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RESEARCH ARTICLE

# Experimentally Elevated Levels of Testosterone Advance Daily Onset of Activity in Short-Day Housed Male House Sparrows (*Passer domesticus*)

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## ABSTRACT

Seasonal changes in sleep/wake cycles and behaviors related to reproduction often co-occur with seasonal fluctuations in sex hormones. Experimental studies have established that fluctuations in circulating testosterone mediate circadian rhythms. However, most studies are performed under constant lighting conditions and fail to investigate the effects of testosterone on the phenotypic output of circadian rhythms, that is, chronotype (daily activity patterns under light:dark cycles). Here, we experimentally elevated testosterone with implants during short nonbreeding daylengths in male house sparrows (*Passer domesticus*) to test if observed seasonal changes in chronotype are directly in response to photoperiod or to testosterone. We fitted individuals with accelerometers to track activity across treatment periods. Birds experienced three treatments periods: short day photoperiods before manipulation (SD), followed by testosterone implants while still on short days (SD + T). Implants were then removed. After a decrease in cloacal protuberance size, an indicator of low testosterone levels, birds were then photostimulated on long days (LD). Blood samples were collected at night, when testosterone peaks, to compare testosterone levels to daily onset/offset activity for experimental periods. Our results indicate that experimentally elevated testosterone under short nonbreeding photoperiods significantly advanced daily onset of activity and total daily activity relative to daylength. This suggests that testosterone, independent of photoperiod, is responsible for seasonal shifts in chronotypes and daily activity rhythms. These findings suggest that sex steroid hormone actions regulate timing of daily behaviors, likely coordinating expression of reproductive behaviors to appropriate times of the day.

## 1 | Introduction

Circadian rhythms facilitate the coordination of daily behaviors across the 24-h day in nearly all organisms (Kreitzman and Foster 2004). Under natural conditions timing of daily activities and changes in physiology are entrained (i.e., synchronized) by external environmental signals, with daylength (photoperiod) acting as the strongest cue (Aschoff 1981; Dominoni et al. 2013; Edmunds 1983; Kreitzman and Foster 2004). Chronotype, or the phenotypic output of these circadian rhythms, is influenced by entrainment with environmental cues such as photoperiod as well as other endogenous factors (Elderbrock, Hau, and Greives 2021; Graham et al. 2017; Samson et al. 2017). Under natural conditions circadian rhythms are hypothesized to enable individuals to anticipate for regular changes in the environment to coordinate behaviors related to foraging,

**Summary**

- Experimentally elevated testosterone advanced onset of daily activity and increased total activity during the photophase during short days.
- Photostimulation delayed daily onset activity and reduced total activity during the photo-phase.

migration, and reproduction at appropriate/optimal times of the day and year (Decoursey, Walker, and Smith 2000; Elderbrock, Hau, and Greives 2021; Hau et al. 2017; Kronfeld-Schor and Dayan 2008; Wikelski and Hau 1995).

The timing of daily behavioral rhythms of birds has been observed to vary across seasons, with individuals changing the timing of their daily activities relative to sunrise and sunset (Hinde 1952, pp. 159–174; Steinmeyer et al. 2010). For example, during the spring and summer months birds have been observed to advance their timing of onset of activity relative to sunrise corresponding with behavioral and physiological changes related to reproduction (Dawson et al. 2001; Hinde 1952, pp. 159–174; Steinmeyer et al. 2010). The seasonal shifts in the relative timing of sleep/wake cycles may correspond with the seasonal changes in hormones levels, specifically sex steroids.

In many seasonally breeding vertebrates, such as songbirds, the lengthening photoperiods of spring and summer are associated with increases in sex steroids (androgens and estrogens) due to the photostimulation of molecular timekeepers of the hypothalamus–pituitary gonadal axis (Dawson et al. 2001; Li et al. 2001; Loubser, Van Niekerk, and Botha 1983; Zena et al. 2019). Indeed, several studies on a variety of temperate breeding avian species have observed that annual peak levels in reproductive hormones co-occur with the increased daylengths that are experienced during the breeding season (Foerster et al. 2002; Nottebohm et al. 1987; Valdez et al. 2014; Wingfield and Farner 1976; Wingfield et al. 1990). This increase in sex hormones coordinate the seasonal phenotypic expression of reproductive-related traits and behaviors (i.e., sexual signaling, gonadal growth, and parental care) that are critical for reproduction, ultimately effecting reproductive success (Adkins-Regan 2007; Dawson et al. 2001; Foerster et al. 2002; Gwinner 1989; Laucht, Kempenaers, and Dale 2010; Poesel et al. 2006; Schlicht, Santema, and Kempenaers 2023).

In addition to these above studies, research conducted in captivity where photoperiod can be controlled has also observed changes in timing of daily behavior rhythms associated with photoperiod-induced changes in testosterone. Eurasian tree sparrows (*Passer montanus*) that were subjected to natural fluctuation in photoperiod exhibited earlier onset and a delay in offset activity during longer photoperiods, a time when they also had elevated levels of testosterone (Dixit and Singh 2016). Additionally, male birds that were photostimulated before being transferred to constant light conditions exhibited longer active days ($\alpha$) and maintained elevated levels of testosterone and larger testes compared to birds that were also transferred to constant light but that were not photostimulated before transfer (Dixit and Singh 2016; Gwinner 1975).

