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Up to the challenge? Hormonal and behavioral responses of free-ranging male Cassin's Sparrows, *Peucaea cassinii*, to conspecific song playback

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ABSTRACT

(208 words)

The Challenge Hypothesis postulates that male vertebrates can respond to social challenges, such as simulated territorial intrusions (STI), by rapidly increasing their concentrations of plasma androgens, such as testosterone (T). This increase may facilitate the expression of aggressive behavior and lead to persistence of this behavior even after withdrawal of the challenge, thus potentially promoting territoriality and the probability of winning future challenges. The validity of the Challenge Hypothesis was investigated in socially monogamous free-ranging male Cassin's Sparrows, *Peucaea cassinii*. Exposure to STI at the beginning of the vernal nesting season stimulated aggressive behavior but did not increase plasma T. Furthermore, plasma T did not correlate with the duration of exposure to STI and the behavioral response to STI did not differ in males that were challenged a second time shortly after the first challenge. As birds were investigated at a stage of their reproductive cycle when plasma T is presumably seasonally high due to photostimulation, the lack of hormonal response to STI may have been due to the hypothalamo-pituitary-gonadal axis secreting hormones at maximum rates. This was not the case, however, because administration of gonadotropin-releasing hormone I (GnRH-I) rapidly stimulated the secretion of luteinizing hormone (LH) and T, and treatment with ovine LH rapidly stimulated T secretion.

Introduction

In many birds the activity of the hypothalamic-pituitary gonadal (HPG) axis changes seasonally under the influence of photoperiod (Wingfield et al., 1997) and is facultatively modulated by non-photoperiodic factors that include social interactions, including male-male aggressive interactions (Wingfield et al., 1987, 1990). The influence of social interactions on the HPG axis is suggested by correlative studies of territorial male birds indicating that plasma T is often seasonally highest at the beginning of the breeding season, when male-male interactions are frequent and females are sexually receptive (Wingfield et al., 1987), and is confirmed by experimental studies. One commonly used experimental procedure is to expose individuals to simulated territorial intrusions (STI) consisting of pre-recorded conspecific vocalizations combined with a live (Charlier et al., 2009; Hau and Beebe, 2011; Landys et al., 2007; Moore et al., 2004; Wingfield, 1994a) or stuffed (Apfelbeck and Goymann, 2011; Silverin et al., 2004) decoy. Exposure to STI often elicits a strong aggressive response (Gill et al., 2008; Klukowski and Nelson, 1998; Wingfield, 1994b) and, in some situations, a rapid (within minutes) increase in plasma testosterone (T; Ros et al., 2002; Wingfield, 1984, 1994b). In free-ranging Song Sparrows, *Melospiza melodia*, exposure to conspecific song playback alone or with a live decoy bird has a similar stimulatory effect on aggressive behavior, but plasma T increases significantly only in sparrows that are exposed to both stimuli (Wingfield and Wada, 1989). Whether a combination of auditory and visual stimulation is necessary to rapidly elevate plasma T also in other species has, however, not been investigated.

Testosterone can exert rapid (within minutes to hours) physiological (Sachs and Leipheimer, 1988) and behavioral effects (Lord et al., 2009), and in some situations enhances attention to novel conspecifics and other relevant stimuli (rat, *Rattus norvegicus*: Thor et al., 1982; chicken, *Gallus domesticus*: Archer, 1977). According to the Challenge Hypothesis (Wingfield et al., 1987, 1990), increased T in response to social challenge may normally function not to stimulate aggression *per se*, but rather to enhance the intensity of the behavioral response to STI and the persistence of this response even when an individual is no longer challenged (Oyegbile and Marler, 2005; Wingfield, 1994a,b, 2005), which in turn may promote territoriality (Oliveira et al., 2009).

Social challenges can induce rapid endocrine changes in insects (juvenile hormone: Kou et al., 2008; Tibbetts and Huang, 2010), fish (Oliveira et al., 2002; Pankhurst and Barnett, 1993;

Ros et al., 2003), reptiles (Rubenstein and Wikelski, 2005), birds (Beletsky et al., 1992; Ferree et al., 2004; Smith et al., 2005; see above), and mammals (Buck and Barnes, 2003; Setchell et al., 2008) including humans (Fry et al., 2011; van der Meij et al., 2010). However, other studies found no influence of single or repeated social interactions on plasma androgens (birds: Addis et al., 2010; Apfelbeck and Goymann 2011; Apfelbeck et al., 2011; Gill et al., 2008; lizards: Klukowski et al., 1998; Thompson and Moore, 1992) whereas in the Great Tit, *Parus major*, and Blue Tit, *Cyanistes caeruleus*, plasma T decreased in response to STI (Landys et al., 2007; Van Duyse et al., 2004; reviews: Goymann et al., 2007; Goymann, 2009). To reconcile these apparent discrepancies, it has been proposed that androgen responsiveness to a social challenge varies as a function of a number of factors including the mating system (Wingfield et al., 1990; single- vs. multiple broodedness: Landys et al., 2007), the contribution of males to incubation (Hirschenhauser et al., 2003), and the duration of the breeding season (Goymann, 2009). As proposed by the latter author, the absence of androgen responsiveness to STI in some studies may also reflect the fact that observations were made when the activity of the HPG axis, including T secretion, was at its seasonal highest and physiologically incapable of further activation. This could be the case in males of single-brooded species particularly at the beginning of their breeding season.

The functionality of the HPG axis can be evaluated by measuring hypothalamic gonadotropin-releasing hormone (GnRH-I), the plasma luteinizing hormone (LH) response to administration of GnRH-I or the secretagogue N-methyl-D,L-aspartate (NMA), and by measuring the plasma T response to LH (Dawson, 2005; Deviche et al., 2010) or GnRH (Jawor et al., 2006; Spinney et al., 2006) administration. For example, in male Cassin's Sparrows, *Peucaea carpalis*, the increase in plasma LH in response to NMA injection is less when birds are photostimulated and have elevated plasma LH than when they are exposed to short days and have low plasma LH (Deviche et al., 2008). In the Green Anole, *Anolis carolinensis*, lightweight males have lower plasma T than heavyweight males, but GnRH administration does not increase plasma T in either form, suggesting that intact lightweight and heavyweight males are secreting this hormone at the maximum rate (Husak et al., 2009). To our knowledge, only one study has investigated the HPG axis functionality in the context of the Challenge Hypothesis (Apfelbeck and Goymann, 2011). In this study on male Black Redstarts, *Phoenicurus ochruros*, plasma T increased in response to GnRH injection but not STI, suggesting that the lack of

hormonal response to a social challenge was not due to physiological incapacity to increase T secretion. Additional studies are warranted to test the generality of this conclusion.

The objective of the present study was to test the validity of the Challenge Hypothesis in field experiments using a seasonally breeding, socially monogamous male songbird, the Cassin's Sparrow (Dunning, 1999), as an experimental model. We measured the behavioral response of the sparrows to conspecific song playback (SPB) and determined whether this response is associated with elevated plasma T. Birds were studied at the beginning of their breeding period, when plasma T in other socially monogamous male songbirds generally is at its highest. To test whether the activity of HPG axis at the time of the study was at its maximum, we measured the plasma LH response to acute treatment with GnRH-I, and the plasma T response to a GnRH-I or LH injection. Once exposed to STI, some birds in breeding condition remain highly aggressive even after STI withdrawal (Wingfield, 1994b). The physiological basis of the persistence of this behavior is not known and may involve a short-term sensitization of the pituitary gland to GnRH and/or of the testes to LH. We tested this hypothesis by comparing the effects of GnRH-I or LH treatment described above, prior to and following SPB. Finally, we determined whether the behavioral response of socially challenged males differs between those exposed to SPB for the first or second time.

