



## Experimentally-elevated testosterone, female parental care, and reproductive success in a songbird, the Dark-eyed Junco (*Junco hyemalis*)

Dawn M. O'Neal <sup>a,\*</sup>, Dustin G. Reichard <sup>a</sup>, Katie Pavilis <sup>b</sup>, Ellen D. Ketterson <sup>a</sup>

<sup>a</sup> Department of Biology, Indiana University, Bloomington, IN 47405, USA

<sup>b</sup> Department of Ecology, Evolution and Environmental Biology, Columbia University, New York, NY 10027, USA

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### ABSTRACT

In male dark-eyed juncos (*Junco hyemalis*), experimentally elevated testosterone (T) decreases male parental care and offspring survival, but results in higher overall fitness through greater mating success. To help address the ensuing question of what prevents selection from favoring higher levels of T in male juncos, we manipulated T in female juncos. A previous study demonstrated no effect of experimentally elevated T on female incubation behavior, suggesting that female parental behavior might be insensitive to T. In this study we asked whether experimentally elevated T mediates other female parental behaviors and whether variation in T-mediated parental behavior might influence reproductive success. We videotaped free-living control- and T-females during nesting to quantify brooding behavior when young were 3 days old and provisioning behavior when young were 6 days old. Nest defense was measured by quantifying responses to a mounted predator placed near the nest. Reproductive success was assessed via fecundity, nestling quality, and nest survival. T-females spent less time than control females brooding but did not differ in provisioning rate. T-females performed fewer dives at the predator mount and, unlike controls, failed to increase defense as nesting progressed. T-females also had lower daily nest survival and lower nest success (odds of producing at least one fledgling). We conclude that some aspects of female parental behavior are sensitive to experimentally elevated T while others are not and consider the implications for the evolution of T-mediated characters in both sexes.

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### Introduction

Natural selection often differs in its impact on males and females of a species, leading to sex differences in morphology, physiology, and behavior (Blanckenhorn, 2000; Ketterson et al., 2005; Ricklefs and Wikelski, 2002). Hormonal mechanisms are an important mediator of sex differences, as various aspects of the phenotype can be coordinated by the capacity of hormones to alter gene expression at any number of tissues in the brain and periphery (Arnold, 2004; Cheverud and Routman, 1995; Finch and Rose, 1995). Here, we focus on testosterone (T) because in many vertebrates, T is produced by both sexes and mediates diverse aspects of the adult phenotype (e.g., neural pathways, muscle cell proliferation, and immunity) (Arnold, 2004; Hiipakka and Liao, 1998; Navara et al., 2005; Simerly, 2002; Staub and DeBeer, 1997). Additionally, T is an important mediator of the organization of sex-specific phenotypes (Arnold, 1996; Balthazart and Ball, 1995; Ketterson et al., 2005).

Testosterone has been extensively studied in male birds, but significantly less so in females (Ball and Balthazart, 2004; Balthazart and Ball, 1995; Garamszegi et al., 2005; Hirschenhauser et al., 2003

but see Cordero et al., 2003; Veiga et al., 2004; Ketterson et al., 2005; Sandell 2007). In males of many bird species, T has been shown to mediate aggressive interactions, as well as reproductive and parental care behaviors (Hirschenhauser et al., 2003; Ketterson and Nolan, 1994; Wingfield, 2005; Wingfield et al., 1987). Field studies of seasonal variation in circulating T have shown that in many temperate-zone bird species, T is highest at the start of the breeding season (at the time of territory and mate acquisition) and fluctuates thereafter, depending on challenges from other males or the reproductive status of the mate, but is generally low during offspring care (Wingfield et al., 1990). Several reviews have demonstrated a similar phenomenon in females (Ketterson et al., 2005; Moller et al., 2005; Wingfield et al., 2000), but relatively few studies have addressed the phenotypic or fitness consequences of seasonal or other forms of variation in female T.

One way to investigate the factors underlying the proximate and ultimate causes of sex differences is to manipulate hormone levels and measure the costs and benefits of these altered phenotypes in relation to measures of fitness (Hunt and Wingfield, 2004; Ketterson et al., 1996; Ketterson et al., 2001; Lynn et al., 2005). Experimental elevation of T levels in birds has been shown to decrease male parental care and survival, but to increase male attractiveness to females and numbers of extra-pair fertilizations (Enstrom et al., 1997; reviewed in Ketterson et al., 2001; Ketterson et al., 1992; Raouf et al., 1997; Reed et al., 2006;

\* Corresponding author. Fax: +1 812 855 6705.

E-mail address: [daoneal@indiana.edu](mailto:daoneal@indiana.edu) (D.M. O'Neal).

Schwagmeyer et al., 2005; Van Roo et al., 2003; Wingfield, 1984). The finding that a shift in behavior from parental care to behavior associated with mate acquisition does not reduce overall fitness was unexpected and creates a paradox: if an increase in male T increases overall fitness, what prevents directional selection in favor of higher levels of T in male juncos?

