Hormones and Behavior 62 (2012) 418–425

Contents lists available at SciVerse ScienceDirect

Hormones and Behavior

journal homepage: www.elsevier.com/locate/yhbeh

Robust behavioral effects of song playback in the absence of testosterone or corticosterone release

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ARTICLE INFO

Article history:
Received 24 April 2012
Revised 13 July 2012
Accepted 22 July 2012
Available online 28 July 2012

Keywords:
Testosterone
Playback
Corticosterone
HPA
HPG
Aggression
Androgen responsiveness
Challenge hypothesis
Simulated territorial intrusion
Low-amplitude song

ABSTRACT

Some species of songbirds elevate testosterone in response to territorial intrusions while others do not. The search for a general explanation for this interspecific variation in hormonal response to social challenges has been impeded by methodological differences among studies. We asked whether song playback alone is sufficient to bring about elevation in testosterone or corticosterone in the dark-eyed junco (*Junco hyemalis*), a species that has previously demonstrated significant testosterone elevation in response to a simulated territorial intrusion when song was accompanied by a live decoy. We studied two populations of juncos that differ in length of breeding season (6–8 vs. 14–16 weeks), and conducted playbacks of high amplitude, long-range song. In one population, we also played low amplitude, short-range song, a highly potent elicitor of aggression in juncos and many songbirds. We observed strong aggressive responses to both types of song, but no detectable elevation of plasma testosterone or corticosterone in either population. We also measured rise in corticosterone in response to handling post-playback, and found full capacity to elevate corticosterone but no effect of song class (long-range or short-range) on elevation. Collectively, our data suggest that males can mount an aggressive response to playback without a change in testosterone or corticosterone, despite the ability to alter these hormones during other types of social interactions. We discuss the observed decoupling of circulating hormones and aggression in relation to mechanisms of behavior and the cues that may activate the HPA and HPG axes.

Introduction

Hormones are key mediators of social behavior, in part because they provide a flexible link to allow appropriate responses to changes in the environment, including photoperiod, food, and the presence of conspecifics (Adkins-Regan, 2005). This interaction has bidirectional causality: hormones can facilitate the expression of behavior, and exhibiting a behavior can likewise affect the release of hormones. Among the best-studied hormone-behavior interactions in vertebrates is that between testosterone and aggressive behavior expressed during male-male social challenges in the breeding season. In some species, social challenges activate the hypothalamo–pituitary–gonadal (HPG) axis, leading to the release of testosterone (T) from the gonads and elevating circulating T levels above baseline breeding levels (Archer, 2006; Goymann, 2009; Wingfield et al., 1990). This acute elevation in T is thought to be beneficial during periods of social instability, e.g. by mobilizing energy reserves and shifting stress reactivity, immune function, or behavior (Muehlenbein and Bribiescas, 2005; Wingfield et al., 2001). The hormonal effects of social challenges are best characterized in birds, where, interestingly, a growing number of studies indicate that T does not always elevate in response to social challenges (reviewed in Goymann, 2009). This interspecific variability has been linked with various ecological factors, such as the relative importance of parental care and the length of the breeding season (Goymann, 2009; Landys et al., 2007; Lynn, 2008). In some species that do not elevate T in response to a social challenge, activation of the hypothalamo–pituitary–adrenal (HPA) axis has been observed instead, particularly in species that experience a short breeding season (Landys et al., 2007). These data suggest that socially-induced HPA axis activation may interfere with HPG axis signaling, or that glucocorticoids, such as corticosterone (CORT), mediate physiological and behavioral responses to social challenges in some species. While often thought of as a 'stress hormone', CORT induces a variety of organism-wide metabolic and behavioral effects that would be well suited to social challenges, e.g. increased activity, energy metabolism, or cardiovascular function, and the suppression of sickness behavior (Ashley et al., 2009; Breuner et al., 1998; Haller et al., 2008; Landys et al., 2004; Sapolsky et al., 2000).

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http://dx.doi.org/10.1016/j.yhbeh.2012.07.009

Methodological differences among studies have further complicated interpretations of interspecific variation in the hormonal effects of social challenge (Goymann, 2009). Studies have varied, for example, in the length of time males were exposed to simulated territorial intrusions (STI). While most studies report T or CORT elevation 10 to 30 min after the initiation of an STI (Landys et al., 2007; McGlothlin et al., 2008; Van Duyse et al., 2004; Wingfield and Wada, 1989), other species may require significantly more time to elevate hormone levels (Wikelski et al., 1999). In addition, the type of decoy (taxidermic or live) has varied from study to study and may affect hormone signaling (Scriba and Goymann, 2008, 2010), suggesting that different sensory modalities may influence the degree to which a social challenge leads to an elevation in T or CORT.

A key issue that is not completely resolved is whether song playback alone is sufficient to activate the HPA or HPG axes. Over twenty years ago, two studies suggested that vocal cues may be insufficient to elicit a robust T surge following an STI, though both studies were somewhat limited in interpretive power due to small sample sizes. Wingfield and Wada (1989) reported significant T elevation in free-living song sparrows (*Melospiza melodia*) in response to a live bird supplemented with song playback, whereas the presentation of either a devocalized male or song playback alone only trended toward T elevation. Captive male cowbirds (*Molothrus ater*) housed with either devocalized or normally vocalizing males had higher T than males exposed to silence or auditory playback alone (Dufty and Wingfield, 1990). A few recent studies report no relationship between the duration of playback exposure and the degree of T or CORT elevation (Busch et al., 2008; Deviche et al., 2006; Fokidis et al., 2011) or no effect of song playback on T (Deviche et al., 2012). Because of the high interspecific variation in the hormonal effects of social challenges (Goymann, 2009), however, failure to find an effect of playback alone on T or CORT can be difficult to interpret unless these null results are contrasted with data demonstrating social elevation of T does occur under other conditions. Thus, our study investigates the issue of whether song alone can lead to T or CORT elevation in a species in which past work has shown a rise in T under other circumstances (see below).

