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Experimental elevation of testosterone lowers fitness in female dark-eyed juncos

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ABSTRACT

Testosterone (T) is often referred to as the "male hormone," but it can influence aggression, parental behavior, and immune function in both males and females. By experimentally relating hormone-induced changes in phenotype to fitness, it is possible to ask whether existing phenotypes perform better or worse than alternative phenotypes, and hence to predict how selection might act on a novel or rare phenotype. In a songbird, the dark-eyed junco (*Junco hyemalis*), we have examined the effects of experimentally elevated T in females on fitness-related behaviors such as parental care. In this study, we implanted female juncos with exogenous T and examined its effect on fitness (survival, reproduction, and extra-pair mating) to assess whether T-altered phenotypes would prove to be adaptive or deleterious for females. Experimental elevation of T decreased the likelihood that a female would breed successfully, and T-implanted females had lower total reproductive success at every stage of the reproductive cycle. They did not, however, differ from control females in fledgling quality, extra-pair offspring production, survival, or reproduction in the following year. Previous work in this system has shown that experimental elevation of T in males alters behavior and physiology and decreases survival but increases the production of extra-pair offspring, leading to higher net fitness relative to control animals. Our results suggest that increased T has divergent effects on male and female fitness in this species, and that prevailing levels in females may be adaptive for them. These findings are consistent with sexual conflict.

Introduction

Testosterone (T) has a variety of behavioral and physiological effects, and is therefore likely to be involved in life history evolution (Finch and Rose, 1995). T is frequently considered to be a "male" hormone, and it unquestionably has numerous organizational and activational effects in male vertebrates (Adkins-Regan, 2005). Of course, T is not limited to males in terms of its production or its action; female vertebrates have detectable levels of circulating testosterone, and in some cases female T levels may approach those of males (Ketterson et al., 2005; Wingfield et al., 2000). T has been shown to affect a number of behavioral and physiological traits in females of a wide variety of taxa, including immune function, attractiveness, and sexual behavior (De Ridder et al., 2002; Eens et al., 2000; Langmore et al., 2002; Staub and De Beer, 1997). The ways in which testosterone can affect fitness in males are well characterized (reviewed in Ketterson and Nolan, 1999); relatively less work has been devoted to understanding this relationship in females (although see Ketterson et al., 2005; Staub and De Beer, 1997 and references therein).

Physiological mechanisms such as circulating levels of hormones relate to a suite of behavioral, immunological, and reproductive traits, and can frequently coordinate trade-offs between these traits (Finch and Rose, 1995; Hau, 2007; Ketterson and Nolan, 1992). Because testosterone can mediate a number of different phenotypic traits, each of which may be the target of selection, it is crucial not just to relate each trait to fitness independently, but to assess the net change in overall fitness that results from a change in hormone phenotype. One way to accomplish this is to use exogenous hormone implants to look at the effects of elevated testosterone on overall reproductive success over the course of a breeding season (Ketterson et al., 1996; Lynn et al., 2009; Marler and Moore, 1988; Silverin, 1991; Wingfield, 1984). In this study, we used exogenous testosterone implants to address three related questions: 1) Do males and females respond similarly to elevated T levels with respect to offspring production and survival? 2) Are current levels of T expression in females advantageous or detrimental relative to elevated levels? 3) Is selection on T phenotype in females likely to accelerate or constrain the evolution of T phenotype in males?

Previous work in our study system, the dark-eyed junco (*Junco hyemalis*), has shown that in males, experimental elevation of T decreases survival and the production of surviving within-pair offspring, but increases the production of extra-pair offspring, resulting in a net fitness benefit for implanted males relative to controls (Raouf et al., 1997; Reed et al., 2006). This suggests that selection may favor males with higher testosterone than is currently prevalent in the population. High levels of testosterone may be favored in female juncos as well. Junco females have relatively high levels of testosterone compared to other species of songbirds (Ketterson et al., 2005), and this testosterone peaks during the pre-breeding period, suggesting that these high levels may

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function in mate acquisition. Female juncos with experimentally elevated testosterone are also more aggressive (Zysling et al., 2006), which may confer a reproductive advantage, particularly in years with high intrasexual competition or predation pressure (Cain and Ketterson, 2012; Rosvall, 2013). In contrast, it may be the case that female fitness is relatively unaffected by high levels of testosterone. Because female parental care is critical to offspring survival (Wolf et al., 1988), the theory underlying the behavioral insensitivity hypothesis can be expanded to suggest that females should be relatively insensitive to any negative effects of testosterone on parental care during this period (Lynn, 2008; Lynn et al., 2002; Lynn et al., 2005).

However, there are also some indications in juncos and other species that selection on females may be constraining rather than accelerating the evolution of T in males, and thus that the sexes may be experiencing intersexual conflict (Chapman et al., 2003; Lande and Arnold, 1983; Price and Burley, 1993). Elevated testosterone levels delay clutch initiation or decrease fecundity in female zebra finches (Rutkowska et al., 2005), homing pigeons (Goerlich et al., 2009; Goerlich et al., 2010), red-winged blackbirds (Searcy, 1988), and spotless starlings (Veiga and Polo, 2008; Veiga et al., 2004), and a high level of T decreases incubation behavior and hatching success in tree swallows (Rosvall, 2013). In the spotless starling, T implants decrease female parental care, fledgling production, and attractiveness to males (García-Vigón et al., 2008; Veiga and Polo, 2008). However, they also increase a female's chances of acquiring and maintaining a position in a breeding colony over multiple years, resulting in no net difference between the lifetime reproductive success of T-implanted and control females (Veiga and Polo, 2008). In the dark-eyed junco, artificially elevated T levels in females result in a delay in egg-laying, decreased parental care during the nestling phase (but not during incubation), and overall lower rates of daily nest survival (Clotfelter et al., 2004; O'Neal et al., 2008). While these results certainly suggest that elevated T may be detrimental to female juncos, and thus may potentially constrain the evolution of male T, measures of overall reproductive success and survival are needed to fully address the net selective force experienced by females in this species.