One possibility is that evolution in males is constrained by the negative impact of elevated T in females, if female T were to rise as a correlated response to selection on males (the constraint hypothesis, Ketterson et al., 2005). Alternatively the restriction on male evolution may lie elsewhere, as theory would also predict that selection should favor female insensitivity to T if sensitivity led to negative fitness consequences. This latter idea has been articulated as the behavioral insensitivity hypothesis (Lynn et al., 2002, 2005). To address these alternatives we measured the effect of experimentally elevated T on aspects of the female phenotype and examined consequences for female fitness. We also compared effects in females to those previously described in males to determine whether females were similarly sensitive to elevated T.

Previous studies in the dark-eyed junco have shown that experimentally elevated T in females leads to increased aggression and increased deposition of T in egg yolk, but decreased body mass, immune function, choosiness for mates, and brood patch formation (Clotfelter et al., 2004; McGlothlin et al., 2004; Zysling et al., 2006). Some of these effects would be expected to reduce fitness (e.g., suppressed immune function); others might be advantageous (e.g., aggression). Treatment with T had no effect, however, on female incubation behavior or nest defense during the egg stage (Clotfelter et al., 2004), suggesting that female parental behavior is insensitive to elevated T.

In this study we focused on the effects of experimentally elevated T on female care of nestlings, a stage of reproduction that has not been addressed previously and one for which theory would predict that the level of parental investment would be greater than during incubation given the higher reproductive value of the offspring (Clutton-Brock, 1991; Clutton-Brock and Godfray, 1991; Trivers, 1972). We also sought to extend our comparison of phenotypic effects of T in females to those previously described in males, to determine traits for which females are similarly sensitive to elevated T. We considered three behaviors associated with the nestling stage: brooding, nestling feeding, and nest defense. We hypothesized that if the impact of T on female juncos resembled that seen in males of numerous species, then experimentally elevated T would reduce the amount of time females spent brooding nestlings, the amount of time spent on the nest, and the rate of nestling provisioning (Ketterson et al., 2001; Reed et al., 2006; Schwagmeyer et al., 2005; Van Roo, 2004). Conversely, if females are insensitive to the suppressive effect of T on parental behavior during the nestling stage, we would predict no effect on any of these measures (Clotfelter et al., 2004; Lynn et al., 2005). Similarly, for nest defense during the nestling stage, based on previous results in males (Cawthorn et al., 1998), we predicted that females treated with T would defend more than control females (reviewed in Wingfield et al., 1987). If, however, females are insensitive to T with respect to defense, we would expect no effect. Finally, we considered three variables related to female fitness: nestling mass [an indirect measure of female fitness and an indicator of nestling quality and survival; Stienen and Brenninkmeijer, 2002], nest survival, and reproductive success (the number of fledglings produced). If treatment with T reduces parental care, we would expect a significant reduction in nestling growth along with declines in nest success and the number of young produced.

## Methods

### Study species and site

The dark-eyed junco (*Junco hyemalis carolinensis*) is a socially monogamous songbird that breeds throughout the southern Appa-

lachian Mountains. Only females brood, but feeding and nest defense are a bi-parental effort.

This study took place at the University of Virginia's Mountain Lake Biological Station and the nearby grounds of Mountain Lake Hotel (Giles County; 37°22'N, 80°32'W) between early June and mid-July 2005 and 2006. The environs of the station, located in the Appalachian Mountains, consist of mountainous deciduous forest in which juncos are abundant (Chandler et al., 1994). Since 1983, almost all juncos on the study site have been uniquely color banded as part of long term study of their breeding biology. All procedures used in this study were approved by the Bloomington Institutional Animal Care and Use Committee.

### Hormone implants

In 2005 and 2006, starting 1 May through 15 June, we captured 190 adult female juncos in mist nets and Potter traps and transported them to a central location where they were weighed, aged (estimated by looking at plumage color in conjunction with mark and recapture data from previous years), and bled for a baseline hormone sample. They were then anesthetized (metofane in 2005 and halothane in 2006) and implanted subcutaneously along the left flank with one 7 mm silastic tube (Dow Corning, 1.47 mm internal diameter, 1.96 mm outer diameter). For the 90 females receiving testosterone (T-females), 5 mm of the tubes were packed with crystalline testosterone (Sigma Chemical) and sealed with silastic glue. One hundred control females (C-females) received empty tubes sealed with glue. Females were implanted during various stages of the nesting cycle (laying stage, egg stage, or nestling stage) but had been implanted for a minimum of two weeks before behavioral observations occurred. The treatment of each female was determined randomly at each capture site by coin toss. Females were returned to their capture sites within 30 min of implantation. Observers were blind to the treatment of each female to avoid any biases in data collection. Details of the implantation procedure for birds implanted in 2001 and 2002 have been reported in Clotfelter et al. (2004) and are similar to the methods employed here with the exception of date; in 2001 and 2002, birds were implanted between 15 April and 15 May.

