosterone in females of a socially monogamous songbird: Evidence of constraints on male evolution? *Hormones and Behavior*, *46*, 171–178. <https://doi.org/10.1016/j.yhbeh.2004.03.003>
- Crawley, M. J. (2013). *The R book*, 2nd ed. Chichester, UK: John Wiley & Sons Ltd.
- Cristol, D. A., & Johnsen, T. S. (1994). Spring arrival, aggression and testosterone in female red-winged blackbirds (*Agelaius phoeniceus*). *The Auk*, *111*, 210–214. <https://doi.org/10.2307/4088527>
- Crowe, S. A., Kleven, O., Delmore, K. E., Laskemoen, T., Nocera, J. J., Lifjeld, J. T., & Robertson, R. J. (2009). Paternity assurance through frequent copulations in a wild passerine with intense sperm competition. *Animal Behavior*, *77*, 183–187. <https://doi.org/10.1016/j.anbehav.2008.09.024>
- Dawson, R. D., Lawrie, C. C., & O'Brien, E. L. (2005). The importance of microclimate variation in determining size, growth and survival of avian offspring: Experimental evidence from a cavity nesting passerine. *Oecologia*, *144*, 499–507. <https://doi.org/10.1007/s00442-005-0075-7>
- De Ridder, E., Pinxten, R., & Eens, M. (2000). Experimental evidence of a testosterone-induced shift from paternal to mating behaviour in a facultatively polygynous songbird. *Behavioral Ecology and Sociobiology*, *49*, 24–30. <https://doi.org/10.1007/s002650000266>
- Double, M., & Cockburn, A. (2000). Pre-dawn infidelity: Females control extra-pair mating in superb fairy-wrens. *Proceeding of the Royal Society of London B: Biological Sciences*, *267*, 465–470. <https://doi.org/10.1098/rspb.2000.1023>
- Drevon, T., & Slagsvold, T. (2005). When and from whom do female pied flycatchers (*Ficedula hypoleuca*) solicit copulations. *Behaviour*, *142*, 1059–1076. <https://doi.org/10.1163/156853905774405335>
- Dunn, P. O., & Whittingham, L. A. (2005). Radio-tracking of female Tree Swallows prior to egg-laying. *Journal of Field Ornithology*, *76*, 259–263. <https://doi.org/10.1648/0273-8570-76.3.259>
- Field, A., Miles, J., & Field, Z. (2012). *Discovering statistics using R*, 4th ed. Thousand Oaks, CA: Sage Publications Ltd.
- Forstmeier, W. (2007). Do individual females differ intrinsically in their propensity to engage in extra-pair copulations? *PLoS ONE*, *2*, e952. <https://doi.org/10.1371/journal.pone.0000952>
- Forstmeier, W., Nakagawa, S., Griffith, S. C., & Kempenaers, B. (2014). Female extra-pair mating: Adaptation or genetic constraint? *Trends in Ecology and Evolution*, *29*, 456–464. <https://doi.org/10.1016/j.tree.2014.05.005>
- García-de Blas, E., Mateo, R., & Alonso-Alvarez, C. (2015). Accumulation of dietary carotenoids, retinoids and tocopherol in the internal tissues of a bird: A hypothesis for the cost of producing colored ornaments. *Oecologia*, *177*, 259–271. <https://doi.org/10.1007/s00442-014-3163-8>
- García-Vigón, E., Cordero, P. J., & Veiga, J. P. (2008). Exogenous testosterone in female spotless starlings reduces their rate of extrapair offspring. *Animal Behavior*, *76*, 345–353. <https://doi.org/10.1016/j.anbehav.2008.01.019>
- Gerlach, N. M., & Ketterson, E. D. (2013). Experimental elevation of testosterone lowers fitness in female dark-eyed juncos. *Hormones and Behavior*, *63*, 782–790. <https://doi.org/10.1016/j.yhbeh.2013.03.005>
- Golberg, M. A., & Cho, H. A. (2004). *Introduction to regression analysis*. Billerica, MA: WIT Press.
