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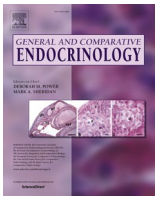

Research paper

Early nighttime testosterone peaks are correlated with GnRH-induced testosterone in a diurnal songbird

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ABSTRACT

Experimental manipulation has established testosterone as a potent, pleiotropic regulator coordinating morphology, physiology and behavior. However, the relationship of field-sampled, unmanipulated testosterone concentrations with traits of interest is often equivocal. Circulating testosterone varies over the course of the day, and recent reports indicate that testosterone is higher during the night in diurnal songbirds. Yet, most field studies sample testosterone during the morning. Sampling at times when levels and individual variation are low may be one reason relationships between testosterone and other traits are not always observed. Testosterone is regulated by the hypothalamic-pituitary–gonadal axis, with gonadotropin-releasing hormone (GnRH) initiating the endocrine cascade. Research has examined GnRH-induced testosterone levels with traits of interest, yet the relevance of these induced levels and their relationship with endogenously produced levels are not fully clear. Using photostimulated male great tits (*Parus major*) we tested the hypotheses that circulating testosterone levels peak during the night and that GnRH-induced testosterone concentrations are positively related to nightly testosterone peaks. Blood was sampled during first, middle or last third of night. One week later, baseline and GnRH-induced testosterone levels were sampled during mid-morning. Morning baseline testosterone levels were low compared with night-sampled levels that peaked during the first third of the night. Further, GnRH-induced testosterone was strongly positively correlated with levels observed during the first third of the night. These data suggest that morning testosterone samples likely do not reflect an individual's endogenous peak. Instead, GnRH-induced testosterone levels do approximate an individual's nightly peak and may be an alternative for birds that cannot easily be sampled at night in the field. These findings are likely to have implications for research aimed at relating traits of interest with natural variation in sex steroid hormone levels.

1. Introduction

Sex steroids, including testosterone, are implicated in the regulation and expression of traits and behaviors related to reproduction. Manipulation of testosterone levels demonstrates a clear link between the endocrine system and reproductive related traits, including territorial aggression and song in many bird species (Enstrom et al., 1997; Moore, 1984; Silverin, 1980; Van Duyse et al., 2002, 2000; Van Roo, 2004; Wingfield et al., 1990). However, numerous studies fail to find clear relationships between natural circulating testosterone levels and reproductive traits of interest (Apfelbeck et al., 2013; Book et al., 2001; Hau and Goymann, 2015; Husak et al., 2006; Johnson et al., 2011; Laucht et al., 2011, 2010; McGlothlin et al., 2008; Roulin et al., 2004; Villavicencio et al., 2014; Weatherhead et al., 1993).

One difficulty in relating circulating levels of hormones with behaviors and other traits of interest arises as a result of the labile nature of hormones. Hormone levels vary in response to social and environmental cues (Adkins-Regan, 2005; Wingfield et al., 1990). Hormone levels, including sex steroids, also vary predictably over a 24 h period (i.e., circadian) (Bailey and Silver, 2014; Nelson and Kriegsfeld, 2017). While it is known that circadian/daily rhythms in circulating levels of sex steroids exist, it is almost universal in field endocrinology studies of songbirds that blood samples are collected during the morning hours, when capturing birds is often easiest. Only a small number of studies

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have reported levels of testosterone across the daily cycle in birds. In these studies on diurnal species, testosterone is reported to peak during the nighttime hours (Foerster et al., 2002; Laucht et al., 2011; Needham et al., 2017). The variance among individuals and range of circulating levels of testosterone during the night also appear to be greater than samples obtained during the morning hours of daylight (Foerster et al., 2002; Laucht et al., 2011; Needham et al., 2017). These observations suggest that most studies are collecting samples at times outside of the peak and when variance among individuals is lower, potentially making it more difficult to uncover relationships. To better understand if natural testosterone peaks are related to behavioral and morphological traits of interest, we need to determine a narrower window for sampling to reliably capture these peaks.

