



Reproductive state modulates testosterone-induced singing in adult female European starlings (*Sturnus vulgaris*)



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ABSTRACT

European starlings (*Sturnus vulgaris*) exhibit seasonal changes in singing and in the volumes of the neural substrate. Increases in song nuclei volume are mediated at least in part by increases in day length, which is also associated with increases in plasma testosterone (T), reproductive activity, and singing behavior in males. The correlations between photoperiod (i.e. daylength), T, reproductive state and singing hamper our ability to disentangle causal relationships. We investigated how photoperiodic-induced variation in reproductive state modulates the effects of T on singing behavior and song nuclei volumes in adult female starlings. Female starlings do not naturally produce measurable levels of circulating T but nevertheless respond to exogenous T, which induces male-like singing. We manipulated photoperiod by placing birds in a photosensitive or photorefractory state and then treated them with T-filled or empty silastic implants. We recorded morning singing behavior for 3 weeks, after which we assessed reproductive condition and measured song nuclei volumes. We found that T-treated photosensitive birds sang significantly more than all other groups including T-treated photorefractory birds. All T-treated birds had larger song nuclei volumes than with blank-treated birds (despite photorefractory T-treated birds not increasing song-rate). There was no effect of photoperiod on the song nuclei volumes of T-treated birds. These data show that the behavioral effects of exogenous T can be modulated by reproductive state in adult female songbirds. Furthermore, these data are consistent with other observations that increases in singing rate in response to T are not necessarily due to the direct effects of T on song nuclei volume.

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Introduction

A complex web of endogenous and exogenous cues controls singing in temperate-zone songbirds. External cues such as photoperiod and the social milieu can interact with a bird's hormonal and physiological state (including the state of neural control systems) to determine the rate and quality of singing (Tramontin and Brenowitz, 2000; Catchpole and Slater, 2008; Kroodsma, 2005). For example, increases in photoperiod are known to induce increases in plasma testosterone (T), singing behavior, and the volumes of song nuclei in male European starlings (Bernard and Ball, 1995; Ritters et al., 2000; Dawson et al., 2001; Stevenson and Ball, 2010).

The administration of exogenous T can induce an increase in singing and the volumes of song nuclei in male and some female songbirds independent of photoperiodic cues, indicating that the effects of

photoperiod on song behavior and underlying brain plasticity may be mediated by T (Nottebohm, 1980; Hausberger et al., 1995; Harding, 2004; see Schlinger and Brenowitz, 2002 for a review). There is evidence that reproductive state (e.g. breeding, non-breeding, or pre-breeding) can modulate T-induced behavioral and neural changes in mammals and birds (Campbell et al., 1978; Ellis and Turek, 1983; Bernard et al., 1997; Smith et al., 1997). Data collected from male starlings suggest that a pre-breeding 'photosensitive' state increases sensitivity to exogenous T on song nuclei volumes, resulting in larger volumes of the song nucleus HVC in photosensitive compared with non-breeding 'photorefractory' birds treated with similar doses of T (Bernard and Ball, 1997). This is not true of all temperate-zone songbirds; photoperiod (i.e. reproductive state) does not alter the effects of exogenous T in male song sparrows (*Melospiza melodia*; Nowicki and Ball, 1989; Ball and Nowicki, 1990). The interrelation of photoperiod, steroids, song nuclei plasticity, and song behavior are not well understood.

European starlings are an excellent model system to study the modulatory role of photoperiod on T-induced singing behavior and song quality. Starlings, as in a number of other songbird species, display absolute photorefractoriness after exposure to long day lengths for an extended period of time (weeks to months of exposure to "long days"

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depending on species; Burger, 1947; Nicholls et al., 1988; Dawson et al., 2001). This state of photorefractoriness is characterized by regression of the gonads to a prepubescent-like state, as well as an inability to respond to long day lengths including photoperiods of constant daylight (Burger, 1947; Dawson et al., 1985; Falk and Gwinner, 1988; MacDougall-Shackleton et al., 2009). When males are exposed to short day lengths for an extended period of time, the birds become photosensitive (i.e., responsive) to the stimulating effects of long day lengths (Nicholls et al., 1988; Dawson et al., 2001).

Photoperiod is also involved in the control of the reproductive cycle in female starlings. This regulation does not involve changes in concentrations of T circulating in plasma (Dawson and Goldsmith, 1983; Dawson, 1984, 1997; Stevenson et al., 2012). In addition, natural singing behavior in female starlings is partially regulated by photoperiod (Pradhan et al., 2008). Female starlings tend to sing in the non-breeding season when day lengths are short and significantly reduce song output in the breeding season when day lengths increase and male conspecifics compete for mates (Pavlova et al., 2005; Pavlova et al., 2007a, 2007b).

However, adult female starlings respond to exogenous administration of T by singing male-like songs (Hausberger et al., 1995; De Ridder et al., 2002). These findings suggest that the activational effects of T largely cause sex differences in song behavior in adult starlings rather than organizational effects during ontogeny (Arnold et al., 1996; Wade and Arnold, 2004). Adult female starlings treated with T provide a useful model system to investigate the activational properties of T independent of prior exposure to male-like concentrations of circulating T.

We used female starlings to study how the effects of T on singing behavior, song quality, and song system morphology are modulated by photoperiodic (i.e. reproductive) state. It has been assumed that T-induced changes in females are modulated by the same mechanisms as in males (Madison et al., 2014), but this has not been tested. Based on the hypothesis that reproductive state can modulate the effectiveness of T on brain and behavior, we predicted that photosensitive females would be more responsive to the effects of T and exhibit increased song behavior compared to photorefractory females treated with T and sham-treated controls. Finally, we predicted that the volumes of song nuclei would be larger in T-treated photosensitive female starlings compared with photorefractory females treated with T and sham-treated controls.

Methods

Animals and photoperiodic treatments

Twenty-eight wild-caught adult female European starlings were used in this experiment. All birds were captured using a drop down V-trap in early March 2007 and late February 2013. Upon arrival in the laboratory, birds were group-housed and maintained on a natural photoperiod (8 L:16 days; lights on at 1200 h EDT, lights off at 2000 h). Shortly after arrival all birds were laparotomized and the gonads examined in order to confirm sex and assess reproductive condition. All birds were housed in groups on 8 L:16 days for 6 to 7 months before the start of the experiment to maintain a photosensitive state. Animal husbandry of the starlings was in accordance with guidelines published by the National Research Council (2011). All experimental procedures were approved by the Johns Hopkins University Animal Care and Use Committee and adhered to standards of the Society for Neuroscience.

The twenty-eight female starlings were randomly assigned to one of two photoperiodic conditions: 1) long-day photorefractory or 2) short-day photosensitive. Fourteen birds were transferred to group housing on a long-day photoperiod (16 L:8 days; lights on 0700 h EDT, lights off 2300 h EDT) and fourteen birds remained in group housing on a short-day photoperiod (8 L:16 days; lights on at 1200 h EDT, lights off at 2000 h EDT) to maintain a photosensitive state.