In this study, we address the fitness consequences of chronically elevated T levels in female juncos. We address first the direct effects of T on fitness, i.e. a female's reproductive output and survival, by comparing the number of offspring produced at each stage of the breeding cycle by females treated with T as compared to controls. We also address whether the effects of T treatment have any long-term effects on female fitness; that is, whether elevated levels of T affect female reproduction in the following breeding season. Finally, we examine T's effects on a female's indirect fitness, i.e. the quality of her offspring. By comparing the effects of elevated maternal T not only on nestling quality, but also on nestling paternity (which has been shown to have effects on an offspring's reproductive success as adults, Gerlach et al., 2012) and juvenile survival and recruitment, we can examine the more subtle effects of testosterone on female fitness that extend over multiple generations.

Methods

Study system and general field methods

The dark-eyed junco is a socially monogamous songbird that breeds at high elevations throughout the southern Appalachian Mountains in eastern North America. This study was conducted on the population of juncos (*J. h. carolinensis*) breeding at Mountain Lake Biological Station in Giles County, Virginia (37°22′31″ N, 80°31′24″ W) (Chandler et al., 1994). This population has been studied since 1983, and almost all adult juncos in this population receive numbered USFWS aluminum bands as well as unique combinations of color bands for field identification.

Our field methods are described in detail elsewhere (Reed et al., 2006); in brief, adult juncos were captured at baited mist nets and potter traps during the pre-breeding and early breeding period (typically 15 April–15 May). At this time, each bird was banded, weighed, aged based on its age at banding or by using plumage characteristics (Ketterson, 1979), and checked for reproductive condition (i.e. presence of a brood patch or cloacal protuberance). All birds also had a small blood sample (50–100 μL) collected via alar vein puncture. Blood samples were centrifuged and the plasma was drawn off and stored at −20 °C for later hormone analysis. Longmire's solution was added to the red blood cells for lysis and storage until DNA analysis (Longmire et al., 1988).

Experimental manipulation of testosterone

During the breeding seasons of 2001–2 and 2005–7, implants were given during the early part of the breeding season (during the spring census, 15 April–15 May, in 2001–2; shifted to 1 May–15 June in 2005–7 to focus implanting on resident females rather than late migrants). Captured females were anesthetized and had a 7 mm piece of silastic tubing implanted subcutaneously along their left flank. Implants were sealed at both ends with silastic glue, and contained either 5 mm of packed crystalline testosterone (=0.01 g) (T-implants), or were empty (C-implants). In female juncos, levels of naturally-produced testosterone peak during the pre-breeding season (Ketterson et al., 2005); a T-implant of this size has been shown to maintain females at this peak level throughout the breeding season (Clotfelter et al., 2004; O'Neal et al., 2008).

Treatment group (T or C) was determined randomly by coin flip after blocking by site of capture within the study area. Females that had been implanted in previous years received the same treatment in each subsequent year they were captured. To avoid pseudoreplication, for females that were implanted in multiple years, we considered reproductive success from only the first year they received an implant. Our data set thus included 284 females; 142 with control implants and 142 with testosterone (2001: 16 C/16 T; 2002: 16 C/18 T; 2005: 52 C/47 T; 2006: 28 C/27 T; 2007: 30 C/34 T). Because adult site fidelity in this species is high (Reed et al., 2006; Gerlach et al., unpublished data), we measured survival based on whether a female was sighted and/or captured in our population in subsequent years. For those females that returned to the population and were not implanted in the year following their first implant (primarily females that returned in 2003 or 2008), we quantified annual reproductive success for that returning year in order to examine the potential carryover effects of testosterone treatment.

Nests

Each year we searched for nests daily between 15 May and 15 July. Once a nest was found, the female and the social father (the male that defended the nest and cared for the young) associated with the nest were identified using color bands. Nests that were found during building, laying, or incubation were checked every other day for progress. Once nestlings had hatched, the nests were checked on the day the eggs hatched (hatch day, day 0, D0), D3, D6, D9, and when the young left the nest (fledging; typically D11–12). Nestlings were weighed and measured on D0, D6, and at fledging. On the afternoon of D6, nestlings were individually banded, and a small (~50 μL) blood sample was collected by alar vein puncture for paternity analysis.

Adult females were recaptured at the end of the breeding season (15 July–early August). At this time a blood sample was taken to confirm plasma testosterone levels post-treatment, and their implants were removed. During this period, we also captured juveniles to determine fledgling survival to independence.

All procedures used in this study were approved by the Bloomington Institutional Animal Care and Use Committee.

