Testosterone affects reproductive success by influencing extra-pair fertilizations in male dark-eyed juncos (Aves: *Junco hyemalis*)

SAMRRAH A. RAOUF[1*], PATRICIA G. PARKER[2], ELLEN D. KETTERSON[1], VAL NOLAN JR[1] AND CHARLES ZIEGENFUS[3]

[1]*Department of Biology and Center for the Integrative Study of Animal Behavior, Indiana University, Bloomington IN 47405, USA*
[2]*Department of Zoology, The Ohio State University, Columbus, OH 43210, USA*
[3]*Department of Mathematics, James Madison University, Harrisonburg, VA 22801, USA*

SUMMARY

Monogamous male birds typically allocate less effort to courtship and more to parental behaviour than males of polygynous species. The seasonal pattern of testosterone (T) secretion varies accordingly. Monogamous males exhibit a spring peak in plasma T followed by lower levels during the parental phase, while males of polygynous species continue to court females and maintain T at higher levels. To determine whether testosterone underlies the trade-off between mating and parental effort, we treated male dark-eyed juncos (*Junco hyemalis*) with exogenous T and compared the reproductive success (RS) of T-treated males (T-males) to that of controls. T-males had lower apparent annual RS than controls, probably because elevated T reduced parental care. Nevertheless, annual genetic RS of the treatment groups was similar because (i) T-males suffered fewer losses in genetic RS due to extra-pair fertilizations (EPFs), and (ii) T-males gained more genetic RS through their own EPFs. This is the first hormonal manipulation of an avian phenotype shown to influence male RS through EPFs. Together with other studies, it suggests that testosterone may have mediated the evolution of inter- and intraspecific differences in allocation of reproductive effort to mate attraction and parental care.

1. INTRODUCTION

With the advent of genetic techniques that can accurately determine parentage (Burke & Bruford 1987; Jeffreys *et al*. 1985), we now know that monogamous male birds can enhance their reproductive success (RS) by obtaining copulations with females other than their social partner, and that these copulations can lead to extra-pair fertilizations (EPFs) (Birkhead & Møller 1992; Westneat *et al*. 1990). The results of many studies have shown that, among passerine birds, an average of 20% of nestlings are produced by EPFs (for a review, see Birkhead & Møller (1992)), but few studies have reported the identities of the sires of the young produced by these EPFs (Gibbs *et al*. 1990; Hasselquist *et al*. 1996; Weatherhead & Boag 1995; Westneat 1993). Therefore, we know little about the behavioural, morphological, or physiological determinants of variation in males' abilities to obtain EPFs. In order to investigate a potential physiological basis for this variation, we manipulated the reproductive behaviour of a socially monogamous passerine bird, the dark-eyed junco, by placing silastic tubing that contained crystalline testosterone (T) under the skin of free-living males. Experimentally elevated T, in juncos and other species, suppresses male parental behaviour (Ketterson *et al*. 1992; Oring *et al*. 1989; Silverin 1980) but increases male attractiveness to females (Beletsky *et al*. 1995; Enstrom *et al*. 1997; Wingfield 1984; Zuk *et al*. 1995). These results parallel interspecific variation in patterns of testosterone secretion, mating systems, and male parental care in birds (Wingfield *et al*. 1990). In socially monogamous species, plasma T is high early in the breeding season when territories are established and pair bonds formed, but drops thereafter when males provide parental care (Wingfield & Moore 1987). In contrast, among polygynous species T-levels remain high, as males attract additional social mates while providing comparatively less parental care (Beletsky *et al*. 1995). Thus, it seems likely that testosterone plays a critical role in mediating allocation of male effort between mate attraction and parental care. What is not known is whether testosterone may also affect the ability of males to obtain EPFs at the expense of neighbouring males or to minimize EPFs of their own social mates by other males.

