The Auk 115(3):684–693, 1998

VARIATION IN EJACULATE QUALITY IN DARK-EYED JUNCOS ACCORDING TO SEASON, STAGE OF REPRODUCTION, AND TESTOSTERONE TREATMENT

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ABSTRACT.—To assess natural variation in ejaculate quality of male Dark-eyed Juncos (*Junco hyemalis*), as well as to measure any effect of experimental treatment with testosterone (T), we used cloacal massage to collect sperm samples from captive (1993) and free-ranging (1994, 1995) populations. We made three measurements of ejaculate quality in males, approximately half of which had been treated with T: (1) volume, (2) sperm concentration, and (3) total number of sperm per ejaculate (ejaculate size). Ejaculate volume and concentration varied by year, but ejaculate size did not; therefore, we used ejaculate size as our primary measure of ejaculate quality. Control (C-males) and hormone-treated (T-males) males from the captive population did not differ in any measure of ejaculate quality, but in the free-ranging population, C-males produced larger ejaculates than T-males. Independent of treatment, ejaculate size varied significantly with season and stage of reproduction in the free-ranging population. C-males had significantly fewer sperm at the beginning of the breeding season than in midseason and thereafter, but reserves in T-males did not differ significantly with season. For males in both treatments, ejaculates were smallest when their mates were fertile and increased significantly when their mates were incubating and when the pair was feeding nestlings. We then asked whether the observed patterns were more likely attributable to differences in rate of sperm production or rate of sperm utilization (i.e. copulation frequency). The finding that free-ranging T-males had fewer sperm than C-males, whereas captives did not, suggests that treatment with T may have led to differences in utilization (i.e. by copulation). The observation that in both T- and C-males ejaculates were smallest when their mates were fertile also suggests that frequent copulation depletes sperm reserves. *Received 3 April 1997, accepted 19 December 1997.*

THE MALES OF MANY SOCIALLY MONOGAMOUS BIRDS adopt a mixed reproductive strategy and engage in both parental care and extrapair copulations (EPCs; Westneat et al. 1990, Birkhead and Møller 1992). Because a consequence of EPCs is a mixing of male ejaculates in female reproductive tracts, sperm competition among males may be an important selective force in these apparently monogamous mating systems (Birkhead 1987, Birkhead and Møller 1992). Males may benefit from guarding their mates; however, when guarding is ineffective, selection should favor males that dilute a competitor's sperm through frequent copulations and inseminations by larger numbers of sperm (Birkhead et al. 1987, Møller 1988).

In passerines, sperm available for copulation is stored in the seminal glomera, or sperm reserves, of the cloacal protuberance (Salt 1954, Wolfson 1954, Birkhead 1991, Birkhead et al. 1994). The number of sperm in the glomera at any one time is a function of three variables: the glomera's total capacity, the rate of sperm production, and the rate of sperm utilization, or copulation (Birkhead 1991, Birkhead et al. 1994, Birkhead et al. 1995). When full to capacity, the glomera are thought to contain multiple ejaculates. The number of sperm per ejaculate (ejaculate size) decreases with frequency of successive copulations until eventually the sperm are depleted (Ansah et al. 1984, Birkhead 1991, Birkhead et al. 1994). Although the rate at which sperm are replenished probably varies among bird species, maximum ejaculate size in Zebra Finches (*Taeniopygia guttata*) is not fully restored for up to 24 h after the glomera have been depleted (Birkhead 1991). Variation in any of the variables affecting ejaculate size could greatly influence reproductive success among males.

Testosterone (T) is essential to male reproductive behavior and physiology. It stimulates

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the production and maturation of sperm (Jones and Lin 1993, Maddocks and Setchell 1993), regulates the hypothalamic-pituitary-gonadal axis (Silver and Ball 1989, Maddocks and Setchell 1993), and mediates the expression of reproductive behaviors such as copulation (Beach and Inman 1965, Balthazart et al. 1985, Watson and Adkins-Regan 1989, Balthazart and Ball 1993) as well as the expression of secondary sexual characteristics (Witschi 1961, Sturkie and Opel 1976, Owens and Short 1995). Individuals that lack an endogenous source of T (e.g. because they are hypophysectomized or castrated) or that have low plasma levels of T (e.g. naturally low or depressed with hormones) do not produce sperm (Kumaran and Turner 1949) and do not exhibit copulatory behavior (Schumacher and Balthazart 1983, Balthazart et al. 1985). However, spermatogenesis and copulatory behavior can be restored if exogenous T is administered (Kumaran and Turner 1949, Schumacher and Balthazart 1983). Further, T can suppress sperm production through negative feedback (Ludwig 1950, Sturkie and Opel 1976). Testosterone's involvement in sperm production and copulatory behavior prompts the question whether T influences the number of sperm stored in the glomera and, thus, ejaculate size.