Testosterone assays

Plasma was extracted with diethyl ether, and testosterone levels were measured using an enzyme immune assay (EIA), as described in Clotfelter et al. (2004). Each year was analyzed separately, and because

of the large number of samples within each year, multiple assays had to be performed. In all cases, a female's pre- and post-implant samples were run within a single assay, but the samples were otherwise randomized across assays with respect to treatment. Inter- and intra-assay variation was calculated as the coefficient of variation of values obtained from standard samples of known concentration. Average recoveries and intra- and inter-assay variation are reported in Clotfelter et al. (2004) for 2002 and O'Neal et al. (2008) for 2005–2006. For 2007 samples, 3 assays were run; average recovery was 96.4 ± 0.27%; inter-assay variations were 20.32, 10.41, and 15.98%, and intra-assay variation was 13.58%. The pre- and post-implant T levels for T-treated and control birds were compared separately for each year.

Paternity analysis

DNA was extracted using a standard phenol:chloroform protocol from blood samples of nestlings, mothers, and all adult males from 2001, 2002, and 2005–7. All samples were genotyped at nine microsatellite loci (see Gerlach et al., 2012 for loci information and details of genotyping). Each sample was run at least twice at each locus to account for allelic dropout; only alleles that consistently amplified were considered in paternity analyses.

Paternity analyses were conducted using the program CERVUS (Kalinowski et al., 2007; Marshall et al., 1998). Nestlings from each year were analyzed separately; all males known to be alive during a given year were considered as potential fathers for each nestling. We also used the program COLONY to determine sibling relationships and thus the minimum number of sires among nestlings for which no genetic sire was identified by CERVUS (Jones and Wang, 2010).

We used three measures to quantify a female's level of extra-pair activity. For each female, we asked whether or not she produced any extra-pair offspring, and we quantified the percentage of her genotyped offspring that were sired by extra-pair males. We also quantified the number of males with which females produced offspring, as a measure of the minimum number of males with which a female had copulated successfully.

Statistical analyses

For each female, we quantified the total number of nests, eggs, hatchlings, and fledglings she produced over the course of the breeding season. While some females were implanted in the middle of a nesting bout, only nests for which the female had been implanted prior to laying the first egg were included in counts of female reproduction. Further limiting our data set to exclude females that had begun a nesting bout before they were implanted did not qualitatively affect our results; we therefore include all individuals, regardless of whether they had initiated reproduction prior to implanting, in the analyses below.

Analyses of reproductive success were done using a generalized linear model, with either a Poisson error distribution (for counts of nests, eggs, etc.) or a binary logistic error distribution (for likelihood of reaching a nest stage, female survival, etc.), and including treatment and year as random factors and female age and implant date as covariates. Measurements of nestling weight were compared using a generalized linear model with maternal ID, maternal treatment, and year as factors, and date of measurement as a covariate. Likelihood of juvenile survival and recruitment was analyzed with a generalized linear model with a binary logistic error distribution, including year and maternal treatment as random factors. All statistical analyses were conducted using SPSS Version 19.0.

Results

Implant effectiveness and plasma T

The testosterone implants significantly raised plasma testosterone levels within each year. These values have been previously reported for 2002 (plasma T concentration post-implant; $t = 6.34$, $p < 0.001$), 2005 (mean T concentration ± s.e. post-implant: C-females = 0.79 ± 0.22; T-females = 2.98 ± 0.37 ng/mL; $t = -5.44$, $p < 0.05$), and 2006 (mean T concentration ± s.e. post-implant: C-females = 0.91 ± 0.32; T-females = 2.62 ± 0.36 ng/mL; $t = -3.57$, $p < 0.05$) (Clotfelter et al., 2004; O'Neal et al., 2008). In 2007, testosterone implants similarly significantly raised plasma testosterone levels relative to control implants in post-implant samples (mean T concentrations ± s.e. post-implant: C-females: 1.33 ± 0.22, $n = 11$; T-females: 2.66 ± 0.38 ng/mL, $n = 11$; $t = -3.11$, $p < 0.01$).

Reproductive success

Nests

Testosterone-treated females did not statistically differ from control females in their likelihood of building a nest on our study site (C-females: 82/142 (57.7%), T-females: 68/142 (47.9%); Table 1), but on average built

Table 1
Effect of testosterone treatment on likelihood of reaching a given nest stage and fitness (number of offspring produced) at each stage; all implanted females ($n = 284$).

	Factor	Likelihood of reaching this stage			Number produced		
		β	Wald X^2	p	β	Wald X^2	p
Nests	Intercept	1.30	1.59	0.21	**1.38**	5.22	0.02
	Year of implant	0.92, 0.23, −0.17, 0.25	6.07	0.19	**0.79, 0.48, 0.11, 0.37**	13.95	0.01
	Testosterone treatment	0.41	2.82	0.09	**0.35**	6.93	0.01
	Date of implant	−0.01	1.02	0.31	**−0.01**	5.16	0.02
	Age	−0.08	0.29	0.59	−0.11	1.49	0.22
Eggs	Intercept	1.54	2.09	0.15	**2.47**	41.46	<0.001
	Year of implant	0.46, 0.31, −0.22, 0.31	3.94	0.41	**0.57, 0.42, 0.04, 0.32**	28.21	<0.001
	Testosterone treatment	**0.61**	6.30	0.01	**0.54**	50.54	<0.001
	Date of implant	−0.01	1.65	0.20	**−0.01**	17.16	<0.001
	Age	−0.09	0.37	0.54	−0.06	1.66	0.20
Hatchlings	Intercept	0.99	1.29	0.26	**1.29**	7.27	0.01
	Year of implant	0.86, −0.20, 0.05, 0.78	8.27	0.08	**0.40, −0.27, 0.12, 0.56**	22.26	<0.001
	Testosterone treatment	**1.06**	15.22	<0.001	**0.71**	37.70	<0.001
	Date of implant	−0.02	2.41	0.12	**−0.01**	6.51	0.01
	Age	−0.11	0.36	0.55	−0.02	0.08	0.77
Fledglings	Intercept	−1.57	0.40	0.53	−1.56	1.37	0.24
	Year of implant	**−0.51, −0.96, −0.18, 1.09**	14.25	0.01	**−0.39, −0.78, 0.30, 1.34**	49.20	<0.001
	Testosterone treatment	**0.98**	7.51	0.01	**0.66**	11.34	0.001
	Date of implant	−0.01	0.20	0.66	−0.002	0.09	0.77
	Age	0.04	0.04	0.85	0.08	0.47	0.49