To this end, we tested the hormonal effects of playback in two subspecies of dark-eyed junco (*Junco hyemalis*). The junco is a North American sparrow that has long been a model system for the study of life history, behavior and hormones, particularly the role of T in mediating life history and phenotypic evolution (Ketterson et al., 1992, 2009; Reed et al., 2006). We focused on the well-studied slate-colored junco (*J. h. carolinensis*) breeding in the Appalachian Mountains in Virginia (VA) and the comparatively less well-studied pink-sided junco (*J. h. mearnsi*) that breeds around the Yellowstone plateau and Teton range in Wyoming (WY). During the breeding season, we challenged free-living males with song playbacks previously recorded in their native population, and compared their post-challenge circulating T and CORT levels to controls that were captured rapidly. In one subspecies (WY), we also asked whether restraint stress further elevated CORT to test whether individuals exposed to song playback had reached their maximum CORT levels. Finally, we asked whether hormone elevation (or lack thereof) differed in response to high amplitude, long-range song versus low amplitude, short-range song that has been shown to elicit an extremely aggressive behavioral response in these and other Emberizid sparrows (Beecher et al., 2000; Reichard et al., 2011).

The primary goal of our study was to determine whether T or CORT rises in response to a social challenge consisting solely of a song playback in the absence of a decoy. While not identical to our study, a previous study in the same VA population revealed elevated T in response to a live conspecific and song playback in male juncos captured during the female's fertile period (McGlothlin et al., 2008), suggesting that male juncos can elevate T in response to some social challenges. By measuring the hormonal effect of song playback in two junco populations, we further examined whether geographic differences in latitude and length of breeding season could affect the elevation of T or CORT in response to playback (Landys et al., 2007; Wingfield et al., 2007). Finally, by presenting males with two song stimuli that differ significantly in the strength of the behavioral response they elicit (Reichard et al., 2011), we asked how different types of social challenges may differentially affect the HPA and HPG axes.

Materials and methods

Study sites and populations

We conducted two song playback experiments with free-living, male dark-eyed juncos. The experiments took place in the area surrounding Mountain Lake Biological Station in Pembroke, Virginia (VA; 37°22′N, 80°32′W), from 24 April to 25 May 2011 and in Grand Teton National Park, Wyoming (WY; 43° 56′N, 110°38′W), from 9 June to 19 July 2011. Observations were made between 0600 and 1200 EDT (VA) or MDT (WY). We tested 21 males in VA ($n=11$ controls, $n=10$ males exposed to long-range song), and we tested 60 males in WY ($n=20$ controls, $n=20$ males exposed to long-range song, $n=20$ males exposed to short-range song).

Both populations appear to be similar in life history, but the breeding season is notably shorter in WY (e.g. 6–8 weeks for WY versus 14–16 weeks for VA; pers. obs. DGR). While exact breeding stage was not known for most males, all males were in the early to mid breeding season at each site, as evidenced by concurrent observations of females with nesting material and incomplete to full brood patches for the first few weeks of sampling, and no observations of males caring for nestlings until the latter half of our sampling. Treatments were balanced by date and breeding stage, if known.

To ensure that playbacks and control observations did not occur near territorial boundaries, we noted the locations of song perches or nests of our subjects, and aborted any trials in which more than one male responded to the playback. We did not test neighbors on the same or consecutive days.

Observations and hormone collection from control males

We used free-living males as controls because their hormone levels should reflect the background level of social interactions encountered by free-living juncos in each population. In one population (VA) we observed behavior for 25 min prior to sampling blood for hormones by noting the number of songs sung on previously mapped territories. To capture control males from both populations we used a very brief conspecific playback (latency to capture VA [mean ± se]: 83 ± 24 s, range: 20 to 240 s; WY: 93.2 ± 20.5 s, range: 6 s to 318 s), and we collected blood very rapidly, before hormone levels were likely to have been influenced by this brief playback and/or handling (latency from capture to blood sampling: VA: 68 ± 6 s, range: 35–100 s; WY: 84.7 ± 11.8 s, range: 34 s to 232 s). We eliminated one VA male from analyses because blood sampling occurred after 4 min of handling, leaving $n=10$ controls in VA and $n=20$ controls in WY. While there is some possibility that our capture method (brief playback) may have affected hormone signaling in control males, the total time from initial disturbance to blood sampling was short (VA: 116 ± 9 s, range: 60–159 s; WY: 166 ± 22 s, range: 56–367 s), and we observed no relationship between latency to capture or bleed and any hormone measure (Pearson's $|r|<0.48$, $p>0.16$). Moreover, control hormone levels for both T and CORT fall within the normal range for baseline breeding male juncos (see results, Ketterson et al., 1991; Schoech et al., 1999), suggesting that control samples represent baseline hormone levels. In VA, each male was sacrificed with an overdose of isoflurane, followed by rapid decapitation, as a part of another experiment, and latencies to blood collection include time to sacrifice. We collected up to 300 μL trunk blood from these males directly into heparinized

microtainers (Becton Dickinson #365965, Franklin Lakes, NJ USA). In WY, we collected 50–200 μL of blood from the alar vein using heparinized microhematocrit tubes, and we then banded and released each male. All blood samples were stored on ice in the field. After centrifugation (10 min at approx. 10,000 rpm), plasma was collected with a Hamilton syringe and stored at −20 °C until later hormone assays.