Materials and methods

Animals

We investigated adult male Cassin's Sparrows at the Comanche National Grasslands (Baca Co., Colorado, USA; $37^{\circ} 06' N$; $102^{\circ} 34' W$) between the 14th and 19th of May 2010. The species in the study area is considered migratory, but its secretive habits outside the breeding season limit the amount of information that is available on its migratory patterns (Dunning et al., 1999). During the breeding season, males defend territories mostly through song duels that do not involve physical contact (Dunning et al., 1999). Cassin's Sparrows are thought to be socially monogamous (Dunning et al., 1999) and to establish seasonal breeding territories, and at least in part of their breeding range, to normally be double-brooded (Texas: Schnase et al., 1991). Whether the latter applies to birds in the current study area is, however, conjectural. Males at the time of the study had established territories and were skylarking (= territorial flight singing) at

a high rate (personal observations), a behavior that in this species is most often given in the presence of females (Dunning et al., 1999). Three females caught during the study had partially developed incubation patches, indicating that the birds were at an early stage (preincubation) of their reproductive cycle (Dunning et al., 1999). All birds were caught and observed between 0600 and 1600. A previous study on free-ranging breeding males of a conspecific species, the Rufous-winged Sparrow, *Peucaea carpalis*, found that plasma T does not change consistently during the course of the day (Deviche et al., 2012).

The study included 98 males that we randomly assigned to one of three independent experiments (Expts 1, 2, and 3; see below and Fig. 1). Sparrows in Expt 1 ($n = 48$) and Expt 2 ($n = 32$) were caught in Japanese mist nets in response to SPB; birds in Expt 3 ($n = 18$) were used for a behavioral study but were not caught. Sex was determined based on behavioral observations before capture (only male Cassin's Sparrows sing and skylark; Dunning, 1999) and, after capture, confirmed by the presence of a developed cloacal protuberance (males only) or incubation patch (females only; Pyle, 1997).

All activities were authorized by the Colorado State Department of Natural Resources and the US Fish and Wildlife Service, and approved by the Arizona State University Institutional Animal Care and Use Committee.

Experimental designs

Experiment 1. The first objective of Expt 1 was to determine whether exposure to SPB influences *initial* (= "baseline") plasma T. Birds were exposed to SPB for a short (= SHORT SPB) or long (= LONG SPB) duration using a stationary portable system (MP3 player) equipped with multidirectional speakers that were usually placed 0.5 - 1 m above ground. For exposure to SHORT SPB, a mist net was deployed where a bird was found singing, the SPB system was placed near the net and turned on, and a bird was then caught as soon as possible and sampled. Males in the SHORT SPB group ($n = 24$) were exposed to SPB for 4.1 ± 0.5 (S.E) min before capture. For exposure to LONG SPB, a similar procedure was followed except that the SPB was turned on for 30 min before the mist net was unfurled and the bird then caught as soon as possible. Males in the LONG SPB group ($n = 24$) were exposed to SPB for 36.2 ± 1 min before capture. Within three min of capture and removal from the net, a blood sample (= *initial*

bleed; 100 µl) was collected from the right jugular vein of each sparrow into a heparinized microsyringe. These samples were used to determine *initial* plasma T concentrations.

The second objective of Expt 1 was to determine whether any effect of SPB on plasma T is mediated at the pituitary gland or testicular level. SHORT- and LONG-SPB-exposed sparrows were each divided into three groups ($n = 8$ birds per group). Within 2 min of the initial bleed, birds (one group of SHORT and one group of LONG SPB-exposed sparrows) received an intrajugular injection either of ovine LH (oLH; National Hormone and Peptide Program, Harbor-UCLA Medical Center, CA, USA; 20 µg/bird (≤ 1 mg/kg) in 0.1 ml saline solution (0.9 % NaCl in distilled water)), of synthetic chicken GnRH-I (hereafter GnRH; 500 ng/bird (≤ 25 µg/kg) in 0.1 ml saline solution; Sigma Chemical Co., MO, USA), or of control saline solution (0.1 ml). A second blood sample (= *postinjection* bleed; 100 µl) was collected from each bird 20 min after the injection.

Experiment 2. This study also had two objectives. The first objective was to determine whether SPB exposure influences LH secretion. For this, we used the same method as for Expt 1 to compare *initial* plasma LH of sparrows exposed to SHORT SPB (average duration: 2.3 ± 0.5 min; $n = 14$) or LONG SPB (average duration: 34.9 ± 0.8 min; $n = 18$).

The second objective of Expt 2 was to investigate whether any endocrine effect of SPB exposure on plasma LH involves changes in pituitary gland sensitivity to GnRH. SHORT- and LONG SPB-exposed groups of birds were each divided into two groups. Within 2 min of the initial bleed, sparrows received an intrajugular injection of GnRH (SHORT SPB: $n = 8$; LONG SPB: $n = 9$) or of control saline vehicle (SHORT SPB: $n = 6$; LONG SPB: $n = 9$). All birds were bled 3 min later (= *postinjection* bleeds) as described for Expt 1. The total volume of blood collected from each bird (200 µl) did not make it possible to measure T and LH concentrations in the same plasma samples.

Within each experiment, treatments were administered in a random order. Between the injection and the *postinjection* bleed, birds were held in individual breathable cloth bags. Blood samples were kept on ice until centrifuged later the same day. Plasma was harvested using a glass Hamilton syringe and frozen at -20°C until assayed. After completion of the blood collection, the cloacal protuberance width of each bird was measured to the nearest millimeter and we recorded whether birds were molting. All sparrows had an enlarged cloacal protuberance, indicating that they were in breeding condition, and no bird was undergoing wing

or contour feather molt. Sparrows received a uniquely numbered aluminum tarsal band from the U.S. Geological Survey and were released at the capture site.

Experiment 3. The objective of this study was to determine whether exposure to SPB modifies the behavioral response to subsequent SPB. Males were located by their spontaneous singing behavior (i.e., no SPB was used) and observed for several minutes. The SPB system was then placed at the estimated center of the area where a bird had been observed singing and was either left off (control group; $n = 8$) or turned on (experimental group; $n = 10$) for 30 min, i.e., for the same time as that to which LONG SPB birds were exposed to SPB before capture in Expts 1 and 2. During the first 10 min of SPB (or silence), an observer located 15-20 meters from the SPB speakers quantified five well defined, discrete, and easily identifiable behaviors: latency to first approach speakers within 5 meters; number of approaches within 5 meters of speakers; cumulative time spent within 5 meters of speakers; number of skylarking-associated songs; and number of non skylarking-associated songs. The latter songs were generally heard while a bird was perched rather than in flight.

At the end of the 30 min SPB (or silence) period, the observer walked to the speakers and either turned on the SPB system (control group) and then walked away, or left the SPB system on and then walked away (experimental group). The same behaviors as described above were quantified for the following 10 min, thus allowing us to determine whether the behavioral response of sparrows to SPB was influenced by their having been exposed to SPB for the previous 30 min (experimental group) or not (control group).

Hormone assays

Plasma samples collected during Expts 1 and 2 were assayed for T and LH, respectively.

Testosterone. Plasma T was measured as previously described (Deviche et al., 2006) using commercial enzyme-linked immunoassay kits (Enzo Life Sciences, Farmingdale, NY). All samples were assayed in duplicate, on the same day and on three assay plates, using the manufacturer's recommended procedure and after 10x dilution in assay buffer. Samples were randomly assigned to assay plates except that the *initial* and *postinjection* samples from the

same sparrow were assayed on the same plate. The primary antibody used in the assay has less than 5% crossreactivity with 17 β -estradiol, dihydrotestosterone, CORT, and progesterone (manufacturer's specifications). The mean interassay and intrassay coefficients of variation were 17.5% (2 samples assayed on each plate) and 2.5% ($n = 95$ samples), respectively, and the assay sensitivity was 44 pg/ml. The slope of a plasma dilution curve did not differ from that of a standard curve on the same assay plate ($p = 0.13$).