T-implantation

Previous data have shown that housing starlings on long-days for a minimum of 8 to 10 weeks can induce photorefractoriness, as determined by gonad size, molt and beak score (Dawson and Goldsmith, 1983). After 10 weeks, both the photosensitive and the photorefractory groups received either a single 10 mm length Silastic capsule (1.47 mm i.d., 1.96 mm o.d.) containing crystalline T ($n_{\text{photosensitive}} = 7$; $n_{\text{photorefractory}} = 7$) or a 10 mm length empty Silastic capsule (1.47 mm i.d., 1.96 mm o.d.) blank implant ($n_{\text{photosensitive}} = 7$; $n_{\text{photorefractory}} = 7$). Implants were inserted through a small incision (approx. 2–3 mm) over the left flank subcutaneously. Immediately following implantation, birds were transferred to individual sound attenuated chambers. Photoperiod was held constant for each individual bird. Birds were implanted for a total of 3 weeks and during that time behavioral measurements were taken daily.

Behavioral measurements

Bird vocalizations were recorded daily using an electret microphone (Radioshack Model 33-3013) and digitized using a custom software package with 16-bit resolution and 44.1 kHz sampling rate. Over the 3-week experimental manipulation, song samples were automatically recorded for a 2-hour period starting when the lights were turned on in the morning. Recordings were high pass filtered with a cutoff frequency of 900 Hz.

Songs were operationally defined as periods of at least 5 s of singing with no more than 3 s of silence (see Bernard, Eens, and Ball, 1996). The total number of songs per recording per day was tallied. The daily count was averaged across the week of treatment (i.e. week 1, week 2 and week 3). In addition, birds that sang complete song bouts were identified for further behavioral analysis. Complete song bouts were defined as songs with at least three of the four phrase-types characteristic of male starling song. These songs were then quantified for a motif analysis to provide the relative size of the bird's vocal repertoire.

As previously stated, only complete song bouts (i.e. songs where at least three of the four phrase types characteristic of starling song were present) were used for the motif analysis. Not all birds sang complete song bouts. As a result only a subset of the birds were included in this analysis (photosensitive + T, $n = 5$; photorefractory + T, $n = 4$). One photosensitive control and one photorefractory control were observed singing complete bouts during the 3-week treatment period. However, since there was only 1 animal in each group, we could not include them in the statistical analysis of repertoire and do not present their data, as we cannot make a valid comparison to the T-treated groups.

The motif analysis was used to estimate the repertoire size of individual birds. Sound analysis was done using Ravenlite (Cornell Ornithology Lab, Ithaca, NY) and each song ($N = 1,849$) was visualized using oscillograms and spectrograms. Each unique motif/phrase was counted and categorized into one of four phrase types characteristic of starling song (whistle, variable/warble, rattle, and high frequency phrase types; Eens, 1997; see Fig. 1).

Peripheral physiology

Beak color was assessed before and after hormonal implantation. In starlings, beak color ranges from yellow to black (Hicks, 1934; Kessel, 1957). Bright yellow beaks indicate that testosterone is present in the blood while black beaks indicate that little or no T is available in the blood (Ball and Wingfield, 1987). The beak is therefore an indicator of hormonal condition. Beak scoring was as follows: 0 – completely black beak; 1 – 2/3 black beak with 1/3 yellow at the base; 2 – 2/3 yellow beak with 1/3 black toward the tip; and 3 – completely yellow beak (Wydoski, 1964).

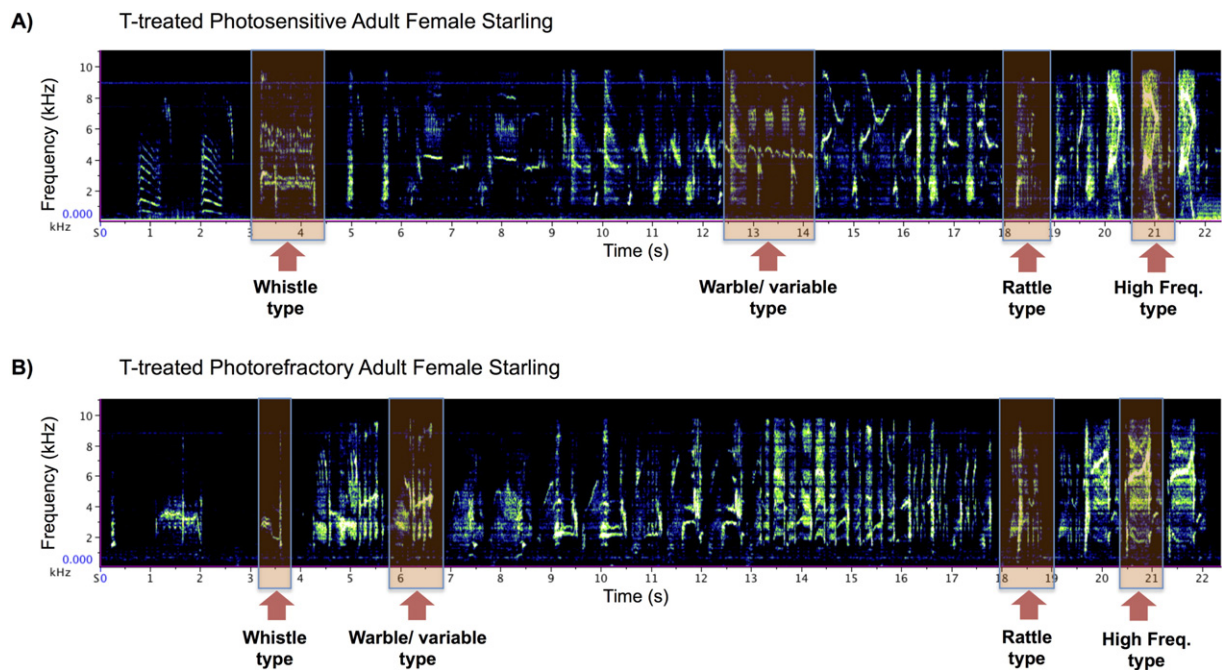


Fig. 1. Sound spectrographs of T-treated adult female starling songs. The x-axis represents time and the y-axis frequency. Amplitude is represented by the brightness of the vocalization (dark = low amplitude, bright = high amplitude). Displayed are songs from two different T-treated females in two different photoperiodic conditions taken from the same day of study, A) a photosensitive T-treated female starling and B) a T-treated photorefractory female starling. As illustrated, starling song is a complex arrangement of harmonically variable vocalizations and though rich in acoustic diversity the various syllable phrases that make up starling song can be characterized into one of four motif or phrase types. The labeled boxes highlight exemplars of the four phrase types; whistle, warble/variable, rattle, and high frequency phrases.