Captive studies have indicated a bidirectional relationship between sex steroids and circadian mechanisms. Sex steroids fluctuate predictably over the 24-h period regulated by endogenous timekeepers (Bailey and Silver 2014; Greives et al. 2021). In addition to the circadian fluctuation of circulating sex steroid hormones, sex hormones may mediate daily activity patterns (Gamble et al. 2014; Hagenauer and Lee 2011; Hatcher, Royston, and Mahoney 2020; Kriegsfeld and Silver 2006; Tonsfeldt and Chappell 2012; Yan and Silver 2016). Gonadectomized rodents and birds kept under constant darkness exhibited changes in their circadian behavioral rhythms following gonadectomy, including increased circadian period length ($\tau$) and reduced precision of onset daily activity and activity bout lengths; these changes were reversed with testosterone replacement (Butler et al. 2012; Karatsoreos et al. 2007; Lumineau et al. 1998; Model et al. 2015).

While these previous studies observed correlations between testosterone and behavioral rhythms under natural or experimental light:dark conditions and that manipulation of testosterone has effects on circadian rhythms under constant lighting conditions, few studies, and none we are aware of in birds, have investigated the direct effects of testosterone manipulation on the expression of chronotype under light:dark conditions. Further, while observations identify relationships between photoperiod-induced reproductive status and changes in daily rhythms, it is not clear if these changes in chronotype are directly in response to changing photoperiod, or if they are in response to changing testosterone levels.

The current study aims to address whether observed seasonal changes in daily activity rhythms are a result of increases in sex steroid hormones, specifically testosterone, or are a result from the increases in daylength in a songbird, the house sparrow (*Passer domesticus*). We monitored daily activity rhythms of captive males under short and long photoperiods combined with testosterone manipulations. To precisely obtain onset and offset of daily activity relative to lights on/off and total daily activity levels, birds were fitted with accelerometer data loggers. We predicted that, regardless of daylength, when birds with higher levels of testosterone, both from exogenous (implants) during short days and endogenous (longer photoperiods) sources, they will wake earlier and stop activity later resulting in longer active periods compared to during unmanipulated short days.

## 2 | Material and Methods

### 2.1 | Experimental Animals

Sixteen adult male house sparrows were captured with mist nets from Fargo, ND, USA, in late November 2022 and housed in individual cages ($59.7 \times 39.4 \times 30.5$ cm) in two temperature and light-controlled rooms and held on 8L:16D until the start of the experiment. All birds received food (millet and deshelled sunflower mix, grit, mealworms, and a dog food mix with hard-boiled eggs and carrots) and water ad libitum. Birds' weights were measured weekly. Two birds developed an illness and were removed from the study at the time point of symptoms were observed.

## 2.2 | Cloacae Protuberances (CPs) Assessment and Testes Mass

The width (mm) of birds' CPs were measured weekly (Boersma and Davies 1987). Cloacae tissue is androgen-sensitive and grows in response to the presence of androgens and was used to verify the effectiveness of our experimental design aimed to increase circulating levels of androgens (see Section 2.3) (Balthazart, Bottoni, and Massa 1980; Massa, Davies, and Bottoni 1980). Size changes were also used to inform the time periods to include in our analysis to assess effect of treatment on daily behavioral rhythms. Specifically, we included all days from the time period when CP size was significantly different following treatment manipulation until the end of the treatment (i.e., when implants were removed or photoperiod was switched) (see Section 2.7). At the end of photostimulation, birds were euthanized with an overdose of isoflurane and testes to were removed and weight on an electric scale to the nearest 0.0001 g to confirm that birds were photostimulated (Lombardo and Thorpe 2009). Testes mass following photostimulation was $0.3458 \pm 0.1478$ g with a range of 0.1408–0.6709 g.

## 2.3 | Experimental Design

Once brought into captivity all birds received a BitTag accelerometer to record daily activity (Brown et al. 2023). These accelerometers were placed on the birds back via a leg loop harness made from 0.5 mm elastic cording (Naef-Daenzer 2007). Birds were held on a short-daylight cycle (SD-8L:16D, 08:00–16:00) for at least 2 weeks before any manipulations to allow time for adjustment to captivity. Temperature was kept approximately 21–22°C for the duration of the experiment. After the ≥ 2-week adjustment period a blood sample was taken at 21:00 to measure baseline testosterone before any manipulations (see Section 2.4) (Greives et al. 2021). The day following baseline blood sampling all birds received a single 11 mm silastic subcutaneous testosterone implant with crystalline testosterone (Sigma) (1.47 mm in diameter; 1.96 mm outer diameter; Dow Corning, sealed at both ends with Sil-Bond 100% silicone sealant [Silco Inc.; RTV 4500] on their flank (Hau, Dominguez, and Evrard 2004). Photoperiod was maintained on 8L:16D for 3 weeks following implantation. To confirm effectiveness of the implant a blood sample was taken 3 weeks after implantation at 21:00 (Greives et al. 2021; Hau, Dominguez, and Evrard 2004).

