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RESEARCH ARTICLE

Condition- and context-dependent factors are related to courtship behavior of paired and unpaired males in a socially monogamous songbird

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ABSTRACT

Males of many animal species display to attract and stimulate potential mates. In socially monogamous species, males will court females both to establish a pair bond and to solicit extrapair copulations. We investigated whether paired and unpaired male Dark-eyed Juncos (*Junco hyemalis*) differ in their courtship of a novel female and whether their courtship behavior is related to their morphology or circulating hormones. We conducted simulated courtship interactions (SCI) by presenting free-living paired and unpaired males with a live, caged conspecific female accompanied by playback of a precopulatory trill, a signal of female receptivity, and tested predictions of multiple hypotheses. We quantified courtship behaviors for 20 min and then captured the males, measured them, and collected blood samples to quantify circulating post-SCI testosterone and corticosterone levels as well as restraint-induced corticosterone. Paired males approached the female more rapidly and spent more time in close proximity to the female than unpaired males. Paired males were also more active and spent more time with body feathers fully erect, but sang fewer songs, compared to unpaired males. Unpaired males were smaller in mass than paired males and had higher post-SCI corticosterone and restraint-induced corticosterone than paired males, but the groups did not differ in post-SCI testosterone. We discuss whether these findings should be interpreted as differences in how males court when seeking a social mate vs. an extrapair mate or as inherent differences between successful and unsuccessful males.

Keywords: corticosterone, Dark-eyed Junco, eavesdropping avoidance, extrapair courtship, sexual selection, signal, testosterone

Los factores dependientes de la condición y del contexto se relacionan con el comportamiento de cortejo de machos con y sin pareja en un ave canora socialmente monógama

RESUMEN

Los machos de muchas especies de animales realizan despliegues para atraer y estimular a sus potenciales parejas. En las especies socialmente monógamas, los machos cortejarán a las hembras para establecer un vínculo de pareja y para obtener copulaciones extra-pareja. Aquí investigamos si los machos con y sin pareja de *Junco hyemalis* difieren en el cortejo de una nueva hembra y si sus comportamientos de cortejo se relacionan con sus morfologías o sus hormonas circulantes. Realizamos simulaciones de interacciones de cortejo (SIC) exponiendo machos libres con y sin pareja a una hembra viva de la misma especie ubicada en una jaula, reproduciendo al mismo tiempo el sonido del trino precopulatorio, una señal de receptividad de la hembra, y evaluamos las predicciones de múltiples hipótesis. Los comportamientos de cortejo fueron cuantificados durante 20 minutos, y luego capturamos a los machos, los medimos y colectamos muestras de sangre para medir los niveles circulantes de testosterona post-SCI y de corticoesterona, así como de corticoesterona inducida por restricción. Los machos en pareja se acercaron a la hembra más rápidamente y pasaron más tiempo en proximidad de la hembra que los machos sin pareja. Los machos con pareja también fueron más activos y pasaron más tiempo con las plumas del cuerpo totalmente erectas, pero cantaron menos canciones que los machos sin pareja. Los machos sin pareja fueron más pequeños que los machos con pareja y tuvieron más corticoesterona post-SCI y corticoesterona inducida por restricción que los machos con pareja, pero no difirieron en la testosterona post-SCI. Discutimos si estos hallazgos deberían ser interpretados como diferencias en cómo los machos cortejan cuando están buscando una pareja social versus un compañero extra-pareja, o como diferencias inherentes entre machos exitosos y no exitosos.

Palabras clave: cortejo extra-pareja, corticoesterona, evasión de espionaje, *Junco hyemalis*, selección sexual, señal, testosterona

INTRODUCTION

Understanding the sources of variation in reproductive success is central to the study of evolution (Darwin 1859, 1871). To that end, a large body of research on sexual selection and mate choice has been focused on identifying the ornaments and courtship behaviors that distinguish individuals that pair or reproduce successfully from those that do not (Andersson 1994, Andersson and Simmons 2006). The factors underlying these differences in reproductive success are complex because both male courtship signaling and female mating decisions are affected by a variety of intrinsic and extrinsic factors (Cotton et al. 2004, 2006).

It is well established that male phenotypic traits and courtship behaviors can be condition-dependent (Andersson 1994, Jennions et al. 2001), and consequently, males that are in better condition court more effectively than males that are in poor condition. Condition-dependent hypotheses, such as the handicap principle (Zahavi 1975, 1977), are particularly appealing because they provide a clear mechanism ensuring the signal's honesty (Grafen 1990, Searcy and Nowicki 2005; but see Számadó 2011). Alternatively, male courtship behavior may also differ as a result of extrinsic factors such as the social environment in which the courtship event occurs (e.g., the presence or absence of eavesdroppers; McGregor 2005), the quality of the female being courted (Jones et al. 2001, Lihoreau et al. 2008, Heinig et al. 2014), and whether the male is courting an extrapair mate or social mate (Davis 2002).

In socially monogamous species, males defend territories from rival males and court females throughout the breeding season in a variety of contexts. When a paired male encounters a novel female, any courtship behavior that follows is, by definition, extrapair courtship. The behavior of an unpaired male toward a female is more difficult to classify. Novel females that appear on the territories of unpaired males will likely be courted as potential social mates. However, if the female is familiar and known to be mated to a neighbor, unpaired males may exhibit extrapair courtship. Whether courtship behavior differs between these 2 types of males and mating contexts remains poorly understood (but see Davis 2002).