Radioimmunoassay

Blood samples were taken before T implantation and at the end of the experiment via puncture of the alar wing vein with a 25-gauge needle. 300–500 μ l of blood was collected into caraway capillary tubes at both sampling times. The blood samples were transferred into tubes and centrifuged at 9000 rpm for 15 min. The plasma was removed and stored in vials at -20° prior to T assay. Plasma T concentrations were analyzed in a single run of duplicates (50 μ l) using a commercially available 125I Coat-A-Count kit for total testosterone (Siemens Medical Solutions Diagnostics, Los Angeles, CA). This kit provides reliable hormone concentrations and has been validated for and previously used in starlings (Stevenson et al., 2008; Stevenson and Ball, 2009; Cornil et al., 2009). The antiserum is highly specific for testosterone (i.e. 100 pg/ml) and shows negligible cross reactivity with other steroids including dihydrotestosterone (<3.5%); 17β -estradiol (<0.01%); and corticosterone (<0.01%).

Perfusion & peptide immunocytochemistry

At the end of the experiment, birds were deeply anesthetized with secobarbital (50 mg/ml IM) and perfused transcardially with heparinized 0.1 M phosphate buffered saline (PBS) pH 7.5, followed by 4% paraformaldehyde. The brains were extracted, weighed, and placed in 4% paraformaldehyde and left overnight at 4° C. Immediately following that the ovaries and oviducts were dissected out and the wet weight was measured and recorded. The following morning, the brains were transferred into a sucrose solution (30% sucrose in 0.1 M PBS) and left overnight at 4° C. The brains were frozen with dry ice for 2 min and then stored in a freezer (-70° C) until sectioning. Brains were sectioned coronally (40 μ m thick) using a cryostat.

Every third section was mounted onto gelatin-coated slides and Nissl-stained with thionin. Adjacent sections were processed with immunocytochemical techniques for enkephalin (ENK). The boundaries of the forebrain nucleus lateral magnocellular nucleus of the anterior

nidopallium (LMAN) and medial magnocellular nucleus of the anterior nidopallium (MMAN) cannot be reliably discerned in Nissl-stained material but can be distinguished in ENK positive material (Ball et al., 2008; Bottjer and Alexander, 1995; Stevenson and Ball, 2010). The boundaries of HVC are clearly defined by both Nissl and ENK immunoreactive fiber staining (Stevenson and Ball, 2010).

The brains were processed in random order such that the time from tissue collection to processing was similar across groups. Brain sections were washed in 0.1 M PBS twice, once in 0.5% H_2O_2 for 15 min., then washed three times in 0.1 M PBS and left overnight in normal goat serum (20% solution in 0.3% PBS/T [Triton X]) at 4° C. Sections were incubated in primary antibody (1:2000 for ENK) for 24 h. In the morning the sections were washed three times with 0.1% PBS/T, then incubated in biotinylated secondary antibody (goat anti-rabbit IgG, 1:250) for 1 h, washed three times in 0.1% PBS/T, incubated in avidin biotin horseradish-peroxidase complex (Vectastain ABC, Elite Kit 1:200) for 1 h, and then washed again three times in 0.1% PBS/T. The sections were then incubated in biotinylated tyramine (1:150 in PBS/T) for 1 h, washed three times in 0.1% PBS/T, incubated in streptavidin horseradish peroxidase (1:200 in PBS/T) for 1 h, and then washed another three times in 0.1% PBS/T. The antibodies were visualized by incubating the sections with diaminobenzidine (Sigma Fast DAB) for 6 min. Finally, sections were washed three times with 0.1 M PBS and mounted onto gelatin coated microscope slides. Sections were then serially dehydrated in ethanol and then placed in xylene for 5 min. The slides were then coverslipped using Permount (Fisher).

Song nuclei volume reconstruction

Digital images of brain sections containing the regions of interest were captured using a microscope with a CCD camera connected to a computer. Digital images of the brain images were analyzed and the peptidergic-defined boundaries for each nucleus were traced using Openlab (Improvision® a Perkin-Elmer company). The regions of interests were Area X, the lateral magnocellular nucleus of the anterior nidopallium (LMAN), HVC (used as its proper name), and the robust

nucleus of the arcopallium (RA). The volume of each region of interest was reconstructed; the areas of sections containing the region of interest were summed with the sampling interval (120 μm) using the formula for a truncated cone (developed by Smith et al., 1995). This method has been used previously in European starlings (Bernard and Ball, 1995; Bentley et al., 1999; Bernard and Ball, 1997). For each bird, both the left and right hemispheres were measured and the average between hemispheres was calculated. Volumes of each nucleus were measured and normalized to the post-perfusion brain weight of the individual, which was weighed immediately after brain extraction, just prior to post-fixation in 4% paraformaldehyde. After the raw value of the nuclei volume was calculated. The raw value was normalized by dividing the nuclei volume by the total brain weight in grams. Prior to normalization, the mean volumes of the song nuclei were well within the previously reported range(s) for untreated adult female starlings (Ball et al., 1994; Bernard et al., 1993).

Statistical analysis

The open source programming language R (version 3.1.0) was used for statistical analyses; we used the statistical, nlme, and ggplot2 packages (R Core Team, 2013; Pinheiro et al., 2014; Wickham, 2009). We analyzed the song rate data using a mixed-design analysis of variance (ANOVA). For the repeated measure song rate (mean daily song count by week) and interaction variables (week by photoperiod, week by T-treatment, and week by photoperiod by T-treatment), *p*-values for effects were corrected using a Greenhouse–Geisser correction for non-sphericity. Since the variables photoperiod and T-treatment are binary, post-hoc tests were not necessary for these main effects. Post-hoc pairwise comparisons were made for the main effect of the repeated measures variable, as well as all significant interaction effects. All pairwise comparisons were corrected for multiple comparisons using Westfall's procedure (Westfall, 1997; Bretz et al., 2010). All possible pairwise combinations for the main and interaction effects were tested.

For the physiological measures (i.e. T-RIA, beak score, ovary and oviduct weight) and the brain volume data, a MANOVA was used to test for significant effects of treatment and interaction effects. For statistically significant interaction effects in the MANOVA, post-hoc pairwise comparisons were made and were corrected for multiple comparisons using Westfall's procedure (Westfall, 1997; Bretz et al., 2010). All possible pairwise combinations for significant interaction effects were tested.

The number of song samples used for motif analysis varied dramatically between individuals and groups, which resulted in a multimodal distribution of songs per sampling period. Further, not all birds sang complete bouts during the course of treatment, leaving unequal sizes for final motif analyses. This non-normal distribution of singing and unequal group sizes made parametric statistical analysis inappropriate. We used non-parametric statistics (Wilcoxon rank sum test) to test for effects of treatment on the number of unique phrases because equal sample size and normal distribution are not required for assessment of independent observations. All statistically significant differences reported were evaluated with respect to $\alpha = 0.05$. For statistically significant main and interaction effects, generalized eta squared values are reported. For statistically significant pairwise comparisons of the main and interaction effects, Cohen's *d* values are reported.