Treatment with testosterone prolongs naturally occurring high levels of testosterone in females throughout the breeding season (Ketterson et al., 2005). We recaptured females after each successful nesting attempt and to remove implants in the late summer. During these captures we drew blood to measure testosterone concentrations and to check the status of the implant. For both captures and recaptures we measured the duration of capture and bleeding time with a stopwatch. Following blood collection, we centrifuged samples for 5 min and stored the separated plasma at 20 °C until we performed enzyme immunoassays (EIA).

### Nest monitoring

Beginning 15 May each year we searched daily for nests. Once found, nests were marked and the social pair (defined as the pair seen defending the nest and caring for young) was identified. Nests found during the building stage were monitored daily until the clutch was complete and then every 3 days afterward until eggs hatched. Nests found with eggs were monitored every other day until eggs hatched. Nests with nestlings were checked on hatch day (day 0), and on days 3, 6, 11 and fledging day (day 12). Nestlings were weighed in the afternoon on days 0, 6, and 12 with the exception of nests found for the first time after nestlings had hatched, in which case we weighed young immediately to determine age.

### Nestling brooding and feeding behaviors

In 2006, we quantified brooding and feeding rates in females and males caring for day 3 nestlings. On day 3 after counting the number of

young, cameras were set up approximately 2–3 meters from the nest. We then video recorded behavior for 4 h to measure the amount of time females spent brooding nestlings (sitting directly on nestlings) and the number of times females brought food to the nest. Additionally, we quantified the number of times males brought food to the nest.

In 2005 and 2006, we also quantified provisioning behavior in females caring for day 6 young. Prior to observations, males were captured near the nest in a mist net or Potter trap and held, until completion of the feeding trial, allowing us to be certain of the identification of the parent feeding the young and to control for interactions between mated pairs (Clotfelter et al., 2007). After male removal, the number of young was counted. For 4 h we recorded the number of times females brought food to the nest. Nestlings were weighed on day 6 after nest defense trials. To reduce inter-observer error and bias, all day 3 tapes were analyzed by D.G.R. and all day 6 tapes by D.M.O.

#### Nest defense

Using standard nest defense protocols (Cawthorn et al., 1998; Clotfelter et al., 2004), in 2005 and 2006, we measured the female's response to a taxidermic mount of a known junco nest predator, the eastern chipmunk (*Tamias striatus*). Trials were conducted in the early afternoon when nestlings were 6 days old, following provisioning trials. Females were flushed off the nest and the mount, covered by a piece of camouflage cloth attached to string, was placed approximately 0.5 m away from the nest opening. The observer (K.P. in 2005 and D.M.O in 2006) concealed herself in vegetation approximately 10–15 m away and waited for the female to resume feeding before uncovering the chipmunk. The frequency of dives (swoops at predator with no contact), hits (swoops with contact), and nest checks (female enters nest or stands on rim) were recorded for a 10-minute period. Using previously published data from Clotfelter et al. (2004), we also compared nest defense during the nestling stage to nest defense during the egg stage to determine whether defense rates differed by treatment or increased as reproductive value of young increased.

#### Nest survival and female reproductive success

We monitored 415 junco nests of implanted females over four years (2001–2002, 2005–2006). Of this total, 139 had insufficient data for nest survival analyses for reasons including uncertain dates of nest initiation or failure, having been found during building stage but never producing eggs, or association with females that were implanted after nest initiation. Removing these nests from the analysis resulted in a final sample size of 276 nests used to estimate nest survival, which we report as the probability that a nest survived a single day, using the program MARK (White and Burnham, 1999). The nest survival model in program MARK is an extension of the model developed by Bart and Robson (1982), allowing flexibility in modeling daily nest survival using individual, group, and time-specific covariates and allows intervals between nest visits to vary. Nests were assigned to one of 16 groups based on female treatment (T or C), nest stage (eggs or nestlings), and year (2001, 2002, 2005, 2006). The model allowed nest survival to vary between years but due to the small number of nests, kept daily survival within a year constant.

As a direct measure of female fecundity, we estimated fledgling production for each female within a year. A female was considered successful if she produced at least one fledgling. Nests were included in fecundity analysis only if we could be certain of the number of nestlings that left the nest on fledgling day (day 12 April–June, day 11 July–August).