In addition to possible effects of paired vs. unpaired status on courtship behavior, condition-dependent factors may affect courtship and may or may not be confounded with pairing status. Another complication is that the costs of courting behavior may be greater for paired males if the behavior is detected by his social mate, which can result in the dissolution of the pair bond and a loss of fitness (Cezilly and Nager 1995, Lazarus et al. 2004). This potential cost of courtship by paired males is likely absent in mating systems that lack long-term pair bonds. Finally, because females in a number of species are known to

prefer different male traits when selecting a social mate as compared to an extrapair copulatory partner (Regan et al. 2000, Foerster et al. 2003, Mays and Hill 2004), males may modify their courtship signaling according to whether they are courting a potential long-term, social mate or a short-term, extrapair mate, in order to align their signaling tactics with the female preferences for the social context.

Observational data that directly compare the courtship of paired and unpaired males represent an essential first step for developing testable hypotheses to disentangle how these concurrent selective pressures drive variability in male courtship behavior and fitness. In the present study, we developed a behavioral assay to quantify the courtship behavior of free-living male songbirds, which we refer to as a "simulated courtship interaction" (SCI; after Balsby and Dabelsteen 2005). We conducted our SCIs with a socially monogamous species, the Dark-eyed Junco (*Junco hyemalis*), in which both paired and unpaired males defend territories and court females throughout the breeding season (Nolan et al. 2002). Male Dark-eyed Juncos perform a variety of easily quantifiable courtship behaviors when presented with a female conspecific. Acoustically, males produce 2 distinct types of song: long-range song and short-range song, the latter functioning predominantly in courtship (Titus 1998, Reichard et al. 2011, 2013). Visually, males will change perches, erect their body feathers (ptilorection), and spread their tails to reveal white outer tail feathers that are sexually selected in this species (Enstrom et al. 1997, Hill et al. 1999).

Combining our SCI protocol with the Dark-eyed Junco study system allowed us to investigate the relationship between mating status and courtship behavior in order to observe whether any differences were consistent with 3 hypotheses, not mutually exclusive, to explain male courtship. Under the "condition-dependent hypothesis," we predicted that paired and unpaired males would differ in size, condition, and circulating hormone levels, and that those aspects of male phenotype would correlate with courtship behavior (Andersson 1994). Specifically, we predicted that paired males would be larger, be in better condition, have higher levels of circulating testosterone, and have lower levels of circulating corticosterone.

Under the "eavesdropping avoidance hypothesis" (*sensu* Dabelsteen 2005), we predicted that both paired and unpaired males would produce fewer long-range songs, which transmit long distances, and more short-range songs and visual signals, which transmit short distances, to avoid attracting the attention of eavesdroppers. With respect to paired males, we predicted that a male whose mate was present during the SCI would exhibit reduced courtship behavior compared to males whose mates were absent.

Under the "mate type hypothesis," we predicted that unpaired males would invest more heavily in courtship

behaviors that are important for attracting social mates, whereas paired males would invest more heavily in behaviors important in stimulating extrapair mates. Our understanding of female preferences for social and extrapair mates is limited, which restricts our ability to make specific, well-supported predictions for this hypothesis. Previous research in Dark-eyed Juncos has shown that unpaired females in a y-maze choice test prefer males that are more active, sing more short-range song, engage in more tail-spreading displays, and have greater amounts of white on their outer tail feathers (Enstrom et al. 1997, Hill et al. 1999). These results may be indicative of the traits preferred by females when selecting social mates and represent our predictions for the courtship behavior of unpaired males. Additionally, females in our study population choose males with larger body mass and longer wings as extrapair sires (Gerlach 2010), which suggests that paired males courting extrapair mates should emphasize body size. Although male mass is a condition-dependent trait, males can vary investment in size signaling by adjusting their ptiloerection during courtship. As such, we predicted that paired males would spend more time with their body feathers fully erect.

METHODS

Study Site, Territory Mapping, and Mating Status

We conducted our experiment at Mountain Lake Biological Station and the adjacent grounds of Mountain Lake Hotel in Pembroke, Virginia, USA (37°22'N, 80°32'W). Dark-eyed Juncos (*J. h. carolinensis*) were captured via mist nets and Potter traps and banded with unique color combinations as part of a long-term study (see Reed et al. 2006). We located male territories by observing aggressive interactions, song perches, and nesting sites. Territory boundaries were determined by moving a conspecific song playback until the male stopped responding or was chased by a competitor. We determined male mating status by monitoring nesting attempts of all breeding pairs. Unpaired males were easily identifiable because they sing significantly more long-range song than paired males (Nolan et al. 2002).

Captive Females and Playback Stimuli

We captured 5 females near our study site and housed them together in an outdoor aviary to serve as stimuli in our behavioral trials (see below). Each day, we selected a stimulus female in a random, stratified order to ensure that all females were sampled equally. Additionally, we created playback tapes of a female precopulatory trill, a vocal signal of receptivity produced by females when soliciting copulations. To create our playbacks, we had access to only one high-quality female trill recording, but trills are stereotyped and vary minimally among females (Reichard

et al. 2013). Our trill recording included deviations in frequency (<800 Hz) among its repeated notes that allowed us to artificially create distinct trills to limit pseudoreplication.