Results

Effect of T on song rate and syllable repertoire

The latency to sing varied widely by individual and by treatment. Indeed, some birds did not sing over the course of treatment; those birds were included in the analysis of the data unless otherwise noted. There was a significant main effect of photoperiod ($F(1,24) = 4.39$, $p < 0.05$, $\eta^2 = 0.12$) on the overall rate of singing (mean number of

song bouts per recording period); photosensitive birds sang more than photorefractory. Likewise, there was a significant main effect of T-treatment ($F(1,24) = 5.31$, $p < 0.05$, $\eta^2 = 0.14$) on the overall rate of singing; T-treated birds sang more than blank-treated birds. Though there was a trend in the interaction between photoperiod and T-treatment, the interaction effect was not statistically significant ($F(1,24) = 3.72$, $p = 0.07$).

Interestingly, there was a significant main effect of the repeated measures variable week of observation ($F(2,48) = 4.20$, $p < 0.05$, $\eta^2 = 0.09$). Likewise, there were significant interaction effects with this variable; namely, there was a significant interaction between week and photoperiod ($F(2,48) = 5.87$, $p < 0.05$, $\eta^2 = 0.13$), week and T-treatment ($F(2,48) = 4.84$, $p < 0.05$, $\eta^2 = 0.11$), and a three way interaction of week, photoperiod, and treatment ($F(2,48) = 6.03$, $p < 0.05$, $\eta^2 = 0.13$; Fig. 2).

The main effect of week was further investigated. Pairwise post-hoc comparisons showed that birds sang more during week 3 of treatment compared to week 1 ($z = 2.354$, $p < 0.05$, $d = 0.44$). However, there was no difference between week 3 and week 2 of treatment ($z = 1.04$, $p = 0.30$) or week 2 and week 1 of treatment ($z = 1.31$, $p = 0.19$). The two-way interaction between week and photoperiod was further investigated. Pairwise post-hoc comparisons showed that during week 1 and week 2, the effect of photoperiod was not statistically significant; however, the effect of photoperiod during week 2 approached a statistical trend ($z_{\text{week1}} = 0.81$, $p = 0.42$; $z_{\text{week2}} = 1.92$, $p = 0.05$). Further, during week 3 the effect of photoperiod was significant; photosensitive birds sang more than photorefractory birds ($z = 1.98$, $p < 0.05$, $d = 0.37$). The two-way interaction between week and T-treatment yielded a similar pattern. Pairwise post-hoc comparisons showed that during week 1 and week 2, the effect of T-treatment was not statistically significant and only approached a statistical trend ($z = 1.69$, $p = 0.09$; $z = 1.83$, $p = 0.07$). Conversely, during week 3, T-treated birds sang more than blank-treated control birds ($z = 2.22$, $p < 0.05$, $d = 0.42$).

Furthermore, the three-way interaction of week of observation, photoperiod and T-treatment yielded a specific pattern of behavior in post-hoc comparisons. During weeks 2 and 3, differences in the interaction between photoperiod and T-treatment were significant. Specifically, photosensitive T-treated birds sang more than photorefractory T-treated ($z_{\text{week2}} = 3.05$, $p < 0.05$, $d = 0.82$; $z_{\text{week3}} = 3.08$, $p < 0.01$, $d = 0.82$), photosensitive blank-treated birds ($z_{\text{week2}} = 2.99$, $p < 0.05$, $d = 0.80$; $z_{\text{week3}} = 3.25$, $p < 0.01$, $d = 0.87$), and photorefractory blank-treated birds ($z_{\text{week2}} = 2.99$, $p < 0.05$, $d = 0.80$; $z_{\text{week3}} = 3.403$, $p < 0.01$, $d = 0.91$). Conversely, photorefractory T-treated birds did

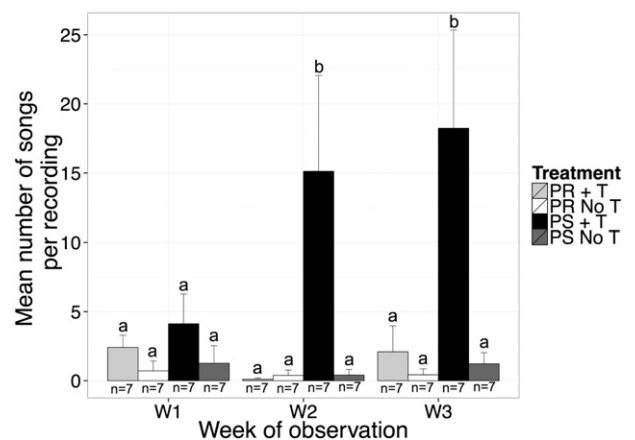


Fig. 2. Bar graph illustrating the mean number of songs produced per 2 hour recording session. Black bars represent T-treated photosensitive birds. Dark gray bars represent photosensitive control birds. Light gray bars represent T-treated photorefractory birds. White bars represent photorefractory controls. In general T was much more effective in inducing high rates of singing in photosensitive than in photorefractory female starlings. See Results section for more details.

not differ from photorefractory blank-treated birds ($z_{\text{week}2} = 0.06$, $p = 1.00$; $z_{\text{week}3} = 0.32$, $p = 0.95$) or photosensitive blank-treated birds ($z_{\text{week}2} = 0.06$, $p = 1.00$; $z_{\text{week}3} = 0.17$, $p = 0.95$) during week 1. Furthermore, photosensitive blank-treated birds did not differ from photorefractory blank-treated birds ($z_{\text{week}2} = 0.00$, $p = 1.00$; $z_{\text{week}3} = 0.15$, $p = 0.95$).

However, during week 1, differences in the interaction between photoperiod and T-treatment were not significant, specifically, photosensitive T-treated birds did not differ from photorefractory T-treated ($z = 0.88$, $p = 0.61$), photosensitive blank-treated birds ($z = 1.47$, $p = 0.31$), and photorefractory blank-treated birds ($z = 1.75$, $p = 0.30$) during week 1. Likewise, photorefractory T-treated birds did not differ from photorefractory blank-treated birds ($z = 0.87$, $p = 0.66$) or photosensitive blank-treated birds ($z = 0.59$, $p = 0.66$) during week 1. Finally, photosensitive blank-treated birds did not differ from photorefractory blank-treated birds ($z = 0.28$, $p = 0.78$) during week 1.

Starling song is composed of a highly variable number of syllables and syllable/phrase types arranged into either long or short song bouts. Long song is complex, male-typical, and is composed of a diverse array of syllable/phrase types. We measured the relative repertoire size of birds that sang complete song bouts to further assess the quality/complexity of songs in birds that sang complete song bouts. Photorefractory T-treated singers tended to have a larger vocal repertoire compared with photosensitive T-treated singers (total number of unique phrases; Wilcoxon rank sum test, $W = 18.5$, $p < 0.05$, $d = 1.67$; Fig. 3A). However, they did not actually differ in the number of specific phrase types including whistle phrases ($W = 16$, $p = 0.17$), variable/warble phrases ($W = 7$, $p = 0.52$), rattle phrases ($W = 15$, $p = 0.25$), and high frequency phrases ($W = 14$, $p = 0.33$; Fig. 3B).