Bold values are significant at $p < 0.05$. βs for year of implant are for 2001, 2002, 2005, and 2006, respectively; βs for testosterone treatment are for T-implants = 0; C-implants = 1.

fewer nests overall (Fig. 1A; Table 1). However, among those females that built at least one nest, testosterone treatment did not significantly affect the number of nests they built (Fig. 1C; Table 3). Almost all females that built a nest laid at least one egg; however, all seven females that built a nest but abandoned it prior to egg-laying were T-females (see below).

Eggs

Overall, testosterone-treated females were significantly less likely to lay eggs (C-females: 82/142 (57.7%), T-females: 61/142 (43.0%); Table 1), and on average laid 41% fewer eggs than did control-treated females (Fig. 1A; Table 1). When considering only those females that built at least one nest (i.e. females that could potentially have laid eggs), T-females did not differ significantly in their likelihood to lay eggs (C-females: 82/82 (100%), T-females: 61/68 (89.7%); Table 2), but they did produce 29% fewer eggs than C-females (Fig. 1B; Table 2). Even among those females that laid at least one egg, testosterone treatment resulted in significantly fewer eggs laid during the breeding season (Fig. 1C; Table 3).

Hatching

Testosterone treatment significantly decreased the likelihood that a female would produce any hatched offspring (C-females: 61/142 (43.0%), T-females: 30/142 (21.1%); Table 1). On average, C-females produced almost twice as many hatchlings as did T-females (Fig. 1A; Table 1). Within the subset of females that could have produced hatchlings (i.e. those females that laid at least one egg), T-females were significantly less likely to hatch any offspring (C-females: 61/82 (74.4%), T-females: 30/61 (49.2%); Table 2), and on average produced one fewer hatched offspring than did C-females (Fig. 1B; Table 2), likely due in part to their smaller number of eggs (see above). The number of hatchlings produced by females that successfully hatched at least one offspring was not affected by testosterone treatment (Fig. 1C; Table 3).

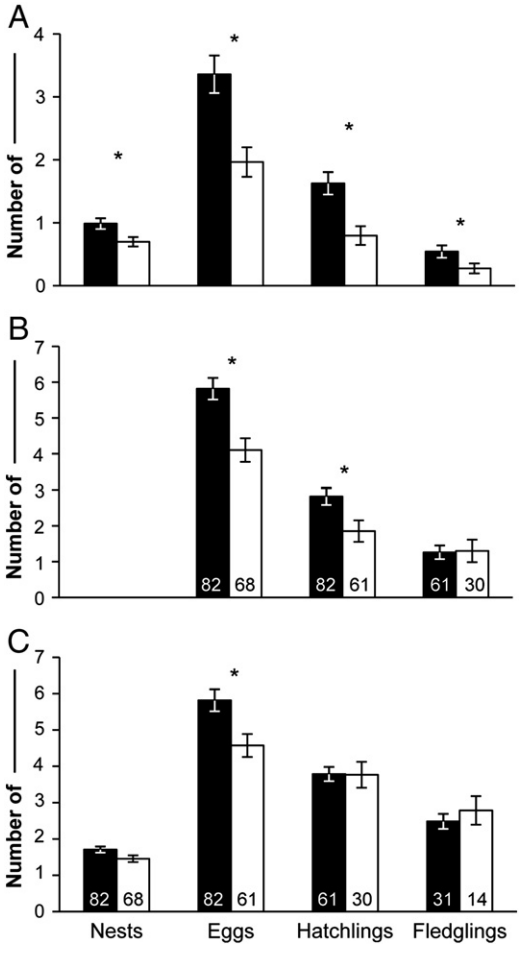

Fig. 1. Reproductive success of female dark-eyed juncos according to hormone treatment (C-implants = black bars; T-implants = white bars; $n = 142$ per treatment in A, indicated on bars for B and C). A) All implanted females. B) Females that reached the previous reproductive stage (e.g. mean number of eggs among females that produced at least one nest). C) Females that reached the reproductive stage in question (e.g. mean number of eggs among females that produced at least one egg). Graphs depict raw means ± SE; asterisks indicate comparisons that were significantly different at $p < 0.05$ when controlling for year, female age, and date of implant.

Fledging

Testosterone-treated females on the whole were significantly less likely to produce any fledglings (C-females: 31/142 (21.8%), T-females: 14/142 (9.9%); Table 1), and on average produced only half as many fledged offspring as control females (Fig. 1A; Table 1). However, when considering only females that successfully hatched offspring, there was no effect of treatment on whether or not a female successfully reared any offspring to fledging (C-females: 31/61 (50.8%), T-females: 14/30 (46.7%); Table 2), or on the number of fledglings she produced (Fig. 1B; Table 2). The number of fledglings produced by females that successfully fledged at least one offspring was not affected by testosterone treatment (Fig. 1C; Table 3).