#### Testosterone enzyme-linked immunoassay (EIA)

Testosterone concentrations were determined using a commercial enzyme immune assay (EIA) (catalog # 901-065; Assay Designs, IN., Ann Arbor, MI) as described in Clotfelter et al. (2004). Briefly, 20  $\mu$ l of plasma were diluted 6-fold in distilled water and 2000 cpm [ $^3$ H] testosterone (NET-553; New England Nuclear Corp., Boston, MA) were added to allow the calculation of recoveries following extractions with diethyl ether. Extracts were then evaporated and reconstituted in 50  $\mu$ l of 100% ethanol, and diluted to 350  $\mu$ l with assay buffer; 100  $\mu$ l were used for recoveries and 100  $\mu$ l as samples in duplicate. Due to the large number of samples, four assays were performed in 2005 and two in 2006. In 2005, inter-assay variation was 6.992% and intra-assay variation was 6.032%, 6.208%, 6.377% and 2.893%. In 2006, inter-assay variation was 4.120% and intra-assay variation was 27.832% and 3.587%. Mean percent recovery was 95.8% and 97.2% for 2005 and 2006 respectively. The manufacturer's instructions were otherwise followed throughout. Testosterone concentrations were determined with the aid of a four parameter logistic curve-fitting program (Microplate Manager; Bio-Rad Laboratories, Inc., Hercules, CA), and concentrations were corrected for incomplete recovery.

#### Statistics

We analyzed female concentrations of plasma testosterone, behavior (provisioning, brooding, nest defense), and fecundity using SPSS version 14.0. We compared testosterone levels before and after treatment using an independent samples *t*-test. To examine the effects of hormone treatment on feeding and brooding, we used an ANCOVA correcting for female age, brood size, year, and Julian Date. Data for individual defense behaviors (hits, dives, checks) were square root transformed for normality and compared by treatment (*t*-test). In addition we employed a principal components analysis (PCA) to reduce variation in the three defense behaviors to a single variable (PC1) and to derive a single defense score for each individual. Using current data and previously published data from Clotfelter et al. (2004), we used a two-way ANOVA to compare PC1 defense scores between treatments across the nestling and egg stages. The effect of female treatment on nestling mass was analyzed using a mixed model ANOVA including brood size, female age, year, and Julian date in the analysis. We excluded nestlings from mass analysis if the female was implanted with T after egg laying and in instances where hatch date was unknown. For each analysis, tests were two-tailed and significance was calculated at the  $P=0.05$  significance level. Covariates were removed from the final model if they fell above the 0.05 significance level.

We evaluated support for 10 candidate models developed to explain variation in nest survival with respect to female treatment (Table 1). Competing nest survival models were evaluated using an information-theoretic approach (Burnham and Anderson, 2002; Dinsmore et al., 2002) in program MARK. Akaike's information criterion for small sample sizes (AIC) was used to rank models from the most to least supported. Additionally, we used Akaike weights ( $w_i$ ) calculated in program MARK to determine estimates of the relative importance of our predictor variables (treatment, stage, and year) across all the models in the set. Akaike weights were summed across all models in the set in which each predictor variable occurred ( $w_+$ ) and then ranked in their importance; the larger  $w_+$ , the more important the predictor variable is relative to other variables (Burnham and Anderson, 2002).

We analyzed the effect of treatment on number of young produced using a logistic regression. We performed 4 separate regressions for each of the four years with female treatment as the independent variable and a single regression combining all four

**Table 1**

Summary of model selection results for nest survival of Dark-eyed Juncos, 2001–2002 and 2005–2006

Model	AIC <sub>c</sub>	$\Delta AIC_c$	w <sub>i</sub>	Model likelihood	K	Deviance
Treatment+year	1287.473	0	0.33603	1	5	1277.454
Year	1288.546	1.0723	0.19657	0.585	4	1280.533
Treatment+stage+year	1289.217	1.7437	0.14052	0.4182	6	1277.19
Treatment*year	1289.273	1.7995	0.13665	0.4067	8	1273.227
Treatment	1291.238	3.7641	0.05117	0.1523	2	1287.234
Stage*year	1291.356	3.8828	0.04822	0.1435	8	1275.31
Intercept	1291.816	4.3421	0.03833	0.1141	1	1289.814
Treatment*stage*year	1292.431	4.9573	0.02818	0.0839	16	1260.255
Stage	1293.732	6.2584	0.0147	0.0437	2	1289.728
Treatment*stage	1294.578	7.1043	0.00963	0.0287	4	1286.565

Models are ranked by ascending  $\Delta AIC_c$ ; w<sub>i</sub> is the model weight and K is the number of parameters. Factors included in models were treatment, year, stage, and a model with constant daily nest survival (intercept). Deviance is computed as  $-2[\log_e(L(\theta)) - \log_e(L_s(\theta))]$ , where [L(θ)] represents a maximum likelihood estimate whose log-likelihood is evaluated for the model in question and for the saturated model [L<sub>s</sub>(θ)].

years together. The resulting log(odds) were then converted to the probability of producing young by treatment for each year and all years combined.