While sampling individuals during the time of peak circulating hormone levels would be ideal, this may not always be feasible or realistic in field studies, especially if the peak occurs during the night while the animal is sleeping or roosting. One current method used to determine individual variation in peak production and output of sex steroids is through exogenous activation of the reproductive endocrine axis by injecting a standardized dose of gonadotropin-releasing hormone (GnRH), which is endogenously released from the hypothalamus (Jawor et al., 2006; Needham et al., 2017). This method stimulates the hypothalamic-pituitary–gonadal (HPG) axis, where GnRH then stimulates the production and secretion of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), from the pituitary. These gonadotropins in turn act on the gonads to stimulate the generation and release of the sex steroids, androgens and estrogens (Nelson and Kriegsfeld, 2017). Exogenous injections of GnRH enable researchers to assess variation in the collective response of the pituitary and gonad to upstream stimulation. While this method of exogenous activation of the HPG axis stimulates sex steroid production and secretion to a level much greater than normal daytime circulating levels of testosterone, it is unclear if these induced testosterone levels reflect endogenous levels of circulating testosterone that an individual would be exposed to, aside from increases in response to social challenges observed in some bird species (McGlothlin et al., 2008; Wingfield et al., 1990).

While the available evidence suggest that testosterone levels are elevated at night in diurnal birds, data are still scarce. Further, if and how these levels vary across the night, how strongly they relate with GnRH-induced levels, and how they compare with daytime levels are not known. Having a better knowledge of these relationships will be important for future investigations of potential testosterone-related traits and will prove useful for this and other systems when sampling during the window of peak endogenous testosterone production is not feasible. Using the great tit (*Parus major*), a common songbird model for research in field behavioral and evolutionary ecology, we aim to establish whether testosterone peaks in morning, or during the early, mid, or late, night hours. Further we aimed to assess relationships between endogenous levels of testosterone circulating at night and GnRH-induced testosterone levels sampled during the morning hours. To address these aims we captured wild great tit males and held them in captivity under standardized conditions. All birds were photostimulated while housed in individual cages at constant temperature with ad libitum food. We collected baseline (i.e. non-stimulated) testosterone samples at several daily time points and injected GnRH to collect GnRH-stimulated testosterone samples from the same individuals.

2. Methods

2.1. Study species, capture and husbandry

The great tit is a well-studied avian species, with much already known regarding aspects of their reproductive physiology and behavior (Caro et al., 2019; Charmantier et al., 2008; Helm and Visser, 2010; Hinde, 1952; Lack, 1964; Nussey, 2005; Perfito et al., 2012; Silverin, 1994; Silverin et al., 1993; te Marvelde et al., 2012; Verhagen et al., 2019). Twenty-seven wild male great tits were captured using playback and mist-nets in mid-late October 2018 in Upper Bavaria near the Max Planck Institute for Ornithology, Seewiesen, Germany. Birds were brought into captivity and housed in two rooms where they were individually held in L × W × H: 81x50x40 cm cages that were visually but not acoustically isolated. Birds were maintained on daylengths closely mimicking natural daylength at time of capture (10:14 L:D) until the end of October when daylength was shortened to 8:16 to ensure breaking of photorefractoriness (Silverin et al., 1993). On 14 November photostimulation was induced with 14:10 L:D for four weeks. All birds received ad libitum food (seed mixture, live mealworms) and water.

2.2. Blood sampling and groupings

Each individual was sampled at one of three nighttime points: 2 h after lights off (21:00, n = 9), the mid-point of the dark-phase (0:00, n = 9) and 2 h before lights on (03:00, n = 9). Night sampling was distributed across two nights (Dec. 9/10 and 10/11) and the two rooms to ensure that individuals sampled were not previously disturbed that night. Briefly, birds were captured from their home cage using red-illuminated headlamps and removed to a separate room to minimize disturbance in the housing room. A small blood sample (~50–60 μl) was collected from the wing vein and stored on ice. Birds were then returned to their home cage. For the night sampling, all blood samples were collected within 22 min of entering the room. All nine birds in the 21:00 group were sampled on the first night from the same room and did not experience disturbance prior to sampling. Five of the birds in the 0:00 group were sampled from an undisturbed room on the first sampling night, the other four were sampled from the room where the 21:00 group birds were sampled (i.e. there was a mild disturbance ~ 3 h prior to sampling). Prior disturbance did not influence circulating testosterone levels in these birds (mean and SEM of birds undisturbed prior to sampling = 1.13 ng/ml +/- 0.24; mean SEM of birds whose room was entered at 21:00 = 1.55 ng/ml +/- 0.89; *t*-test $p > 0.05$). All birds from the 03:00 group were sampled on the second night and did not experience any nighttime disturbance on the day of sampling. Birds not being sampled remained inactive in their cages while researchers were in the room (TJG personal obs.).