Offspring quality

Offspring produced by T-females did not differ significantly in mass from offspring produced by C-females (Fig. 2) when measured at hatching (D0) (treatment: Wald $X^2 = 1.06$, $p = 0.30$; maternal ID: Wald $X^2 = 128.64$, $p < 0.001$; Julian date of measurement: Wald $X^2 = 0.02$, $p = 0.90$), D6 (treatment: Wald $X^2 = 1.32$, $p = 0.25$; maternal ID: Wald $X^2 = 346.76$, $p < 0.001$; Julian date of measurement: Wald $X^2 = 0.78$, $p = 0.38$), or at fledging (treatment: Wald $X^2 = 0.12$, $p = 0.73$; maternal ID: Wald $X^2 = 139.09$, $p < 0.001$; Julian date of measurement: Wald $X^2 = 0.27$, $p = 0.61$).

However, and perhaps unexpectedly, among those offspring that fledged successfully, individuals produced by T-females were significantly more likely to survive to independence (offspring of C-females: 17/71 (23.9%), offspring of T-females: 14/31 (45.2%); treatment: Wald $X^2 = 3.94$, $p = 0.05$; year: Wald $X^2 = 0.31$, $p = 0.99$). Offspring of T-females were more than twice as likely as offspring of C-females to return to the population as adults, although this difference was non-significant, perhaps owing to overall low rate of offspring return (offspring of C-females: 6/71 (8.5%), offspring of T-females: 6/31 (19.4%); treatment: Wald $X^2 = 2.85$, $p = 0.09$; year: Wald $X^2 = 1.97$, $p = 0.74$). Because it was common that more than one fledgling from a clutch would survive to independence, counts of fledglings may suffer from non-independence based on mother ID. These analyses do not control for maternal identity, and are therefore presented with less strength of assertion. However, of the 8 T-females that produced banded fledglings, 7 had at least one fledgling survive to independence, as compared to 10 of 26 C-females, which is at least suggestive that T-females, when they produce offspring, may produce offspring that are higher quality and/or less likely to disperse.

Offspring paternity and female mate choice

T-females did not differ significantly from C-females in whether or not they produced any extra-pair offspring (C-females: 18/41 (43.9%), T-females: 9/15 (60.0%); treatment: Wald $X^2 = 1.34$, $p = 0.25$; year: Wald $X^2 = 4.74$, $p = 0.32$; date of implant: Wald $X^2 = 3.59$, $p = 0.06$; age: Wald $X^2 = 0.03$, $p = 0.87$), in the number of males with which they produced offspring (Fig. 3; treatment: Wald $X^2 = 0.12$, $p = 0.73$; year: Wald $X^2 = 0.95$, $p = 0.92$; date of implant: Wald $X^2 = 0.08$, $p = 0.78$; age: Wald $X^2 = 0.19$, $p = 0.66$), or in the percentage of their offspring that was sired by extra-pair males (Fig. 3;

Table 2
Effect of testosterone treatment on likelihood of reaching a given nest stage and fitness (number of offspring produced) at each stage; females that reached the previous nest stage.

	Factor	Likelihood of reaching this stage			Number produced		
		β	Wald X^2	p	β	Wald X^2	p
Eggs	Intercept	−10.37	0.00	1.00	**2.64**	31.44	<0.001
$n = 150$	Year of implant	1.95, −19.24, 1.14, −18.83	2.11	0.72	0.18, 0.32, 0.09, 0.18	6.29	0.18
	Testosterone treatment	−20.25	0.00	1.00	**0.32**	17.17	<0.001
	Date of implant	0.05	1.57	0.21	**−0.01**	6.35	0.01
	Age	0.18	0.14	0.71	−0.02	0.17	0.68
Hatchlings	Intercept	0.68	0.38	0.54	1.00	2.65	0.10
$n = 143$	Year of implant	0.82, −0.64, 0.29, 1.04	7.34	0.12	**0.14, −0.43, 0.18, 0.40**	14.46	0.01
	Testosterone treatment	**1.11**	8.47	0.004	**0.38**	10.64	0.001
	Date of implant	−0.01	0.14	0.71	−0.004	0.46	0.50
	Age	−0.09	0.13	0.72	0.01	0.04	0.85
Fledglings	Intercept	−5.12	2.24	0.13	**−3.50**	6.14	0.01
$n = 91$	Year of implant	**−1.80, −1.51, −0.36, 1.04**	11.83	0.02	**−1.03, −0.75, 0.29, 0.91**	30.74	<0.001
	Testosterone treatment	0.24	0.22	0.64	−0.02	0.01	0.94
	Date of implant	0.04	1.71	0.19	**0.02**	5.13	0.02
	Age	0.31	0.66	0.42	0.20	1.71	0.19

Bold values are significant at $p < 0.05$. βs for year of implant are for 2001, 2002, 2005, and 2006, respectively; βs for testosterone treatment are for T-implants = 0; C-implants = 1.

treatment: Wald $X^2 = 1.56$, $p = 0.21$; year: Wald $X^2 = 10.15$, $p = 0.04$; date of implant: Wald $X^2 = 3.73$, $p = 0.05$; age: Wald $X^2 = 0.35$, $p = 0.56$).