Testosterone implants have been used to manipulate plasma T levels in investigations of the evolution of mating systems (Watson and Parr 1981, Wingfield 1984, Ketterson and Nolan 1992, Ketterson et al. 1996) and of breeding behaviors such as parental care (Silverin 1980, Hegner and Wingfield 1987, Ketterson et al. 1992, Saino and Møller 1995), song (Silverin 1980, Ketterson et al. 1992), territorial aggression (Silverin 1980, Watson and Parr 1981, Wingfield 1984), and use of space (Chandler et al. 1994, 1997). We used T implants to investigate the physiological and behavioral mechanisms influencing variation in ejaculate quality.

Our objectives were to assess natural variation in ejaculate quality in Dark-eyed Juncos (*Junco hyemalis*) and to determine whether experimentally elevated T influenced ejaculate quality. The Dark-eyed Junco is an appropriate species for such a study because much is known about the interactions among T, behavior, and correlates of fitness (Ketterson et al. 1991, 1992, 1996, Nolan et al. 1991, Ketterson and Nolan 1992). Furthermore, extrapair fertilizations (EPFs) have been documented in this species, and their frequency is known to be affected by whether a male is treated with testosterone (Ketterson and Nolan 1992, Ketterson et al. 1996, Raouf et al. 1997). Thus, sperm competition occurs in juncos, suggesting that it may play a selective role in the processes influencing sperm production, ejaculate size, and rate of copulation, and that variation in ejaculate quality may affect reproductive success.

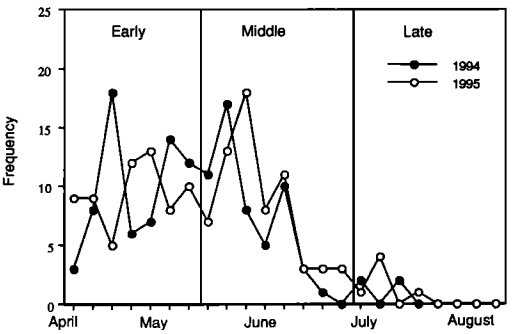

FIG. 1. Frequency of clutch initiation (laying egg 1 in nest) by Dark-eyed Juncos according to date during the 1994 and 1995 breeding seasons (laying dates combined over 5-day intervals). Breeding season is divided into early (20 April to 26 May), middle (27 May to 2 July), and late (3 July to 9 August) periods.

METHODS

Study site and species.—We studied captive juncos from April to July 1993 and free-ranging juncos from April to August 1994 and May to July 1995 at Mountain Lake Biological Station, Virginia (37°22'N, 80°32'W). See Wolf (1987), Wolf et al. (1988), and Chandler et al. (1994) for descriptions of the study site. Juncos have been color banded at this site since 1983. Juncos are socially monogamous, and males defend breeding territories. Males and many females enter breeding condition in late March or April and occupy their previous year's home range. Females build the nest and incubate the eggs, and both sexes feed and defend the nestlings and fledglings. Nesting usually begins in early May, and all females tend to begin nesting at the same time early in the season (Fig. 1). As the season progresses, however, nest predation forces many females to renest, and nesting becomes asynchronous at the level of the population. We identified breeding pairs and determined the locations of territories, checked territories every one or two days for nesting activity, and monitored active nests about every two or three days until the young fledged.

Sampling sperm.—We collected sperm samples in 5-

μL microcapillary tubes (Volupette pipette, Baxter Scientific Products) using cloacal manipulation, i.e. simultaneously massaging the cloacal protuberance and uropygial gland (Gee and Temple 1978, Tuttle et al. 1996). Although most samples were obtained within 1 to 2 min, we standardized all massage and collection times to 3 min. We estimated the volume of ejaculate by comparing contents of the collection tube with a calibrated tube. We diluted the sperm sample using Minnesota Turkey Semen Extender (Ogasawara and Ernst 1970), a pH-buffered solution that maintains sperm viability, and thoroughly mixed the dilution by flushing it in and out of the tip of a micropipetter. We used a standard Improved Neubauer hemacytometer to determine the concentration of sperm in the ejaculate, taking two readings of each sample and averaging the readings for analyses. We counted the total number of cells instead of the number of live (i.e. motile) cells, because we could not be certain of the effectiveness of the semen extender in maintaining the viability of junco sperm. We calculated ejaculate size (i.e. total number of sperm) by multiplying the volume of the ejaculate by its concentration. All sperm samples were collected and counted by T.K.