We generated a spectrogram (digitized at 44.1 kHz, 32 bits, WAV format) using Adobe Audition 1.5 (Adobe Systems, San Jose, CA, USA) and extracted consecutive, 3-note segments from the trill. Each segment was repeated consecutively to create 5 novel trills that were identical in length and trill rate to the original recording. We then resampled each recording such that the duration and frequency bandwidth was either decreased to 95% or increased to 105% of the original value to create a total of 15 playback tapes (5 original, 5 compressed to 95%, and 5 expanded to 105%). All tapes were processed using a high-pass equalizer to remove background noise below 5.5 kHz and were standardized to 90% of the peak amplitude. Given our sample size (39 subjects), each trial playback tape was used at least twice, and 9 tapes were used 3 times.

Simulated Courtship Interactions

Between May 22 and July 19, 2012, we conducted SCIs consisting of a live female conspecific accompanied by playback of a female precopulatory trill on the territories of 27 paired and 12 unpaired, free-living male Dark-eyed Juncos. We conducted trials between 0600 and 1200 hours EST in a stratified order, such that neighboring males were not sampled on the same day and both paired and unpaired males were sampled throughout the breeding season to control for seasonal effects. Both groups included individuals in their first breeding season (12 paired and 4 unpaired) and individuals in their second breeding season or later (15 paired and 8 unpaired).

To prepare each SCI, we placed a covered, caged female on the focal male's territory. An amplified speaker (Pignose 7-100) covered in camouflage cloth was placed next to the cage and connected to an Apple iPod Nano. A shotgun microphone (Audio-Technica AT835b) connected to a Marantz digital recorder (model PMD660) was mounted 1–2 m away from the cage to record vocalizations. A mist net was set up and furled nearby for capturing the male after the SCI.

To initiate each SCI, we revealed the female conspecific and began a precopulatory trill playback. All subjects received the same initial trill playback, which consisted of one trill every 10 s at 90–95 dB SPL. Amplitude was measured at 1 m from the speaker with a Radio Shack sound level meter (model 33-2055; C-weighting) at another location before the trial. After the male approached within 10 m of the female, we switched to one of the 15 artificial-trill playback tapes (see above) and reduced the amplitude to 70–75 dB SPL to mimic the natural amplitude of a female trill (D. G. Reichard personal observation). These playbacks consisted of one trill every

15 s for the first minute, one trill every 30 s for the next 9 min, and one trill every minute for the remaining 10 min. We chose to conduct 20 min trials because males vary substantially in the persistence of their response to an SCI (<1 min to >30 min; Reichard et al. 2011). Additionally, the trial playback was designed to intensely stimulate the male initially while limiting habituation later in the trial.

During the SCI, 2 observers sat 10–15 m away, dictating the behaviors of the focal male into a lapel microphone. One observer noted all distance-based behaviors, including movements >0.25 m (referred to as “perch changes”), time spent within 5 m and 1 m of the female, and the closest approach to the female. The other observer noted all vocal and visual courtship behaviors. Long-range songs, which are discrete trills, were counted individually. The duration spent singing short-range song, which is sung continuously (Titus 1998), was noted during the trial and later confirmed using recordings from the shotgun microphone. The male’s ptiloerection (puffing of body feathers) was recorded on an ordinal scale (0 = no feather erection, 1 = feathers noticeably elevated, 2 = feathers fully erect), and a similar scale was followed for the male’s tail spreading (0 = no spread, 1 = slight spread with some tail white visible, 2 = fully spread). We calculated the total time the male spent in each level of visual display. We also noted the presence or absence of the focal male’s mate when applicable. The behavior of the caged female was not directly quantified; however, females never solicited copulations and rarely vocalized in response to the male (D. G. Reichard personal observation).

Bird Capture, Hormone Sampling, and Measurements

After each SCI, we opened the mist net, moved the caged female near the middle of the net, and attempted to capture the focal male (time from SCI initiation to capture: mean \pm SE = 2,034 \pm 110.0 s; range: 1,390–3,695 s). We alternated our capture playback between the female trill, long-range songs, and short-range songs to overcome the male’s habituation to the trial stimulus. Playback of these vocalizations has no detectable effect on testosterone or corticosterone levels in Dark-eyed Juncos (Rosvall et al. 2012b, 2014). After capture ($n = 30$, including 21 paired and 9 unpaired), we rapidly collected a blood sample in <3 min (time from capture to completed blood sample: mean \pm SE = 166 \pm 5.1 s) to limit the effect of handling on corticosterone levels. Then the male was placed in a bag to simulate restraint-induced stress, and a second blood sample was taken at 15 min post-capture to measure the stress reactivity of each male ($n = 29$, including 20 paired and 9 unpaired).

A.A.K. measured each male’s body mass, tarsus length, flattened wing chord, and tail length and estimated the percentage of white on the outer tail rectrices following an established protocol (see Reed et al. 2006). Male condition

was assessed by calculating the residuals of a regression between mass and tarsus length. We categorized males as first-year or older on the basis of plumage or previous banding history (Nolan et al. 2002). Males that were not captured following the SCI ($n = 9$) were captured at a later date for morphological measurements.

Hormone Assays

To determine circulating levels of plasma testosterone, we used an enzyme immunoassay kit (Enzo Life Sciences, no. 901-065; assay sensitivity = 5.67 pg mL⁻¹) that was previously optimized for use in Dark-eyed Juncos (see Clotfelter et al. 2004). Both control and post-SCI samples were randomized and spread evenly by treatment and date across 2 plates from the same kit lot. Intra-plate variability was 0.7% and 19.2% (mean = 9.9%), and inter-plate variability was 9.6%.