- Griffith, S. C., Owens, I. P. F., & Thuman, K. A. (2002). Extra pair paternity in birds: A review of interspecific variation and adaptive function. *Molecular Ecology*, *11*, 2195–2212. <https://doi.org/10.1046/j.1365-294X.2002.01613.x>
- Groothuis, T. G. G., Müller, W., von Engelhardt, N., Carere, C., & Eising, C. (2005). Maternal hormones as a tool to adjust offspring phenotype in avian species. *Neuroscience and Biobehavioral Reviews*, *29*, 329–352. <https://doi.org/10.1016/j.neubiorev.2004.12.002>
- Hall, Z. J., & MacDougall-Shackleton, S. A. (2012). Influence of testosterone metabolites on song-control system neuroplasticity during photostimulation in adult European starlings (*Sturnus vulgaris*). *PLoS ONE*, *7*, e40060. <https://doi.org/10.1371/journal.pone.0040060>
- Hau, M., Wikelski, M., Soma, K. K., & Wingfield, J. C. (2000). Testosterone and year-round territorial aggression in a tropical bird. *General and Comparative Endocrinology*, *117*, 20–33. <https://doi.org/10.1006/gcen.1999.7390>
- Hoi, H., Krištofík, J., & Darolová, A. (2013). Experimentally simulating paternity uncertainty: Immediate and long-term responses of male and female reed warblers (*Acrocephalus scirpaceus*). *PLoS ONE*, *8*, e62541. <https://doi.org/10.1371/journal.pone.0062541>
- Hussell, D. J. T. (1983). Age and plumage color in female tree swallows. *Journal of Field Ornithology*, *54*, 312–318.
- IBM Corp. (2011). *IBM SPSS statistics for Windows, version 20.0*. Armonk, NY: IBM Corp.
- Imwalle, D. B., & Katz, L. S. (2004). Divergent roles for estrogens and androgens in the expression of female goat sexual behavior. *Hormones and Behavior*, *46*, 54–58. <https://doi.org/10.1016/j.yhbeh.2004.01.008>
- de Jong, B., Lens, L., Amininasab, S. M., van Oers, K., Darras, V. M., Eens, M., ... Groothuis, T. G. G. (2016). Effects of experimentally sustained elevated testosterone on incubation behaviour and reproductive success in female great tits (*Parus major*). *General and Comparative Endocrinology*, *230–231*, 38–47. <https://doi.org/10.1016/j.ygcen.2016.02.017>
- de Jong, B., Lens, L., van der Velde, M., Korsten, P., Groothuis, T., & Komdeur, J. (2017). Testosterone reduces promiscuity of female blue tits (*Cyanistes caeruleus*): An experimental study. *Ethology*, *123*, 69–82. <https://doi.org/10.1111/eth.12574>
- Kempenaers, B., Congdon, B., Boag, P., & Robertson, R. J. (1999). Extrapair paternity and egg hatchability in tree swallows: Evidence for the genetic compatibility hypothesis? *Behavioral Ecology*, *10*, 304–311. <https://doi.org/10.1093/beheco/10.3.304>
- Kempenaers, B., Verheyen, G. R., Van den Broeck, M., Burke, T., Van Broeckhoven, C., & Dhondt, A. (1992). Extra-pair paternity results from female preference for high-quality males in the blue tit. *Nature*, *357*, 494–496. <https://doi.org/10.1038/357494a0>
- Ketterson, E. D., & Nolan, V. Jr (1999). Adaptation, exaptation, and constraint: A hormonal perspective. *American Naturalist*, *154*, S4–S25. <https://doi.org/10.1086/303280>
- Ketterson, E. D., Nolan, V. Jr, & Sandell, M. (2005). Testosterone in females: Mediator of adaptive traits, constraint on sexual dimorphism, or both? *American Naturalist*, *166*, S85–S98. <https://doi.org/10.1086/444602>
- Lakens, D. (2013). Calculating and reporting effect sizes to facilitate cumulative science: A practical primer for t-tests and ANOVAs. *Frontiers in Psychology*, *4*, 863. <https://doi.org/10.3389/fpsyg.2013.00863>
- Leboucher, G., Béguin, N., Mauget, R., & Kreuzer, M. (1998). Effects of fadrozole on sexual displays and reproductive activity in the female canary. *Physiology and Behavior*, *65*, 233–240. [https://doi.org/10.1016/S0031-9384\(98\)00079-1](https://doi.org/10.1016/S0031-9384(98)00079-1)
- Leffelaar, D., & Robertson, R. J. (1984). Do male tree swallows guard their mates? *Behavioral Ecology and Sociobiology*, *16*, 73–79. <https://doi.org/10.1007/BF00293106>
- Leffelaar, D., & Robertson, R. J. (1985). Nest usurpation and female competition for breeding opportunities by Tree Swallows. *The Wilson Bulletin*, *97*, 221–224.