We refer to the sperm samples we obtained as "ejaculates." We do not know whether these ejaculates are larger or smaller than the natural ejaculate size delivered by juncos during copulation, nor do we know how they relate in size to the total number of sperm stored in the glomera. We assumed that the physiological response to the fixed amount of stimulation we provided by cloacal massage was the same across treatments and throughout the season and stages of reproduction. Thus, we assumed that variation in ejaculate size reflected variation in total number of sperm stored in the glomera.

Captive males.—In 1993 we captured male juncos early in the breeding season (17 to 18 April) and assigned them to one of two treatment groups: control (C-males) or testosterone (T-males). C-males ($n = 8$) were implanted with two empty 10-mm Silastic tubes (1.473 mm inside diameter, 1.956 mm outside diameter) sealed with Silastic glue, and T-males ($n = 10$) received two 10-mm Silastic tubes filled with crystalline testosterone (Sigma Chemical; Ketterson et al. 1992). Throughout the breeding season, this dose maintains plasma T at levels higher than the levels of C-males, but within the physiological range that is normal in unmanipulated males during territory establishment and pair formation (Ketterson et al. 1991, Ketterson and Nolan 1992, Chandler et al. 1997). Males were implanted from 21 to 23 April; implants were inserted subcutaneously along the left or right flank. Males were housed in individual cages (0.6 × 1.2 × 2.4 m) in an outdoor aviary and were fed seed, meal worms, and water *ad libitum*. We collected sperm samples during the morning hours (0800 and 1200 EST) over the period 6 June to 8 July 1993. Each male was sampled only once.

Free-ranging males.—We caught males early in the season (15 April to 11 May 1994 and 14 April to 20 May 1995) when they were establishing territories and acquiring mates. Individuals were taken to the laboratory, assigned to a treatment group, implanted, and immediately released onto their territories. In 1995, males that had been studied in 1994 were assigned the same treatment that they had been randomly assigned in 1994. In each year, males new to the study site were separated according to age (one year old and older than one year) and capture location, then randomly assigned to a treatment. When individuals were recaptured later in the field season for collection of sperm (see below), we checked to verify that their implants were still in place. Males with territories on the study site that were not caught during the time that implanting took place received no implants. Sperm of such males, when they were caught later, was sampled only in 1994; these cases comprised <15% of the 1994 samples and <11% of the combined 1994 and 1995 samples. These males did not differ significantly from C-males (i.e. males with empty implants) in any measure of ejaculate quality (total number of sperm: $t = 0.39$, df = 24, $P = 0.70$; concentration: $t = 0.62$, df = 24, $P = 0.54$; volume of ejaculate: $t = 0.25$, df = 24, $P = 0.81$), so the two groups were pooled for analyses.

We divided the nesting cycle into three stages of reproduction based on when an individual male's mate was: (1) fertile, (2) incubating, or (3) feeding nestlings. We defined the fertile stage as the 10 days before the laying of the last egg in the clutch (Birkhead and Møller 1992). In juncos, this period includes nest building and egg laying; 10 days is also the minimum observed amount of time necessary for a female to begin building a nest after her previous nest has failed (Wolf et al. 1991, Ketterson et al. 1992). Nine of the 14 males sampled during the fertile stage were caught when there were either one or two eggs in the nest. The other five were caught one or two days before the laying of the first egg. Because female juncos generally begin to incubate on the night before their last egg is laid, we defined the incubation stage as the period from the laying of the last egg to the hatching of the first egg, inclusive; this interval averages 12 days in juncos. Males captured during the incubation stage were caught between day 6 and day 10 of incubation. The nestling stage also lasts approximately 12 days, at the end of which time the young fledge. All males caught during the nestling stage were captured when nestlings were between 6 and 11 days old (hatching day = day 0).

We caught all individuals in the morning (0600 to 1200). Because males were present at the nest more frequently during the nestling stage, we could intercept them as they approached to feed young. However, the location of males was less predictable dur-

ing the fertile and incubation stages, and to catch them we used playback of song near a caged male placed beside a mist net.

Data analysis.—For all analyses we used JMP (SAS Institute 1988) and SYSTAT (Wilkinson 1992). Data were square-root transformed to meet assumptions of normality before any parametric tests were used, and all tests were two-tailed. When transformation did not improve normality, we used nonparametric tests.