In juncos, previous studies have shown that treatment of free-ranging males with exogenous testosterone (T-males) leads to behaviour that could increase a male's extra-pair mating, while not necessarily increasing his

*Author for correspondence (sraouf@indiana.edu).

losses to EPFs. T-males sing more than controls (hereafter C-males) (Ketterson *et al.* 1992), and captive female juncos prefer to associate with, and solicit copulations from, T-males (Enstrom *et al.* 1997). When their social partners are not fertile, T-males occupy larger home ranges, thereby potentially increasing encounter rates with fertile female neighbours (Chandler *et al.* 1994); but when their social partners are fertile, T-males have home ranges similar in size to those of C-males, and males of both treatments spend equal time in the vicinity of their fertile social partners (Chandler *et al.* 1997). Together, these results suggest that T-males may have a higher probability of inseminating the social partners of other males without lowering their own effectiveness at defending against EPFs. On the other hand, observations that T-males feed their young less frequently than controls (Ketterson *et al.* 1992), and are less effective at nest defence (Ketterson *et al.* 1996), predict higher mortality of young in these nests which may be a potential cost to elevated T.

During four breeding seasons we compared free-living T- and C-males with respect to the following five measures of reproductive success. (1) Annual apparent reproductive success. This is the number of eggs, hatchlings and fledglings recorded in the nests of a socially mated pair during one breeding season. We predicted that T-males would have lower apparent reproductive success than C-males. (2) Annual losses of reproductive success owing to EPFs. We predicted either that losses by T-males and C-males would be similar because males of these two groups did not differ in propensity to accompany (mate-guard) their fertile social mates, or if there was a difference, that losses by T-males would be fewer because they are more attractive to females. (3) Annual home genetic reproductive success. This is the number of fledglings produced from matings with the social mate, i.e. apparent RS minus losses caused by EPFs. (4) Annual gains in RS resulting from EPFs. We predicted that gains would be greater for T-males. (5) Net annual genetic RS. This is annual genetic RS at home plus gains in RS from EPFs. We predicted that this measure would be larger for C-males, as they represent the 'normal' phenotype.

2. METHODS

(*a*) *Species and study area*

From 1990–1993, we studied the subspecies *J. h. carolinensis*, which breeds above *ca.* 1000 m in the Appalachian Mountains of Virginia, USA (for details on study site, see Chandler *et al.* (1994)). These juncos are territorial and socially monogamous. Pairs can rear as many as three broods per season, but nest predation prevents many from rearing any young. Females build the nest, incubate the eggs, and brood nestlings; both parents contribute to nest defence and feeding of young (Ketterson *et al.* 1992, 1996). All birds reported on were individually colour-marked.

(*b*) *Manipulation of hormone profile*

T-males were implanted with two 10 mm lengths of silastic tubing (Dow Chemical, inner diameter = 1.47 mm, outside diameter = 1.95 mm) packed with crystalline testosterone (Sigma Chemical). C-males received empty tubing. Implanting occurred while birds were anaesthetized (for details see Ketterson *et al.* (1992, 1996)) and took place upon a male's first capture, early in the breeding season prior to onset of egg-laying. At this time, and again later in the season, we took blood from the alar vein for paternity analysis and hormone analysis. The effect of T-implants was to elevate and maintain circulating T within levels that occur naturally early in the breeding season (6.24 ng ml^{-1}). In C-males, after the natural peak of T in late April, levels drop to an average of 2.1 ng ml^{-1} (Chandler *et al.* 1997; Ketterson & Nolan 1992). At the end of the breeding season, males were caught, and their implants were removed.

Yearling males (*ca.* 50% of our sample), as well as older males not previously banded, were assigned randomly to treatment groups by a coin toss. Males implanted in a previous year were assigned to the treatment group opposite that of the previous year. The result was that T-males and C-males were equally represented among yearling and older males and randomly distributed across the study site.

(*c*) *The sample*

All territories were routinely searched to determine whether males had acquired mates and, if so, the stage of their reproductive cycle. Because all measures of RS are annual, it was necessary to document all outcomes of a pair's reproductive attempts. We determined season-long success in 58 cases of T-males and their 156 young, and 67 cases of C-males and their 259 young. Included in this total of 125 male-years are data from 18 individuals that were present for two years, two individuals for three years, and one individual for all four years. Because an individual's treatment alternated between years, we counted each year's result as an independent event. Furthermore, for the individuals that were studied in more than one year, there were no significant across-year correlations in measures of RS. Three T-males and one C-male never acquired social mates, and ten C-males and nine T-males had social mates but produced no offspring that left the nest. We included these males because all could have produced young through EPFs. All statistical comparisons report two-tailed p values.