Ten days after nighttime sampling, each bird had a blood sample collected (~60 μl) to assess morning baseline testosterone levels. Morning sampling (between 09:00–11:00) was distributed across two days and the two rooms to ensure that individuals being sampled were not disturbed prior to sampling that day. All birds had their baseline blood sample collected within 30 min of researchers first entering the room. Immediately following the baseline blood sample, each individual received an intramuscular injection (~50 μl) of 2 mg/kg of body weight dose of chicken GnRH-I dissolved in phosphate buffered saline (concentration of 25 ng/μL; Bachem Americas Inc, product #H-3106.0005, Vista, CA, USA) (Jawor et al., 2007; Needham et al., 2017). Birds were held in opaque cloth bags until a second blood sample (~50 μl) was collected 30 min post-injection.

All procedures were carried out in accordance with German and EU regulations and the animal care and use protocol was approved by the authorities of Upper Bavaria, Germany and North Dakota State University.

2.3. Testosterone assays

Plasma testosterone levels were measured following the manufacturer's guidelines of a commercially available enzyme immunoassay kit (Enzo Life Sciences, ADI-900–065). Hormones were extracted (2x) from 30 μl of plasma using diethyl ether, dried under nitrogen gas at 25 °C, and reconstituted with assay buffer overnight (1:10 dilution). Concentration was adjusted for samples that did not have 30 μl of plasma available. Reconstituted samples were plated in duplicate (100 μl per

well) and concentrations were determined with a five-parameter logistic curve-fitting program (Microplate Manager, Bio-Rad Laboratories, Inc.). The sensitivity of the assay reported by the manufacturer is 5.67 pg/mL. Intra-plate (n = 3 plates) %CVs, calculated from a standard of known concentration run 3x across the plate, were all below 8%. The inter-plate %CV was 8.3%.

2.4. Statistical analysis

To determine whether testosterone levels varied at different daily time points and after GnRH injection, we ran a linear mixed-effects model with sampling time point as the fixed effect (as a categorical variable) and individual ID as a random effect in order to account for repeated sampling from the same individuals. All birds were sampled for morning basal and morning post-GnRH, but at night individuals were sampled at only one of three time points (21:00; 0:00; 3:00). Following a significant result, pair-wise post-hoc comparisons between sampling time points were performed using the Tukey-Kramer correction. To test for differences in variances of testosterone values between the sampling time point groups we conducted an Analysis of Means for Variances with Levene test. This analysis compares the group means of the absolute deviations from the median (ADM) with the overall sample mean ADM (Brown and Forsythe, 1974; Nelson et al., 2005). To explore relationships between nightly testosterone and GnRH-induced testosterone levels, we conducted a separate linear regression for each of the three nighttime points: before lights off (21:00), mid-point of night (0:00), and before lights on (03:00) relative to that individual's GnRH-induced level. All analyses were performed using JMP Pro 14 (SAS Institute Inc.).