Song playbacks to experimental males

Immediately after entering the territory of each experimental male, we placed a speaker (Altec Lansing model iM237 in VA; Pignose model 7–100 in WY) on the ground with its cone facing up. We then set up and furled a mist net approximately 15 m away. The observers (KAR in VA; DGR and SMF in WY) then retreated at least 15 m before beginning the playback. Each playback stimulus consisted of high-quality songs (high signal-to-noise ratio) recorded at least 1 km away in the bird's native population using an Audio-Technica shotgun microphone (model AT835b) and a Marantz digital recorder (model PMD660). We used Adobe Audition 1.5 (Adobe Systems, San Jose, California) to create all playback tapes and to standardize each playback to 90% of the peak amplitude. Male juncos sing a small repertoire of 1–8 unique song types, depending on the population sampled, and thus each playback is likely to mimic a prolonged but otherwise natural intrusion (Cardoso et al., 2009; Titus, 1998, Reichard, unpub. data). Some playback methods differed slightly by location as detailed below, but methods generally proceeded through the following steps: (1) playback period (2) post-playback observation period, and (3) capture and blood sampling (see Fig. 1), comparable to a previous study using males from the VA population (McGlothlin et al., 2008).

Virginia

We presented playback tapes consisting of 5 long-range song types that were chosen randomly from a pool of 22 song types, such that each male heard a unique set of songs. Each song type was repeated at a rate of 6 songs/min for 2.5 min before transitioning to the next song type for a total playback length of 12.5 min/tape. This song rate and playback structure mimics the normal production of long-range song in juncos (Titus, 1998). All males appeared in the arena quickly (35 ± 10 s; range: 9 to 107 s) and remained present for the duration of the trial. All tapes were processed through a high-pass equalizer to remove low-frequency background noise below 800 Hz. We used a Radio Shack digital sound level meter (model 33-2055) to standardize each playback to a natural amplitude of 85–90 dB sound pressure level (SPL) measured at 1 m. Playback tapes were presented in a random order.

Wyoming

We presented playback tapes consisting of either long-range song or short-range song. Long-range song (LRS) playback tapes were comparable in structure to the VA tapes but each tape contained only one song type and all tapes were 10 min in length. Short-range song (SRS) playback tapes contained a unique 30-sec segment of slow short-range song (see Reichard et al., 2011) repeated continuously for 10 min. Junco LRS and SRS differ substantially in complexity and structure, and our playback tapes were designed to mimic those natural differences. In both song treatments, each playback tape was unique and none were repeated between individuals such that each male heard a unique song stimulus. LRS tapes were processed through a high-pass equalizer to remove low-frequency background noise below 1200 Hz. SRS tapes were only filtered below 700 Hz to avoid degrading any low-frequency song elements. We used the same amplitude meter as above to standardize LRS playbacks to a natural amplitude of 85 db SPL and short-range song playbacks to 65 dB SPL (Anderson et al., 2007). To ensure that focal males were close enough to hear SRS stimuli, every WY trial began with the same high amplitude LRS playback to attract the focal male. After the focal male was within 10 m of the speaker (<5 min), we stopped the attraction playback and began a 1-min silent observation period before starting the 10-min LRS or SRS playback.

Quantifying behavior in experimental males

We quantified male behavior during playback and for approximately 10–12 min post-playback before capture. We observed and recorded several standard measures of aggressive behavior, including the number of flights over the speaker ('flyovers,' VA), flights longer than 1 m ('flights,' WY), number of songs sung, latency to first song, latency to approach the speaker within 1 and 5 m, time spent within 1 and 5 m of the speaker, and closest approach. These behavioral measures are established measures of aggressive response or the likelihood of attack in this and other species of songbirds (McGlothlin et al., 2008; Reichard et al., 2011; Searcy and Beecher, 2009; Searcy et al., 2006).

Bird capture, hormone sampling, and restraint stress

At the conclusion of each experimental trial in both populations (i.e., at least 25 min after the start of playback), we unfurled the mist net, revealed a mount (VA) or live conspecific (WY) placed directly underneath it, and re-started conspecific playback. We opted to reveal a lure or mount at this point to provide a visual target for focal males that would facilitate rapid capture. As expected, we captured experimental males rapidly and took a blood sample (latency from playback re-starting to blood sampling: VA: 254 ± 50 s, range: 60 to 520 s; WY:

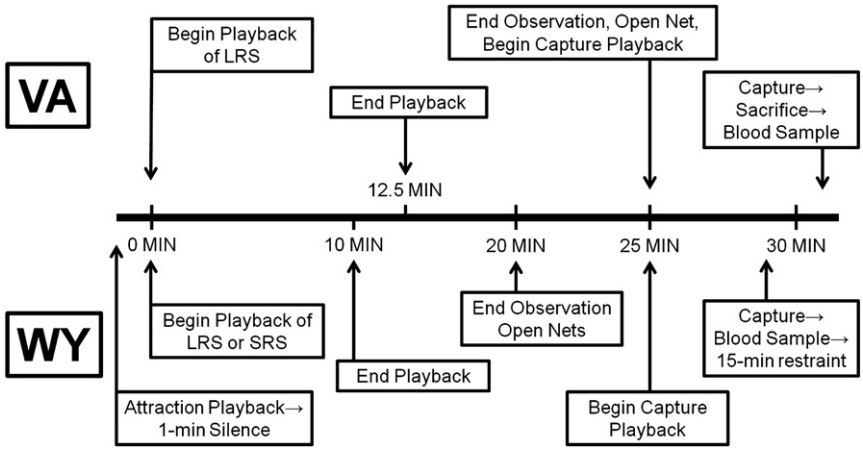

Fig. 1. Schematic of timeline for playback, post-playback and capture in the two populations (VA and WY). Not to scale.