Similarly, we measured circulating levels of plasma corticosterone with an enzyme immunoassay kit (Cayman no. 500655; assay sensitivity = 30 pg mL⁻¹) that also was previously optimized for use in Dark-eyed Juncos (see Rosvall et al. 2012b). Samples were run on 3 plates from the same kit lot. We again randomized all samples and spread the samples evenly across plates according to treatment and date. Repeated stress samples from the same individuals were always run on the same plate. Intra-plate variability was 13.6%, 17.8%, and 12.4% (mean = 14.6%), and inter-plate variability was 10.4%.

Statistics

We compared all behavioral responses, morphological measures, and other phenotypic measures of paired and unpaired males using separate independent-samples *t*-tests. All phenotypic measures were related to male behavior using *t*-tests (categorical variables) or Pearson’s correlations (continuous variables). We tested for an effect of the identity of the female stimulus and of Julian date on male behavior using one-way analyses of variance and Pearson’s correlations, respectively. We also tested for an effect of the presence of a male’s mate on the behavior of paired males using *t*-tests. Four of the behavioral measures, including the latency to approach within 1 m and within 5 m, the closest approach, and time spent with the tail fully spread (tail spread 2), required a square-root transformation to achieve normality.

We used general linear models to test for a difference between paired and unpaired males in plasma testosterone (post-SCI) and corticosterone levels (post-SCI and after 15 min of restraint) because sampling procedure often influences hormone measures. Julian date and length of time from initiation of the SCI to capture were included as covariates, and handling time between capture and completion of the blood sample was also a covariate in the post-SCI corticosterone model. All hormone data were

TABLE 1. Means (\pm SE), effect sizes, and 95% confidence intervals (CI) for behavioral responses of paired and unpaired male Dark-eyed Juncos during a simulated courtship interaction at Mountain Lake Biological Station, Pembroke, Virginia, USA. *P* values were calculated using an independent-samples *t*-test. Bold indicates that confidence intervals do not overlap zero.

Behavior	Paired	Unpaired	<i>d</i> ^a	95% CI	<i>P</i>
Latency to 1 m (s)	378.81 \pm 100.25	797.75 \pm 146.11	0.794	0.091 to 1.496	0.028
Latency to 5 m (s)	84.78 \pm 41.88	372 \pm 128.42	0.727	0.038 to 1.426	0.054
Closest approach (m)	1.00 \pm 0.25	2.88 \pm 1.13	0.557	-0.134 to 1.248	0.131
Time within 1 m (s)	233.19 \pm 53.92	34.08 \pm 26.34	-1.155	-1.881 to -0.428	0.002
Time within 5 m (s)	459.56 \pm 61.99	199.5 \pm 58.07	-0.892	-1.601 to -0.184	0.014
Perch changes (count)	24.48 \pm 3.96	12.92 \pm 2.39	-0.868	-1.574 to -0.161	0.017
Long-range song (count)	38.89 \pm 6.88	72.58 \pm 13.23	0.864	0.157 to 1.570	0.017
Short-range song ^b (s)	294.15 \pm 57.84	176.92 \pm 86.95	-0.383	-1.068 to 0.303	0.178
Tail spread 1 (s)	217.15 \pm 38.78	261.83 \pm 76.59	0.198	-0.483 to 0.880	0.572
Tail spread 2 (s)	70.07 \pm 22.98	95.58 \pm 50.09	0.185	-0.496 to 0.867	0.723
Feather erection 1 (s)	259.52 \pm 49.90	334.58 \pm 92.20	0.294	-0.389 to 0.977	0.402
Feather erection 2 (s)	225.59 \pm 39.75	84.75 \pm 34.15	-0.933	-1.643 to -0.222	0.011

^a Small, medium, and large effect sizes correspond to $|d| \approx 0.2$, $|d| \approx 0.5$, $|d| \approx 0.8$, respectively (Cohen 1988).

^b There was a significant effect of Julian date ($r = 0.380$, 95% CI: 0.07–0.62, $P = 0.017$) on short-range song data. These data result from a general linear model controlling for date.

normally distributed except for the post-SCI corticosterone data, which required a log transformation to achieve normality. Each hormone measure was also related to male behavior using Pearson's correlations.

All statistics were calculated with IBM SPSS Statistics 20.0 (IBM, Armonk, New York, USA). Given that our study is predominantly descriptive, we avoided multivariate or data-reductive approaches (e.g., principal component analysis) when possible, in order to facilitate a simpler, more transparent interpretation of our data. We also did not apply a Bonferroni correction or other, similar procedures that would reduce our statistical power substantially and increase the likelihood of Type II errors to unacceptable levels (Nakagawa 2004). Instead, we focus on effect sizes and 95% confidence intervals (CIs) for the majority of our comparisons (measured as Cohen's *d*) and correlations between male behavior and phenotype (measured as Pearson's *r*; Nakagawa 2004, Nakagawa and Cuthill 2007). We used the following guidelines for assessing effect size: small effect, $d \approx 0.20$ or $r \approx 0.10$; medium effect, $d \approx 0.5$ or $r \approx 0.30$; and large effect, $d \approx 0.80$ or $r \approx 0.50$ (Cohen 1988, Møller and Jennions 2002). In cases where the CIs do not overlap zero, the comparison or relationship can be considered significant (Nakagawa and Cuthill 2007); however, we also report *P* values for the sake of comparison.