Three measures of ejaculate quality were compared across treatment groups within the captive and the free-ranging populations: volume of ejaculate, concentration of sperm, and total number of sperm. We determined whether males from the 1994 and 1995 free-ranging populations differed significantly between years in any measure of ejaculate quality. With treatment groups combined, we found that 1995 males had significantly fewer sperm per microliter of ejaculate ($\bar{x} = 0.53 \times 10^6 \pm$ SE of 0.05×10^6) than 1994 males ($\bar{x} = 0.82 \times 10^6 \pm 0.06 \times 10^6$). That is, ejaculates in 1995 were less concentrated ($t = 2.77$, df = 64, $P < 0.01$). However, 1995 samples had significantly higher volumes (1994 males: $\bar{x} = 1.61 \pm 0.15$ μL; 1995 males: $\bar{x} = 2.47 \pm 0.29$ μL; $t = 2.91$, df = 64, $P < 0.01$). Consequently, samples from the two years did not differ in ejaculate size (total number of sperm per ejaculate for 1994 males: $\bar{x} = 1.46 \times 10^6 \pm 0.20 \times 10^6$; 1995 males: $\bar{x} = 1.35 \times 10^6 \pm 0.23 \times 10^6$; $t = 0.02$, df = 64, $P = 0.99$). Therefore, we pooled the years for subsequent analyses of ejaculate size and drew inferences about ejaculate size and the effect of season, stage of reproduction, and treatment based only on this variable.

We quantified the effect of T on sperm number both with respect to advance of season (defined below) and to stage of reproduction (defined above). We included in the stage analyses only males whose stage of reproduction we knew, whereas analyses by advance of season included all males sampled, whether or not we knew their stage of reproduction. For the season-advance analyses, we counted the days between first and last sampling dates (both years combined), inclusive, and divided that number into three equal periods: early (20 April to 26 May), middle (27 May to 2 July) and late (3 July to 9 August). Only a small number of males was captured in all periods of the season (five C-males, two T-males) or all stages of reproduction (three C-males, one T-male), so we were unable to perform repeated-measures analyses. Instead, we performed one two-way ANOVA of hormone treatment/season and one of hormone treatment/stage of reproduction. For these analyses in cases in which males had been sampled in more than one period of the season or stage of reproduction, we chose one sample at random to represent each male in each ANOVA in order to avoid pseudoreplication.

Some of the 1994 males did not produce an ejaculate upon manipulation (33% of 120, all sampling events); in 1995 all ($n = 22$) males delivered an ejaculate. The absence of sperm in the cloacal protuberance probably indicates that the male is not producing sperm (i.e. "true" infertility), or that he has depleted his reserves through copulation (i.e. "temporary" infertility). The former explanation seems unlikely, because true infertility is considered rare in birds (see Birkhead and Møller 1992). It is also possible that birds that did not produce sperm when sampled simply did not respond to massage. The conditions under which all males were sampled were the same, but there may have been individual differences in responsiveness to massage. Thus, the reason for empty reserves is uncertain.

To assess variation in ejaculate size with and without the effect of instances of sampling that produced no sperm, we proceeded as follows. First, we defined the sample as all males that produced sperm on at least one of the occasions that they were sampled. We thus excluded any occasions in which individuals had empty reserves. Next, from among the occasions on which sperm were produced, we randomly selected one occasion to represent each individual male for which we had more than one sample (season: 37 C-males, 29 T-males; stage: 32 C-males, 21 T-males). We then performed both two-way ANOVAs (i.e. hormone treatment by stage and by season) on the remaining samples. Second, we defined the sample as all males whether or not they produced sperm on at least one occasion. This procedure included a larger number of males than the one used to produce the first sample and also required reconsideration of males belonging to the first sample, because it was now appropriate to consider occasions when males did not produce sperm (season: 43 C-males, 37 T-males; stage: 35 C-males, 27 T-males). Again to avoid pseudoreplication, we repeated the procedure of choosing a single occasion at random to represent each male in the second sample. The effect of this resampling was that a male might enter the analysis of the first sample as a sperm producer during a particular period of the season or stage of reproduction, and enter the analysis of the second sample as a nonproducer during a different period of the season or stage of reproduction. For the second sample, which included many "zero" ejaculates, assumptions of normality were not met and were uncorrectable by transformation. Therefore, we used Kruskal-Wallis one-way ANOVA and when appropriate performed post-hoc multiple comparisons (Siegel and Castellan 1988, Sokal and Rohlf 1996).