Implants were removed 36 h after this second blood sample. Birds were allowed to clear the exogenous testosterone for 3 weeks following removal of implants, while remaining on 8L:16D. To confirm that testosterone had been cleared after this 3-week period, we collected a blood sample (between 11:00 and 13:00) and confirmed that CP values were reduced and were not different from their baseline CPs from the start of the study.

Following confirmation that CP size had been reduced indicating that testosterone had been cleared over these 3 weeks postimplant removal, birds were photostimulated (LD-16L:8D, 04:00–20:00) for 4 weeks. This period of photostimulation has been demonstrated to elevate endogenous production of testosterone (Farner et al. 1966; Needham, Dochtermann, and Greives 2017; Small, Sharp, and Deviche 2007). A blood sample was taken at 22:00 at the end of the 4-week photostimulation period to confirm that photostimulation had increased circulating testosterone levels (Figure 1).

## 2.4 | Blood Sampling

Blood (~115 μL) was collected with microhematocrit heparinized capillary tubes (Fisher Scientific) from the brachial vein following venipuncture with a 26-g needle and placed immediately on ice. Plasma was separated by centrifuging the samples and extracted plasma was stored at −20°C until assayed for testosterone.

We chose to collect nighttime samples during the first 1/3 of the dark phase of the bird's circadian rhythm at 21:00 (SD) and 22:00 (LD) because testosterone fluctuates predictably over the 24-h day and exhibits a diel peak during this time frame (Foerster et al. 2002; Greives et al. 2021; Laucht et al. 2011; Needham, Dochtermann, and Greives 2017). Night sampling occurred over four nights for both rooms, where birds were captured from their home cage using red-illuminated headlamps and removed to a separate room for sampling (Greives et al. 2021). Two birds per room were sampled per night, with birds remaining in their cage until sampling. Birds were kept under red light and all nighttime samples were collected within an average of 7.91 (range: 3–22) min upon first entrance to the room. Following sampling, birds were returned to their cage. Daytime samples were collected after implant removal between 11:00 and 13:00 to verify that testosterone levels had dropped.

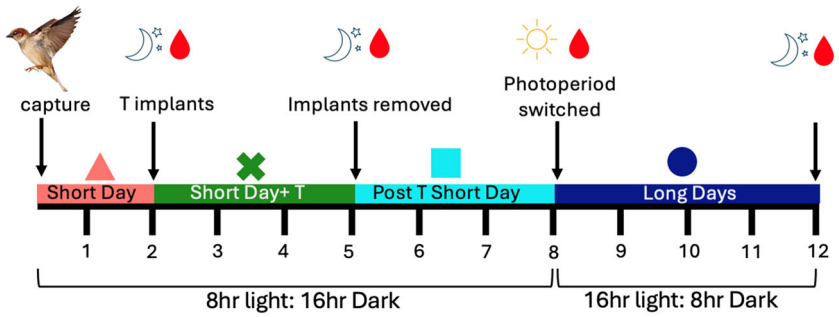


**FIGURE 1** | Experimental design, each color represents each treatment period (salmon/triangle: short days [SD]; green/X-mark: short days with testosterone implants [SD + T]; light blue/square: implants removed still on short days [post-T SD]; dark blue/circle: long days [LDs]). Blood sampling is indicated at what time of day they were taken at the end of each treatment period. Additionally, short days consisted of 8:16 light/dark cycles and long days consisted of 16:8 light/dark cycles.

## 2.5 | Testosterone Assays

Plasma testosterone was measured using an enzyme immunoassay (Enzo Life Sciences, ADI-900-065) following the manufacture's guidelines. Testosterone was extracted (2×) from 30 μL of plasma with diethyl ether and dried under nitrogen gas at 25°C (Greives et al. 2021). Samples were then reconstituted overnight in 300 μL assay buffer (1:10 dilution). Samples that were less the 30 μL dilutions were recorded and that volume was used in the calculations for sample concentration. All reconstituted samples were randomly assigned across six plates and were plated in duplicate (100 μL/well). Testosterone concentrations were calculated with a five-point parameter logistic curve. A standard curve was calculated by using the observed optical densities across all plate standards using MyAssays Ltd., an online data analysis tool (Arbor Assays) (Gomes et al. 2023). All sample testosterone concentrations were calculated by using the standard curve obtained by MyAssays Ltd. (Gomes et al. 2023). The coefficients of variation (CVs) for intraplate testosterone ranged from 1.05% to 10.54% and interplate CV was 18.09% (Supporting Information S1: Table 2). Samples that were above the detection limit of our assay were assigned a value of the highest kit sensitivity and corrected for plasma volume (20,000 pg $mL^{-1}$). All five samples above the detection limit were from samples from the testosterone implant sampling period.

## 2.6 | Behavioral Data

BitTags collect behavioral activity by recording percent activity each minute by tracking acceleration changes with a programmable motion detector to set active/inactive threshold. BitTags were used to determine animal activity by aggregating activity data, counting the number of active seconds during a 1 min aggregation period. For this experiment, the threshold active/inactive status was set to an acceleration of 0.5× gravity with an inactivity time set to 0.2 s if no changes in acceleration occurred (Brown et al. 2023). BitTags recorded continuously throughout the duration on the experiment.