(*d*) *DNA fingerprinting and paternity analysis*

Following previously established methods (Piper & Parker-Rabenold 1992; Rabenold *et al.* 1990), we used minisatellite multilocus DNA fingerprinting to determine parentage. Briefly, 5 μg of DNA were digested with Hae III at 37 °C for 3–5 h, and the fragments separated on an 0.8% agarose gel for 65–70 h at 20 V. DNA of putative parents and their offspring was placed on gels, such that samples were not separated by more than three lanes. Membranes were hybridized to Jeffreys's 33.6 or 33.15 probes (Jeffreys *et al.* 1985) overnight at 62 °C.

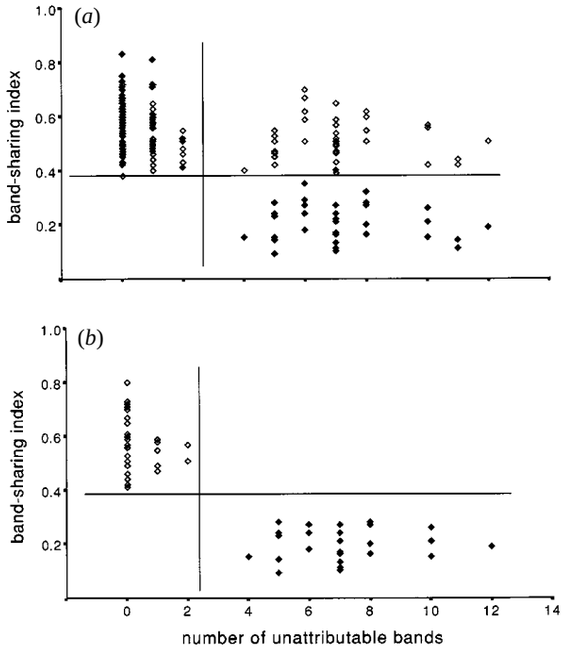

Figure 1. Relationship between band-sharing index and unattributable bands in parent–offspring dyads. Each diamond represents a dyad with respect to percentage of bands shared between parent and offspring (summed over probes) and collective number of offspring bands not attributable to either social parent. The lines distinguish purely social parent–offspring dyads (lower right quadrant) from genetic parent–offspring dyads (upper two quadrants). (*a*) Social and genetic mother–offspring dyads (open diamonds) and social father–offspring dyads (closed diamonds). (*b*) Genetic sire–offspring dyads (open diamonds) and social father–offspring dyads (closed diamonds).

To determine parentage, we scored 25.6 ± 0.5 bands per individual with Jeffreys's probe 33.6 and 18.8 ± 0.3 bands per individual with Jeffreys's probe 33.15. Following the methods of Rabenold *et al*. (1990) and Westneat (1993), we assigned or excluded a male as the genetic sire of a nestling based on the band-sharing index (Wetton *et al*. 1987) and on the number of nestling bands that were unattributable to either of its putative parents. Using the lower 95% confidence interval of the band-sharing indices between young and their genetic parents (identified as those parent–offspring dyads producing fewer than two unattributable bands), we excluded any putative parent that had a band-sharing index of less than 0.37 with the nestling and, in combination with the other putative parent, produced more than two unattributable bands (figure 1*a*).

In cases in which we first determined that one parent (invariably the male) was not the genetic parent, we then produced additional gels that contained the DNA of the excluded offspring, the mother, the excluded father, and several neighbouring and non-neighbouring males. Using the same criteria as above, we scored the autoradiographs to determine whether a match could be made (figure 1*b*). On average, we ran three additional gels and screened 8–12 males per offspring before we found the genetic sire.

3. RESULTS

(*a*) *Annual apparent RS*

There was no detectable effect of treatment on the number of nests initiated, the number of successful nests, the numbers of eggs laid or hatched in the nests of T- and C-males (table 1). However, nests of C-males produced more nestlings that survived to the sixth day after hatching ($p = 0.03$), as well as significantly more young that survived to leave the nest ($p = 0.007$) (table 1).