3. Results

There was a significant fixed effect of sampling time point on circulating levels of testosterone ($F_{4,74.67} = 16.35$, $p < 0.001$; Fig. 1). Pair-wise post-hoc comparisons found that, as expected, testosterone levels following GnRH injection administered in the morning were higher than the morning baseline testosterone sample ($t = 6.70$, $p < 0.001$). These post-GnRH injection samples were also higher than unstimulated testosterone levels collected at 3:00 in the morning ($t = 3.21$, $p = 0.016$). Following GnRH stimulation, testosterone levels were not different than testosterone levels sampled during the dark hours at 21:00 (2 h after lights off) and 0:00 (midpoint of dark phase; both $p > 0.05$). Testosterone levels from samples collected at 21:00 were significantly higher than the morning baseline levels ($t = 6.34$, $p < 0.001$), and higher than testosterone samples collected at 0:00 ($t = 3.56$, $p < 0.01$) and the 03:00 sampling time point ($t = 3.85$, $p = 0.022$). All other pairwise comparisons were not significantly different (all $p > 0.05$; see supplemental table 1). The average absolute deviation from the median (ADM) of all samples was 0.72 ng/ml. The analysis of means test for variances test indicated that the baseline morning group displayed significantly lower variance than the overall sample mean variance, with a group mean ADM of 0.12 ng/ml, exceeding the lower deviation limit, while the testosterone samples from the post-GnRH group mean (group mean ADM of 1.22 ng/ml) and 21:00 group (group mean ADM of 1.56 ng/ml) exceeded the upper deviation limit, displaying significantly greater variance than the mean (supplemental Fig. 1 and supplemental table 3).

We found a significant and strong positive relationship between an individual's circulating testosterone at 21:00 and their GnRH-induced testosterone level ($n = 9$, $R^2 = 0.62$, $\beta = 1.75$, $p = 0.01$), with individuals that exhibited higher levels of testosterone at night also exhibiting higher levels following GnRH-injection (Fig. 2). We did not observe significant relationships between GnRH-induced testosterone and testosterone levels sampled from individuals at either 00:00 ($n = 9$, $R^2 = 0.17$, $\beta = 0.23$, $p = 0.27$) or 03:00 ($n = 9$, $R^2 = 0.16$, $\beta = 0.07$, $p =$