A percent activity threshold of 8% was applied to filter out background noise in the recording by changing all minutes with percent activity < 8%–0% to increase clarity between active and nonactive bouts (see Supporting Information S1: Figure 1). Next using the changepoint R package (Killick and Eckley 2014; Killick, Haynes, and Eckley 2022) changes in activity were detected by using the cpt.meanvar command which identified changes in the mean and variance in percent activity. This cpt.meanvar command includes a pruned exact linear time method and an MBIC penalty. To detect transitions in the subtle changes in activity of captive birds we set the minimal segment length to 75 for detecting a changepoint, meaning changes in activity would not be detected for a minimum of 75 observations following an identified changepoint. If a changepoint occurred during the 75-min minimum segment length, that changepoint would be identified at the end of the minimum segment length. To determine the optimal minimal segment length, we visually inspected a subset of the raw and filtered activity data with output from the changepoint R package using multiple segment lengths ranging from 50 to 350 min. The minimal segment length of 75 min more consistently predicted activity changes between active and inactive bouts. Additionally, all changepoint outputs for onset activity, along with a subset of offset activity changepoint outputs, were visually assessed for any points that fell within 75 min before the set onset window (see below; Supporting Information S1: Figure 2) or before offset activity times (Supporting Information S1: Figure 3), which could potentially identify inaccurate onset/offset (predicting too early/late). If a changepoint occurred 75 min prior, activity times were checked against the raw and filtered data to confirm the accuracy of predicted changepoints and any poor predictions which were then removed. For onset activity 10.79% of points were removed and none were eliminated for offset times.

Onset and offset activity were selected from the changepoint output within a set time window before and after lights on/off for each treatment period. Onset and offset windows were set by approximately doubling the average of previous field observations of house sparrow roosting times exhibited across the year and under various physiological states (Anderson 2006, pp. 293–294; Patel and Dodia 2021; Singh et al. 2013). House sparrows were observed singing 91 min before sunrise and departed roosts between 65 min before sunrise and up to 30 min after and returned to their roots between 72 min before sunset and up to 25 min after (Anderson 2006, pp. 293–294; Patel and Dodia 2021; Singh et al. 2013). Based on these observations, the onset time window across all treatment periods was set to encompass 150 min before lights on to 60 min after lights on, to capture potential behavioral changes related to testosterone treatment or photoperiod. The first changepoint that fell within the established onset time window for each day was taken as the onset of activity. For offsets times, the window was the same across all treatments and was set to 140 min before lights off to 60 min after lights off (Anderson 2006, pp. 293–294; Patel and Dodia 2021; Singh et al. 2013). The last changepoint that was within the offset time range each day was taken as the offset of activity time.

To calculate the percent of total daily activity relative to daylength, the total duration of daily activity was calculated by subtracting onset activity time from offset activity time for each day and divided by the duration of lights-on for each photoperiod length. For example, a bird housed under 8:16 that was active for 8 h and 40 min would have a value of 108.3% active period.

## 2.7 | Statistical Analysis

All analyses were performed in R studio (R Core Team 2023, Version 4.2.2). All linear mixed effects models were fitted using the package "lme4" (Bates 2010; Bates et al. 2015) and we checked model assumptions using the "check_model" function from the performance package (Lüdecke et al. 2021). We then tested the significance of fixed effects of linear mixed models using a Type III ANOVA, and the statistical significance level of all tests was set at $\alpha = 0.05$. Degrees of freedom were calculated for all models using the Satterthwaite's method. Following the linear mixed models, we did a post hoc pairwise comparison of significant fixed effects using the lsmeans command from the "emmeans" package with a Holm–Bonferroni adjustment for all comparisons (Lenth 2023).

## 2.8 | Effect of Treatment on Circulating Testosterone

To determine if treatment had an effect on circulating testosterone, we performed a linear mixed-effects model with treatment period

(short days prior manipulation (SD), short days with testosterone implants (SD + T), short days postimplantation (post-T SD) and long days (LD) as a categorical variable) and assay plate number (samples were run six plates) as fixed effects and individual identity was included as a random effect. Testosterone values were log transformed to meet the assumption of normality. Following a significant main effect a pair-wise post hoc comparison between treatment periods was performed.

### 2.9 | Selection of Treatment Sampling Windows

As noted above, we used treatment-induced change in CP size to identify the range of days to include in the analysis of treatment effects on behavioral activity. To identify the periods where CP width differed, we performed a linear mixed model with week of experiment set as a fixed effect and individual identity set as a random effect. Following a significant main effect a pairwise post hoc comparisons of weekly CP size was conducted to identify when CP size significantly differed. We set the window for activity measures to begin at the point where CP size following treatment was significantly different from the prior week and the end of the activity window was set as the date the treatment period ended (i.e., implants removed).

### 2.10 | Effect of Treatment on Daily Activity Patterns

To determine treatment effects on onset activity, offset activity and total daily activity percentage relative to daylength we ran separate linear mixed-effects models for each behavioral measure with treatment period; SD, SD + T, and LD (as a categorical variable) as a fixed effect and individual identity a random effect. To explore differences in these parameters between treatment periods we performed a pair-wise post hoc following a significant main effect.