- Lenth, R. V. (2016). Least-squares means: The R package lsmeans. *Journal of Statistical Software*, 69, 1–38.
- Makarewicz, C. A., Stenzler, L. M., Ferretti, V., Winkler, D. W., & Lovette, I. J. (2009). Isolation and characterization of microsatellite markers from three species of swallows in the genus *Tachycineta*: *T. albilinea*, *T. bicolor* and *T. leucorhoa*. *Molecular Ecology Resources*, 9, 631–635. <https://doi.org/10.1111/j.1755-0998.2008.02484.x>
- Montgomery, D. C., Peck, E. A., & Vining, G. G. (2012). *Introduction to linear regression*, 5th ed. Hoboken, NJ: John Wiley & Sons Inc.
- Moore, I. T., Walker, B. G., & Wingfield, J. C. (2004). The effects of combined aromatase inhibitor and anti-androgen on male territorial aggression in a tropical population of rufous-collared sparrows, *Zonotrichia capensis*. *General and Comparative Endocrinology*, 135, 223–229. <https://doi.org/10.1016/j.ygcen.2003.09.012>
- Moreira, F., Beja, P., Morgado, R., Reino, L., Gordinho, L., Delgado, A., & Borralho, R. (2005). Effects of field management and landscape context on grassland wintering birds in Southern Portugal. *Agriculture, Ecosystems, and Environment*, 109, 59–74. <https://doi.org/10.1016/j.agee.2005.02.011>
- O'Brien, E. L., & Dawson, R. D. (2007). Context-dependent genetic benefits of extra-pair mate choice in a socially monogamous passerine. *Behavioral Ecology and Sociobiology*, 61, 775–782. <https://doi.org/10.1007/s00265-006-0308-8>
- Petrie, M., & Kempenaers, B. (1998). Extra-pair paternity in birds: Explaining variation between species and populations. *Trends in Ecology and Evolution*, 13, 52–58. [https://doi.org/10.1016/S0169-5347\(97\)01232-9](https://doi.org/10.1016/S0169-5347(97)01232-9)
- Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D. & R Core Team (2016). nlme: Linear and nonlinear mixed effects models. R package version 3.1-124. Retrieved from <http://CRAN.R-project.org/package=nlme>
- R Development Core Team (2015). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.
- Rissman, E. F., Clendenon, A. L., & Krohmer, R. W. (1990). Role of androgens in the regulation of sexual behavior in the female musk shrew. *Neuroendocrinology*, 51, 468–473. <https://doi.org/10.1159/000125376>
- Riters, L. V., & Alger, S. J. (2011). Hormonal regulation of avian courtship and mating behaviors. In D. O. Norris & K. H. Lopez (Eds.), *Hormones and reproduction of vertebrates* (Vol. 4, pp. 153–180). London, UK: Academic Press.