RESULTS

Mating Status and Courtship Behavior

Paired and unpaired males differed significantly in 7 of the 12 behaviors quantified, and each of those differences was of either a medium (2 of 7) or a large (5 of 7) effect size (Table 1). Paired males approached to within 5 m and 1 m

of the female more rapidly than unpaired males. They also approached more closely to the female, but this difference was not statistically significant despite a medium effect size. Paired males spent more time within 5 m and 1 m of the female than unpaired males and were more active, with more perch changes. Unpaired males sang more long-range songs than paired males, but there was not a significant difference between the 2 groups in the amount of short-range song produced. With respect to visual displays, there were no significant differences in the amount of time spent with the tail spread partially or fully, or in the time spent with body feathers partially erect. However, paired males spent more time with their body feathers fully erect than unpaired males.

The presence of a male's social mate (mate present, $n = 13$; mate absent, $n = 14$) did not have any significant effects on the courtship behavior of paired males. All observed effect sizes were small, with 2 exceptions. Paired males whose mates were present took longer to approach within 5 m of the stimulus female (medium effect size: $d = 0.51$, 95% CI: -0.26 to 1.28, $P = 0.20$) and performed more perch changes (medium effect size: $d = 0.74$, 95% CI: -0.04 to 1.52, $P = 0.07$).

Julian date was positively related to the production of short-range song ($r = 0.38$, 95% CI: 0.073 to 0.621, $P = 0.02$), but we did not detect any other significant relationships with other behaviors ($P > 0.05$ in all comparisons). To control for the effect of Julian date in our short-range song comparison, we conducted a separate general linear model with Julian date as a covariate, but short-range song still did not differ significantly between paired and unpaired males (Table 1). The behavior of the female stimulus bird was not directly quantified during the trial; however, there were no significant effects of the

TABLE 2. Means (\pm SE), effect sizes, and 95% confidence intervals (CI) for phenotypic traits of paired and unpaired male Dark-eyed Juncos that experienced a simulated courtship interaction at Mountain Lake Biological Station, Pembroke, Virginia, USA. *P* values were calculated using an independent-samples *t*-test. Bold indicates that confidence intervals do not overlap zero.

Phenotypic measure	Paired	Unpaired	<i>d</i> ^a	95% CI	<i>P</i>
Mass–tarsus residual	0.027 \pm 0.07	−0.060 \pm 0.11	−0.237	−0.919 to 0.121	0.498
Mass (g)	21.49 \pm 0.19	20.68 \pm 0.32	−0.775	−1.477 to −0.074	0.032
Tarsus (mm)	21.79 \pm 0.07	21.63 \pm 0.12	−0.429	−1.116 to 0.258	0.224
Tail white (sum of %)	2.36 \pm 0.06	2.28 \pm 0.10	−0.264	−0.947 to 0.419	0.451
Tail length (mm)	70.76 \pm 0.36	71.46 \pm 0.57	0.365	−0.320 to 1.050	0.299
Wing length (mm)	82.09 \pm 0.36	82.25 \pm 0.45	0.089	−0.591 to 0.770	0.799

^a Small, medium, and large effect sizes correspond to $|d| \approx 0.2$, $|d| \approx 0.5$, and $|d| \approx 0.8$, respectively (Cohen 1988).

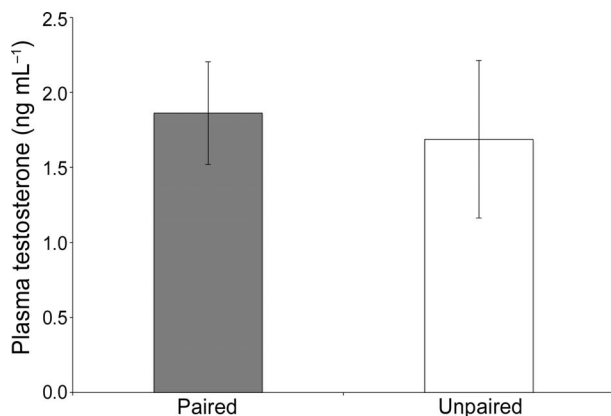
identity of the caged, female stimulus on any of the behaviors measured ($P > 0.05$ in all comparisons).

Mating Status and Male Phenotype

Paired and unpaired males did not differ significantly in their tarsus, tail, or wing lengths, and there was not a significant difference between the 2 groups in the percentage of white on the outer tail feathers (Table 2). However, paired males were significantly larger in mass than unpaired males, and this difference was of a medium effect size (Table 2). With respect to body condition, there was not a significant difference between paired and unpaired males (Table 2).

Mating Status and Hormones

Paired and unpaired males did not differ significantly in their post-SCI plasma testosterone levels ($F_{1,26} = 0.077$, $P = 0.783$; Figure 1). Time to capture had no significant effect on post-SCI testosterone ($F_{1,26} = 1.242$, $P = 0.275$), and Julian date trended toward a seasonal decline in post-SCI testosterone levels ($F_{1,26} = 3.652$, $P = 0.067$). By contrast,

**FIGURE 1.** Estimated marginal mean (\pm SE) plasma testosterone levels of paired ($n = 21$) and unpaired ($n = 9$) male Dark-eyed Juncos after simulated courtship interaction at Mountain Lake Biological Station, Pembroke, Virginia, USA. Testosterone levels did not differ detectably between paired and unpaired males ($P = 0.783$).

post-SCI plasma corticosterone levels were significantly higher in unpaired males than in paired males ($F_{1,25} = 5.037$, $P = 0.034$; Figure 2A). There was not a significant effect of time to capture ($F_{1,25} = 0.119$, $P = 0.733$), handling time between capture and the completion of the blood sample ($F_{1,25} = 1.217$, $P = 0.280$), or Julian date ($F_{1,25} = 0.567$, $P = 0.458$) on post-SCI corticosterone.