Luteinizing hormone. Plasma LH concentrations were determined using a micromodification of the radioimmunoassay described previously (Sharp et al., 1987). The assay has been validated for use in the Cassin's Sparrow (Deviche et al., 2008). Briefly, the reaction volume was 60 μ l, comprising 20 μ l of plasma sample or standard, 20 μ l of primary rabbit LH antibody, and 20 μ l of I¹²⁵-labelled LH. The primary antibody was precipitated to separate free and bound I¹²⁵ label using 20 μ l of donkey anti-rabbit precipitating serum and 20 μ l of non-immune rabbit serum. All samples were measured in duplicate and in a single assay. The intra-assay coefficient of variation was 7.3% and the minimum detectable dose was 0.15 ng/ml.

Statistical analyses

We analyzed data using SigmaPlot (version 12.0; Systat Software, Inc., San Jose, CA), Statistica (version 10; StatSoft, Inc., Tulsa, OK), and the R freeware. Effects of the experimental treatments and SPB duration before capture on plasma T (Expt. 1) and LH (Expt. 2) were analyzed by two-way analysis of variance (ANOVA). Data sets that were not normally distributed or homoscedastic were transformed as necessary before these analyses (see Results). Correlations between the duration of exposure to SPB and hormone levels were analyzed by linear regression. We used non-parametric Mann-Whitney U-tests to analyze behavior data obtained in Expt. 3. The significance level of all statistical tests was set at $p = 0.05$.

Results

Experiment 1

Birds in this experiment were sampled over a 10 hour-long period (between approximately 0600 and 1600). As shown in Fig. 2, *initial* plasma T during this period did not undergo a consistent daily change.

Figure 3 shows plasma T levels in sparrows exposed to SHORT or LONG SPB and treated with GnRH, oLH, or control vehicle.

We analyzed *initial* plasma T using two-way analysis of variance (ANOVA) with duration of exposure to SPB (SHORT or LONG SPB) and hormonal treatment (GnRH, oLH, or control vehicle) as the main factors. *Initial* plasma T did not differ in SHORT and LONG SPB-exposed sparrows ($F_{1,47} = 0.003, p > 0.9$), indicating that song playback did not influence T secretion. Furthermore, sparrows of the three experimental groups had similar *initial* plasma T ($F_{1,47} = 1.565, p > 0.2$). To further test the possibility that exposure to SPB influenced plasma T, we investigated whether *initial* plasma T levels were related to the individual duration of SPB exposure. There was no statistical relationship between these variables in SHORT SPB-exposed sparrows (linear regression: coefficient of determination: $r^2 = 0.001, p = 0.88, n = 24$; Fig. 4), suggesting that plasma T did not increase during the initial (i.e., within a few minutes) period of SPB exposure. No relationship between *initial* plasma T and SPB duration was found in LONG SPB-exposed sparrows either ($r^2 < 0.001, p = 0.91, n = 24$; Fig. 4).

To determine the effects of SPB across experimental groups and the effects of the experimental treatments on plasma T, we analyzed data using a simple two way ANOVA on a single (non-repeated) dependent variable consisting of the change in plasma T (*postinjection* minus corresponding *initial* plasma T; Table 1). As there was considerable variation among subjects, we analyzed these data in a mixed model framework with subjects as a random effect. Changes in plasma T were normally distributed (Lilliefors test: $D = 0.11, p = 0.14$) and homoscedastic (from visual inspection of residuals) after log 10 transformation. Exposure to SPB did not influence plasma T (Table 1; Fig. 3), but plasma T differed between hormone treatments: GnRH- and oLH-treated sparrows had higher plasma T than control birds 20 min post-injection. Thus, sparrows at the time of capture were not secreting LH or T at the maximum rate. There was no SPB duration x hormonal treatment interaction, showing that the SPB duration did not influence the effects of the experimental treatments on plasma T.

We used Expt. 1 data to also determine whether capture and restraint for 20 min influenced plasma T. For this, we used two-way ANOVA to compare *initial* and *postinjection* plasma T in control sparrows belonging to the SHORT and LONG SPB groups. Consistent with

the above analyses, there was no effect of the SPB duration on plasma T levels in control sparrows ($F_{1,31} = 0.709$, $p > 0.7$). However, capture, injection of saline solution, and restraint for 20 min decreased plasma T by 34.8% ($F_{1,14} = 23.772$, $p < 0.001$; average plasma T: *Initial* bleed: 3.65 ± 0.92 ng/ml; *postinjection* bleed: 1.35 ± 0.26 ng/ml). There was no statistical interaction on plasma T between the SPB duration and the effect of capture and restraint ($F_{1,31} = 0.020$, $p > 0.8$).

Experiment 2

As was the case for Experiment 1, birds in this experiment were sampled over a 10 hour-long period. Similar to the situation for *initial* plasma T, *initial* plasma LH did not change consistently during the course of the day (Fig. 2).

Initial and *postinjection* plasma LH levels were positively correlated in the control group (Spearman rank correlation coefficient = 0.789; $p < 0.0001$; $n = 15$) and in GnRH-treated birds (correlation coefficient = 0.618; $p = 0.008$; $n = 17$). There was no statistical relationship between SPB duration and *initial* plasma LH in SHORT (linear regression: $p = 0.068$; $n = 14$) or LONG (id.: $p = 0.406$; $n = 18$; Fig. 4) SPB-exposed birds.

The effects of SPB duration and GnRH administration on plasma LH were analyzed by two way ANOVA as described for plasma T in Experiment 1. This analysis was performed on log 10-transformed data, which met assumptions of normality (Lilliefors test: $D = 0.146$, $p = 0.08$) and equal variance. There was no effect of SPB exposure on plasma LH, but plasma LH increased in response to GnRH injection (Table 2, Fig. 5). There was no statistical interaction between exposure to SPB and the effect of GnRH injection on plasma LH. Thus the stimulatory effect of GnRH treatment on plasma LH was similar whether birds had been exposed to SHORT or LONG SPB before hormone administration (Fig. 5).

Taken together, the results of Experiments 1 and 2 indicate that exposure to SPB did not influence plasma LH or the pituitary gland responsiveness to GnRH.

Experiment 3

Exposure to SPB had marked behavioral effects. Compared to control sparrows that were not exposed to SPB, experimental birds responded to SPB by decreasing the latency to approach speakers, increasing the number of approaches to the speakers, and increasing the amount of time spent within five meters of the speakers (Mann-Whitney rank sum tests: p 's <

0.05; Fig. 6). Exposure to SPB markedly decreased skylarking-associated songs (id., $p < 0.05$), but there was no difference between control and experimental birds in the number of songs not associated with skylarking.

No behavioral differences were observed between control males exposed to SPB for the first time and experimental males that had just been exposed to SPB for 30 min (Fig. 7). Exposure to SPB had, therefore, no detectable influence on the behavioral response to subsequent SPB.

Discussion

Consistent with studies on other avian species, male Cassin's Sparrows increased their aggressive behavior when challenged with SPB. The Challenge Hypothesis led us to predict that this increase would be associated with elevated plasma T concentrations, but we found no evidence in support of this hypothesis. A failure to reject the null hypothesis (i.e., no effect of SPB on plasma T) does not lead us to accept it. However, we note that relatively large sample sizes (Expt. 1: $n = 48$), robust statistical analyses that account for individual variation, and multi-pronged statistical analyses strengthen our conclusion that SPB did not influence plasma T in the present study. Our failure to reject the null hypothesis is, therefore, not likely a result of low statistical power or the masking effect of individual variation. Birds were investigated at the beginning of their nesting season when plasma T in males of socially monogamous species usually is seasonally high. We hypothesized that the lack of endocrine response to SPB results from the HPG axis already secreting LH and T at the maximum physiological rate. This was, however, not the case because GnRH administration rapidly increased plasma LH and T, and oLH administration rapidly increased plasma T. Furthermore, the endocrine effects of GnRH or LH treatment did not differ whether sparrows had been exposed to SHORT or LONG SPB, showing that this exposure does not influence the sensitivity of the pituitary gland to GnRH or of the testes to LH.