- Rosvall, K. A. (2008). Sexual selection on aggressiveness in females: Evidence from an experimental test with tree swallows. *Animal Behavior*, 75, 1603–1610. <https://doi.org/10.1016/j.anbehav.2007.09.038>
- Rosvall, K. A. (2013). Life history trade-offs and behavioral sensitivity to testosterone: An experimental test when female aggression and maternal care co-occur. *PLoS ONE*, 8, e54120. <https://doi.org/10.1371/journal.pone.0054120>
- Rosvall, K. A., Bergeon Burns, C. M., Hahn, T. P., & Ketterson, E. D. (2013). Sources of variation in HPG axis reactivity and individual consistent elevation of sex steroids in a female songbird. *General and Comparative Endocrinology*, 194, 230–239. <https://doi.org/10.1016/j.ygcen.2013.09.015>
- Sandell, M. I. (2007). Exogenous testosterone increases female aggression in the European starling (*Sturnus vulgaris*). *Behavioral Ecology and Sociobiology*, 62, 255–262. <https://doi.org/10.1007/s00265-007-0460-9>
- Schielzeth, H. (2010). Simple means to improve the interpretability of regression coefficients. *Methods in Ecology and Evolution*, 1, 103–113. <https://doi.org/10.1111/j.2041-210X.2010.00012.x>
- Schlicht, L., Valcu, M., & Kempenaers, B. (2015). Male extraterritorial behaviour predicts extrapair paternity pattern in blue tits, *Cyan caeruleus*. *Behavioral Ecology*, 26, 1404–1413. <https://doi.org/10.1093/beheco/arv076>
- Schwabl, H., Lindsay, W. R., Barron, D. G., & Webster, M. S. (2014). Endocrine correlates of mate choice and promiscuity in females of a socially monogamous avian mating system with alternative male reproductive phenotypes. *Current Zoology*, 60, 804–815. <https://doi.org/10.1093/czoolo/60.6.804>
- Seutin, G., White, B. N., & Boag, P. T. (1991). Preservation of avian blood and tissue samples for DNA analyses. *Canadian Journal of Zoology*, 69, 82–90. <https://doi.org/10.1139/z91-013>
- Sheldon, B. C. (1994). Sperm competition in the chaffinch: The role of the female. *Animal Behavior*, 47, 163–173. <https://doi.org/10.1006/anbe.1994.1018>
- Smith, E. R., Damassa, D. A., & Davidson, J. M. (1977). Hormone administration: Peripheral and intracranial implants. In R. D. Myers (Ed.), *Methods in psychobiology Vol III: Advanced laboratory techniques in neuropsychology and neurobiology* (pp. 259–279). New York, NY: Academic Press. <https://doi.org/10.1016/B978-0-12-461003-3.50014-3>
- Soma, K. K., Sullivan, K., & Wingfield, J. (1999). Combined aromatase inhibitor and antiandrogen treatment decreases territorial aggression in a wild songbird during the nonbreeding season. *General and Comparative Endocrinology*, 115, 442–453. <https://doi.org/10.1006/gcen.1999.7334>
- Staley, M., Vleck, C. M., & Vleck, D. (2011). Plasma testosterone concentrations in adult tree swallows during the breeding season. *The Wilson Journal of Ornithology*, 123, 608–613. <https://doi.org/10.1676/10-142.1>
- Stapleton, M. K., & Robertson, R. J. (2006). Female tree swallow home-range movements during their fertile period as revealed by radio-tracking. *The Wilson Journal of Ornithology*, 118, 502–507. <https://doi.org/10.1676/05-079.1>
- Staub, N. L., & De Beer, M. (1997). The role of androgens in female vertebrates. *General and Comparative Endocrinology*, 108, 1–24. <https://doi.org/10.1006/gcen.1997.6962>
- Stutchbury, B. J., & Robertson, R. J. (1988). Within-season and age-related patterns of reproductive performance in female tree swallows (*Tachycineta bicolor*). *Canadian Journal of Zoology*, 66, 827–834. <https://doi.org/10.1139/z88-122>
- Tomaszycski, M. L., Banerjee, S. B., & Adkins-Regan, E. (2006). The role of sex steroids in courtship, pairing and pairing behaviors in the socially monogamous zebra finch. *Hormones and Behavior*, 50, 141–147. <https://doi.org/10.1016/j.yhbeh.2006.02.005>
- Van Duyse, E., Pinxten, R., Snoeijs, T., & Eens, M. (2005). Simultaneous treatment with an aromatase inhibitor and an anti-androgen decrease the likelihood of dawn song in free-living male great tits, *Parus major*. *Hormones and Behavior*, 48, 243–251. <https://doi.org/10.1016/j.yhbeh.2005.02.013>
- Vandermeer, C. (2013). *The effect of testosterone on the spring migratory phenotype of a north American songbird (Zonotrichia albicollis)*. MSc thesis. University of Western Ontario, London, Canada.