## Results

### Testosterone treatment

T manipulation data for birds implanted in 2001 and 2002 has been reported in Clotfelter et al. (2004). In 2005 and 2006, plasma testosterone did not differ between treatments before implantation (2005 and 2006 respectively; t=0.456, 1.31, P>0.05; mean T concentration±s.e.: C-females=0.5383±0.0299, 0.774±0.093; T-females=0.563±0.047, 1.1289±0.255, ng ml<sup>-1</sup>) but implantation with T significantly increased plasma T levels in T-females compared to C-females (t=-5.44, -3.57, P<0.05; mean T concentration±s.e. for 2005 and 2006: C-females=0.787±0.220, 0.910±0.318; T-females=2.982±0.365, 2.622±0.360, ng ml<sup>-1</sup>). Testosterone levels induced by the implant were comparable to peak female levels observed in the field (Ketterson et al., 2005).

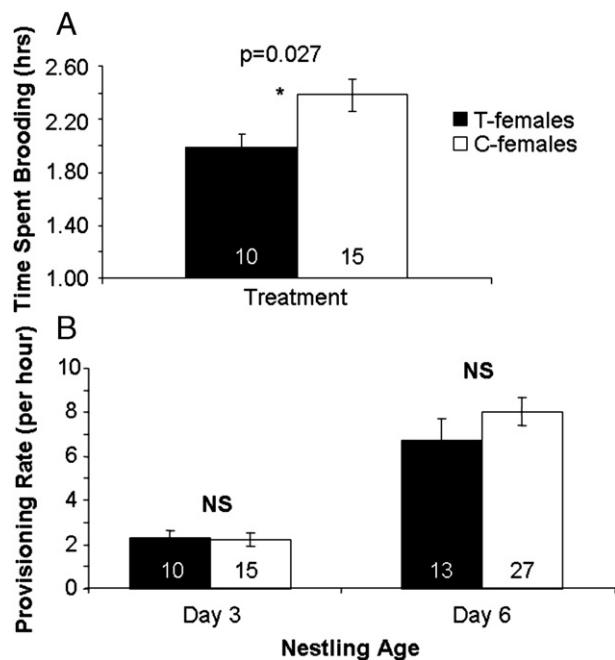
### Nestling brooding and feeding behaviors

After controlling for female age ( $F(1,25)=3.98, P=0.050$ ) and brood size ( $F(1,25)=11.975, P=0.02$ ), T-females spent significantly less time than C-females brooding day 3 young (mean brood time (hours)±s.e. T-females: 1.99±0.97, C-females: 2.38±0.121) (Fig. 1). Consistent with provisioning of day 6 nestlings, there was no significant difference in female feeding rate on day 3 ( $F(1,25)=1.97, P=0.175$ ). When we analyzed male feeding rate on day 3 with respect to female treatment, we found a significantly higher rate of provisioning in males mated to T-females (mean provisioning rate±s.e. mates of T-females: 3.51±0.293, mates of C-females: 2.61±0.312,  $F(1,25)=8.55, P=0.008$ , when the analysis was run using brood size as a covariate,  $F=7.305, P=0.013$ ).

Female provisioning rate of day 6 nestlings did not differ significantly by treatment (mean feeding rate (per hour)±s.e. T-females=6.76±0.95, C-females=8.04±0.64,  $F(1,40)=2.01, P=0.164$ ) (Fig. 1). Of the covariates analyzed for provisioning rate of day 6 nestlings, none was a significant predictor of female feeding rate.

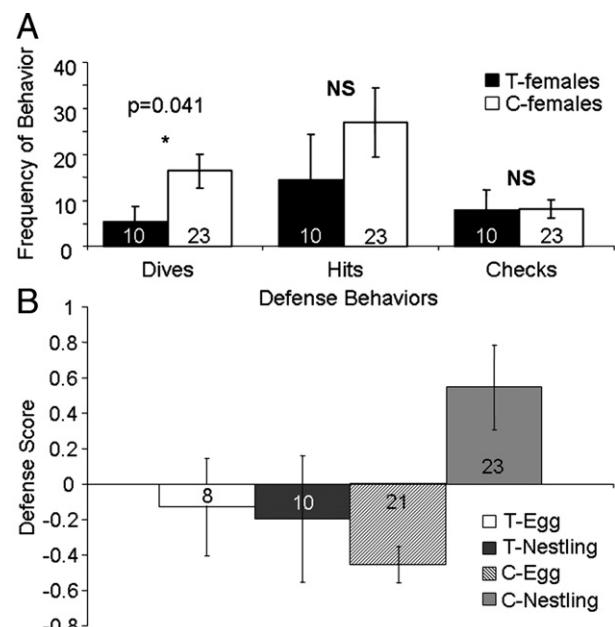
### Nest defense

T-females performed fewer dives at the predator mount than C-females ( $t=2.136, P=0.041$ ) (Fig. 2A), but did not differ in hits ( $t=1.430, P=0.163$ ) or nest checks ( $t=0.702, P=0.488$ ). The three defense responses of hits, dives, and nest checks contributed almost