186 ± 21 s, range: 64 to 630 s). While we cannot eliminate the possibility that this short exposure to a visual stimulus may have affected hormone signaling, our data suggest this effect is unlikely due to the short latencies between revealing the visual stimulus and sampling blood, and due to the lack of any observed correlation between latency and hormone levels (Pearson $|r|<0.21$, $p>0.13$ for all comparisons). The mean (± se) total time from initial playback to blood sampling of experimental males was 34.9 ± 0.8 min in VA and 27.0 ± 0.3 min in WY, falling firmly within the time frame when T levels have previously been elevated following a social challenge (McGlothlin et al., 2008, and unpub. data, see Discussion). Males exposed to LRS and SRS did not differ in latency to capture (unpaired *t*-test, $t_{1,38} = -0.27$, $p = 0.79$). In WY, each experimental male was transferred to a bag post-blood sampling to simulate restraint stress. At 15 min post-capture we took a second blood sample to measure restraint-induced CORT levels. Centrifugation and storage for these samples were identical to the methods for control males, described above.

Hormone assays

Testosterone

We quantified plasma testosterone using an enzyme immunoassay kit (Enzo Life Sciences, #901-065; previously known as Assay Designs; assay sensitivity = 5.67 pg/mL) that has been validated for use in this species (Clotfelter et al., 2004). In brief, we added ^{3}H-labeled T to each 20 μL plasma sample. We then extracted plasma twice using diethyl ether, and we calculated extraction efficiencies to account for incomplete recoveries (efficiencies: 90.0 ± 0.2%). T concentrations were obtained using a logistic standard curve and curve-fitting program (Microplate Manager, Bio-Rad Laboratories). VA samples were confined to one plate; WY samples were spread across two plates, balanced by date and treatment. Intra- and inter-plate variability was 1.8–6.3% (mean: 4.0%) and 0.7% respectively, and all three plates were from the same kit lot.

Corticosterone

We quantified plasma corticosterone using an enzyme immunoassay kit (Cayman #500655; assay sensitivity = 30 pg/mL) that has low cross-reactivity with 11-dehydrocorticosterone (11%) and 11-deoxycorticosterone (7%) and negligible cross-reactivity with other steroid hormones (e.g. <0.5% with cortisol, aldosterone, testosterone, androstenedione, DHEA). We found no previously published validation for this EIA using songbird plasma, so we validated the assay for use in the dark-eyed junco. We found high assay parallelism ($R^2 = 0.99$) between a standard curve based upon reference standards provided by the kit and a displacement curve based upon pooled plasma from juncos used in this study. Recovery of known amounts of CORT added to a dilute pool of plasma extract revealed high accuracy (121%, $R^2 = 0.99$, slope = 1.01). We followed the manufacturer's instructions with minor modifications. Briefly, we added 200uL dH_20 and 20uL of tritiated CORT (roughly 2500 CPM) to each 20 μL of plasma and allowed the samples to equilibrate overnight. We extracted the samples three times with diethyl ether, dried with N_2, and reconstituted with 30uL ethanol and 270 μL assay buffer. Samples were further diluted 1:4 prior to plating in duplicate. CORT concentrations were obtained using a four-point logistic standard curve and curve-fitting program (Microplate Manager) corrected for incomplete recoveries (extraction efficiencies: 90.7 ± 0.3%). VA samples were confined to one plate; WY samples were spread across three plates, balanced by date and treatment. Intra-assay variability was between 5.1% and 11.45% (mean: 8.6%), and inter-plate variability was 4.4%.

Statistical analyses

All statistical tests were performed in JMP 9.0.2 (SAS Institute Inc. Cary, NC). We performed a principal component analysis (PCA) on the WY behavioral data by combining eight behaviors to generate composite response scores for each individual and treatment. We used unpaired t-tests to compare PC scores between song treatments (SRS, LRS) in WY and to compare song rate between playback vs. control in VA (song vs. silence). Baseline T and CORT levels were natural log-transformed for normality and compared between treatments via unpaired t-tests or a one-way analysis of variance (ANOVA). We used paired t-tests to compare CORT in the initial and post-handling blood samples in WY. We related plasma hormone levels to individual behaviors and PC scores using Spearman rank correlations, and we related plasma hormone levels to latencies and date using Pearson's correlations.