(*b*) *Annual genetic RS losses and genetic success at home*

Nests of T-males contained 0.74 ± 0.20 EPF-produced fledglings, while nests of C-males contained significantly more: 1.4 ± 0.27; $t = 2.04$, $p = 0.04$ (figure 2). Because the apparent RS of C-males was greater by 1.17 fledglings but the losses to EPFs were also greater, the net result was that treatment had no detectable effect on home success: T-males raised 1.95 ± 0.22 genetically related fledglings and C-males raised 2.45 ± 0.27 related fledglings; $t = 1.42$, $p = 0.16$ (figure 2).

Table 1. *Measures of apparent reproductive success of control (C-males) and of males implanted with testosterone (T-males)*

(Initiated nests are those that were built by males' social partners, summed over the whole season. Successful nests are those that produced at least one fledgling. Eggs laid and eggs hatched are the season-long total numbers of eggs laid and eggs that survived to hatch, produced by the males' social partners. Nestlings are young that survived to the sixth day after hatching, and fledglings are young that survived to leave the nest, 11–12 days after hatching. An asterisk indicates differences that are statistically significant after sequential Dunn–Šidák adjustments for multiple comparisons, with an experiment-wide error rate of 0.05 (Sokal & Rolf 1995). All p values are two-tailed.)

	T-males mean ± s.e. (*n*)	*C-males* mean ± s.e. (*n*)	
initiated nests	2.2 ± 0.16 (57)	2.0 ± 0.13 (67)	$t = 0.72$, 122 d.f., $p = 0.47$
successful nests	1.0 ± 0.09 (57)	1.17+0.08 (67)	$t = 1.55$, 122 d.f., $p = 0.13$
eggs laid	7.6 ± 0.51 (50)	7.4 ± 0.41 (64)	$t = 0.18$, 112 d.f., $p = 0.86$
eggs hatched	4.6 ± 0.35 (52)	5.5 ± 0.36 (65)	$t = 1.73$, 115 d.f., $p = 0.08$
nestlings	3.4 ± 0.33 (58)	4.5 ± 0.32 (67)	$t = 2.26$, 123 d.f., $p = 0.03$
fledglings	2.7 ± 0.27 (58)	3.9 ± 0.32 (67)	$t = 2.73$, 123 d.f., $p = 0.07$*

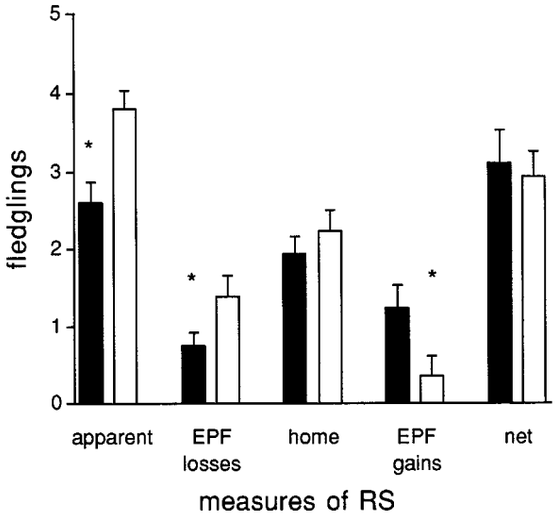

Figure 2. Five measures of reproductive success (RS; means ± 1 s.e.): apparent success, EPF losses, home success (apparent minus EPF losses), EPF gains, and net genetic success, measured as the number of fledglings produced over the breeding season by 58 T-males (black bars) and 67 C-males (white bars). Asterisks above histogram bars indicate statistical significance as determined using the Dunn–Šidák sequential correction for multiple comparisons (Sokal & Rohlf 1995) with an experiment-wide error rate set at 0.1, based on *a priori* predictions and two families of comparison: (1) apparent success, EPF losses, and home success and (2) EPF gains and net success.