The Challenge Hypothesis posits that birds can respond to social challenges by rapidly and transiently increasing their T secretion (Wingfield et al., 1990). It has been proposed that this increase promotes aggressive behavior and the persistence of this behavior even after the challenge has been withdrawn (Lynn et al., 2008; Wingfield, 1994b), thereby potentially

enhancing territoriality and the probability of winning future interactions (Oyegbile et al., 2005). Exposure to STI, indeed, increases plasma T in territorial males of some species (McGlothlin et al., 2008; Wingfield and Wada, 1989; Wingfield and Hahn, 1994), but not in others (Apfelbeck and Goymann, 2011; Goymann, 2009; Landys et al., 2007; Lynn et al., 2007, 2008). Several factors may account for these differences.

One such factor is the subjects' HPG axis sensitivity to stimulation at the time of the study. Specifically, lack of hormonal responsiveness to STI in some studies may result from subjects being tested when their HPG axis was maximally active (Goymann, 2009). This could be the case particularly in monogamous birds studied at the beginning of their breeding season, as was the case here, when plasma T is at its seasonal highest (Horton et al., 2010; Van Duyse et al., 2003; Wingfield and Farner, 1978). If the HPG axis is operating at its physiologically maximum, exposure to a social challenge or other types of stimuli would be predicted not to enhance its activity. Generally supporting this idea, there is evidence that the HPG axis responsiveness varies across reproductive stages. For example, free-ranging male Dark-eyed Juncos, *Junco hyemalis*, have similar plasma T concentrations early and late in their nesting season, yet the increase in plasma T in response to GnRH injection is greater at the beginning than towards the end of the breeding season (Jawor et al., 2006). In captive male Cassin's Sparrows, administration of the GnRH secretagogue NMA to photostimulated birds with high plasma LH had little effect on the plasma concentrations of this hormone (Deviche et al., 2008). However, this treatment to short day-exposed males with low plasma LH stimulated LH release. A single study has, to our knowledge, tested the avian HPG axis functionality in the context of the Challenge Hypothesis. In this study on the male Black Redstart, plasma T did not increase in response to STI, but did so after GnRH injection (Apfelbeck and Goymann, 2011). Thus the lack of endocrine response to STI could not be accounted for by the HPG axis being maximally active. The present work supports and extends this conclusion. In the male Cassin's Sparrow, GnRH injection increased plasma LH and T, indicating that the pituitary gland and testes of experimental birds responded to GnRH and endogenous LH, respectively. The rapid increase in plasma T following oLH injection also supports the view that the testes of intact sparrows were not secreting T at the physiologically maximum rate. As in redstarts, the absence of T response to SPB in Cassin's Sparrows in the present study did, therefore, not stem from insensitivity of the pituitary gland or testes to relevant secretagogues.

Methodological differences between studies may also contribute to the fact that a social challenge rapidly increases plasma T in some situations but not others. Most avian studies examining the effect of STI on plasma T in males exposed birds to SPB concurrently with a conspecific decoy (see Introduction). The separate contribution of auditory and visual stimulation to the T response of challenged birds has to our knowledge been researched only in the male Song Sparrow (Wingfield et al., 1989). In this species, exposure to SPB increases plasma T less than exposure to SPB plus a live decoy bird. Cassin's Sparrows in the present investigation were exposed to auditory (SPB) but not visual and/or other (e.g., tactile) stimulation. Sparrows responded strongly to auditory stimulation behaviorally but not hormonally. Thus, as in the Song Sparrow, visual (and potentially tactile) stimulation may be necessary to induce a hormonal response in male Cassin's Sparrows. It should, however, be pointed out that as described above, plasma T does not always increase following exposure to STI consisting of SPB together with a decoy (Apfelbeck et al., 2011; Charlier et al., 2009; Gill et al., 2008; Moore et al., 2004; Schwabl et al., 2005). In two species (Great and Blue Tits), this exposure in fact decreases plasma T (Landys et al., 2007; Van Duyse et al., 2004). A combined auditory and visual challenge is, therefore, frequently insufficient or altogether ineffective to rapidly elevate plasma T. It has also been proposed that the number of broods regulates the response to STI, with males of multi-brooded but not single-brooded species increasing plasma T in response to STI (Gill et al., 2008; Landys et al., 2007). Cassin's Sparrows in part of their range (Texas; Schnase et al., 1991) are thought to commonly be double-brooded, but the reproductive biology of the species remains incompletely known and it has not been determined whether this is the case also for sparrows of the northern population investigated here. Thus, the present data neither support nor contradict the hypothesis that the androgen responsiveness to STI is related to the mating system.

As is commonly the case in other songbirds (Lynn et al., 2007; Schwabl et al., 2005; Silverin et al., 2004), male Cassin's Sparrows responded to SPB by increasing aggressive behavior, as shown by decreased latency to approach and increased amount of time spent close to the song playback device. Furthermore, sparrows responded similarly to SPB when exposed to this stimulus for the first or second time, indicating no short-term habituation or sensitization. As exposure to SPB increased aggressive behavior but this increase was not associated with elevated plasma T, we conclude that plasma T does not regulate short-term changes in aggressive behavior such as induced by SPB exposure. The results of other studies

led to a similar conclusion (Schwabl et al., 2005; Silverin et al., 2004). For example, when repeatedly exposed to STI over the course of several days, male Black Redstarts gradually increase the intensity of their aggressive response, but this increase is not associated with a change in plasma T (Apfelbeck et al., 2011). In Sonoran Desert songbirds, population differences in expression of territorial behavior are related to habitat characteristics but not plasma T (Fokidis et al., 2011) and in free-ranging Rufous-winged Sparrows, acute T administration does not increase aggressive behavior (Deviche, Davies, Gao, and Bittner, unpublished data). Rather, it seems that neural signaling mechanisms may control rapid changes in aggressive behavior, such as those induced by STI. Support for this proposition is provided by recent research in non-breeding Song Sparrows, *Melospiza melodia*, demonstrating that exposure to STI stimulates the brain conversion of the prohormone dehydroepiandrosterone to androstenedione (Pradhan et al., 2010). Estradiol acts centrally to mediate many behavioral effects of T (e.g., Schlinger and Callard, 1989, 1990) and in the White-crowned Sparrow, *Zonotrichia leucophrys*, stimulation of aggressive behavior by STI is associated with rapid changes in concentration of estrogen and aromatase, the enzyme that converts androgens into estrogens, in discrete brain regions (Charlier et al., 2011). Species in which short-term changes in aggressive behavior are not paralleled with changes in plasma T may be particularly well suited to pursue research on the rapid neuroendocrine signaling mechanisms that control this behavior.

As discussed above, much research in behavioral endocrinology has investigated the facultative regulation of plasma androgens, in particular the potentially stimulatory role of social interactions on the circulating levels of these hormones. Mounting evidence indicates that plasma androgens can also rapidly and facultatively decrease, e.g., in response to acute stress (Deviche et al., 2010, 2012; Lynn et al., 2010; Moore et al., 2002; Wingfield et al., 1982). Consistent with these studies, we found in Cassin's Sparrows that plasma T decreases by approximately 35% in response to capture and restraint for 20 minutes. As the above studies also show, the inhibitory effect of stress on plasma T was not associated with a decrease of plasma LH, suggesting that it did not result from impairment of pituitary gland function. The mechanism by which acute stress rapidly decreases plasma T in birds and the role of this decrease are poorly understood (Deviche et al., 2010, 2012). Nevertheless, these observations emphasize the fact that plasma T, besides being seasonally regulated, is susceptible to facultative modulation not only by stimulatory factors, but also by rapidly acting inhibitory

factors. More research is warranted to identify the factors that account for individual variation in plasma hormone concentrations within a given population and the potential role of this variation in the control of physiological and behavioral responses.