**Fig. 1.** Mean time (in hours) (±SE) females spent brooding 3-day old nestlings (A) and the mean number of times (±SE) females brought food to nestlings 3 days in age and 6 days in age (B). T-females spent significantly less time brooding compared to C-females (A). Neither measure of provisioning rate (day 3 or day 6) was significantly affected by the female's hormone treatment (B).

equally to the first principal component of nest defense behavior (PC1), which explained 65.9% of the total variation. Based on values of PC1, we found a significant interaction between treatment and



**Fig. 2.** Mean number (±SE) of hits, dives, and nest checks performed by females exposed to an eastern chipmunk mount at the nest site (A). T-females performed significantly fewer dives than C-females, but did not differ in hits or nest checks. Mean response scores (±SE) extracted from a principal components analysis combining hits, dives, and nest checks performed by T- and C-females during the egg and nestling stages in response to an eastern chipmunk mount at the nest site (B). A comparison across stages and between treatments revealed a significant interaction between nest stage and treatment, indicating that the effect of treatment depends on stage: T-females defended less than C-females but only during the nestling stage. Data from egg stage from Clotfelter et al. (2004).

nest stage ( $F(1, 61)=4.252, P=0.044$ ), indicating that C- but not T-females increased intensity of defense at the later stage of reproduction (Fig. 2B).

#### Nestling mass

We used a mixed model analysis to analyze average nestling mass in nests of treated females. Correcting for year ( $F(1, 62.22)=17.244, P=0.000$ ), nestlings of T-females weighed significantly less at day 6 of nestling life than those of control females (average mass $\pm$ s.e. nestlings T-females= $12.208\pm 0.439$ , nestlings C-females= $13.349\pm 0.313$ ,  $F(1, 33.73)=4.140, P=0.050$ ).

#### Nest survival and female reproductive success

The most supported model of nest survival ( $w_i=0.33$ ) included the additive effects of year and treatment, but no interaction term. This model was 1.07 AICc units better than the second-best model which included year alone, and was >1.7 AICc units better than models including either the additive effects of treatment, stage, and year or interactions between treatment and year (Table 1). The best supported model of nest survival indicated that nest survival varied by year (each year compared to 2001:  $\beta_{2002}=-0.336, SE=0.243, 95\% CL=-0.811, 0.141$ ;  $\beta_{2005}=0.265, SE=0.232, 95\% CL=-0.188, 0.719$ ;  $\beta_{2006}=0.291, SE=0.215, 95\% CL=-0.131, 0.713$ ) and was lower in T-females when compared to C-females ( $\beta=0.279, SE=0.158, 95\% CL=-0.030, 0.589$ ) (Fig. 3). With respect to the relative importance of our three predictor variables (treatment, stage, and year), the model suggests that year had the strongest effect on nest survival, followed by treatment, and then by stage ( $w_+ \text{year}=0.89, w_+ \text{treatment}=0.70, w_+ \text{stage}=0.21$ ).

One hundred fifty-five females were included in the logistic regression analysis of female treatment in relation to female production of at least one fledgling over the course of the breeding season. Combined across all four years, C-females had a significantly higher probability of success compared to T-females, 49% vs. 30% (Wald statistic=5.334,  $df=1, P=0.021$ ) (Table 2). Considering each year separately, the relative success of C-females was greatest in 2005 when C-female probability of success was 57% as compared to the 14% success rate of T-females (Wald stat=6.217,  $df=1, P=0.013$ ). There was no significant difference between treatments in probability of success in any of the other three years, although C-females tended to have greater success in 2006: 55% vs. 40% (Wald stat=1.196,  $df=1, P=0.274$ ).

#### Discussion

We asked whether experimentally elevated T in female dark-eyed juncos influenced any of three components of female parental behavior and whether any alterations in behavior could be related

**Table 2**  
Probability of a female producing at least one fledgling

Year	C(N)	T(N)
2001	38%(13)	31%(13)
2002	23%(11)	27%(13)
2005	57%(14)*	14%(33)*
2006	55%(25)	40%(33)
2001–2002; 2005–2006	49%(63)*	30%(92)*

N indicates the number of females that fit the fledgling criterion.

Significant differences ( $P<0.05$ ) in probability are indicated by an asterisk.

to fitness. We found that treatment with T significantly reduced the amount of time females spent brooding nestlings. We also found reduced intensity of nest defense by T-females in that T-females swooped at the predator mount less often and did not increase defense of nestlings over eggs. We found no effect of T on the rate of provisioning of offspring, although we did observe enhanced provisioning on day 3 by the mates of T-females that may have been compensatory for reductions in brooding. Nestlings of T-females weighed significantly less than those of C-females, and treatment with T reduced both nest survival and the probability of producing at least one fledgling. Collectively, our findings suggest female juncos with naturally higher levels of T may be selected against. Our findings also provide provisional support for the hypothesis that response to selection favoring higher T in males might be constrained if it led to a correlated response in females.