(*c*) *EPF gains and net annual RS*

Treatment had an effect on the probability of gaining RS from EPFs. T-males sired significantly more fledglings by EPFs (1.21 ± 0.29) than C-males (0.36 ± 0.12, $t = -2.66$, $p = 0.009$; figure 2). However, when these gains in RS were added to home success, there was no detectable difference in net annual genetic RS between T-males (3.19 ± 0.43) and C-males (2.94 ± 0.33; $t = -0.47$, $p = 0.64$; figure 2).

4. DISCUSSION

As predicted, male juncos treated with testosterone had lower apparent annual output of fledglings than did control males. The difference was probably attributable to predation and/or to starvation of nestlings, as the numbers of nests built, eggs laid, and eggs hatched did not differ with treatment (table 1). This apparent disadvantage to T-males is misleading, however, because genetic RS of T-males at home was statistically indistinguishable from that of C-males. This, in turn, was because C-males lost twice as many potential offspring to EPFs as did T-males. There are several possible explanations for this result. One is that T-males may be more effective than C-males at mate-guarding their fertile social partners. Although Chandler *et al.* (1997) found no detectable effect of treatment on measures of male proximity to their female mates when these females were fertile, increased song production by T-males may have deterred intrusions by potential extra-pair sires (Yasukawa 1981).

A second possible explanation is that the social mates of T-males may have been less inclined than the mates of C-males to participate in extra-pair copulations (EPCs). While we have no data to support this possibility, data from other species have shown that females mated to males of a preferred phenotype (e.g. a particular leg-band colour or tail length) are less likely to participate in EPCs than are females mated to males with a less preferred phenotype (Burley *et al.* 1996; Saino *et al.* 1997).

A third possible explanation is that, compared to C-males, T-males may copulate more frequently with their fertile female partners. Sperm reserves of free-ranging T-males are significantly lower than sperm reserves of C-males during their respective females' fertile stage (Kast 1996). Yet, when T- and C-males were held captive and not allowed access to females, there was no detectable effect of treatment on sperm volume or sperm density (Kast 1996). Together, these results suggest that T-males may use their sperm reserves at a higher rate, i.e. by copulating more frequently and thus, compared to C-males, fertilizing a larger proportion of their partner's eggs.

T-males' larger home ranges (Chandler *et al.* 1994) could explain why T-males obtained more EPFs than control males. That is, their more extensive movements may allow T-males to encounter more fertile females and/or to keep better track of the nesting status of neighbouring females.

The greater attractiveness of T-males to females may also have contributed to their greater success at obtaining EPFs. In choice experiments, females prefer T-males to C-males, perhaps because T-males are more active and sing more songs (Enstrom *et al.* 1997). Female preference for males with higher song rates has been demonstrated both for males as social partners (Gottlander 1987) and as EPF partners (Hasselquist *et al.* 1996).

The net result of the various gains and losses in RS was that there was no detectable difference in net season-long genetic RS of T- and C-males. However, it is notable that they obtained their RS via different pathways. T-males obtained a greater proportion of their RS by EPFs, while C-males obtained comparatively more RS by providing parental care to young in their own nests. This is an example of a physiologically mediated trade-off between parental care and mating effort in which alternative experimentally induced phenotypes resulted in equal pay-offs. Further, our findings mirror the interspecific differences in allocation of reproductive effort that characterize socially monogamous and polygynous male birds. Monogamists attract one social mate and obtain RS primarily by providing parental care to related young in their home nests, while polygynists attract more than one social mate and provide comparatively less parental care.

Although a relationship between T and the expression of sexually selected characters that influence mate choice has been well established (Ligon *et al.* 1990; Zuk *et al.* 1995), our study appears to be the first experimental manipulation of T that has shown an effect on RS as the result of altering the number of EPFs, both gained and lost. Further, the phenotypic traits that we

manipulated were not the sexually selected characters typically studied (e.g. bill or plumage colour, tail length). Rather, we manipulated a single trait (T-level), which then had cascading effects on male behaviour, such as elevating song rate and territory size, and which in turn appears to have affected male attractiveness to both social and extra-pair mates.

Clearly there is need for more studies that are designed to explain the often large individual variation in RS caused by EPFs (Gibbs *et al*. 1990; Whittingham & Lifjeld 1995). Our data suggest that further investigation of the role of testosterone will be useful in this regard.

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