Adult return rate

Testosterone treatment did not significantly affect a female's likelihood of returning in the next breeding season (C-females: 50/142 (35.2%), T-females: 41/142 (28.9%); treatment: Wald $X^2 = 1.24$, $p = 0.27$; year: Wald $X^2 = 2.31$, $p = 0.68$; date of implant: Wald $X^2 = 0.37$, $p = 0.55$; age: Wald $X^2 = 0.64$, $p = 0.42$).

Carryover effects

Within the limited sample of females that returned to our population in the breeding season following implant, testosterone treatment in the previous year decreased the number of eggs they laid in the following year by approximately 46% but did not affect their reproductive success at any other stage across the breeding cycle (Fig. 4; Table 4).

Discussion

Testosterone and female fitness

Females suffered a substantial fitness cost to high levels of testosterone, which arose via testosterone's effect on female reproduction rather than via decreased survival. T-females were less likely to build a nest than control females, laid fewer eggs once they did build a nest, and were less likely to have any of those eggs hatch. As a result, C-females had almost twice the total reproductive success (measured as the number of fledglings) of T-females. This suggests that despite the potential benefits of elevated T levels for mate acquisition or predator defense (Cain and Ketterson, 2012), maintaining peak levels of T continuously throughout the breading season is detrimental to females due to T's interference with reproductive function.

While elevated T decreased female reproductive success, its effects were not evenly distributed over all nesting stages, but were concentrated in the early stages of nest-building and egg-laying. We found that T-females built fewer nests than C-females, and once they had built a nest, they laid fewer eggs and had fewer hatchlings. This suggests that elevated levels of testosterone may be interfering with reproductive development or egg maturation. A delay in clutch initiation by

Table 3
Effect of testosterone treatment on female reproductive success; females that reached the current nest stage.

	Factor	β	Wald X^2	p
Number of nests	Intercept	**1.62**	4.32	0.04
$n = 150$	Year of implant	0.42, 0.39, 0.19, 0.24	3.81	0.43
	Testosterone treatment	0.12	0.83	0.36
	Date of implant	−0.01	2.13	0.14
	Age	−0.08	0.75	0.39
Number of eggs	Intercept	**2.43**	26.56	<0.001
$n = 143$	Year of implant	0.30, 0.27, 0.13, 0.16	6.39	0.17
	Testosterone treatment	**0.22**	8.02	0.005
	Date of implant	**−0.01**	3.84	0.05
	Age	−0.02	0.11	0.74
Number of hatchlings	Intercept	1.51	2.88	0.09
$n = 91$	Year of implant	−0.19, −0.11, 0.06, 0.07	2.09	0.72
	Testosterone treatment	−0.01	0.01	0.91
	Date of implant	−0.002	0.11	0.74
	Age	0.07	0.74	0.39
Number of fledglings	Intercept	−0.28	0.01	0.91
$n = 45$	Year of implant	0.03, 0.04, 0.50, 0.53	4.91	0.30
	Testosterone treatment	−0.19	0.72	0.40
	Date of implant	0.01	0.46	0.50
	Age	0.01	0.001	0.97

Bold values are significant at $p < 0.05$. βs for year of implant are for 2001, 2002, 2005, and 2006, respectively; βs for testosterone treatment are for T-implants = 0; C-implants = 1.

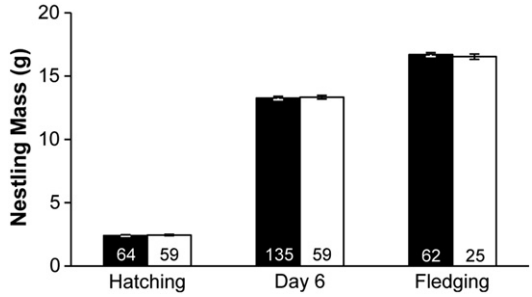

Fig. 2. Offspring mass based on mother treatment (C-implants = black bars; T-implants = white bars). Sample size (number of offspring) is indicated on the bars. Graph depicts raw means ± SE; all comparisons had $p > 0.05$ when controlling for maternal ID, year, and date of measurement.

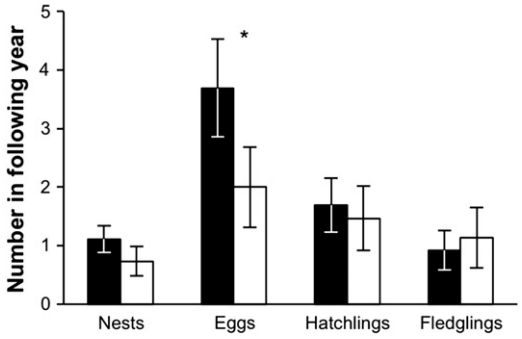

Fig. 4. Carryover effects of testosterone treatment on female reproduction in the following (unimplanted) year (C-implants = black bars, $n = 26$; T-implants = white bars, $n = 15$). Graph depicts raw means ± SE; asterisks indicate comparisons that were significantly different at $p < 0.05$ when controlling for year, female age, and date of implant.

T-females has been previously demonstrated in the junco (Clotfelter et al., 2004), which further supports the idea that T is primarily having its effects early in the breeding cycle. The exact mechanisms of testosterone's effects on reproductive development and egg production in the junco are unknown, but high levels of T have been linked with lower vitellogenin (Crossin et al., 2012) and delayed follicular maturation (Goerlich et al., 2010) in other species.