#### Brooding behavior and nestling growth

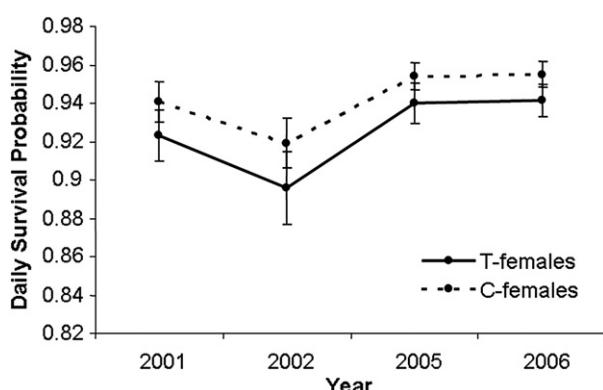
Reduction in brooding bouts by T-females is consistent with the effects of T on males and females of other species (Schwagmeyer et al., 2005; Van Roo, 2004). However, earlier studies of female juncos found that T did not interfere with incubation behavior, leading to the conclusion that incubation behavior is insensitive to T (Clotfelter et al., 2004). Because nestlings seem likely to provide a stronger stimulus to parental behavior than eggs (Trivers, 1972), we would have predicted even less suppression of brooding by T, not greater suppression as observed.

The longer brooding bouts by control females seem likely to have elevated nestling body temperature, allowing nestlings to devote less energy towards thermoregulation and more energy towards growth and development and thus accounting for their greater body mass. Since mass has been shown to be an important indicator of nestling and fledgling survival (Dawson et al., 2005; Monros et al., 2002; Perrins and McCleery, 2001; Stienen and Breninkmeijer, 2002), reductions in the mass of nestlings belonging to T-females suggests an indirect fitness loss to females with elevated T.

The fact that T-females spend less time brooding raises the question of how females allocate additional time away from the nest. Testosterone has been reported to elevate activity levels and metabolic rate in at least one species of bird (Buchanan et al., 2001; but see Lynn et al., 2000; Wikelski et al., 1999), so treatment with T may have increased female food requirements and caused females to spend more time on self maintenance and gathering food for themselves. However, preliminary data from radio tracking of T-females has provided little support for an increase in time spent foraging during the nestling stage (Reichard and Ketterson, unpublished data). Future studies should investigate possible alternative behavioral outlets for females implanted with T to determine whether increased time off the nest is indeed correlated with greater food consumption (Markman et al., 2002).

#### Sensitivity of provisioning behavior to the suppressive effects of testosterone

T had no detectable effect on offspring provisioning, as measured by feeding rate, indicating that provisioning behavior may be



**Fig. 3.** Daily nest survival for T- and C-females. Values depicted are the probability of a nest surviving a single day ( $\pm$ SE). Nest survival varied with year but T-females had consistently lower nest survival than C-females.

insensitive to T in female juncos. This result differs from males of many songbird species, including juncos (Ketterson et al., 1992; Schwagmeyer et al., 2005; Stoehr and Hill, 2000) though not from males of all species (Lynn et al., 2002; Van Duyse et al., 2002). Two ecological factors have recently been identified as predictive of whether experimentally elevated T will suppress male parental behavior: the degree to which male care is essential to offspring survival (Lynn et al., 2005), and the number of broods the species or population ordinarily produces (Goymann et al., 2007). In species in which male care is essential to offspring survival, male care has been shown to be insensitive to experimentally elevated T (Lynn et al., 2002; Van Duyse et al., 2002; for an important exception see Van Roo, 2004). In single-brooded species in which males are not likely to benefit from effort devoted to extra-pair fertilizations (EPFs) due to limited availability of fertile females, male care may also be insensitive to elevated T (Hunt et al., 1999).

In juncos, male care increases fledgling survival but is not essential to reproductive success (Wolf et al., 1988) and EPFs are frequent (Raouf et al., 1997). Further, female juncos typically lay replacement clutches after nest loss to predation, and also produce second broods if their first broods are successful. Both factors predict male sensitivity to the suppression of parental behavior by T so as to foster direction of effort towards extra-pair mating (Ketterson et al., 1992). Female parental behavior, on the other hand, is essential to reproductive success; females are the sole incubators in juncos and, unlike males, do not seek EPFs while rearing offspring. These behavioral and physiological differences between the sexes may help to explain in an ultimate sense why provisioning behavior in females is insensitive to the suppressive effects of T, while male provisioning is not.