## 3 | Results

### 3.1 | Treatment Effect on Circulating Testosterone Concentrations

There was a significant main effect of treatment period on circulating levels of testosterone ($F_{3,48} = 86.54$, $p < 0.0001$; Figure 2A). Pair-wise post hoc comparison found that testosterone levels after implantation (SD + T) and photostimulation (LD) where significantly higher compared to initial levels during short days before any manipulation (SD) (post hoc: SD-(SD + T) $t_{(df=34.98)} = -15.43$, $p < 0.0001$; SD-(LD) $t_{(df=36.20)} = -9.31$, $p < 0.0001$; Supporting Information S1: Table 3). Additionally, testosterone levels during implantation were significantly higher compared to levels during LDs (post hoc: SD + T − (LD) $t_{(df=36.17)} = 5.22$, $p < 0.0001$; Supporting Information S1: Table 3). After implant removal testosterone levels were significantly lower (SD + T-(post-T SD) $t_{(df=36.22)} = 10.50$, $p < 0.0001$; Supporting Information S1: Table 3). Short days prior manipulation (SD) had an average testosterone level of $0.11 \pm 0.01$ ng mL$^{-1}$. Testosterone treatment (SD + T) increased the average testosterone level to $13.22 \pm 1.84$ ng mL$^{-1}$ while still on a short-day photoperiod. Following removal of testosterone implants (post-T SD), average levels decreased to $0.36 \pm 0.06$ ng mL$^{-1}$ while still on short days. When moved to LD photoperiods, the average levels increased to $2.22 \pm 0.47$ ng mL$^{-1}$.

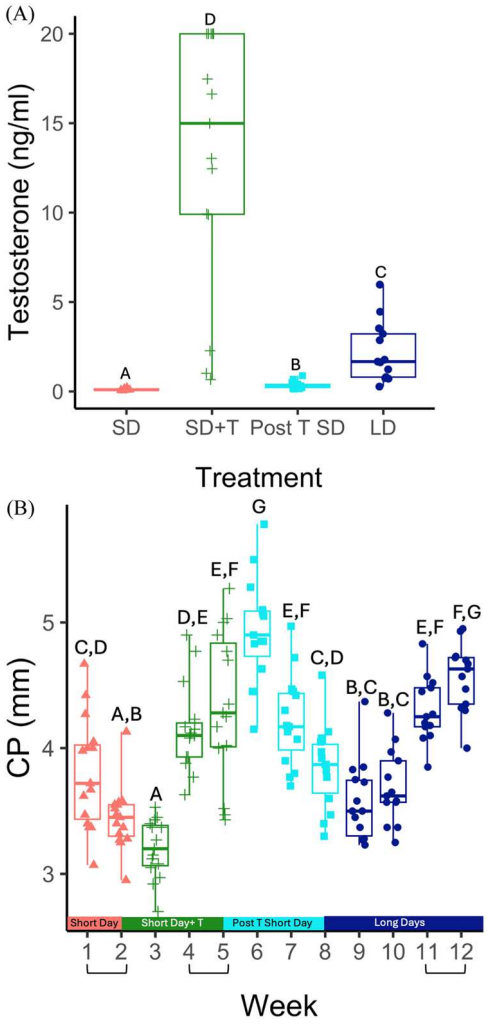


**FIGURE 2** | (A) Photoperiod and testosterone implants had an effect on testosterone levels for each treatment type (short days [SD]; short days with testosterone implants [SD + T]; short days post-implant removal [post-T SD]; and long days [LDs]). Data points represent the raw testosterone data for a single individual. (B) Individual CP size varied across the study period in relation to testosterone treatment and photoperiod length. Black brackets on the *x* axis indicate the weeks that were included in the behavioral analyses for each treatment based on when CP size was significantly different following treatment manipulation (i.e., implants and photostimulation) until the end of the treatment period. Letters indicate significant differences between weeks/groups.

### 3.2 | Effect of Treatment on CP Size

CP size measured each week during the entire duration of the experiment showed an overall main effect of sampling week ($F_{11,146.44} = 41.65$, $p < 0.0001$; Figure 2B). Pair-wise post hoc comparison found that CP significantly