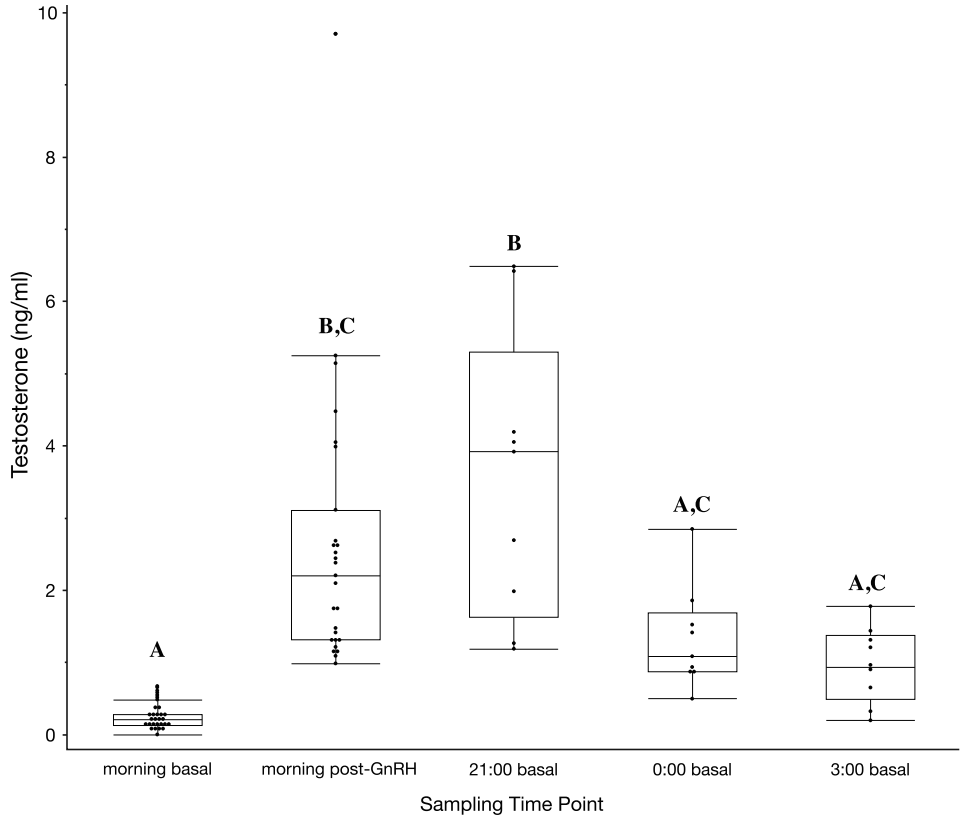

Fig. 1. Box plots of circulating levels of testosterone observed at all sampling time points. A significant main effect of time of sampling on testosterone levels was found. Groups that did not differ in the post-hoc pair-wise comparisons are indicated by the same letter; significant pair-wise differences ($p < 0.05$) are denoted by having different letters.

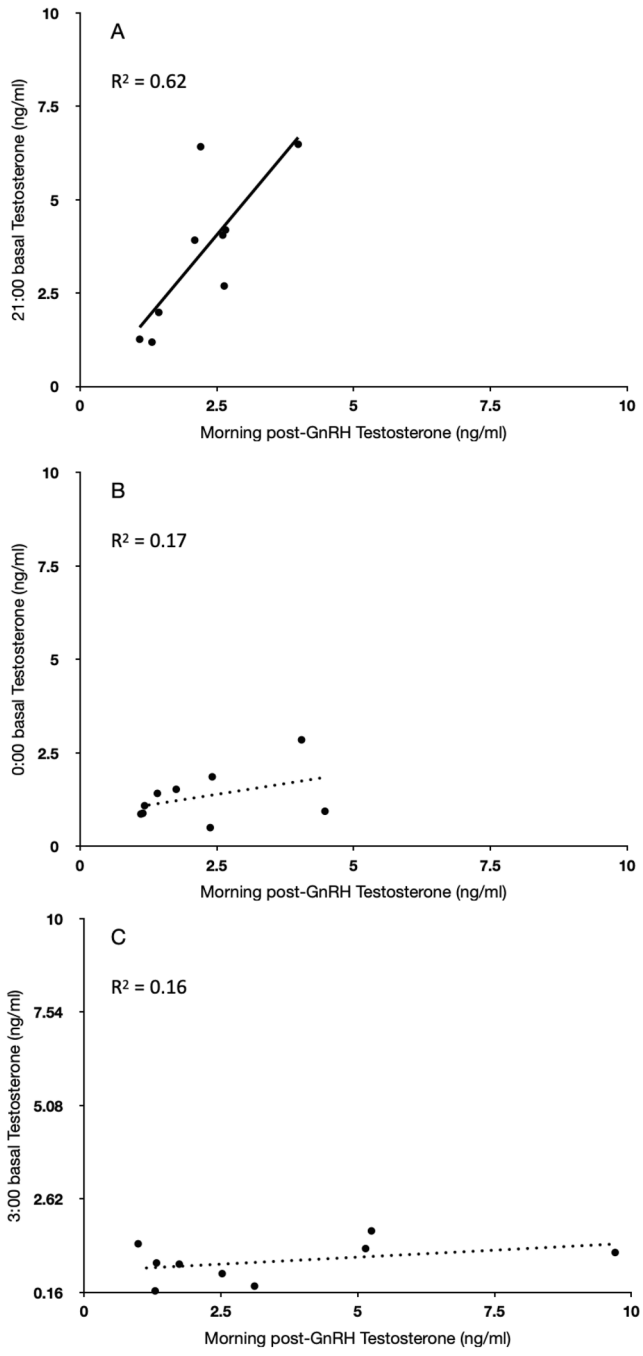

Fig. 2. Correlation between testosterone levels (ng/ml) from morning post-GnRH testosterone levels and circulating testosterone at during the dark hours sampled at 21:00 (a), 0:00 (b), or 3:00 (c). A solid trend-line indicates a significant ($p < 0.05$) relationship between testosterone values at the two time points. A dashed line indicates a non-significant relationship.

0.28).