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Figure legends

Figure 1. Schematic diagram of the experimental design. Ninety-eight adult male Cassin's Sparrows, *Peucaea cassini*, were randomly divided into three groups used for Experiments 1, 2, and 3. Birds were exposed to a short (SHORT) or long (LONG) period of conspecific song playback (SPB). Short and long SPB-exposed sparrows in Experiments 1 and 2 were then randomly divided into three (Experiment 1) or two (Experiment 2) groups that received an injection of gonadotropin-releasing hormone (GnRH), ovine luteinizing hormone (oLH) or control saline solution (Sal) as indicated in the figure. Birds were bled before and after hormone administration. Plasma samples were assayed for testosterone (T, Experiment 1) or luteinizing hormone (LH; Experiment 2). Numbers in parentheses indicate sample sizes.

Figure 2. Plasma testosterone (ng/ml; upper panel; $n = 48$) and luteinizing hormone (LH, ng/ml; lower panel; $n = 32$) of intact adult male Cassin's Sparrows, *Peucaea cassini*, as a function of the time of day when birds were caught. Blood was collected from each bird within 3 min of capture. Each point represents a different individual.

Figure 3. Plasma testosterone (means \pm S.E.; $n = 8$ birds/group) of adult male Cassin's Sparrows, *Peucaea cassini*, after exposure to a short (average: 4.1 min; SHORT SPB) or long (average: 36.2 min; LONG SPB) period of conspecific song playback (SPB). Males were bled within 3 min of capture (initial bleed), received an intravenous injection of control saline, ovine luteinizing hormone (oLH), or gonadotropin-releasing hormone (GnRH), and were bled again 20 min later (postinjection bleed).

Figure 4. Relationship between duration of exposure to SHORT or LONG duration conspecific song playback (SPB) and plasma testosterone (left panels) and luteinizing hormone (LH, right panels) in intact adult male Cassin's Sparrows, *Peucaea cassini*. Birds at the end of the SPB exposure were bled within 3 min of capture. Each point represents one individual.

Figure 5. Plasma luteinizing hormone (LH; means \pm S.E.; $n = 6-9$ birds/group) of adult male Cassin's Sparrows, *Peucaea cassini*, after exposure to a short (average: 2.3 min; SHORT SPB) or long (average: 34.9 min; LONG SPB) period of conspecific song playback (SPB). Males were

bled within 3 min of capture (initial bleed), received an intravenous injection of control saline or gonadotropin-releasing hormone (GnRH), and were bled again 3 min later (postinjection bleed).

Figure 6. Behavioral response (median + 0.5 interquartile interval of five independent behavioral patterns) of free-ranging adult male Cassin's Sparrows, *Peucaea cassinii*, to conspecific song playback for 10 min. Experimental birds ($n = 10$) were exposed to song playback. Control birds ($n = 8$) were exposed to the same setting as Experimental birds, but without song playback. * denotes a statistically significant between Control and Experimental birds (Mann-Whitney rank sum test; $p < 0.05$).

Figure 7. Behavioral response (median + 0.5 interquartile interval of five independent behavioral patterns) of free-ranging adult male Cassin's Sparrows, *Peucaea cassinii*, to conspecific song playback (SPB) exposure for 10 min. Experimental birds ($n = 10$) had been exposed to SPB for 30 consecutive minutes before the behavioral test (see Fig. 5) whereas Control birds ($n = 8$) had not been exposed to SPB prior to this test.