Similarly, the increase in plasma corticosterone concentration after 15 min of restraint-induced stress was significantly greater in unpaired males than in paired males (Figure 2B; $F_{1,25} = 6.293$, $P = 0.019$). Both time to capture ($F_{1,25} = 5.837$; $P = 0.023$) and Julian date ($F_{1,25} = 7.555$, $P = 0.011$) had a significant effect on the change in corticosterone. Time to capture was positively related to restraint-induced corticosterone (medium effect size: $r = 0.333$, 95% CI: -0.03 to 0.62 , $P = 0.077$), such that males with longer times to capture responded with a larger increase in plasma corticosterone after restraint-induced stress. Julian date was weakly negatively related to restraint-induced corticosterone (small effect size: $r = -0.276$, 95% CI: -0.58 to 0.10 , $P = 0.147$), which is consistent with a seasonal decline in the magnitude of response.

Correlates of Courtship Behavior

Age, male phenotype, and condition. The majority of the courtship behaviors measured were not related to male age ($P > 0.20$ in all cases). In one exception, males in their first breeding season spent significantly more time within 5 m of the female than males in their second breeding season or later (medium effect size: $d = 0.659$, 95% CI: 0.005 to 1.31 , $P = 0.05$). Male courtship behavior was also largely unrelated to male phenotype and condition. Mass, wing length, tail length, and amount of tail white were only weakly related to courtship behavior ($r < 0.20$, $P > 0.05$ in all cases). Male condition (medium effect size: $r = 0.40$, 95% CI: 0.10 to 0.75 , $P = 0.01$) and male tarsus length (medium effect size: $r = 0.37$, 95% CI: 0.06 to 0.71 , $P = 0.02$) were positively related to the amount of time spent with tail feathers fully spread. However, none of the other courtship behaviors was significantly related to condition or tarsus length ($r < 0.20$, $P > 0.05$ in all cases).

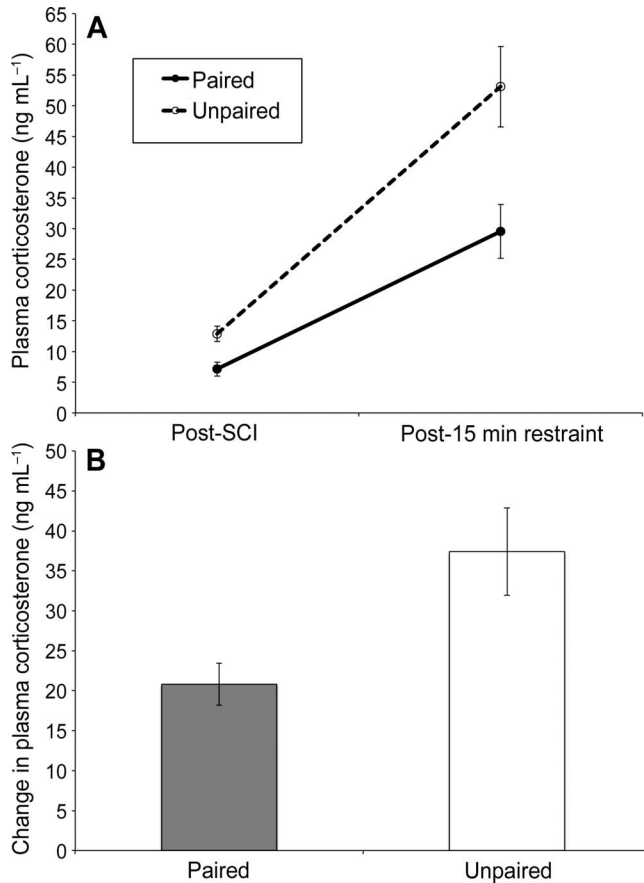


FIGURE 2. (A) Back-transformed estimated marginal mean (\pm SE) plasma corticosterone levels after simulated courtship interaction (SCI), and estimated marginal mean (\pm SE) stress corticosterone levels after 15 min restraint, in paired and unpaired male Dark-eyed Juncos at Mountain Lake Biological Station, Pembroke, Virginia, USA; unpaired males had significantly higher post-SCI plasma corticosterone ($P = 0.034$, $n = 21$ paired, $n = 9$ unpaired) and post-15 min restraint corticosterone ($P = 0.006$, $n = 20$ paired, $n = 9$ unpaired) than paired males. (B) Estimated marginal mean (\pm SE) changes in corticosterone in response to restraint stress; unpaired males experienced a significantly larger increase in plasma corticosterone after restraint stress than paired males ($P = 0.019$, $n = 20$ paired, $n = 9$ unpaired).

Hormone levels post-SCI or after 15 min of restraint.

Significant relationships of a medium or large effect size were rare between courtship behaviors and post-SCI hormone levels. Post-SCI testosterone was negatively related to latency to approach within 1 m of the female (medium effect size: $r = -0.40$, 95% CI: -0.66 to -0.04 , $P = 0.03$), time spent within 5 m of the female (medium effect size: $r = -0.38$, 95% CI: -0.65 to -0.02 , $P = 0.04$), and time spent with body feathers partially erect (medium effect size: $r = -0.32$, 95% CI: -0.61 to 0.05 , $P = 0.08$). Post-SCI corticosterone was negatively related to time spent within 1 m (medium effect size: $r = -0.31$, 95% CI: -0.61 to 0.05 , P

$= 0.09$) and 5 m (medium effect size: $r = -0.38$, 95% CI: -0.65 to -0.03 , $P = 0.04$) of the female. The change in corticosterone after restraint-induced stress was negatively related to time spent singing short-range song (medium effect size: $r = -0.35$, 95% CI: -0.64 to 0.02 , $P = 0.06$). All other behavior-hormone relationships were not significant and were of weak effect size ($r < 0.20$, $P > 0.05$ in all cases).