4. Discussion:

Here we provide evidence of a nighttime testosterone peak in males of a diurnal songbird, the great tit. This nightly peak in endogenous circulating testosterone levels was observed during the first part of the dark-phase (2 h after lights off) and was significantly higher than baseline levels observed during the morning. We also observed that GnRH injections significantly elevated morning testosterone levels and these GnRH-induced levels were significantly positively correlated with testosterone levels observed during the early nighttime (2 h after lights off) endogenous peak, indicating that GnRH challenges induce levels indicative of an individual's peak level expressed endogenously on a daily cycle (at night). This suggests that GnRH-induced testosterone may be used to assess an individual's daily (nighttime) peak, enabling investigations of relationships between natural variation in traits of interest and testosterone.

We, and others previously, observed elevated levels of testosterone during the inactive phase of diurnal birds (i.e. night; Balthazart, 1976; Foerster et al., 2002; Laucht et al., 2011; Needham et al., 2017; Schanbacher et al., 1974). This same pattern of peak levels of testosterone in circulation during the inactive period has also been reported in nocturnal mammals (e.g. during daytime), such as the lesser mouse lemur *Microcebus murinus* (Perret, 1985) and the owl monkey *Aotus trivirgatus* (Dixson and Gardner, 1981). In humans, testosterone also exhibits a circadian rhythm and higher circulating levels are associated with sleep phases (Andersen et al., 2011; Axelsson et al., 2005). In addition, rat testes cultured *in vitro* display robust circadian rhythms, with a peak in testosterone during the early morning (i.e. inactive phase) and a nadir in production occurring during the nighttime hours (i.e. the behavioral active phase) (Chan et al., 1977). In a nocturnal bird, the Indian spotted owlet (*Athene brama*), testosterone levels in males and progesterone and estradiol in females were highest during the daylight hours when activity levels were lowest (Guchhait and Haldar, 1999). Interestingly, in captive house sparrows, males that were sampled 30–60 min after being woken and disturbed had significantly lower levels than males sampled immediately after being woken (Laucht et al., 2011). However this same pattern of disturbance was not observed during the day (Laucht et al., 2011). Together this suggests that testosterone levels are regulated by circadian processes, but that disruption during the inactive phase along with a redistribution of energy to other processes interferes with peak production.

Why testosterone levels are elevated and peak during the inactive phase is not yet known. One possible reason for higher testosterone production during these times may be that testosterone is facilitating sperm production and maturation during this time period – when resources are not being directed towards other functions as they are during the day. For example, sperm levels in house sparrows are depleted during the day and replenished at night (Birkhead et al., 1993). Furthermore, in house sparrows and house finches (*Haemorhous mexicanus*) a daily peak in number of sperm are present in the pre-dawn hours (Quay, 1987). Data on sperm count was not collected in the current study and we are not aware of studies of the daily pattern of spermatogenesis in great tits. An alternative, but not mutually exclusive, possibility may be that glucocorticoids suppress testosterone levels during the active phase. Indeed, most animals demonstrate a peak in glucocorticoid levels just prior to the onset of their active phase and a nadir during their inactive phases (Breuner et al., 1999; Rich and Romero, 2001; Romero and Remage-Healey, 2000), and elevated levels of glucocorticoids are capable of suppressing the HPG axis (Deviche et al., 2010; Vernasco et al., 2019) (see below for further discussion on interactions between glucocorticoids and testosterone).

It has been observed that extra-pair copulations in songbirds are related with activity during the *peri*-dawn period, the time of the dawn chorus (Dolan et al., 2007; Double and Cockburn, 2000; Halfwerk et al., 2011; Poesel et al., 2006). A downstream effect of elevated testosterone at night may be to facilitate adaptive reproductive behaviors such as song, courtship and territoriality in the early morning. Testosterone is implicated in regulating reproductive traits and coordinating morphology, physiology, and behaviors. Interestingly, a report in house sparrows found that badge size was related with night, but not daytime testosterone levels (Laucht et al., 2011). Pharmacologically blocking sex steroid hormonal signaling decreases dawn singing in great tits (Van Duyse et al., 2005). Intensive, long-term field experiments have demonstrated that experimental elevations in sex steroid concentrations influence the extra-pair mating success of male songbirds (Raouf et al.,