Results

Playback elicited strong aggressive behavioral responses in both populations (Fig. 2). In VA, experimental males sang at a significantly higher song rate than control males (Fig. 2a, unpaired *t*-test: $t_{1,18} = 4.61$, $p<0.0002$). There was no significant difference between song rate during and after the playback (paired *t*-test: $t_{1,18} = -0.61$, $p = 0.55$), demonstrating that experimental males continued to behave aggressively after the playback had ended. In WY, a PCA extracted three components that explained 72.1% of the total variance in behavioral response observed during playback (Table 1). Measures of approach to the speaker loaded most strongly onto PC1, flights and time within 1 and 5 m of the speaker loaded onto PC2, and vocal behavior loaded onto PC3 (Table 1). To facilitate an intuitive interpretation of our data we multiplied both the loading and PC scores for PC1 by (−1). Males exposed to SRS trended toward a greater PC1 score than males exposed to LRS (Fig. 2b; $t_{1,38} = 1.98$, $p = 0.058$), which is indicative of a faster, closer approach to the speaker. SRS also elicited a significantly higher PC2 score than LRS (Fig. 2c; $t_{1,38} = 5.70$, $p<0.001$), which is indicative of more time spent in close proximity to the speaker and fewer flights. The two song treatments did not detectably differ in PC3 score (Fig. 2d; $t_{1,38} = 0.19$, $p = 0.85$), which related to vocal behavior. As in VA, WY males continued to behave aggressively during the 15 min post-playback period, as evidenced by persistent singing (LRS: 6.1 ± 0.9 songs/min, SRS: 9.2 ± 0.7 songs/min) and time spent within 5 m of the playback speaker (LRS: 149 ± 42 s, SRS: 243 ± 47 s).

Playback did not elicit any detectable elevation of testosterone or corticosterone. In VA, experimental and control males did not differ in plasma testosterone (Fig. 3a; $t_{1,18} = -0.33$, $p = 0.74$) or plasma corticosterone (Fig. 4a; $t_{1,18} = -1.41$, $p = 0.18$). In WY, males hearing long-range song and short-range song did not differ from each other or from controls in plasma testosterone (Fig. 3b; ANOVA, $F_{2,57} = 0.48$, $p = 0.62$) or corticosterone (Fig. 4b; $F_{2,55} = 0.66$, $p = 0.52$). Importantly, all of these post-playback and control hormone levels were comparable to published accounts of baseline T and baseline CORT for male juncos in the early to mid-breeding season (Ketterson et al., 1991; Schoech et al., 1999). Neither T nor CORT levels in either population related to Julian date (Pearson's $|r|<0.33$, $p>0.15$). Handling restraint in WY significantly increased CORT above baseline levels (paired *t*-test: $t_{1,35} = -19.75$, $p<0.001$), but there was no detectable difference between SRS and LRS males in restraint-induced CORT (unpaired *t*-test: $t_{1,35} = 0.032$, $p = 0.98$).

Because males exposed to different playbacks did not differ from controls in hormone levels, we pooled all males within a population when analyzing inter-individual patterns of co-variation between hormones and behavior. In VA, we did not detect a significant relationship between the number of songs sung during the 25-min observation and post-playback T ($r_s = 0.007$, $n = 20$, $p = 0.98$) or CORT ($r_s = -0.16$, $n = 20$, $p = 0.50$). In WY, we noted a significant positive relationship between PC1 and plasma T ($r_s = 0.50$, $p = 0.0011$, $n = 40$), such that males with higher plasma T post-playback approached the speaker more closely and rapidly than males with lower T (Fig. 5). There was no detectable relationship between plasma T and PC2 or PC3 (both $|r_s|<0.23$, $p>0.15$, $n = 40$). We also found no detectable

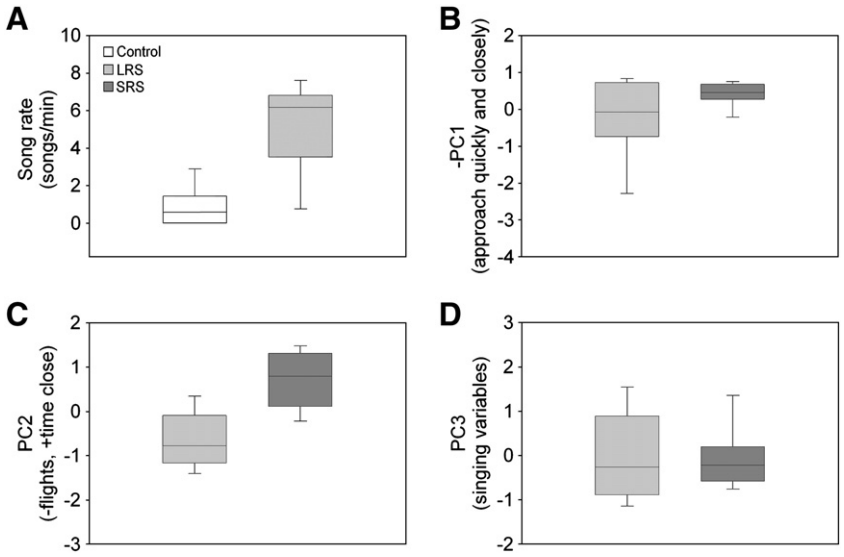

Fig. 2. Song playback elicited strong behavioral responses. Box plots show median + 95% confidence intervals. LRS = long-range song. SRS = short-range song. (A) Virginia, (B)–(D) Wyoming. All LRS and SRS data come from the playback period.

relationships between plasma CORT and any PCs (all $|r_s|<0.27$, $n=40$, $p>0.10$).

Discussion

In two populations of free-living dark-eyed juncos, we presented males with song in the approximate center of their territory, and observed robust behavioral effects of the playback (Fig. 2), coupled with no apparent elevation of plasma testosterone or corticosterone above levels observed in control males (Figs. 3 and 4). Males sang roughly 500% more songs during the song playback than during control observations (VA), and males spent roughly 575% more time in close proximity to the speaker during short-range song playback than during long-range song playback (WY), demonstrating strong and differential behavioral responses to these stimuli, without any detectable hormonal effects. These results differ from a previous account from this species that demonstrated elevated T following a simulated territorial intrusion with a live conspecific and song playback during the fertile period in VA (McGlothlin et al., 2008), suggesting that breeding male juncos can elevate T in response to social challenges, but that song stimuli alone may not be sufficient to induce rapid activation of the HPG axis. The lack of CORT elevation suggests that the failure of the experimental birds to elevate T was not a consequence of the HPA axis suppressing the HPG axis. Furthermore, handling stress rapidly elevated plasma CORT in WY (Fig. 4), confirming previous research in this and other species (Romero, 2002; Schoech et al., 1999). This decoupling of hormones and social behavior has important implications for the cues governing HPA and HPG axis activation, the mechanisms underlying behavioral regulation, and the physiological effects of playback induced by birdsong researchers and amateur birders.

