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SYMPOSIUM

Onset of Daily Activity in a Female Songbird Is Related to Peak-Induced Estradiol Levels

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Synopsis Research in captive birds and mammals has demonstrated that circadian (i.e., daily) behavioral rhythms are altered in response to increases in sex-steroid hormones. Recently, we and others have demonstrated a high degree of individual repeatability in peak (gonadotropin-releasing hormone [GnRH]-induced sex) steroid levels, and we have found that these GnRH-induced levels are highly correlated with their daily (night-time) endogenous peak. Whether or not individual variation in organization and activity of the reproductive endocrine axis is related to daily timing in wild animals is not well known. To begin to explore these possible links, we tested the hypothesis that maximal levels of the sex steroid hormone estradiol (E_2) and onset of daily activity are related in a female songbird, the dark-eyed junco (*Junco hyemalis*). We found that females with higher levels of GnRH-induced E_2 departed from their nest in the morning significantly earlier than females with lower stimulated levels. We did not observe a relationship between testosterone and this measure of onset of activity. Our findings suggest an interaction between an individual's reproductive endocrine axis and the circadian system and variation observed in an individuals' daily activity onset. We suggest future studies examine the relationship between maximal sex-steroid hormones and timing of daily activity onset.

Background

Biological rhythms are highly conserved across taxa (Dunlap et al. 2004) and daily timing of behaviors in the laboratory have been shown to correlate with internally driven circadian (i.e., daily) rhythms (Aschoff and Wever 1966; Duffy et al. 2001). In free-living populations, the timing of daily activity is likely important for survival strategies (Pittendrigh 1954). For example, being active at a time when predators are hunting may attract unwanted attention and reduce survival (DeCoursey et al. 2000). Similarly, daily timing and seasonal expression of reproductive behavior and morphology likely have a strong influence on mating success (Hau et al. 2017). While nearly all animals express daily rhythms in behavior, a large degree of variation within species and populations are observed (Aschoff and Wever 1966; Duffy et al. 2001; Horne and Östberg 1977). The mechanisms that give rise to this variation in timing of daily activities are not yet fully understood.

The influence of sex-steroid hormones on circadian rhythms in captivity are well studied (e.g., Gwinner 1974; 1975; Daan et al. 1975; Morin et al. 1977; Ellis and Turek 1979; Takahashi and Menaker 1980; Guyomarc'h and Guyomarc'h 1994). For example, in captive female hamsters, estradiol (E_2) implants lead to earlier awakening times when

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compared to females with empty implants (Morin et al. 1977; Takahashi and Menaker 1980). In male birds, experimentally induced and naturally increasing (i.e., seasonal) levels of testosterone (T) have been found to lengthen the active period of captive individuals during the breeding season (Gwinner 1974, 1975). Awakening time and levels of E_2 are correlated in women; females with higher levels of E_2 have an earlier awakening time than females with lower levels of E_2 (Bracci et al. 2014). Additional work has shown that in mammals, including mice and humans, the suprachiasmatic nucleus (SCN) of the hypothalamus, which is the master circadian clock controlling endogenous rhythms, contains androgen receptors (AR) and estrogen receptors (ER), therefore suggesting hormonal regulation of rhythms of daily activity (Kruijver and Swaab 2002; Vida et al. 2008; Model et al. 2015). Gonadectomy has been found to reduce AR expression on the SCN and result in changes to locomotor activity (Karatsoreos et al. 2007; Iwahana et al. 2008; Model et al. 2015). It is not yet fully established if, similar to mammals, birds possess AR and ERs in the core circadian machinery (e.g., SCN or pineal gland). However, it is likely that they do possess these receptors in some or all components of the circadian system since evidence in both birds and mammals (above) suggest that manipulation of sex steroid hormones influence circadian rhythms and daily patterns of behavior.

Interestingly, during the early breeding season just prior to egg laying female birds generally emerge from nest cavities before the sun rises (Schlicht et al. 2014), which is approximately the same time of year when baseline E_2 levels peak, as more yolky follicles are developed (Williams et al. 2004). Female mice have significantly higher expression of ERβ in SCN neurons compared to males (Vida et al. 2008), therefore suggesting that E_2 may influence their timing of daily behaviors. Yet, there is a lack of information about the relationships between individual variation in E_2 and behavioral timing of rhythms in non-human, free-living female vertebrates.