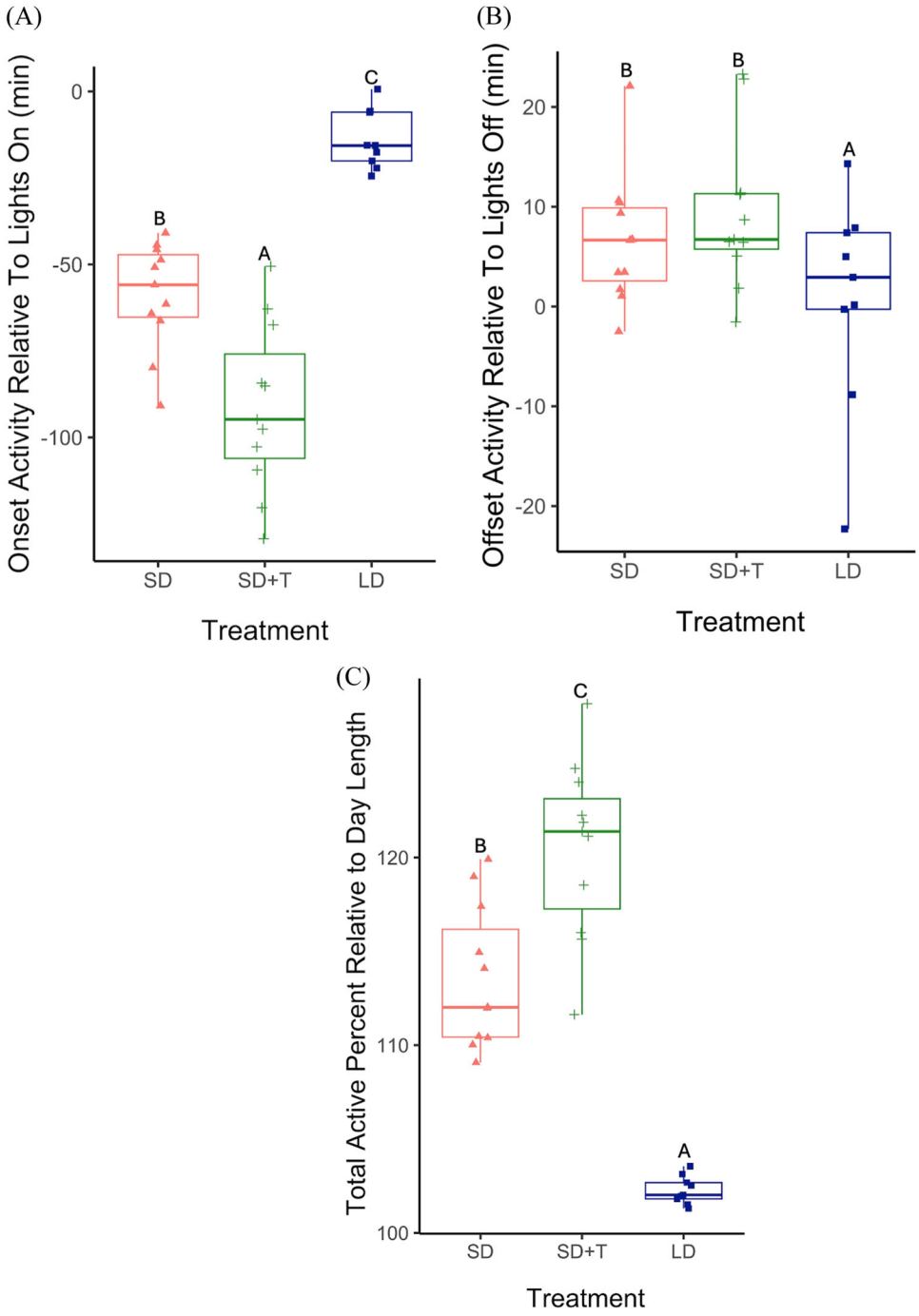


**FIGURE 3** | (A) Daily individual mean onset activity relative to lights on, indicated by zero on the *y* axis, for individuals across all treatments (short days [SD]; short days with testosterone implants [SD + T]; long days [LDs]). (B) Daily individual mean offset activity relative to lights off, indicated by zero on the *y* axis, for individuals across all treatments (SD; SD + T; LDs). (C) Individual mean of total active percent relative to daylength experienced for each treatment period for individuals across all treatments (SD; SD + T; LDs).

differed from Weeks 3 to 4 (i.e., 1 week after implanting with testosterone), denoting the start of the sampling window for behavioral data for short days with testosterone (post hoc: Weeks 3 to 4 $t_{(df=146.01)} = -8.80$, $p < 0.0001$). Following photostimulation CPs from Weeks 10 to 11 were significantly different, denoting the start of the sampling window for behavioral data for LDs (post hoc: Weeks 10–11 $t_{(df=146.01)} = -5.28$, $p < 0.0001$). The sampling window for behavioral data for shorts day before manipulations included both weeks before testosterone implantation.

### 3.3 | Effect of Treatment on Onset Daily Activity

Onset of daily activity significantly varied across the treatment periods ($F_{2405.85} = 203.01$, $p < 0.0001$; Figure 3A). When males were implanted with testosterone, they exhibited significant advancement in onset of daily activity compared to short days before any manipulation (post hoc: SD-(SD + T) $t_{(df=401.72)} = 10.68$, $p < 0.0001$; Supporting Information S1: Table 4). However, onset activity for LDs exhibited the opposite relationship than was predicted, exhibiting a significant delay compared to short days before

manipulation and short days with implants (post hoc: SD − (LD) $t_{(df=407.96)} = -11.60$, $p < 0.0001$; SD + T − (LD) $t_{(df=408.00)} = -19.90$, $p < 0.0001$; Supporting Information S1: Table 4). Daily individual mean onset activity relative to lights on was $-57.65 \pm 2.23$ min for short days before any manipulation (SD). Then daily individual mean onset activity was advanced during testosterone implantation (SD + T) to $-90.99 \pm 2.37$ min. When moved to LDs, daily individual mean onset times were delayed to $-15.83 \pm 3.50$ min before lights on.