Results

Captive Birds

Captive C-males and T-males did not differ in any measure of ejaculate quality. The aver-

age volume of ejaculate obtained from each treatment group was very similar (C-males: $\bar{x}$ = 0.75 ± SE of 0.18 μL; T-males: $\bar{x}$ = 0.78 ± 0.20 μL; t = −0.05, df = 16, P = 0.96). T-males appeared to have somewhat less concentrated ejaculates ($\bar{x}$ = 0.44 × 10^6 ± 0.09 × 10^6 sperm/μL) than did C-males ($\bar{x}$ = 0.71 × 10^6 ± 0.14 × 10^6 sperm/μL), although the difference was not statistically significant (t = 1.62, df = 16, P = 0.13). Also, C-males ($\bar{x}$ = 0.53 × 10^6 ± 0.17 × 10^6) and T-males ($\bar{x}$ = 0.39 × 10^6 ± 0.16 × 10^6) did not differ in total ejaculate size (t = 0.80, df = 16, P = 0.44).

FREE-RANGING BIRDS

Volume and concentration of ejaculate.—Ejaculate volumes of C-males and T-males in 1994 were significantly different. C-males yielded a mean volume of 1.91 ± 0.19 μL of ejaculate, and T-males produced volumes that were 38% smaller ($\bar{x}$ = 1.18 ± 0.20 μL; t = 2.56, df = 42, P = 0.014). Treatment groups did not differ in concentration of sperm (C-males: $\bar{x}$ = 0.85 × 10^6 ± 0.09 × 10^6 sperm/μL; T-males: $\bar{x}$ = 0.78 × 10^6 ± 0.10 × 10^6 sperm/μL; t = 0.61, df = 42, P = 0.55).

In 1995, the two treatments did not differ significantly in volume of ejaculate (C-males: $\bar{x}$ = 2.85 ± 0.44 μL; T-males: $\bar{x}$ = 2.10 ± 0.35 μL; t = 1.43, df = 20, P = 0.17) or in concentration of ejaculate (C-males: $\bar{x}$ = 0.55 × 10^6 ± 0.08 × 10^6 sperm/μL; T-males: $\bar{x}$ = 0.51 × 10^6 ± 0.07 × 10^6 sperm/μL; t = 0.40, df = 20, P = 0.69).

Ejaculate size and advance of season.—Estimated ejaculate sizes for the three periods of the season are shown in Figure 2. When the analyses included only the males that had sperm in their reserves on at least one occasion, ejaculate sizes of both treatment groups were small during the early period (Fig. 2A). Ejaculate sizes for C-males increased in the middle period and remained large in the late period. Ejaculates in T-males remained small throughout the season. Independent of advance of season, T-males had smaller ejaculates than C-males (F = 4.19, df = 1 and 65, P = 0.04). Independent of treatment, advance of season did not have a significant effect on ejaculate size (F = 1.39, df = 2 and 65; P = 0.26), nor was there a significant interaction between treatment and advance of season (F = 1.81, df = 2 and 65, P = 0.17).

When males with empty reserves (i.e. no ejaculate) were included in the analyses, both treatment groups again had fewer sperm during the early than during the later periods of the breeding season (Fig. 2B). Ejaculate size for C-males changed significantly over the season (H = 7.66, df = 2, P = 0.02), and post-hoc tests revealed significantly smaller ejaculates during the early than the middle period (P < 0.05). Season did not have a significant effect on ejaculate size in T-males, and ejaculates remained of similar size throughout the season (H = 2.41, df = 2, P = 0.30).

When treatments were combined, 8 of the 22 (36%) males sampled early in the breeding season had empty reserves, and there was a trend for fewer (n = 6, 14%) to have empty reserves in the middle of the season (Fisher's exact test, P = 0.06). The proportion of individuals with empty reserves did not differ significantly between the middle and the end (n = 4, 25%) of the season (Fisher's exact test, P = 0.44). Within each treatment group, some individuals had