Table 1
Principal components loading and eigenvalues for WY behavioral data. Cumulative % variance explained = 72.166%.

	Component		
	1	2	3
Latency to 1 m	.866	−.021	.223
Latency to 5 m	.549	−.131	−.108
Closest approach	.921	−.112	.092
Latency to song	.140	.123	.858
Flights	−.130	−.823	−.009
Long range songs	.049	.220	−.877
Time within 5 m	−.252	.775	−.234
Time within 1 m	−.428	.755	.121
Eigenvalue	2.761	1.571	1.442
% variance explained	34.507	19.638	18.020

What conditions are necessary for activation of the HPG or HPA axes?

If males in this study perceived song playback as a challenge, as indicated by their strong behavioral response, the question arises why circulating T and CORT levels were apparently unaffected. One possibility is that hormones respond to simulated intrusions more slowly than behavior, but for several reasons, we think that this is not a likely explanation. First, activation of the HPG axis via injection of exogenous GnRH or LH typically leads to elevated T within 20–30 min in this species as well as other sparrows (Deviche et al., 2012; Jawor et al., 2006), similar to the relatively short timeframe for socially induced T elevation seen in many vertebrates (e.g. 10–60 min after a social encounter, Gleason et al., 2009; Hirschenhauser et al., 2003; Oliveira et al., 2002). Likewise, circulating CORT levels rise rapidly following HPA axis activation (e.g. after ~3 min, Romero and Reed, 2005). Thus, if song playback induces a rise in T or CORT in juncos, the ~30 min latency from playback initiation to blood sampling employed in this study should have been sufficient to yield an elevation. We cannot dismiss the possibility that playback would have induced T or CORT elevation given a more extended exposure time. Spotted antbirds (*Hylophylax n. naevioides*), for example, require at least two consecutive hours of song exposure before T elevates (Wikelski et al., 1999), though it is unclear if this finding reflects a generally difficult-to-perturb HPG axis in antbirds and other tropical species, or whether additional cues are needed to elicit rapid HPG axis activation (see below). A previously published account of T elevation in dark-eyed

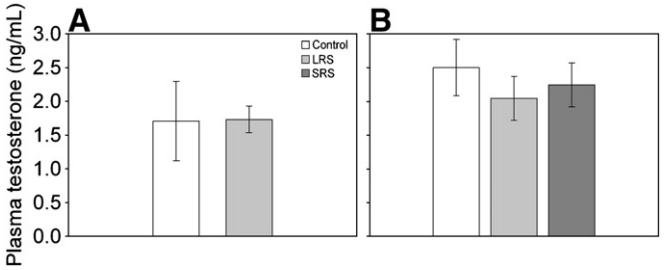

Fig. 3. Back-transformed means (± se) for circulating testosterone were similar for control birds and birds exposed to song playback. LRS = long-range song. SRS = short-range song. (A) Virginia. (B) Wyoming.

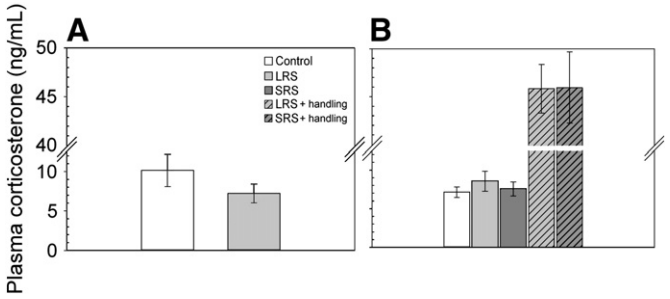

Fig. 4. Back-transformed means (±se) for circulating corticosterone were similar for control birds and birds exposed to song playback. 15 min of handling stress elevated CORT. LRS = long-range song. SRS = short-range song. (A) Virginia. (B) Wyoming.

juncos in the VA population employed a comparable timeframe (36.9 ± 6.4 min from initiation of challenge to blood sampling; McGlothlin et al., 2008). It is important to note that McGlothlin et al. (2008) inadvertently reported the latency from *restarting* playback to blood sampling as 36.9 min; however these data actually represent the latency from STI *initiation* to blood sampling (McGlothlin, pers. comm.). While McGlothlin et al. (2008)'s latencies to blood sampling show a larger range than our study (12 to 83 min vs. 25.1 to 38.9 min after the initiation of playback), they also observed no detectable relationship between plasma T and latency to blood sampling (Pearson's $r = 0.11$, $n = 10$, $p = 0.75$, McGlothlin et al., 2008 and unpublished data), suggesting that a longer exposure to a simulated intruder may not lead to further T elevation.