Circulating steroids and reproductive physiology

At the end of the experiment blood was collected and the concentration of testosterone (T) was measured. We found that there was a significant effect of T-treatment on the amount of T measured in plasma; T-treated birds had more T than blank-treated ($F(1,24) = 39.06$, $P < 0.001$, $\eta^2 = 0.59$; Fig. 4A). There was no effect of photoperiod on the amount of T measured in plasma ($F(1,24) = 1.80$, $p = 0.19$). Likewise, there was no effect of the interaction between photoperiod and T-treatment ($F(1,24) = 1.80$, $p = 0.19$).

Exogenous T in male starlings is known to change the color of the beak from black to yellow (Goldsmith and Nicholls, 1984). We measured the beak scores of all birds both pre- and post-treatment. All of the birds started with black, low-score beaks. At the end of the study, there was a significant main effect of photoperiod on beak scores

($F(1,24) = 66.15$, $p < 0.001$, $\eta^2 = 0.28$; Fig. 4B). In addition, there was a significant main effect of T-treatment on beak scores ($F(1,24) = 79.35$, $p < 0.001$, $\eta^2 = 0.34$). Likewise, there was a significant interaction effect between photoperiod and T-treatment ($F(1,24) = 66.15$, $p < 0.001$, $\eta^2 = 0.28$). Photosensitive T-treated females had higher beak scores than T-treated photorefractory ($z = 11.50$, $p < 0.001$, $d = 3.07$), photorefractory controls ($z = 12.05$, $p < 0.001$, $d = 3.22$), and photosensitive controls ($z = 12.05$, $p < 0.001$, $d = 3.22$). Photorefractory T-treated females were not different from photorefractory controls ($z = -0.55$, $p = 0.85$) or photosensitive controls ($z = -0.55$, $p = 0.85$) and the controls were not different from one another ($z = 0.00$, $p = 0.99$).

Ovaries and oviducts were extracted after transcardial perfusion and weighed (Fig. 5). We found that there was a significant main effect of photoperiod on the wet weight of the ovary; photosensitive birds had larger ovaries than photorefractory birds ($F(1,24) = 36.93$, $p < 0.001$, $\eta^2 = 0.40$). There was a significant main effect of T-treatment; T-treated birds had smaller ovaries than blank-treated birds ($F(1,24) = 14.75$, $p < 0.001$, $\eta^2 = 0.16$). In addition, there was a significant interaction effect of photoperiod and T-treatment ($F(1,24) = 16.92$, $p < 0.001$, $\eta^2 = 0.18$). Photosensitive controls had the heaviest ovaries compared with T-treated photosensitive females ($z = 5.62$, $p < 0.001$, $d = 1.50$), T-treated photorefractory females ($z = 7.01$, $p < 0.001$, $d = 1.87$), and photorefractory controls ($z = 7.21$, $p < 0.001$, $d = 1.93$). Photorefractory control females were not different from photorefractory T-treated birds ($z = -0.19$, $p = 0.85$) or photosensitive T-treated birds ($z = 1.58$, $p = 0.25$) and the T-treated birds were not different from one another ($z = 1.39$, $p = 0.25$). There was a significant main effect of T-treatment on the weight of the oviduct; T-treated birds had smaller oviducts compared with blank-treated birds ($F(1,24) = 5.73$, $p < 0.05$, $\eta^2 = 0.19$). There was no effect of photoperiod on oviduct weight ($F(1,24) = 1.18$, $p = 0.29$). Likewise, there was no effect of the interaction of photoperiod and T-treatment ($F(1,24) = 0.06$, $p = 0.80$).

Effect of T on volumes of song nuclei

We measured the volumes of the song nuclei Area X, LMAN, HVC, and RA, and found a significant main effect of T-treatment (Area X, $F(1,24) = 152.83$, $p < 0.001$, $\eta^2 = 0.86$; LMAN, $F(1,24) = 44.84$, $p < 0.001$, $\eta^2 = 0.64$; HVC, $F(1,24) = 117.38$, $p < 0.001$, $\eta^2 = 0.80$; RA, $F(1,24) = 115.79$, $p < 0.001$, $\eta^2 = 0.79$; Fig. 6). T-treated birds had larger song nuclei volumes. There was no effect of photoperiod on the volumes of these brain regions (Area X, $F(1,24) = 0.71$, $p = 0.41$; LMAN, $F(1,24) = 0.29$, $p = 0.60$; HVC, $F(1,24) = 0.10$, $p = 0.75$; RA, $F(1,24) = 3.03$, $p = 0.09$). Furthermore, the interaction effect of photoperiod and T-treatment was not significant for the volumes of Area X

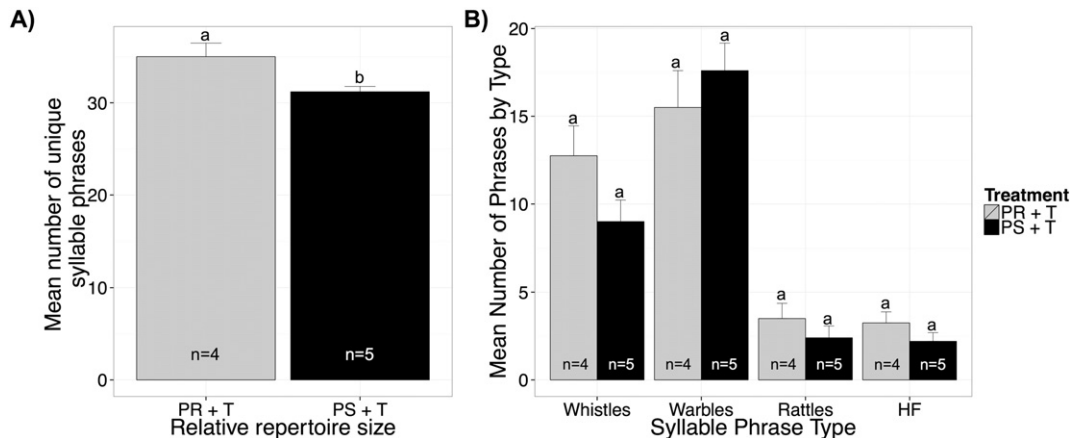


Fig. 3. Bar graphs illustrating the analysis of song repertoire in the photosensitive and photorefractory T-treated female starlings. A) Differences in the relative repertoire size in the T-treated photosensitive (black bars) vs the photorefractory females (gray bars). Too few blank-treated females sang to perform this analysis in the control birds. B) Mean number of phrases of each of the four types defined in Fig. 1 in the T-treated photosensitive (black bars) vs the photorefractory females (gray bars).