Once females had successfully reached the nestling stage, there was no effect of T-treatment on the number of fledglings they were able to produce. Offspring quality during the nestling stage similarly showed no significant effect of maternal treatment. Previous work in this species has shown that T-treated females do not differ from control females in the size or mass of their eggs (Clotfelter et al., 2004) or in their provisioning behavior (O'Neal et al., 2008), so the similarity in offspring mass is perhaps unsurprising. However, offspring of T-implanted mothers had significantly higher post-fledging survival, and were marginally more likely to return to the population as adults. While we must interpret these results with caution – rates of recruitment reflect processes such as dispersal, as well as survival – they may suggest that the increased yolk testosterone deposited by T-treated females (Clotfelter et al., 2004) has positive effects on post-fledging survival (Schwabl et al., 2012), as well as lifelong consequences for offspring fitness (Müller et al., 2008, 2009; Strasser and Schwabl, 2004). Alternatively, increased juvenile survival may be due to increased parental care provided by males mated to T-females (O'Neal et al., 2008), particularly if this increased care continues into the post-fledging period. Whatever the cause, it is unlikely that the gain in indirect fitness to females that is represented by an increase in juvenile survival and return is sufficient to offset the near two-fold cost to a female's direct reproductive success that results from elevated testosterone levels.

This study represents only the second time that the long-term fitness consequences of elevated female testosterone have been examined in a free-living avian species. Concerning female reproductive success, our results match those of previous work; in female spotless starlings, testosterone implants also led to decreased overall reproductive success

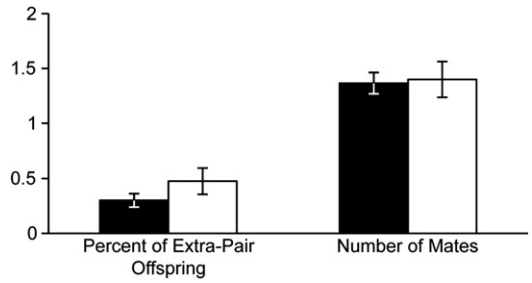

Fig. 3. Extra-pair offspring production and mate number of implanted females (C-implants = black bars, $n = 41$; T-implants = white bars, $n = 15$). Graph depicts raw means ± SE; all comparisons had $p > 0.05$ when controlling for year, female age, and date of implant.

(fewer eggs and fewer fledglings) relative to controls (López-Rull and Gil, 2009; Veiga et al., 2004). However, testosterone also had effects in female starlings that we did not see in the dark-eyed junco. For example, we found no effect of female testosterone on extra-pair paternity, but starling T-females had a decreased percentage of extra-pair offspring, an effect that lasted beyond the year of treatment (García-Vigón et al., 2008). These differences may be due to the differences in social regulation of breeding territory acquisition between the two species. Additionally, starling fledglings produced by C-females were more likely to recruit to the population (Veiga and Polo, 2008), which was the opposite of the pattern we found. This may reflect different mechanisms of maternal transfer of T to egg yolks, or different developmental effects of T in the two species; further studies would help elucidate this answer.

Timing of T effects in relation to stage of reproduction

There are several non-mutually exclusive explanations as to why testosterone affected female reproductive success during the early (pre-hatching) but not late (nestling) stages of the nest cycle. It may be the case that egg production is mediated by testosterone, whereas the behaviors involved in nestling care are not. The behavioral insensitivity hypothesis argues that the degree to which elevated testosterone decreases parental care will be proportional to the relative importance of the investment in parental care in determining reproductive success (Lynn, 2008; Lynn et al., 2002, 2005). While this hypothesis was originally developed to explain variation in male behavior, we can apply similar reasoning to the stages of the nesting cycle in females: nestlings represent a greater investment of parental time and energy than do eggs (Montgomerie and Weatherhead, 1988; Redondo, 1989; Salamolard and Weimerskirch, 1993), so parental behaviors at this stage should be the least sensitive to disruption by fluctuations in hormone levels. On a more mechanistic level, the stimuli presented by a nest full of begging nestlings may be potent enough to counteract any potentially suppressive effects of testosterone and elicit appropriate parental behavior (Brown, 1993; Storey and Joyce, 1995). However, these explanations do not conform with findings from earlier research on the junco which showed that testosterone implants decreased brooding during the nestling phase, but had no effect on incubation (Clotfelter et al., 2004; O'Neal et al., 2008) — results which are seemingly at odds with our own findings (but see below).

One potential explanation that could account for this disparity in findings is that the main source of variation in sensitivity to testosterone may not be between different stages of reproduction and associated behaviors, but rather between individuals (Williams, 2008). If females vary in their sensitivity to testosterone, i.e. the degree to which their reproductive behavior is suppressed by T, then females that are highly sensitive to increased T may have their reproductive behavior suppressed altogether, while females that are only moderately sensitive to elevated testosterone may build nests and lay eggs, but have decreased incubation

Table 4
Carryover effects of testosterone treatment on female reproductive success in the following year ($n = 41$).