Another possible explanation for the lack of T or CORT elevation may relate to the perceived threat of the stimulus. Experimentally staged social encounters run the risk of being perceived as a loss or tie by the focal animal (Goymann et al., 2007; Oliveira et al., 2005), though our behavioral data do not support the interpretation that the males in this study had submitted or given up in any way (e.g. males continued to sing at high rates and remained close to the speaker during the post-playback period). Furthermore, in VA, we challenged males with a playback including repeated song-type switches, a stimulus likely to be perceived as highly aggressive (Searcy and Beecher, 2009). In WY, we challenged males with two different classes of song, both of which are strong behavioral stimuli (Reichard et al., 2011). Evidence to date from this species suggests that short-range song in particular is an extremely potent signal that elicits a very aggressive response, in part because this low-amplitude song may function in courtship, such that short-range song playback simulates an intruding male pursuing an extra-pair copulation. Based upon these data and paired with the strong behavioral responses we observed (Figs. 2a–c), we think it is unlikely that the lack of hormonal response relates to a lack of motivational impact of the stimuli.

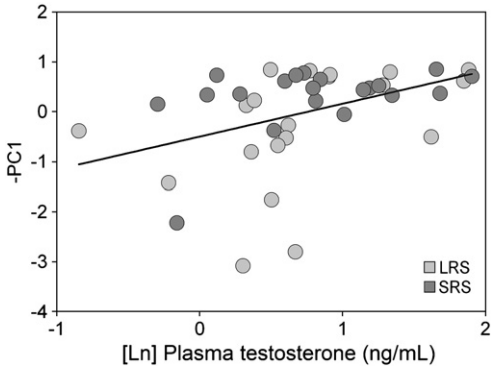

Fig. 5. Testosterone and PC1 were correlated in WY, suggesting males with higher T approached the speaker more closely and rapidly. LRS = long-range song. SRS = short-range song.

Several lines of evidence suggest that auditory stimuli alone may not be sufficient to rapidly upregulate gonadal T production. A few previous studies have suggested that auditory stimuli may not be effective at elevating T (Dufty and Wingfield, 1990; Wingfield and Wada, 1989), though some have lacked comparisons indicating that social challenges are capable of elevating T under any conditions (Deviche et al., 2012; Fokidis et al., 2011). Here, we show that song playback does not affect T elevation in two subspecies of dark-eyed junco. Paired with previous research in the VA subspecies showing a elevated T in response to an STI with a live male decoy and song playback (McGlothlin et al., 2008), our results are suggestive that the absence of a visual cue may contribute to the lack of elevation in T or CORT reported here. With the addition of a second treatment in the WY portion of the study, we have shown for the first time that the null effects of song playback extend to low-amplitude song, a class of particularly challenging stimuli (Searcy and Beecher, 2009).

There is evidence to suggest that the HPG and HPA axes may show signs of activation from auditory stimuli, though auditory stimuli may not always be sufficient to promote T or CORT release. For example, in birds and frogs (both taxa that rely heavily on auditory communication), exposure to conspecific vocalizations can rapidly activate neuronal activity in the hypothalamus (Maney et al., 2007) and increase the number of GnRH-immunoreactive neurons (Burmeister and Wilczynski, 2005), but sound alone may not be sufficient to trigger the release of all signaling molecules along the HPG axis (e.g. Meddle et al., 2002; Small et al., 2008). A recent study demonstrated a lack of co-variation between an individual's T response to exogenous GnRH and to a social challenge (Apfelbeck and Goymann, 2011), which suggests that social activation of the HPG axis may be a selective process. There are currently rather limited data to address whether conspecific song alone is sufficient to bring about an increase in CORT (see Fokidis et al., 2011), though it is clear that some auditory stimuli can activate the HPA axis, as indicated by the strong CORT responses to predator calls, conspecific alarm calls, or other jarring sounds (Avey et al., 2011; Nephew et al., 2003). Similar to the HPG axis, though, song playback or social challenges may initiate preparation of the HPA axis for activation, by changing hypothalamic signaling of vasotocin and corticotrophin releasing factor (Fokidis and Deviche, 2012; Goodson and Evans, 2004).

It is tempting to conclude that the lack of T or CORT elevation that we observed relates to missing visual cues that would have been present had males been challenged with a live decoy (e.g. as in McGlothlin et al., 2008). However, there are a number of issues that have not yet been resolved in understanding inter- and intra-specific variation in social modulation of hormones. For example, we do not know how variation in breeding stage may affect the degree to which social challenges affect T or CORT in juncos, and all males in McGlothlin et al.'s (2008) study had fertile mates, whereas males in our study varied in breeding stage. It is also not yet clear whether species or populations are consistent in their hormonal response to social challenges, or whether annual variation in resource availability or territory sizes might shift a population along a continuum of social modulation of the HPA and HPG axes.

Social challenges and the role of testosterone

The challenge hypothesis proposes that socially induced surges in T prepare individuals for success in extended aggressive encounters by shifting energy toward greater activity and energy mobilization (Wingfield et al., 1990). Of course, prolonged T elevation can be costly, e.g. by decreasing immune function or parental care (Wingfield et al., 2001). Thus, from an ultimate perspective, it may be adaptive that T should elevate only when likely to lead to an advantageous outcome, i.e. after the "threat" posed to a male's defended resources (e.g., mate, territory, etc.) exceeds some critical threshold. Our result suggests that song playback alone may not always exceed this threshold. During the breeding season, male songbirds hear other males singing frequently

(Titus, 1998) and as such, would experience frequent, potentially maladaptive surges of T if simply hearing another male singing was sufficient to activate the HPG axis. The observation that playback alone does not initiate a rapid HPG axis response suggests that a more threatening stimulus, such as playback combined with a live decoy (Wingfield and Wada, 1989) or an intrusion during the female's fertile period (McGlothlin et al., 2008), may be needed for T elevation.