### 3.4 | Effect of Treatment on Offset Daily Activity

Treatment period had an overall main effect on daily offset of activity ($F_{2436.71} = 8.93$, $p = 0.0002$; Figure 3B). Post hoc comparisons revealed that daily activity ended significantly earlier on LDs compared all other treatments (post hoc: SD − (LD) $t_{(df=438.10)} = 3.17$, $p = 0.0032$; SD + T − (LD) $t_{(df=438.22)} = 4.18$, $p = 0.0001$; Supporting Information S1: Table 5). No other significant pair-wise differences were identified. Daily individual mean for offset of activity relative to lights off was $5.55 \pm 1.23$ min for short days before any manipulation (SD) and $7.43 \pm 1.25$ min for testosterone implantation (SD + T). On LDs daily individual mean offset of activity was advanced to $0.24 \pm 2.08$ min.

### 3.5 | Effect of Treatment on Percentage of Total Daily Activity Relative to Daylength

Total daily percent activity relative to daylength was significantly affected by treatment period ($F_{2383.57} = 249.79$, $p < 0.0001$; Figure 3C). When males were implanted with testosterone, they exhibited a significant increase in the percent amount of time they were active during respective daylengths compared to short days before any manipulations (post hoc: SD-(SD + T) $t_{(df=379.31)} = -11.03$, $p < 0.0001$; Supporting Information S1: Table 6). However, total daily percent activity for LD treatments exhibited the opposite relationship, experiencing a significant decrease in the percentage of the daily activity compared with short days before any manipulation and to short days with implants (post hoc: SD-(LD) $t_{(df=385.88)} = 13.74$, $p < 0.0001$; SD + T − (LD) $t_{(df=385.98)} = 22.14$, $p < 0.0001$; Supporting Information S1: Table 6). Daily individual mean of total activity percent relative to daylength was altered by treatment where on short days prior manipulation (SD) activity percent was $113.02 \pm 0.56\%$. During implantation (SD + T) daily individual mean total activity percent increased to $120.18 \pm 0.51\%$. When moved to LDs the daily individual mean total percent activity decreased to $102.33 \pm 0.45\%$.

## 4 | Discussion

Here, we provide evidence that even under reproductively inhibitory short-photoperiods experimentally elevated levels of testosterone lead to a significant advancement in daily onset activity and increased total percent activity relative to daylength. Testosterone implants in birds held on short days had significancy increased testosterone concentrations compared to initial levels during short days before any manipulation. Interestingly, while photostimulation did significantly increase circulating testosterone compared to short days before manipulation, contrary to our hypothesis, we did not observe a similar advance in onset of activity in birds.

Our study asked if testosterone advances onset of activity and increases the percentage of their day that male birds are active even under reproductively inhibitory short-day photoperiods. Indeed, we observed these predicted effects, and these observations were similar to previous captive studies that investigated the effects of testosterone implants under breeding photoperiods, (as opposed to the inhibitory photoperiods used in the current study). In a variety of vertebrates testosterone implants under breeding photoperiods increased locomotor activity and extended activity patterns by increasing the time between onset activity and lights on (Klukowski, Ackerson, and Nelson 2004; Lynn et al. 2000; Pohl 1994). These combined observations suggest that testosterone, and not changes in photoperiod are driving changes in chronotype. Indeed, in nonavian species, pubertal rises in testosterone have been associated with earlier chronotypes (Hagenauer, Ku, and Lee 2011).

The effects of testosterone on chronotype even under reproductively inhibitory photoperiods in our study indicate that sex-steroid hormones directly influence chronotype. This suggests that selection may have acted to functionally link testosterone and chronotype. Testosterone is responsible for coordinating an array of reproductive behaviors (i.e., song) that affect reproductive success. For instance, male song output and structure during the dawn chorus plays an important role in mate attraction and is positively correlated with androgens (Foerster et al. 2002; Galeotti et al. 1997; Hunt, Hahn, and Wingfield 1997). Additionally, diurnal singing activity peaks during the breeding season corresponding with the seasonal increase in testosterone indicating testosterone is key in coordinating reproductive behaviors important for reproductive success (Quispe et al. 2016; Van Duyse, Pinxten, and Eens 2003). However, while our results suggest that testosterone and not daylength drives changes in chronotype, is not yet established if testosterone's influence on chronotype influences reproductive success. Previous observations are consistent with the hypothesis that testosterone is coordinating not only the behaviors itself, but the timing of these behaviors to enhance reproductive success. For example, extra-pair copulations in birds occur early in the morning and males who had increased extra-pair paternity joined the dawn chorus the earliest (Dolan et al. 2007; Double and Cockburn 2000; Kempenaers et al. 2010; Poesel et al. 2006). Additionally, males who were experimentally treated with elevated levels of testosterone sang more, increased territorial aggression/mate guarding and had a greater number of extra-pair fertilizations (Kurvers et al. 2008; Raouf et al. 1997; Reed et al. 2006). Our results, combined with observational and hormonal manipulation reports indicate that testosterone is both responsible for coordinating the timing (i.e., onset of activity) and expression of reproductive behaviors (i.e., song and territorial aggression). Future work will be needed to establish functional links more clearly between testosterone, chronotype, and male reproductive success.