#### *Indirect effects of elevated T in females on their mates*

Results presented here show that males may provide compensatory nestling care (increased provisioning rate) in response to reductions in female care (brooding behavior), similar to the previously reported increase in parental care by females mated to males with experimentally elevated T (Ketterson et al., 1992). The compensation observed must not have been complete, however, because T-nestlings weighed less than C-nestlings. The compensation is nonetheless consistent with models of stable bi-parental care that predict incomplete compensation by one partner when confronted with reduction in care by the other partner (Houston and Davies, 1985; Jones et al., 2002; Ratnieks, 1996).

#### *Nest defense*

Similar to the effect of experimentally elevated T on junco male nest defense behavior (Cawthorn et al., 1998), we found evidence for a reduction in nest defense behavior in females treated with T. This result conforms to the generalization that T reduces investment in dependent offspring as seen in males of this and several other species (Ketterson and Nolan, 1994; Wingfield et al., 1990). Females may be responding to experimentally elevated T similarly to males by shifting their emphasis from offspring survival to their own survival.

Independent of any influence of experimentally elevated T on nest defense, we predicted that intensity of defense would increase as the nest cycle progresses because older offspring are of greater reproductive value, having already survived periods of high mortality (Knight and Temple, 1986; Montgomerie and Weatherhead, 1988; Palestis, 2005). Therefore, as offspring age, parents should take greater risks and defend their nests more intensely as the cost of losing offspring outweighs the potential for re-nesting or even parental survival (Knight, 1997; Listoen et al., 2000; Palestis, 2005; Tryjanowski and Golawski, 2004).

We combined the data in this study with earlier data from Clotfelter et al. (2004) and found a significant interaction between

treatment and nest stage in their effect on nest defense. As offspring aged from eggs to nestlings, intensity of defense increased in C-females, but not in T-females. Thus, C-females appear to respond to the enhanced reproductive value of their older offspring and adjust their defense accordingly, while treatment with T appears to reduce female risk-taking in relation to dependent offspring, perhaps owing to a shift in emphasis to adult survival.

#### *Nest survival and female reproductive success*

In conjunction with a reduction in brooding and defense behaviors, we also saw a negative effect of T on both nest survival and fledgling production, with this effect more pronounced in some years as compared to others. Year seemed to play a major role in predicting differences between treatments, a role we attribute to differences in predation rates between years. In some years nest predation may be less dependent on the behavior of the parents and more dependent on environmental factors such as the number of potential predators, the amount of available ground cover, and food supply (Chalfoun et al., 2002; James et al., 2006; Martin and Joron, 2003; Rastogi et al., 2006; Tirpak et al., 2006; Reed et al., 2006). During "good years" (2005–2006, pers. obs.), however, when predation is low behavioral differences associated with treatment with T appear to have detrimental effects on nest survival of T-females, which suggests that C-females are better able to take advantage of such opportunities when compared to T-females. Thus, in good years C-females may have increased reproductive success with the production of more fledglings. Reduced nest survival and fledgling success in T-females, on the other hand, indicates a fitness loss in females treated with T, which may have implications concerning the evolution of T in females and, by association, in males.

#### *Implications for evolution of T-mediated characters in male juncos*

The constraint hypothesis predicts that because the sexes share most of their genes, selection on one sex may lead to a correlated response in the other sex. If females respond to selection as males do but that response reduces female fitness, females may constrain male evolution so that the successful male phenotype reflects a compromise between what would be optimal for each sex if they were free to evolve independently (Ketterson et al., 2005; Lande and Arnold, 1983; Mank, 2007). Alternatively, as we have demonstrated in previous studies regarding the effects of experimentally elevated T on parental care, females could evolve insensitivity to T if the fitness costs associated with sensitivity are prohibitive as predicted by the behavioral insensitivity hypothesis (Hunt et al., 1999; Lynn et al., 2002). In that case, the sexes could evolve independently. During the egg stage, the parental care behaviors of incubation and nest defense in the female junco seem to be unaffected by treatment with T (Clotfelter et al., 2004), yet as reported here several other behaviors were sensitive to T, including brooding and nest defense, but not provisioning. These data would seem to suggest that sensitivity to T varies with behavior and context, but much remains to be learned about the possible mechanisms underlying this variation.

Our current data on elevated testosterone in female juncos suggests that high T has several negative fitness consequences in females: reducing immune function (Zysling et al., 2006), body mass, readiness to reproduce (as measured by the time taken to develop a brood patch, build nests, and lay eggs) (Clotfelter et al., 2004), choosiness for mates (McGlothlin et al., 2004), attractiveness to mates (Parker-Rena et al. unpublished data) and parental care behavior when caring for nestlings (this study). These negative fitness consequences associated with elevated T suggest that selection on females may act to constrain the evolution of high T in males. Further study must be made of the direct and indirect fitness effects of high female T including information on female return rate and year to year survival, as well as measures of offspring response to high maternal T,

and possible mechanisms behind female insensitivity to T before conclusions can be drawn with regard to correlated responses to selection on T in the junco.

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