The males in these experiments showed strong aggressive responses to playback, consistent with the view that acute T surges are not required for aggressive behaviors to be produced or sustained during a short social challenge. All males had breeding baseline levels of T that are clearly above the levels needed for reproductive and aggressive behaviors (Goymann, 2009; Wingfield et al., 1990). Our data also show patterns of co-variation between levels of T and proxies of aggression in WY (Fig. 5), consistent with previous research in this species showing that a male's hormonal phenotype may be an important predictor of its behavioral phenotype (McGlothlin et al., 2007). Neural sensitivity to T also may play an important role in the immediate aggressive response to an intruder (Soma et al., 2008), and social challenges are known to affect neural androgen synthesis, at least during the non-breeding season when T is low (Charlier et al., 2011; Pradhan et al., 2010). The degree to which breeding season social challenges alter neurosteroidogenesis or sex steroid sensitivity is less clear (Mukai et al., 2009), though aggressive behavior has been linked with the expression of androgen receptor and aromatase in many species, including juncos (Rosvall et al., 2012; Soma et al., 2008). What is clear from our study is that strong aggressive responses, even those that are specific to different song classes, can occur without acute T elevation. While it is tempting to conclude that T elevation is therefore unnecessary for the expression of aggression, it remains to be seen if previous T surges better prepare males for prolonged bouts of social instability (either behaviorally or physiologically), as predicted by the challenge hypothesis (Wingfield et al., 1990).

Corticosterone and social challenges

Corticosteroids also may play a role in the regulation of aggressive behavior, and social challenges may activate the mobilization of resources via glucocorticoids instead of T (Landys et al., 2007, 2010; Mikics et al., 2004; Van Duyse et al., 2004). Across species, some of the variable hormonal effects of a social challenge or STI are thought to relate to life history or ecological factors, such as the importance of parental care or the length of the breeding season (Goymann, 2009; Landys et al., 2007; Lynn, 2008). Here, we observed no detectable elevation of CORT in response to playback in either subspecies of junco. While the WY junco is less well characterized than the VA subspecies, the breeding season is shorter in WY (e.g. 6–8 weeks for WY versus 14–16 weeks for VA; pers. obs. DGR). Thus, our population comparison suggests that the failure of song playback to lead to an elevation of T is unlikely to relate to a potentiated glucocorticoid response or dampened steroid response in the shorter breeding season subspecies (WY). We also saw a strong handling-induced glucocorticoid response in WY that was comparable in magnitude to previous reports in the VA population (Fig. 4 for WY; see Schoech et al., 1999 for VA data). In both populations, the lack of socially induced CORT elevation also minimizes the possibility that our lack of T elevation may have been caused by CORT-induced suppression of the HPG axis (Goymann, 2009; Van Duyse et al., 2004). We cannot discount the possibility that our playback treatment may have caused fluctuations in corticosteroid-binding globulin (CBG) that could affect the immediate expression of behavior. Social challenges may alter CORT or T signaling by changing CBG binding capacity or the percent of free versus bound steroid (Charlier et al., 2009; Landys et al., 2007, but see Lynn et al., 2007), though direct tests of the influence on song playback are lacking, to our knowledge.

Implications for songbird researchers and amateur birders

In addition to the implications for our understanding of behavioral mechanisms and the cues regulating HPA/HPG axis activation, the results reported here may have implications for songbird researchers and amateur birders who regularly stimulate free-living birds with song playback. Our results show that song playback had little effect on T or CORT in two populations of junco, though we urge caution in extending these results to other species based upon large interspecific variation in socially-induced changes in hormones (Goymann, 2009). Our study also cannot address whether more extensive or persistent playback would lead to elevated T or CORT, or whether playback can change other aspects of organismal physiology (Newman and Soma, 2011), but they do suggest that short playbacks used to assess the salience of particular song characters may not always alter T or CORT.

The potentially deleterious effects of song playback have been the center of recent debate in American and European birding circles, with the rise in popularity of smartphones and birding playback applications. Several major groups, including the National Audubon Society and the Royal Society for the Protection of Birds, have discouraged the use of playback for a variety of reasons, chief among them the potential negative effect on bird behavior, physiology, and fitness. Our results suggest that two obvious physiological costs (unnecessary elevation of T or CORT) should not be assumed, though playback still may affect the behavior of prospecting females (Mennill et al., 2002) or eavesdropping juveniles (Templeton et al., 2010). The behavioral effects of playback likewise may be harmful, since a bird that is preoccupied with an imaginary intruder is a bird that is not being watchful of predators, guarding his mate, foraging, or caring for young.

Funding

This project was made possible by an NIH NRSA to KAR (T32HD049336 and F32HD068222), an NSF graduate research fellowship and DDIG to DGR (IOS-1011145), the College of Wooster's Henry J. Copeland Fund for Independent Study to SMF, an NSF Cooperative Agreement DBI-0939454 supported DJW, and an NSF to EDK (IOS-0820055), including an REU supplement.

Acknowledgments

Many thanks to Mountain Lake Biological Station, University of Virginia, University of Wyoming-National Park Service (UW-NPS) Research Center at the AMK Ranch, and Grand Teton National Park for access to field sites. We are also grateful to M. Drouilly, R.E. Koch, and E.M. Schultz for assistance in the field, to R.A. Stewart and the Center for the Integrative Study for Animal Behavior for assistance with hormone validation, and to R.C. Anderson, S.E. Lynn, and members of the Ketterson Lab for helpful feedback.

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