Even though we did not investigate the causal pathways by which the testosterone implants led to changes in chronotype, it

is likely that it was via actions on the suprachiasmatic nucleus (SCN) and underlying circadian timekeeping mechanisms. Within the SCN, the ventrolateral core subregion is densely packed with androgen receptors (ARs) providing a neuroendocrine mechanism whereby androgen can alter circadian and behavioral rhythms (Fernández-Guasti et al. 2000; Karatsoreos et al. 2007; Kashon et al. 1995; Model et al. 2015). Estrogen receptors (ER) are also found in the dorsal SCN offering another potential pathway that androgens can neuromodulate the SCN via aromatization (Forlano, Schlinger, and Bass 2006; Hutchison 1993; Schlaeger et al. 2024).

While our data with testosterone implants under short days supported our hypothesis, our observation of a delay in onset activity and decrease in total percent activity relative to daylength, despite active days being longer, exhibited during photostimulation is opposite of what was predicted. This observation that birds held on LDs did not begin their daily activity earlier relative to lights on than when they were unimplanted and held on short-days may be due to potential physiological limitations in the maximum total duration they are capable of being active per day. An observational study performed in blue tits showed that birds used a smaller proportion of available daylight during summer months (i.e., waking later to relative sunrise and ending activity earlier relative to sunset) even though birds were more active in the total number of hours compared to winter months (Schlicht and Kempenaers 2020). In a study of arctic songbirds—it was observed that Lapland longspurs (*Calcarius lapponicus*) have a period of quiescent, even though there was available daylight (Ashley et al. 2013). Additionally, while the shift in chronotype to earlier is observed leading up to and the beginning part of the breeding season, in temperate breeding birds, the onset of activity steadily becomes later after sunrise as photoperiod approaches its annual maximum (Maury, Serota, and Williams 2020; Schlicht and Kempenaers 2020). These observation suggest a physical limitation to the number of active hours individuals can sustain under longer daylengths; our manipulations of 16L:8D approximates the natural summer maximum of Fargo.

An alternative explanation for our observation that photostimulation did not lead to earlier onset of activity relative to sunrise may be that the prior testosterone manipulation prevented subsequent photostimulation (Nair 2001; Turek, Wolfson, and Desjardins 1980). However, we consider this unlikely as our birds did respond to the increase in photoperiod at the end of the study as indicated by an increase in CP size and testes masses that were comparable to testes size in breeding wild house sparrow (Lombardo and Thorpe 2009). Additionally, birds exposed to LD treatment had testosterone levels similar to those found in other captive house sparrow studies held on long photoperiods (Needham, Dochtermann, and Greives 2017).

While testosterone concentrations during implantation in our study were elevated to a similar range as seen in previous songbird implant studies (Greenman, Martin, and Hau 2005), levels were elevated to approximately twice the concentrations that are exhibited during the breeding season in free-living male house sparrows potentially leading to supraphysiological levels (Hegner and Wingfield 1987). The exposure to potential supraphysiological testosterone levels, may also have contributed to our unexpected observations of chronotype following photostimulation, via induced effects on gene expression and densities of both AR and ER in the SCN (Krongrad et al. 1991; Lee and Chang 2003; Nair 2001; Turek, Wolfson, and Desjardins 1980; Wolf et al. 1993).

Unfortunately, captive space and time limitations influenced our study design, and we prioritized our ability to test the hypothesis that testosterone, independent of daylength, is driving changes in chronotype. Using birds as their own controls, and not randomizing the order of our treatments may have influenced our unexpected observations. Future studies should either randomize treatments and/or provide a longer duration between implant removal and photostimulation to see if our observations were likely a result of carry over effects of exposure to supra-physiological testosterone levels from implantation.

## 5 | Conclusion

Here we report that experimentally elevated levels of testosterone under short nonbreeding photoperiods significantly advanced daily onset of activity and increased total daily activity relative to daylength. This suggests that testosterone, and not daylength per se, is responsible for seasonal shifts in sleep/wake cycles. Additionally, we observed, opposite of our hypothesis, a delay in daily onset activity and a decrease in total daily activity after photo-stimulation compared to short days without implantations, even though testosterone levels were elevated following photostimulation. The unexpected observations following photostimulation may be a result of carry over effects on the regulation of ARs and ERs in response to the prolonged exposure to elevated levels of testosterone from implants. Overall, the results of our study observed an uncoupling of the effects of sex hormones and photoperiods on the timing of daily activity patterns, providing insight for future studies seeking to relate timing of reproductive behavioral traits and sex steroids.


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### Ethics Statement

All experiments and handling were approved by the North Dakota State University Institutional Animal Care and Use Committee and conforms with the US National Research Council's Guide for the Care and Use of Laboratory Animals, the US Public Health Service's Policy on Humane Care and Use of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals.

## Conflicts of Interest

The authors declare no conflicts of interest.

## Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## Supporting Information

Additional supporting information can be found online in the Supporting Information section.