DISCUSSION

Paired and unpaired male Dark-eyed Juncos differed distinctly in their courtship of a novel female, and the factors underlying these differences appear to be complex. Paired males approached more rapidly and closely, were more active, and spent more time with their body feathers fully erect, whereas unpaired males sang more long-range songs. Phenotypically, unpaired males were smaller in body mass, with higher levels of circulating corticosterone, than paired males; but there were no detectable differences in body condition, ornamentation (amount of tail white), or circulating testosterone levels. We also found very few strong relationships between aspects of male phenotype and behavior. The observed differences in courtship behavior may provide insights into why certain males successfully paired and others did not, as well as whether these data are consistent with predictions of the condition-dependent, eavesdropping avoidance, and mate type hypotheses.

Condition-Dependent Hypothesis

Consistent with the predictions of the condition-dependent hypothesis, we observed differences in male mass and circulating corticosterone levels between paired and unpaired males. However, those phenotypic differences were not strongly related to male courtship behavior, with a few exceptions. Focusing first on male mass, we noted that unpaired males were significantly smaller than paired males, but we detected no relationship between mass and courtship behavior. Although this difference in mass cannot explain the observed behavioral differences, mass may be related to plasma corticosterone levels.

In many songbirds, unpaired males maintain lower-quality territories, owing both to their lesser resource-holding ability and the preferences of females for higher-quality territories (Howard 1974, Yasukawa 1981). Thus, unpaired males may be smaller in body mass as a result of resource limitations of their home territory. If this is accurate, poor territory quality may partially explain the failure of unpaired males to acquire a social mate. In addition, limited food availability can elevate plasma corticosterone levels (Smith et al. 1994, Lynn et al. 2010), which were higher in unpaired males immediately after the SCI and after 15 min of restraint-induced stress.

Elevated corticosterone is associated with declines in courtship behavior in Zebra Finches (*Taeniopygia guttata*; Lynn et al. 2010) and with a more general diversion of energetic resources away from physiological processes associated with reproduction in many species (Sapolsky et al. 2000, Groeneweg et al. 2011). Furthermore, elevated corticosterone has been associated with heightened neophobia in Dark-eyed Juncos and other taxa (Koolhaas et al. 1999, Atwell et al. 2012). In the present study, we observed a negative relationship between post-SCI corticosterone levels and time spent within 5 m and 1 m of the female, which is consistent with heightened neophobia. We also noted that males with a larger restraint-induced increase in corticosterone tended to sing less short-range song, which is consistent with a negative relationship between the stress phenotype and a courtship signal (Reichard et al. 2013).

With respect to circulating testosterone, we found that paired and unpaired males were statistically indistinguishable in their post-SCI level of testosterone. Testosterone is a known mediator of a large number of behavioral and physiological traits related to reproduction and fitness in Dark-eyed Juncos and other species (Ketterson et al. 1992, 2009, Ketterson and Nolan 1999, Adkins-Regan 2005), and males with experimentally elevated testosterone perform more elaborate courtship displays (Enstrom et al. 1997, Hill et al. 1999). However, males with higher testosterone in our study spent less time performing feather erections and less time within 5 m of the female, despite rapidly approaching to within 1 m of the female. We found no significant positive relationships between testosterone and the other courtship signals that we quantified. These results contrast with our initial predictions and suggest that natural variation in circulating testosterone may not be an important mediator of courtship behavior. It is still possible, however, that variation in testosterone plays a role in courtship behavior if males vary in their sensitivity to the hormone, but this possibility is outside the scope of our study (Rosvall et al. 2012a, Bergeon Burns et al. 2013).

Eavesdropping Avoidance Hypothesis

Animal communication occurs in networks of multiple signalers and receivers. Signals that project over long distances, such as loud songs, can attract the attention of both their intended receivers and unwanted eavesdroppers such as conspecific competitors and predators. As a consequence, signalers will benefit from signaling tactics that maximize signal transmission to intended receivers while simultaneously limiting transmission to costly eavesdroppers (Reichard and Anderson 2015). Here, we observed that courting males produced a variety of short-distance signals, including tail spreads, feather erections, and quiet, short-range songs, but unpaired males sang

significantly more loud, long-range songs than paired males.

In many songbirds, including Dark-eyed Juncos (Nolan et al. 2002), unpaired males will advertise broadly for potential mates by singing long-range songs more often than paired males (Spector 1991, Staicer et al. 2006). Producing a long-range signal may seem counterproductive from an eavesdropping perspective, but the greater long-range song production of unpaired males likely served a dual function, as both a deterrent to intrusions by eavesdropping rival males and a stimulatory signal to the female (Searcy and Nowicki 2006, Reichard and Anderson 2015). By contrast, the short-range song production of both paired and unpaired males was quiet and unlikely to project beyond the territorial boundary (Balsby et al. 2003), which limited the likelihood that an eavesdropper would hear this courtship signal (Reichard et al. 2013). Thus, by emphasizing the production of both long- and short-range songs, unpaired males may have been signaling in an optimal way by reducing the potential for intrusions from rival males while simultaneously stimulating the female.