Figure 1

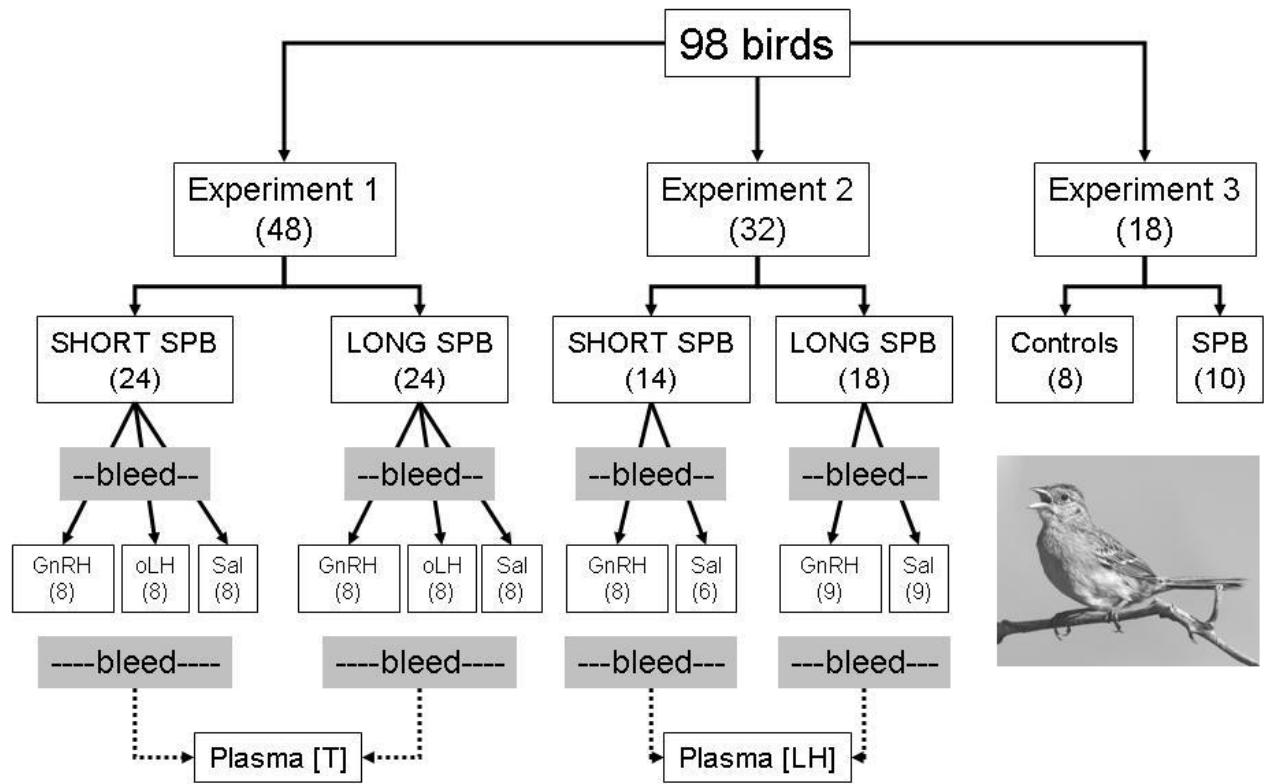


Figure 2

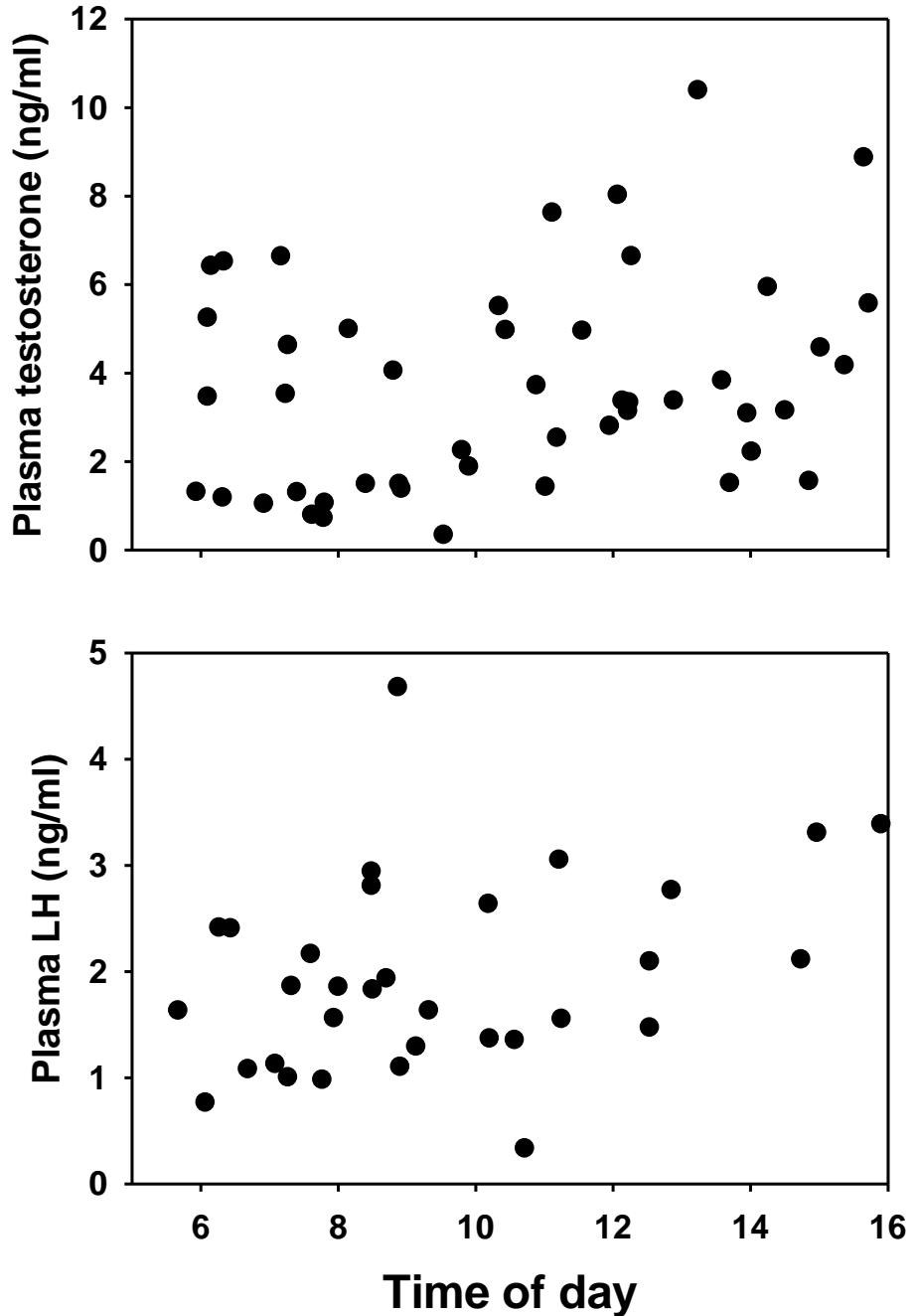


Figure 3

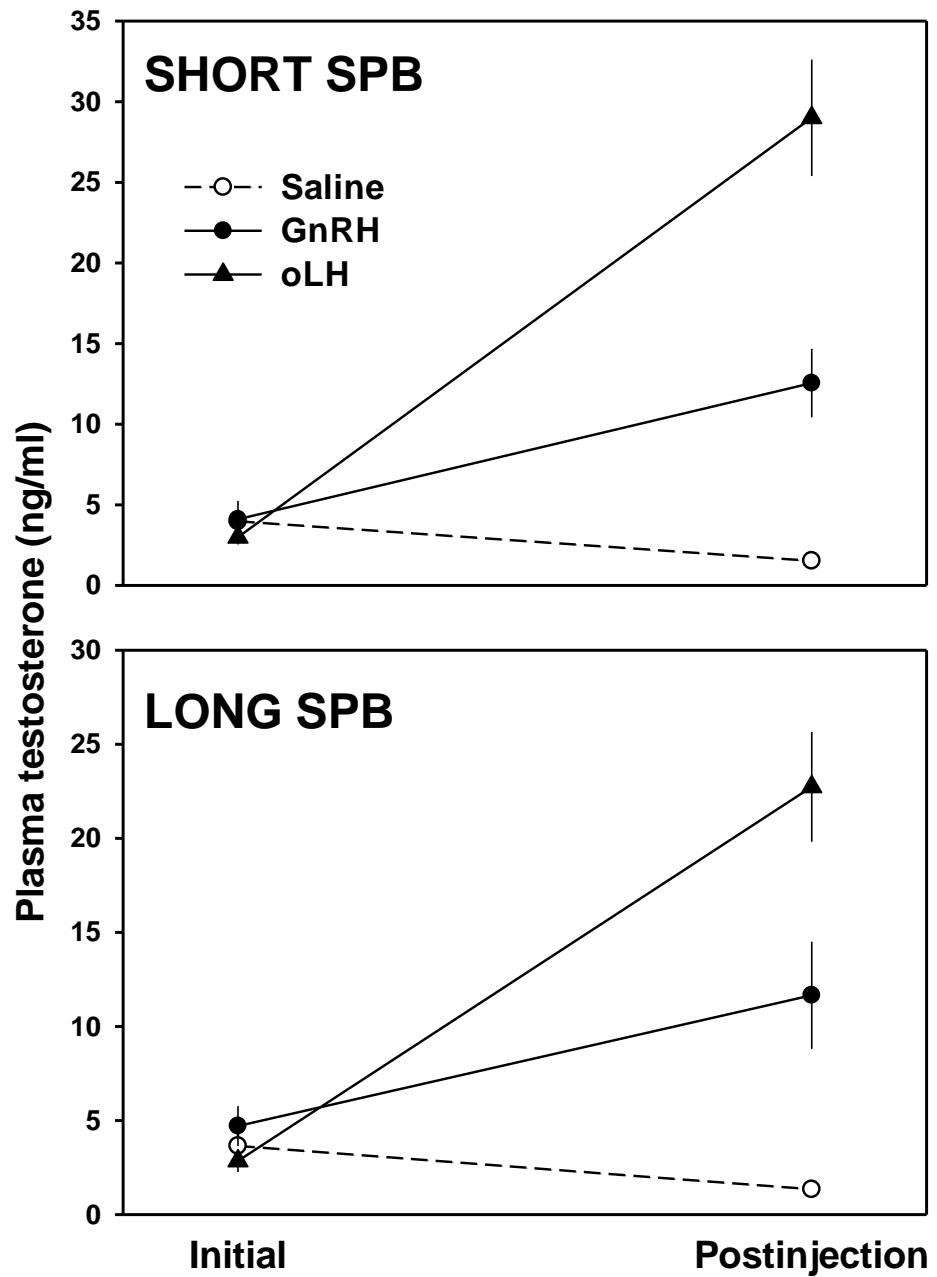


Figure 4

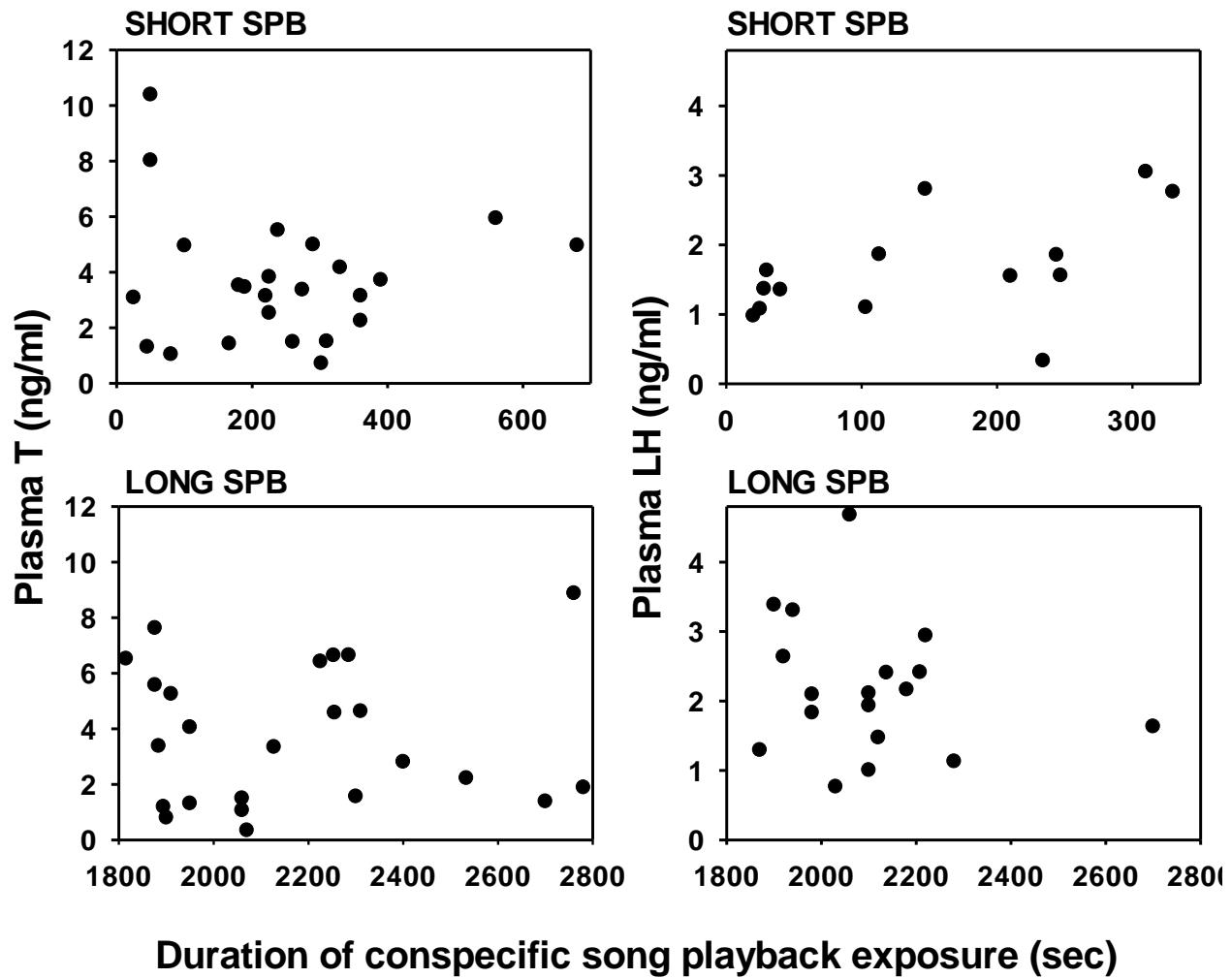


Figure 5

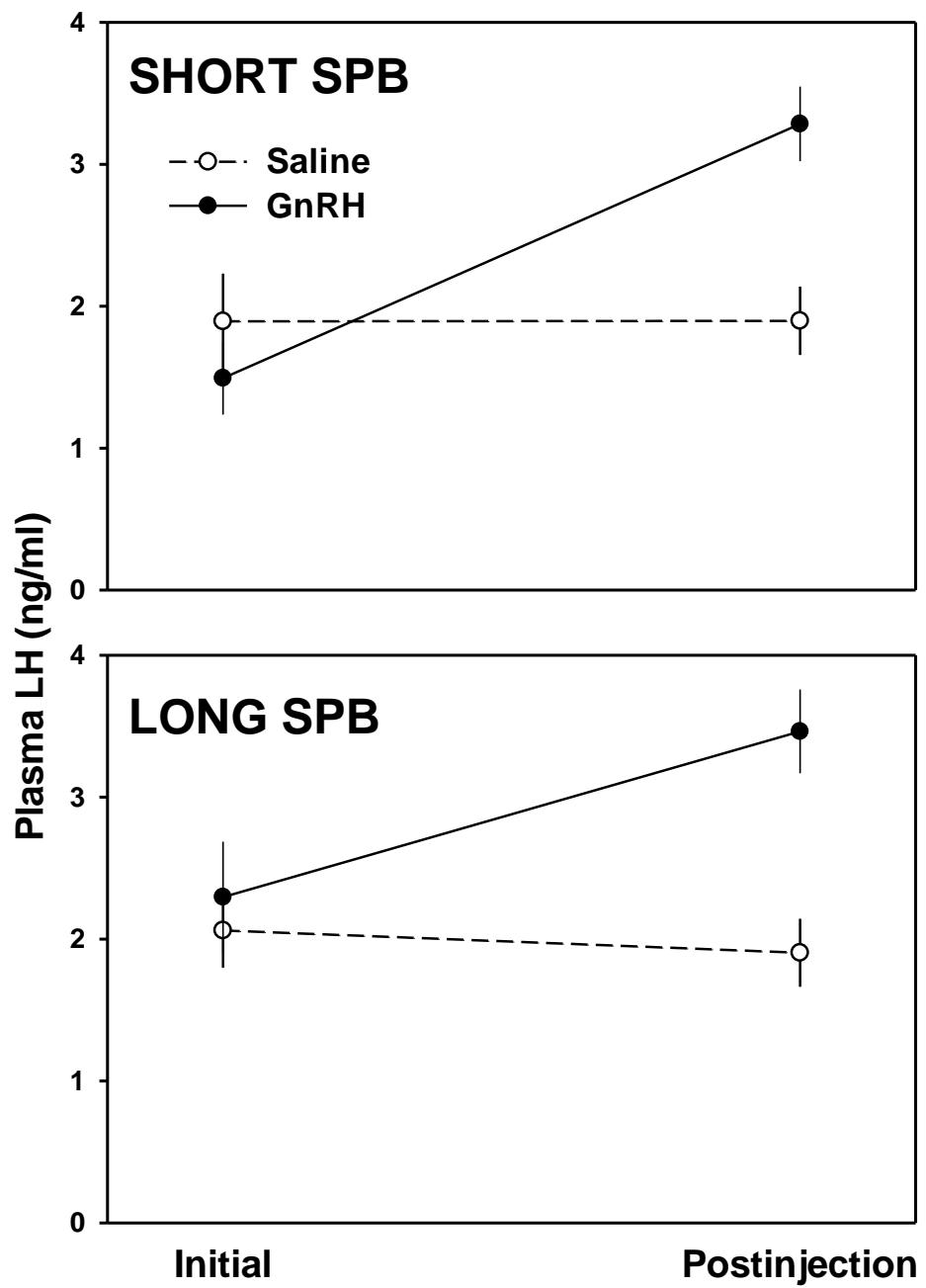


Figure 6

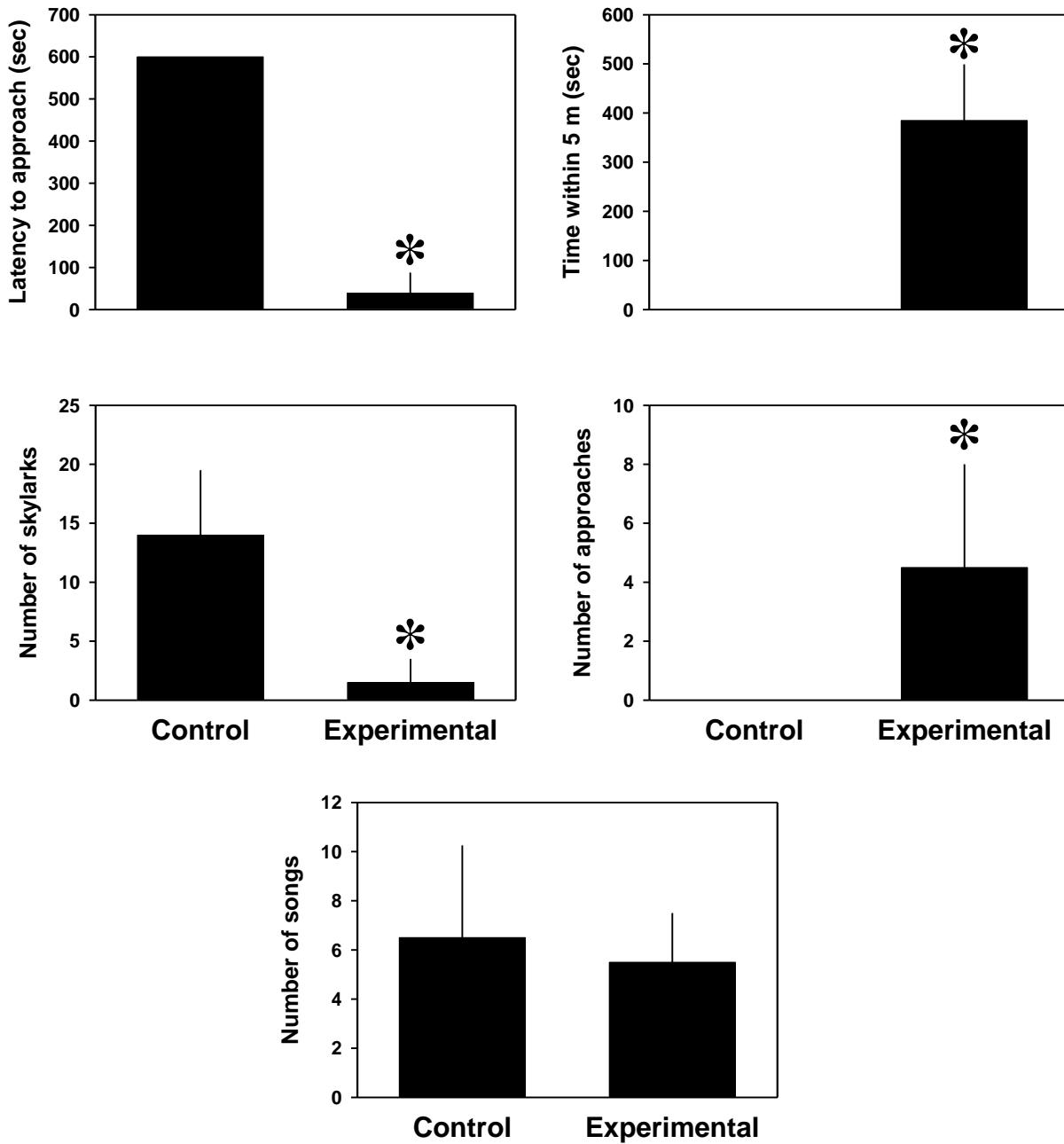


Figure 7

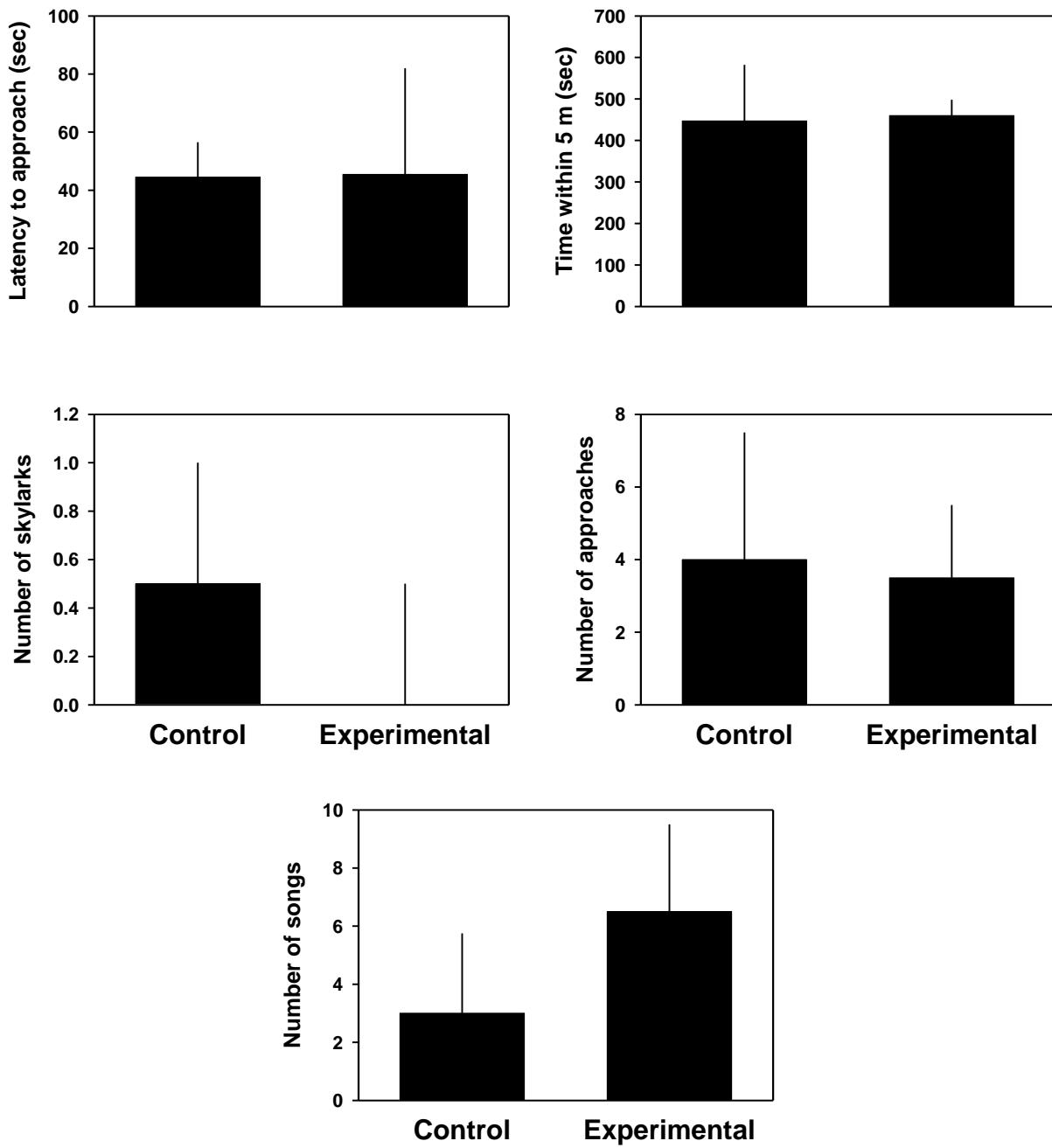


Table 1. Analysis of variance for the plasma testosterone data of Experiment 1. Adult male Cassin's Sparrows, *Peucaea cassinii*, were exposed to short or long duration conspecific song playback (= SPB), bled, injected with ovine luteinizing hormone, gonadotropin-releasing hormone, or control saline solution (= Hormone treatment), and then bled again (see text for details). For each factor and their interaction the table shows the degrees of freedom (DF) and the corresponding F value and probability (p).

Term	Numerator DF	Denominator DF	F	p
(Intercept)	1	42	2783.613	<0.0001
Exposure to SPB	1	42	1.1083	0.2985
Hormone treatment	2	42	66.0967	<0.0001
Exposure to SPB x Hormone treatment	2	42	0.3785	0.6872

Table 2. Analysis of variance for the plasma luteinizing hormone data of Experiment 2. Adult male Cassin's Sparrows, *Peucaea cassinii*, were exposed to short or long duration conspecific song playback (= SPB), bled, injected with ovine luteinizing hormone or control saline solution (= Hormone treatment), and then bled again (see text for details). For each factor and their interaction the table shows the degrees of freedom (DF) and the corresponding F value and probability (p).

Term	Numerator DF	Denominator DF	F	p
(Intercept)	1	28	35942.13	<0.0001
Exposure to SPB	1	28	3.94	0.0571
Hormone treatment	1	28	31.52	<0.0001
Exposure to SPB x Hormone treatment	1	28	0.66	0.4248