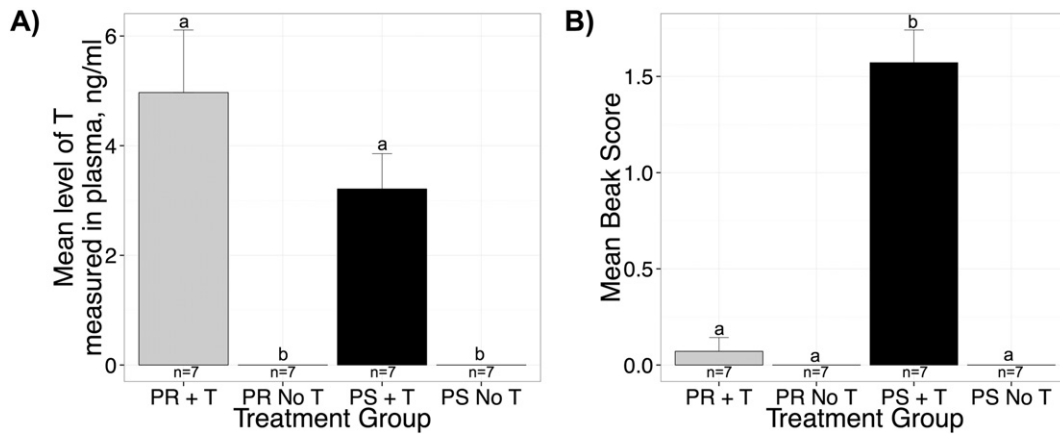


Fig. 4. Bar graphs illustrating different measures of the effectiveness of T-treatment. A) Concentrations of T in the blood based on measurements via RIA on samples collected 3 weeks after implantation just prior to brain collection. T-treated birds had significantly higher T than blank-treated birds. T was not different between T-treated photosensitive (black bars) and T-treated photorefractory (gray bars) females. B) Bar graphs illustrating the mean beak score for females in all the groups. The higher the score the higher the percentage of the beak that is yellow in color rather than black. The beak becomes fully yellow eventually if T is present in the blood. Photosensitive (black bars) T-treated birds had beaks that had a much higher percentage covered in yellow than photorefractory T-treated birds or the blank-treated control groups. See text for more details.

($F(1,24) = 0.62$, $p = 0.43$), LMAN ($F(1,24) = 1.37$, $p = 0.25$), and RA ($F(1,24) = 3.31$, $p = 0.08$) though RA volumes approached a trend. However, there was a significant interaction effect of photoperiod and T-treatment for the volume of nucleus HVC ($F(1,24) = 5.20$, $p < 0.05$, $\eta^2 = 0.04$).

T-treated photosensitive females had significantly larger song nuclei volumes than photorefractory controls (HVC, $z = 9.35$, $p < 0.001$, $d = 2.50$) and photosensitive controls (HVC, $z = -6.52$, $p < 0.001$, $d = 1.74$). T-treated photorefractory females had significantly larger HVC volumes than photorefractory controls ($z = -10.49$, $p < 0.001$, $d = 2.80$) and photosensitive controls ($z = -7.66$, $p < 0.001$, $d = 2.05$). Photosensitive and photorefractory T-treated birds did not differ in HVC volumes ($z = -1.13$, $p = 0.26$). However, photosensitive controls had larger volumes of HVC compared to photorefractory controls ($z = 2.83$, $p < 0.01$, $d = 0.76$).

Discussion

We exposed wild-caught adult female European starlings to two photoperiodic treatment regimens that resulted in the birds being in a photosensitive (i.e., pre-breeding or early breeding state) or photorefractory (i.e., non-breeding state) physiological state. We then implanted birds

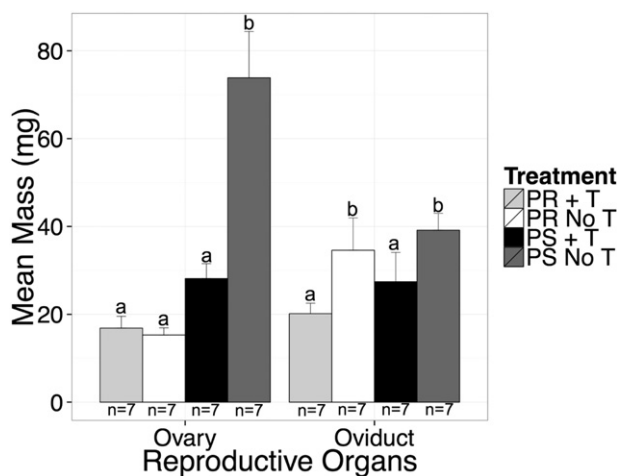


Fig. 5. Bar graph illustrating the mean ovary and oviduct mass for all the experimental groups. Photosensitive females not treated with T (dark gray bars) had the largest ovary mass.

from both groups with silastic capsules filled with T or with blank implants. We found that T-treated photosensitive birds sang significantly more than either the T-treated photorefractory birds or blank treated controls. However, during week 1 of study, T-treated photosensitive females sang only slightly more than the other groups, and this difference was not significant. T-treated photorefractory singers were found to have a small but statistically significant increase in relative song repertoire size compared with T-treated photosensitive singers. There was no effect of photoperiod on the repertoire size of any individual category.

Plasma T was higher 3 weeks post-implantation in the T-treated birds compared with those in the controls. Furthermore, there was no statistical difference between T-treated photosensitive and T-treated photorefractory birds in plasma T, indicating that the T-filled capsules were equally effective in those groups. Beak color in starlings is a sensitive indicator of prolonged exposure to circulating androgens (or lack thereof; Ball and Wingfield, 1987) and we found a significant effect of treatment on beak color scores: T-treated photosensitive females had significantly higher beak scores than photorefractory females and blank-treated birds. However, some photorefractory T-treated birds did appear to be at the beginning phase of responding to elevated T with a change in beak color at the end of the study. It is possible that the beaks of female starlings in a photorefractory state take longer to respond to T than those in a photosensitive state.

Interestingly, we found a significant effect of treatment on measures of reproductive physiology. The ovaries of blank-treated control photosensitive females tended to be heavier than all other treatment conditions. Likewise, there was a significant effect of T-treatment on the weight of the oviduct; T-treated birds had smaller oviducts. This finding is not surprising, as it is well known that exogenous T can reduce the size of gonads and inhibit ovary function in females, perhaps via negative feedback mechanisms (e.g., Barraclough, 1961). There was no effect of photoperiod on the weight of the oviduct. This is most likely due to a floor effect because no bird was in full breeding condition and the oviduct was regressed to its smallest non-functioning state; birds were only in non-breeding and pre-breeding states.

We found that there was a significant effect of T-treatment on the volumes of song nuclei, including Area X, LMAN, HVC, and RA. T-treated birds had larger song nuclei volumes compared with blank-treated birds. Further, there was no effect of photoperiod on song nuclei volume in the T-treated birds, suggesting an equivalent effect of exogenous T on this measure regardless of reproductive state.