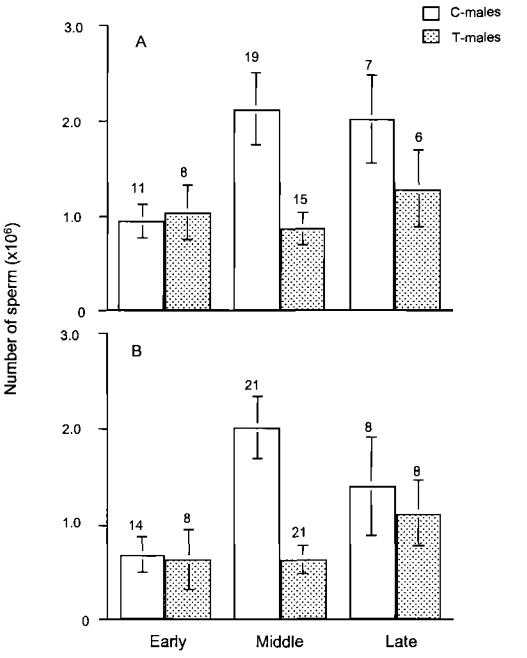

FIG. 2. Seasonal profile of ejaculate size (total number of sperm) in control (C-males) and testosterone-implanted (T-males) male Dark-eyed Juncos. Values are $\bar{x}$ ± SE, with sample sizes displayed above each bar. Season is divided into three periods (see Fig. 1). (A) includes only males that produced an ejaculate during sampling, and (B) includes all males regardless of whether they produced an ejaculate.

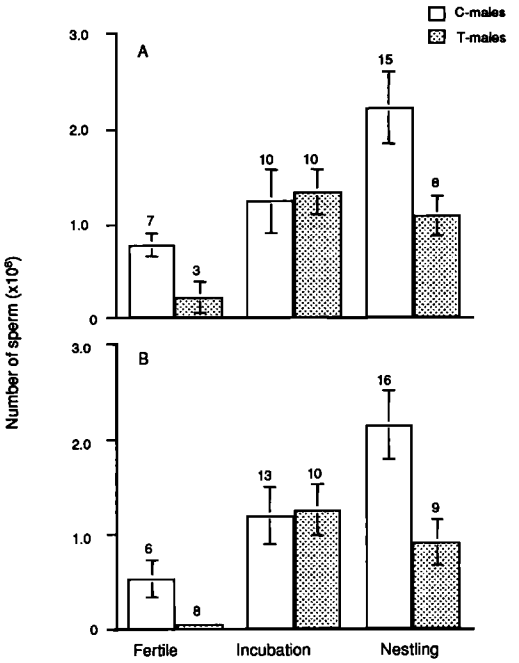

FIG. 3. Ejaculate size (total number of sperm) in control (C-males) and testosterone-implanted (T-males) male Dark-eyed Juncos at three stages of the nesting cycle. Values are $\bar{x} \pm$ SE, with sample sizes displayed above each bar. (A) includes only males that produced an ejaculate during sampling, and (B) includes all males regardless of whether they produced an ejaculate.

empty reserves early in the breeding season (C-males: $n = 5$, 36%; T-males: $n = 3$, 38%), and the treatments did not differ (Fisher's exact test, $P = 1.00$). However, in the middle of the breeding season, six T-males (29%) sampled, but no C-males, had empty reserves (Fisher's exact test, $P = 0.021$). At the end of the season, we found no difference between treatments in the proportion of empty reserves (C-males: $n = 3$, 13%; T-males: $n = 1$, 38%; Fisher's exact test, $P = 0.57$).

Ejaculate size and stage of reproduction.—Ejaculate sizes of C-males and T-males were smallest in the fertile stage (Fig. 3). For males that produced an ejaculate (Fig. 3A), stage of reproduction had a significant effect on ejaculate size independent of the effect of treatment ($F = 5.21$, df = 2 and 52, $P < 0.01$). There was a strong trend for treatment to affect size of ejaculates ($F = 3.73$, df = 1 and 52, $P = 0.06$), but the interaction of stage and treatment was not significant ($F = 1.96$, df = 2 and 52, $P = 0.152$).

The pattern did not change when males with empty reserves were included in the analysis (Fig. 3B). Ejaculate size of C-males increased in a stepwise manner as the nesting cycle progressed: ejaculates were smallest in the fertile stage, larger in the incubation stage, and larger still in the nestling stage ($H = 8.71$, df = 2, $P = 0.013$). Multiple comparison tests showed that C-males had significantly smaller ejaculates in the fertile stage than in both the incubation and nestling stages ($P < 0.05$). In T-males, the effect of stage on ejaculate size also was significant ($H = 11.79$, df = 2, $P = 0.003$); ejaculates were significantly smaller in the fertile stage than in the incubation and nestling stages (ejaculates in the incubation and nestling stages were 20 times larger than those in the fertile stage).