In addition to rival males, paired males may also experience a cost from eavesdropping by their social mate (Cezilly and Nager 1995), which is a unique cost to mating systems that include long-term pair bonds. In the present study, paired males produced significantly fewer long-range songs than unpaired males and relied heavily on short-range acoustic and visual signals during the SCI, which is consistent with the predictions of the eavesdropping avoidance hypothesis. However, our evidence mostly suggests that males do not modify their courtship when their social mate is present. In 2 possible exceptions, males whose mates were present tended to take longer to approach and performed more perch changes. The longer approach latency may be interpreted as an initial avoidance of the SCI, but the likely cause of the increased perch changes was the behavior of each male's social mate, which often involved repeatedly chasing the male from the vicinity of the SCI while also behaving aggressively toward the caged female stimulus (D. G. Reichard personal observation). Future experiments should vary the costs imposed by eavesdroppers in order to fully elucidate the effects of unintended receivers on male courtship behavior.

Mate Type Hypothesis

The ability to modulate courtship behavior according to context and female preferences can have a direct effect on male reproductive success (Patricelli et al. 2002, O'Loughlen and Rothstein 2012). In socially monogamous species, a male's ability to differentiate between potential social and extrapair mates, and to adjust courtship accordingly, may be an important trait that separates males that sire both within-pair and extrapair offspring from those that fail to

sire any extrapair offspring. The mate type hypothesis predicts that males vary their courtship behavior according to whether they are courting a social or an extrapair mate. One limitation of this hypothesis is that it requires knowledge of female preferences for both social and extrapair mates, which can be difficult to acquire.

In our study population, larger males with whiter tails and an ability to elevate testosterone to a level slightly above the population's mean in response to a physiological challenge have the highest fitness as measured by total offspring produced (McGlothlin et al. 2005, 2010). These data include both within-pair and extrapair offspring, making it difficult to differentiate between preferred traits for social and extrapair mates. However, extrapair mates are significantly larger in body mass and wing length than males that fail to sire extrapair offspring (Gerlach 2010), which suggests that size may be important in extrapair mate choice. In lab studies, unpaired female Dark-eyed Juncos in a y-maze choice test preferred males that were more active, sang more short-range song, engaged in more tail-spreading displays, and had larger amounts of white on their outer tail feathers (Enstrom et al. 1997, Hill et al. 1999). Whether these results reflect female preferences for social or extrapair mates is difficult to interpret, given the absence of a second treatment testing the preferences of paired females. Using these data, we predicted that unpaired males should invest more in movement, short-range song, and tail spreading and that paired males should invest more in size signaling by maintaining a full ptiloerection to enhance the perception of body size.

Despite differing in a number of behaviors, paired and unpaired males did not differ significantly in short-range song production, tail spreading, or tail white. These results suggest that paired and unpaired males were emphasizing similar courtship signals that are preferred by female Dark-eyed Juncos when acquiring a social mate. However, it remains possible that paired and unpaired males may have differed in the quality of their short-range song. We also noted that paired males were more active and larger in body mass than unpaired males, which may have contributed to the ability of paired males to establish a pair bond at the beginning of the breeding season. With respect to extrapair courtship, paired males matched our prediction and spent more time with their body feathers fully erect than unpaired males, which suggests that they are investing more heavily in a trait preferred by females seeking extrapair mates.

Evidence from other species, particularly other songbirds, has indicated that paired females prefer extrapair mates that offer indirect benefits such as genetic dissimilarity to enhance offspring heterozygosity (reviewed in Mays and Hill 2004). Paired males in our study approached the novel female faster and spent

more time in close proximity than unpaired males, and close-proximity interactions are essential for the transmission of olfactory signals produced by volatile compounds present in avian preen oil (Soini et al. 2007). Olfactory signals have been widely recognized for their effectiveness as signals of genetic compatibility (Penn 2002). In our study population, males that are more successful in siring extrapair offspring have more "male-like" odor profiles than those that do not sire extrapair offspring (Whittaker et al. 2013). Thus, it seems plausible that olfactory signals play a role in extrapair mate choice in Dark-eyed Juncos, and paired males may approach novel females more closely than unpaired males in an effort to ensure that those olfactory signals are accurately transmitted.

Although we report some evidence suggesting that males may be modifying their behavior according to the courtship context, our data are only observational and must be interpreted with caution. In the future, a more powerful test of the mate type hypothesis would involve manipulating the pairing status of males to quantify their courtship behavior when both paired and unpaired. This experiment can be accomplished by removing female mates to create unpaired males, and by removing male mates to encourage females to pair again with a previously unpaired male. A clearer understanding of female preferences for both social and extrapair mates will also be essential to adequately assess whether males are signaling optimally between contexts.

Conclusions

In summary, we found evidence that paired and unpaired male Dark-eyed Juncos perform different courtship behaviors when presented with a novel female, but the factors underlying those behavioral differences remain unclear and warrant further study. We conclude that some of the behavioral differences are likely condition-dependent and may explain why paired males successfully attracted social mates and unpaired males did not. The courtship tactics of both paired and unpaired males were largely consistent with the predictions of the eavesdropping avoidance hypothesis, but paired males did not appear to decrease their courtship effort when their mate was present. In addition, these differences may also provide evidence that males are modifying their courtship according to whether they are pursuing a short-term or a long-term mate, but more research is needed, particularly on the mating preferences of paired females.

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Author contributions: D.G.R. conceived the idea. D.G.R., A.A.K., and J.F.W. designed the experiment and collected the data. D.G.R. analyzed the data. D.G.R., A.A.K., and E.D.K. wrote the manuscript. E.D.K. contributed substantial resources and funding.

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