Intriguingly, though there was no difference in the volumes of Area X and LMAN in the blank-treated birds, photosensitive controls had larger HVC (and possibly RA) volumes compared with photorefractory

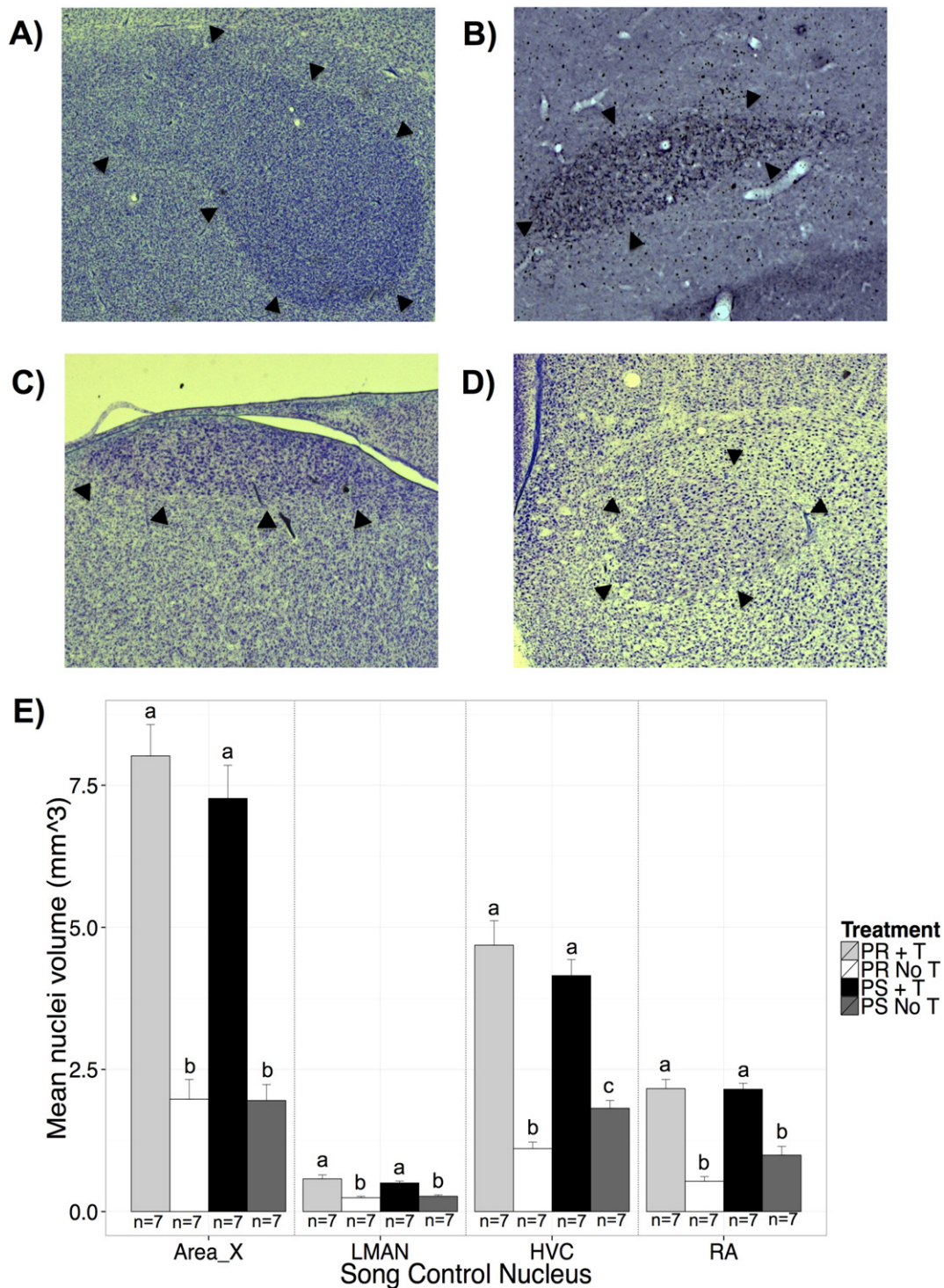


Fig. 6. Photomicrographs of the regions of interest and bar graphs illustrating the mean volume of 4 key forebrain song nuclei in the females in all 4 experimental groups. Representative photomicrographs of the staining quality show the regions of interest for volume reconstruction, namely, song nuclei A) Area X, B) LMAN, C) HVC, and D) RA. E) Bar graphs show that T-treatment resulted in a marked increase in the volume of all four nuclei in both the photosensitive and photorefractory birds compared with blank-treated birds. There were no significant differences in the mean volume of the song nuclei between the T-treated photorefractory and the T-treated photosensitive birds. In the case of HVC and RA nucleus volume was larger in the photosensitive blank-treated control birds than in the photorefractory blank-treated birds. Black bars represent T-treated photosensitive birds. Dark gray bars represent photosensitive control birds. Light gray bars represent T-treated photorefractory birds. White bars represent photorefractory control birds. Abbreviations: HVC used as its proper name; LMAN, lateral magnocellular nucleus of the anterior nidopallium; RA, robust nucleus of the arcopallium.

controls. This finding is consistent with a growing literature based on studies of male songbirds indicating that song nuclei can start growing prior to gonads increasing in size and sex steroid hormone concentrations reaching their maxima (e.g., [Tramontin and Brenowitz, 2000](#); [Caro et al., 2005](#); [Hurley et al., 2008](#)). The song nuclei HVC and RA in

photosensitive female starlings seem to be able to grow, albeit with a relatively small effect size, even when the birds are on short days.

Overall, our findings are consistent with the notion that refractory states attenuate birds' ability to respond to sex steroid hormones. As has been suggested for a variety of photoperiodic species, being in a

'post'-breeding (i.e. non-breeding) state is not only associated with a decrease in gonadal size and secretion, but also with a changes in the signaling properties of target tissues thus affecting the ability to respond to T (Campbell et al., 1978; Ellis and Turek, 1983). Most notably, being in a 'post'-breeding state decreases T's ability to increase singing rate.

Although we did not observe a significant effect of photoperiod on brain region volumes for the T-treated birds, we did observe significant effects of photoperiod on song behavior for T-treated photosensitive and photorefractory birds (e.g. song rate). Importantly, the song system does not regulate all aspects of song behavior (Alward et al., 2013; Ball et al., 2008). Recent data have demonstrated that the rate or motivation to sing in response to steroids is controlled by the preoptic area (POA) and when T is directly implanted into the POA the rate of singing increases similarly to that of systemic treatments (Alward et al., 2013). Other evidence from male starlings indicates that the rate of singing is regulated by the POA (Riters and Ball, 1999; Ball et al., 2002). It may well be that in females song rate is controlled in a similar manner, and thus the POA of photosensitive females is more sensitive to the actions of T thereby increasing their rate of singing. If this is the case it is not surprising that T might have a differential effect on song rate, as a function of reproductive state, despite T retaining its effectiveness in inducing increases in the volume of the song nuclei.