Independent of the effect of treatment, the proportion of individuals with empty reserves was higher in the fertile stage ($n = 8$, 57%) than in the incubation stage ($n = 3$, 13%; Fisher's exact test, $P = 0.008$), but we found no significant difference between the incubation and nestling stages ($n = 1$, 4%; Fisher's exact test, $P = 0.338$). When we considered treatment, a smaller proportion of C-males ($n = 2$, 33%) than T-males ($n = 6$, 75%) had empty reserves during the fertile stage, but this difference between treatment groups was not significant (Fisher's exact test, $P = 0.28$). There were also no significant differences in the proportions of males with empty reserves between treatment groups for the incubation stage (C-males: $n = 2$, 15%; T-males: $n = 1$, 10%; Fisher's exact test, $P = 1.00$) and the nestling stage (C-males: $n = 0$; T-males, $n = 1$, 11%; Fisher's exact test, $P = 0.36$).

DISCUSSION

Ejaculate size varied temporally in male Dark-eyed Juncos. Free-ranging C-males had significantly smaller ejaculates early in the breeding season than later. They also had significantly fewer sperm when their mates were fertile than when their mates were incubating or feeding nestlings.

Free-ranging T-males also showed temporal variation in sperm numbers, but the pattern differed from that of C-males. Numbers of sperm in T-males were low throughout the breeding season and increased slightly, but not significantly, at the end of the season. Similar to C-males, T-males had significantly fewer

sperm during the female's fertile period than during the incubation and nestling stages.

Comparison of C- and T-males.—The number of sperm in the sperm reserves is a function of their capacity, the rate of sperm production, and the rate of utilization (Birkhead 1991; Birkhead et al. 1994, 1995). If, as we have assumed, the amount of sperm in the glomera affects ejaculate size, then observations that ejaculate size was smaller in T- than in C-males could mean that treatment with T suppressed sperm production. However, we believe that this is unlikely for three reasons. First, although T can retard gonadal growth and inhibit spermatogenesis when administered during the early stages of gonadal recrudescence (Burger 1944, Pfeiffer 1947, Lofts 1962), we implanted males during territory establishment, when plasma T levels are already high (Ketterson and Nolan 1992, Chandler et al. 1997) and when T has no known effect on spermatogenesis (see Lofts and Murton 1973). Second, T- and C-males in the captive study had their T-levels elevated for an extended period of time (mid-April to June), yet they did not differ significantly in sperm concentration or ejaculate size, and these birds were alike in that they had no opportunity to copulate. Third, in a separate study of juncos, free-ranging T-males suffered fewer losses of paternity as a result of EPFs of their social mates than did C-males, and T-males were more successful than C-males in fertilizing females that were not their social mates (Raouf et al. 1997). None of these findings suggests that sperm production is suppressed in T-males.

A more likely explanation for the smaller ejaculates of free-ranging T-males during certain stages of the nesting cycle is variation in copulation frequency. In both treatment groups, ejaculate sizes were smallest in the fertile stage, a time when males presumably copulate frequently with their social mates and possibly deplete their sperm reserves. Ejaculates in C-males were larger in the incubation stage and significantly larger during the nestling stage. This fact is consistent with the view that C-males copulate less frequently when their females are incubating than when they are building and laying, and least frequently when the pair is feeding nestlings. Ejaculates in T-males also increased in size from the fertile to the incubation stage, but with no further increase during the nestling stage. This suggests that T-males copulate with equal frequency during the incubation and nestling stages.

The seasonal patterns of ejaculate sizes also indicate that copulation rate may be the more important consideration in explaining treatment-related variation in sperm reserves. At the beginning of the season when nesting among females is synchronous and the proportion of fertile females is therefore high (Westneat et al. 1990; see Fig.1), ejaculate sizes were small in both treatments. These small ejaculates are compatible with the view that males of both treatments copulated most frequently during this period. Later (middle and late periods), when nesting is relatively asynchronous, mean ejaculate sizes increased in C-males, consistent with the view that some of these males (those whose females were incubating eggs or feeding nestlings) copulated less frequently than at the beginning of the season. In contrast, the ejaculates of T-males remained small throughout most of the season (although with a tendency to increase toward the end), which suggests that these males copulated as frequently in the middle of the season as they did earlier, i.e. so long as fertile females were in the population (Fig. 1; note the late-season decline in nest starts, i.e. in females available for insemination).