1997; Reed et al., 2006). How these experimental elevations of testosterone lead to variation in extra-pair copulations is not yet fully known. The classical mechanism by which steroid hormones, like testosterone, influence biological processes is via activation of cytoplasmic receptors that alter transcription of DNA. Thus, steroid hormones have their effects, via their classical cytoplasmic receptors, including transcription of mRNA, translation and post-translational processing, on the order of hours (Adkins-Regan, 2005). This classical action of testosterone may in part explain the temporal dissociation between our observed peak in the early night and androgen-associated behaviors in the morning. Future work will be needed to more directly relate early night peaks with reproductive related traits and the timing of reproductive related behaviors such as song and extra-pair copulations.

In the lab, both acute and chronic manipulations of sex steroids interact with components of the circadian system (e.g. suprachiasmatic nucleus) in turn influencing vertebrate behavioral rhythms (Daan et al., 1975; Ellis and Turek, 1979; Guyomarc'h and Guyomarc'h, 1994; Gwinner, 1975, 1974; Morin et al., 1977; Takahashi and Menaker, 1980). These studies however often fail to consider the daily rhythm of sex steroids or observe effects within a natural environment. Individual variation in peak sex steroid levels at night may influence the timing of behaviors and activities related with reproductive success.

Variation in testosterone levels naturally produced in response to exogenous stimulation of the HPG axis with GnRH has been found to be related with fitness-related traits and reproductive success. Dark-eyed junco (*Junco hyemalis*) males that elevated testosterone the greatest following injection with GnRH have 'sexier' plumage traits (i.e. tail white) (McGlothlin et al., 2008). During the nestling rearing phase, testosterone levels following exogenous stimulation with GnRH, but not baseline testosterone levels, were related to patterns of paternity within social nests in common redstarts (*Phoenicurus phoenicurus*); males with lower GnRH-induced testosterone levels lost paternity in their nest (i.e. cuckolded) while males that did not lose paternity displayed elevated GnRH-induced testosterone (Villavicencio et al., 2014). A previous report from our lab indicated that both midnight and GnRH-induced testosterone levels in house sparrows were repeatable across four sampling periods and a moderate positive relationship was observed between these two time points (Needham et al., 2017). Wild-caught juncos also displayed moderate repeatability in post-GnRH testosterone levels during the breeding season (Jawor et al., 2006). These data suggest that individuals possess, relative to others, consistently high or consistently low activity of the HPG axis during the breeding life-history stage and that the functioning or 'phenotype' of the HPG axis, and its responsiveness to upstream stimulation (i.e. GnRH) is related to reproductive success.

Combined, we suggest that baseline testosterone levels measured at a single time point that is convenient for sampling, but not biologically meaningful, might not capture aspects of individual HPG functioning that are relevant for the expression of traits related to reproductive success. Further, selection may act on peak expression (i.e. daily peak) or peak capacity to express a particular endocrine trait (i.e. maximum sex steroid secretion) (Hau, 2007; Ketterson et al., 2009; McGlothlin et al., 2010). Thus, a greater understanding of whether or how an individual's peak hormone levels, as opposed to a single sample obtained when levels may be lower than their peak, influence expression and timing of behaviors is needed. Our current data suggest that GnRH-induced levels reflect an individual's endogenously produced peak level and thus, these nightly peaks are likely to be related with traits influencing sexual selection. Future work, with targeted nighttime sampling or GnRH injections may reveal relationships between daily timing and the observed underlying individual variation in peak testosterone levels in circulation at night.