The volumetric changes observed in the song system in response to T-treatment, though not directly related to song rate, may nevertheless directly affect syllable stereotypy. Recent data from canaries shows that over a 3-week treatment period, T-treated adult female photosensitive canaries show similar patterns of growth in the song system, and over this treatment period there is a significant linear increase in the stereotypy of individual syllable iterations (Madison et al., 2014). It is likely that we observed the same phenomenon here: T-treated photosensitive female starlings sang with greater frequency and also experienced the general T-induced growth in volume. However, we cannot definitively say that photorefractory T-treated females are also increasing syllable stereotypy; given the low rate of singing, it is not likely that enough song is being produced to properly detect or measure the stereotypy of individual syllables.

As previously mentioned, T-treated photorefractory birds tended to have slightly larger overall repertoires compared to T-treated photosensitive birds. However, upon closer inspection the number of unique phrases in each of the four respective categories did not differ as an effect of photoperiod. Since the song nuclei responded to T in similar manner (i.e. similar volume changes) in photosensitive and photorefractory birds, the quality of songs produced should have been the same overall. We did not find that.

One possible explanation of the song repertoire data is that T-treated photorefractory females were in a different *state* of neuronal excitability and that they had either not engaged sensorimotor learning (or were in a much earlier stage of sensorimotor learning). Castrated and intact male starlings sing at similar rates and have songs of similar lengths throughout the year; however, intact males have a larger syllable repertoire size (Van Hout et al., 2009). Exogenous administration of T in castrated male starlings can induce a turnover in song repertoire, with the addition and deletion of particular syllable phrase types and overall increase in the repertoire size (Van Hout et al., 2009, 2012).

Exogenous T is also thought to recapitulate sensorimotor learning in adult female songbirds that sing in response to treatment resulting in a masculinization of song (Hausberger et al., 1995). Sensorimotor learning is the dynamic modification of the acoustic features and arrangement of syllables/phrases that requires an active and intact song system. Photorefractoriness may inhibit T-induced *state* changes in neuronal excitability, essentially inhibiting the recapitulation of sensorimotor learning. This potentially means that the phrases incorporated into song may include vocal errors that are so different from other iterations that they could be mistakenly identified as unique.

T could be changing the *state* of the neurons in the song system by changing the electrophysiological properties of the cells. This putative

state change would result in a change in the probability of a neuron firing. Evidence from other songbird species supports this notion. In particular, there are data suggesting that T (and putatively its metabolites) play a role in the modulation of the electrophysiological properties of the song system across season (Park et al., 2005; Meitzen et al., 2007, 2009).

In Gambel's white-crowned sparrows, slice preparations of nucleus RA from males in a breeding condition show a more than two-fold increase in spontaneous firing activity relative to slices prepared from males in a non-breeding condition (Park et al., 2005). Further, this seasonal modulation of spontaneous firing activity is reliant on both estrogenic and androgenic signaling (Park et al., 2005). A similar finding was shown in male song sparrows; male birds captured during the spring had a more than three-fold increase in spontaneous RA firing activity relative to males captured in the fall (Meitzen et al., 2007).

Likewise, it was found that the RA-projecting neurons in the HVC of male Gambel's white-crowned sparrows in a breeding condition (i.e. long days plus T) had increases in the membrane time constant, capacitance, and evoked and spontaneous firing rate(s) relative to non-breeding controls (i.e. short days no T; Meitzen et al., 2009). Relatedly, in castrated male zebra finches, relative to intact controls, there was a suppression of spontaneous and evoked firing rates, membrane time constants, and membrane capacitance of RA-projecting neurons (Wang et al., 2014).

Further, in adult female canaries it was found that exogenous administration of T increased the number of neuronal soma-somatic gap junctions in HVC relative to controls (Gahr and Garcia-Segura, 1996). Furthermore, androgens have been shown to hasten the developmental transition of N-methyl-D-aspartate-excitatory postsynaptic currents from slow to fast in the song nuclei of male zebra finches; this finding is not observed in non-song areas (White et al., 1999).

Across seasons, males of a variety of species including starlings add new syllables to their repertoire and ostensibly experience a recapitulation of at least some aspects of sensorimotor learning (Nottebohm and Nottebohm, 1978; Samson, 1978; Bernard et al., 1996). Thus, the difference in phrase repertoire observed between T-treated photosensitive and photorefractory female starlings may not represent a difference in vocal repertoire/ability but rather a difference in the stage of T-induced vocal development. Taken all together, the effects of reproductive state on T-induced song behavior appear not to be related to differences in song nuclei volume, but to actions in other brain regions (in particular the POA) and to potential differences in the excitability of song system neurons.

However, we cannot exclude the possibility that some of the findings observed are due to estrogenic metabolites of T and not androgenic effects. In light of our current findings, it may be that photorefractoriness suppressed estrogenic facilitation of song behavior. Exogenous T can increase the amount of aromatase activity in the brain, meaning that local synthesis of estrogens may be necessary for specific features of T-induced song behavior (Fusani et al., 2001, 2003). Though aromatase expression and activity tends to be sexually dimorphic, exogenous T may be up-regulating aromatase expression and activity in the photosensitive T-treated female starlings, thus facilitating the behavioral differences (Schumacher and Balthazart, 1986; Foidart et al., 1994; Fusani et al., 2001, 2003; Peterson et al., 2005).

This hypothesis is supported by data from female canaries, which show that exogenous T administration can increase aromatase activity in the forebrain (Fusani et al., 2001). Furthermore, blocking aromatase during T-treatment suppresses song masculinization; songs have aberrant duration and a greater proportion of the songs have abnormally slow-repeated syllable phrases (male-typical song has fast repeated syllable phrases; Fusani et al., 2003). Though we did not test this explicitly, it is likely that in addition to being sensitive to the androgenic actions of T, photosensitive-females were also responsive to the actions of estrogenic metabolites (via T-induced up-regulation of brain aromatase). Conversely, photorefractory females were likely insensitive to estrogenic

metabolites, though it is unclear whether photorefractoriness inhibits estrogen synthesis (via suppression of aromatase activity) or inhibits local estrogenic signaling in the brain.

Conclusion

We investigated whether T is equally effective in inducing changes in brain and behavior of adult female starlings in two different reproductive states (pre-breeding and 'post'/non-breeding). The data demonstrate that reproductive state modulates the effects of T. Further, these data show that studies of female song production may shed new light on our understanding of neuroplasticity and neuroendocrine effects on vocal learning. By critically analyzing the changes that occur in response to T we can glean general principles about song behavior and its hormonal regulation. Elevating circulating levels of testosterone to male-like concentrations in females significantly changes song activity. Song becomes more male-like in structure and the rates of singing increases. Changes in song behavior can happen independently of volume changes to song nuclei. However, T may not be equally effective as seasonal photoperiodic conditions change.

Conflict of interest

The authors have nothing to disclose.

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