- Venier, L. A., Dunn, P. O., Lifjeld, J. T., & Robertson, R. J. (1993). Behavioural patterns of extra-pair copulation in tree swallows. *Animal Behavior*, 45, 412–415. <https://doi.org/10.1006/anbe.1993.1050>
- Verhoeven, K. J. F., Simonsen, K. L., & McIntyre, L. M. (2005). Implementing false discovery rate control: Increasing your power. *Oikos*, 108, 643–647. <https://doi.org/10.1111/j.0030-1299.2005.13727.x>
- Washburn, B. E., Millsbaugh, J. J., Morris, D. L., Schulz, J. H., & Faaborg, J. (2007). Using a commercially available enzyme immunoassay to quantify testosterone in avian plasma. *The Condor*, 109, 181–186. [https://doi.org/10.1650/0010-5422\(2007\)109\[181:UACAEI\]2.0.CO;2](https://doi.org/10.1650/0010-5422(2007)109[181:UACAEI]2.0.CO;2)
- While, G. M., Sinn, D. L., & Wapstra, E. (2009). Female aggression predicts mode of paternity acquisition in a social lizard. *Proceeding of the Royal Society of London B: Biological Sciences*, 276, 2021–2029. <https://doi.org/10.1098/rspb.2008.1926>
- Williams, T. D. (2012). *Physiological adaptations for breeding in birds*. Princeton, NJ: Princeton University Press.

- Williams, T. D., Kitaysky, A. S., & Vézina, F. (2004). Individual variation in plasma estradiol-17 β and androgen levels during egg formation in the European starling *Sturnus vulgaris*: Implications for regulation of yolk steroids. *General and Comparative Endocrinology*, 136, 346–352. <https://doi.org/10.1016/j.ygcen.2004.01.010>
- Winkler, D. W., Hallinger, K. K., Ardia, D. R., Robertson, R. J., Stuchbury, B. J., & Cohen, R. R. (2011). Tree swallow (*Tachycineta bicolor*). In A. Poole (Ed.), *Birds of North America*. Ithaca, NY: Cornell Lab of Ornithology. Retrieved from <http://bna.birds.cornell.edu/bna/species/011>
- Winkler, D. W., Ringelman, K. M., Dunn, P. O., Whittingham, L., Hussell, D. J. T., Clark, R. G., ... Ardia, D. R. (2014). Latitudinal variation in clutch size–lay date regressions in *Tachycineta* swallows: Effects of food supply or demography? *Ecography*, 37, 670–678. <https://doi.org/10.1111/j.1600-0587.2013.00458.x>
- Zysling, D. A., Greives, T. J., Breuner, C. W., Casto, J. M., Demas, G. E., & Ketterson, E. D. (2006). Behavioral and physiological responses to experimentally elevated testosterone in female dark-eyed juncos

(*Junco hyemalis carolinensis*). *Hormones and Behavior*, 50, 200–207. <https://doi.org/10.1016/j.yhbeh.2006.03.004>

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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