While we observed a strong positive correlation between testosterone levels sampled during the early portion of the night and GnRH-induced levels, circulating levels of testosterone were often slightly lower in the GnRH-induced samples compared with the nighttime baseline samples. The samples collected at night were obtained very shortly after capture, while the GnRH-induced blood sample was not collected until 30 min following GnRH injection. A hormonal stress response (i.e. glucocorticoid secretion) is induced by capturing and handling and glucocorticoids levels rise above basal levels within 2–3 min of exposure to a stressor such as capture (Romero and Reed, 2005; Small et al., 2017). Acute stress and the corresponding increase in glucocorticoids leads to decreases in testosterone levels (Deviche et al., 2010; Vernasco et al., 2019). Both the pituitary and the gonads possess glucocorticoid receptors, thus, the elevated glucocorticoid levels during the thirty-minute waiting period may have dampened the peak testosterone levels that the birds could produce. Indeed, in dark-eyed juncos, birds that were held in a bag for a longer period of time prior to GnRH injection displayed reduced post-GnRH testosterone levels, suggesting that the stress of being held influences the response to GnRH (Jawor et al., 2006). While the dose of GnRH used in this study was found to induce a peak level in another similarly sized songbird, the dark-eyed junco (Jawor et al., 2006), this dose of GnRH may not have produced a maximal response from the pituitary and gonads of great tits. Regardless, the strong correlation observed suggests the potential utility of GnRH "challenges" to uncover natural variation in peak endogenous testosterone levels without nighttime sampling.

It is worth noting that, due to logistics and restrictions on the volume of blood we were able to collect from a single individual during our study, we did not collect samples during the afternoon (i.e. before lights-off). While we were unable to assess hormone levels during this time period, we do not think that afternoon levels would be higher than the levels observed at 21:00. Lapland longspurs (*Calcarius lapponicus*) sampled in Alaska during June displayed relatively low testosterone levels during the 'bright' day, with an increase observed ~ 21:00, a time light levels become dim as the sun passes closer to the horizon (Hau et al., 2002). Further, in domestic ducks, testosterone levels collected at 18:00 did not differ from samples collected at 10:00 (Balthazart, 1976). In the night-active Indian Spotted Owlet, plasma testosterone levels remained low through the mid- and late-dark periods (Guchhait and Haldar, 1999), a period of the daily cycle similar to the afternoon of diurnal birds like the great tit.

Not only were testosterone levels highest at 21:00 (2 h after lights off), this group also demonstrated the greatest range of values and variance (see supplementary table 2 and 3 and supplementary Fig. 1), while the morning basal testosterone values displayed a comparatively small range of values and lower variance. Statistically, it is difficult to detect linear relationships when variation among individuals is low. Thus, studies aiming to relate traits of interest with testosterone sampled in the morning in diurnal songbirds may be less likely to detect relationships because of the relatively low variance compared with nighttime or post-GnRH testosterone levels.

The current study focused on the pattern of sex steroid levels in male songbirds. Less information is currently available about estrogen levels in female songbirds, although testosterone levels in captive female house sparrows were highest during the night (Laucht et al., 2011). Sex steroids impact daily behavioral rhythms not only in males, but also in females (Hagenauer and Lee, 2011; Yan and Silver, 2016). Increased HPG axis activity in both sexes during the night may indicate increased activity at the level of the hypothalamus or pituitary during periods of inactivity or sleep. Thus, it's possible that circadian rhythms of sex steroids, including peak levels during the night, are involved in the timing of important behaviors and activity but this remains to be explored in the field (Elderbrock et al., 2021).

5. Conclusion

Taken together, we report that morning basal testosterone samples were significantly lower than testosterone levels sampled in the first part of the night. Thus, blood samples collected in the morning are not likely to reflect an individual's endogenous peak. Further, we observed that

testosterone levels two hours after lights off were strongly positively correlated with testosterone levels following a morning injection with GnRH. This suggests that GnRH-induced testosterone approximates an individual's nightly peak and may be an alternative for birds that cannot easily be sampled at night in the field. These observations will be important to consider for studies seeking to relate traits of interest with natural variation in testosterone.

CRediT authorship contribution statement

Timothy Greives: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing - original draft, Visualization, Supervision, Project administration, Funding acquisition. **Michelle Eshleman:** Investigation, Writing - review & editing. **Holland Galante:** Investigation, Writing - review & editing. **Emily Elderbrock:** Investigation, Writing - review & editing. **Caroline Deimel:** Investigation, Writing - review & editing, Resources. **Michaela Hau:** Conceptualization, Methodology, Investigation, Resources, Writing - review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2021.113861.

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