Mechanistic implications.—If, as we have suggested, T influences copulation behavior in male Dark-eyed Juncos, this could result from neurophysiological responses to the presence of more T in the regions of the brain thought to mediate male copulatory behavior (Balthazart and Ball 1993). If this is so, it would imply that natural endogenous T levels, such as those characteristic of C-males (and unmanipulated males), are sufficient to support copulation, but elevated plasma levels may increase its frequency.

We are aware of no work demonstrating an effect of T on copulation rate in birds. However, a correlation exists between copulation frequency and extent of development of morphological characters known to be affected by T. In selecting for mating frequency in Japanese Quail (*Coturnix c. japonica*), Sefton and Siegel (1975) and Cunningham and Siegel (1978) found a positive correlation between copulation frequency and size of the cloacal gland; individuals from "high" mating lines (more frequent copulations) had larger cloacal glands

than those from "low" mating lines. Because growth and maintenance of the cloacal gland require T (Adkins 1977, Adkins and Pniewski 1978, Massa et al. 1980, Deviche and Schumacher 1982, Schumacher and Balthazart 1983), it is plausible (but unknown) that individuals from the high-frequency mating lines had higher levels of plasma T than those from low-frequency mating lines.

Alternatively, the proposed increase in copulation rates could be related to T indirectly if increased levels of T result in more opportunities for copulation. Chandler et al. (1994) placed radio transmitters on male juncos and found that home ranges of T-males were three times larger than those of C-males. In addition, T-males ranged up to twice as far from their nests, on average, than did C-males (Chandler et al. 1994). The use of larger areas may give T-males more opportunities to encounter fertile females (Chandler et al. 1994).

Evolutionary implications.—Male juncos do not incubate, but they make approximately half of the pair's trips to the nest to feed nestlings (Wolf et al. 1988, Ketterson et al. 1992). Because little or no male parental effort is required during the incubation stage, at that time males might be expected to increase mating effort by seeking EPCs (Westneat et al. 1990). If ejaculate size provides a measure of mating effort (i.e. small ejaculate indicates higher frequency of copulation), the stepwise increase in ejaculate size of C-males as they pass through the breeding cycle suggests that they copulate most often when their females are fertile, less during the incubation period when social mates are infertile but males are free to pursue EPCs, and least when feeding and male parental effort is therefore highest. T-males, however, apparently deviate from this pattern. Like C-males, T-males probably pursue EPCs when their mates are incubating; but their relatively low sperm counts during the nestling stage suggest that they allocate more effort than C-males to seeking mating opportunities at that time. In support of this argument, T-males feed nestlings less frequently than C-males (Ketterson et al. 1992, Chandler et al. 1994), and they respond less rapidly to simulated disturbances by predators at the nest (Cawthorn et al. 1998).

Theory suggests that sperm competition should favor large ejaculates (Parker 1970) and rapid replenishment of sperm reserves (Trivers 1972). Data, however, reveal that frequent copulation can lead to small ejaculates or sperm depletion. Because replenishment is not immediate, the effectiveness of males when they engage in subsequent copulations may be compromised (Birkhead 1991, Birkhead and Møller 1992, Birkhead et al. 1994, 1995). Our data suggest that frequent copulation depletes sperm reserves in Dark-eyed Juncos, particularly in those treated with T. Are T-males at a disadvantage in sperm competition?

The recent study by Raouf et al. (1997) suggests that they are not. Compared with mates of C-males, the social mates of T-males lay fewer, not more eggs sired by extrapair males. Furthermore, T-males are more successful than C-males at siring extrapair young in the nests of other males. Despite these findings, the question arises as to whether elevated T and frequent copulation sometimes might cause males to become less-reliable sources of sperm. We conclude that much remains to be learned about the interaction of hormones and behavior in sperm competition.

Acknowledgments

We are grateful to J. Mikesell Zaebst, E. Snajdr, M. Soenkson, and C. Ziegenfus for their expert field assistance. Our special thanks to D. Enstrom for letting us sample ejaculates from his captive birds and to E. Tuttle for sharing her expertise. We also thank D. Sengelaub, B. Sinervo, R. Titus, S. Raouf, L. Klukowski, T. Birkhead, and an anonymous reviewer for comments on earlier versions of the manuscript. We are grateful to the University of Virgina's Mountain Lake Biological Station and to the Mountain Lake Hotel for allowing us to work on their property. This research was supported by the Center for the Integrative Study of Animal Behavior (Indiana University), the Mountain Lake Biological Station, and National Science Foundation grants (BSR-9111498 and IBN-9408061) to E.K. and V.N.

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Associate Editor: M. E. Murphy