	Factor	β	Wald X^2	P
Number of nests	Intercept	−0.66	<0.001	0.99
	Year of implant	**1.65, 0.92, −29.45, −1.01**	11.33	0.01
	Testosterone treatment	0.61	2.55	0.11
	Date of implant	>0.001	0.001	0.98
	Age	−0.02	0.01	0.94
Number of eggs	Intercept	−0.81	0.06	0.81
	Year of implant	**1.27, 1.02, −30.34, −30.24**	26.54	<0.001
	Testosterone treatment	**0.70**	10.54	0.001
	Date of implant	0.01	1.23	0.27
	Age	−0.11	0.45	0.50
Number of hatchlings	Intercept	−2.07	0.97	0.32
	Year of implant	**−0.22, 0.70, −29.86, −29.88**	7.57	0.02
	Testosterone treatment	0.23	0.75	0.39
	Date of implant	0.02	3.09	0.08
	Age	−0.50	3.23	0.07
Number of fledglings	Intercept	4.32	3.57	0.06
	Year of implant	**0.09, 0.76, −29.73, −29.87**	5.43	0.02
	Testosterone treatment	0.04	0.01	0.91
	Date of implant	−0.01	0.39	0.53
	Age	**−2.30**	5.27	0.02

Bold values are significant at $p < 0.05$. βs for year of implant are for 2001, 2002, 2005, and 2006, respectively; βs for testosterone treatment are for T-implants = 0; C-implants = 1.

or nest defense behaviors, such that their nests fail prior to hatching. Under this hypothesis, only those females that are relatively insensitive to high levels of testosterone – i.e. those birds that are functionally similar to control females – will be able to successfully reach the nestling stage. In this view, across the nestling cycle there would be a continual attrition of breeding females based on their inherent sensitivity to testosterone levels, with the females that reach the nestling stage representing those that are least sensitive. This variation in sensitivity may be due to variation in a number of factors, such as the activity of aromatase in converting testosterone to estradiol, or the density of androgen or estrogen receptors in target tissues in the brain or the periphery (Ball and Balthazart, 2008; Canoine et al., 2007); current research by our group is working to elucidate the effects of some of these factors on an individual's response to testosterone (Rosvall et al., 2012).

This model of individual variation in sensitivity to testosterone may account for the differences between our current results and those of a previous study (O'Neal et al., 2008). While the current study considered only reproductive events that were initiated after females were implanted, the previous study included females that had initiated a nesting attempt (i.e. were already incubating eggs) before the implant was placed. Therefore, the earlier study may have included females across a wider range of sensitivity to testosterone, including those whose parental care behaviors were strongly suppressed by T. In essence, that study may have included the nestling-care behaviors of females that might not have reached the nestling stage if they had been implanted prior to clutch initiation.

The reduced size of the group of treated females that reached the mid-nestling stage may also help to explain our observed lack of an effect of testosterone on the paternity of a female's offspring. In this study, we did not quantify female extra-pair copulatory behavior directly, but rather measured the paternity of six-day-old offspring. If the only females that were able to successfully rear offspring to six days old were those that were least sensitive to the effects of testosterone, their mating behavior during the fertile period may have been similarly insensitive to increased testosterone, and therefore would not have differed substantially from that of control females. In the junco, exogenous T has been shown to decrease female choosiness, but not motivation to choose (McGlothlin et al., 2004), suggesting that females with elevated T may be less discriminating in accepting copulations from extra-pair males. Directly measuring the effects of testosterone on female copulatory behavior and fertilization rates rather than realized offspring paternity may yield substantially different results (Birkhead and Møller, 1995; Dunn and Lifjeld, 1994; Hsu et al., 2006; Wojczulanis-Jakubas et al., 2009).

Testosterone and selection in males and females

Testosterone levels have been shown to be correlated between males and females, both between species in maximum value, and within species across the course of the breeding season (Ketterson et al., 2005). If this phenotypic correlation reflects an underlying genetic correlation (Lande, 1980, 1987; Lande and Arnold, 1983), then our findings may explain why implant studies in males suggest that natural male testosterone levels currently fall below the adaptive mean (Reed et al., 2006). Selection against increased testosterone in females may constrain the evolution of testosterone in males, since males with high testosterone (and thus high fitness) would produce daughters with a maladaptively high level of testosterone for females (Ketterson et al., 2009; McGlothlin and Ketterson, 2008). Current testosterone levels may represent a middle ground between opposing selective pressures on male and female levels, and may therefore represent a potential case of sexual conflict. Future research examining the fitness consequences of decreased female testosterone – perhaps via the use of antiandrogen manipulations – would help to resolve the question of whether selection on males has drawn female testosterone levels higher than what would be favored by selection on females alone.

While phenotypic engineering, such as that accomplished with hormone implants, is an important tool for understanding the evolution of complex traits from a whole-organism perspective, it is also clear that the phenotypic consequences of such manipulations do not reflect the range of subtle variation within the natural system (McGlothlin and Ketterson, 2008). We must therefore be cautious in our interpretations about the direction and strength of selection on hormone levels. For example, while implant studies have shown that males with elevated testosterone have lower survival but higher reproductive success than control males, suggesting that net selection should favor increased levels of male T (Reed et al., 2006), selection analyses on natural variation in male T indicate that selection's effects are primarily stabilizing, with only a slight directional component of selection on offspring production (McGlothlin et al., 2010). While the differences between these two studies may be due to temporal variation in the selective regime, they also highlight a potential difficulty in extrapolating the results from experimental manipulations to discuss the selection on natural variation in testosterone production. Our results clearly indicate

that prolonged elevated testosterone has negative consequences on female reproductive success, but the effects of selection on natural levels of female testosterone may be quite different. Future work should continue to focus on the relationship between natural variation in the endocrine system and various components of fitness in females.

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