Circulating levels of baseline sex-steroid hormones are typically measured from samples collected during the daytime or after individuals had been in captivity for several hours. Previous research attempting to relate baseline levels of plasma T and E_2 to reproductive timing (Chastel et al. 2003; Williams et al. 2004), behavior (Damassa et al. 1977, Wells 1984; Eikenaar et al. 2011; Burtka et al. 2016), and other phenotypic traits (Mcglothlin et al. 2008; Huyghe et al. 2009) often do not observe consistent relationships between the sex-steroid hormone levels and the traits of interest. Within males, evidence suggests that reproductive traits are better correlated with variation in response to an injection of gonadotropin-releasing hormone (GnRH), which induces a short term increase in circulating T to maximal levels (Jawor et al. 2006, 2007), rather than variation in baseline levels (McGlothlin et al. 2007). Recently it was found that in a captive songbird, the house sparrow (*Passer domesticus*), nighttime levels of T in both males and females are significantly higher than daytime levels (Laucht et al. 2011; Needham et al. 2017), suggesting hormone levels obtained during the daytime may not be fully reflective of an individual's maximal secretion capacity. While capturing wild individuals at night may not always be feasible, recent evidence suggests that GnRH-induced T levels are reflective of nighttime T levels in captive house sparrows (Needham et al. 2017). Thus, for those situations where nighttime sampling in the wild is not feasible, GnRH challenges may provide an alternative method to assess an individual's maximal levels of T and E_2, which can then be utilized in investigations attempting to relate the influence of sex steroids with traits of interest. Here, we investigate relationships between GnRH-induced sex steroid levels and our trait of interest, onset of daily activity in a wild songbird. While previous studies have observed relationships between activity rhythms and (often) manipulated sex steroid hormone levels (see above), our knowledge of how an individual's natural, maximal levels of hormones like T and E_2 are related to daily activity rhythms in the wild is lacking. Understanding the mechanisms that influence daily timing behaviors in the wild may be important for understanding differences in fitness related traits (DeCoursey et al. 2000; Graham et al. 2017a).

Here we tested the hypothesis that individual variation in maximum E_2 levels is related to female activity onset during the breeding season. Specifically, we predicted that, similar to observations in humans, females departing from the nest earlier in the morning would have higher max E_2 levels compared to females that depart from the nest later in the morning. Based on evidence that supplementary T in males advances activity onset (Gwinner 1974, 1975) and the finding that of T and E_2 were correlated in pooled samples of plasma from dark-eyed juncos (*Junco hyemalis*), (Rosvall et al. 2013), we additionally measured GnRH-induced plasma T. This allowed us to address whether T or E_2 is more closely related to onset of activity in free-living females.

Methods

Ethics

All methods used in the study were approved by the North Dakota State University Institutional Animal Care and Use Committee (IACUC Protocol #A13063).

Pre-breeding trapping and blood collection

We monitored and studied a population of songbirds, dark-eyed juncos (*J. h. aikeni*), near Lead, SD, USA (44o14′38″N, 103o51′55″W). Females were passively trapped prior to the onset of egg laying using seed-baited potter traps from April 17, 2016 to May 9, 2016, a time when the reproductive system should be primed for breeding and responsive to GnRH. Immediately upon capture, females were weighed and given an intramuscular injection 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). Females were held in opaque cloth bags until 30 min post-injection when a single blood sample was collected. Due to the large amount of plasma required by our assay to measure E_2 and regulation limits on volume of blood that can be collected, only post-GnRH samples were collected. A pilot study conducted in this species found that females had elevated levels of E_2 30 min after a GnRH challenge compared to control females (Needham et al. 2019). Blood samples were stored on ice until centrifugation. Plasma samples were stored at −20°C until assayed for plasma E_2. Testosterone was not assayed in these samples because plasma was used to measure levels of very-low-density lipoprotein (Needham et al. 2019). All samples were collected prior to the first egg of the season being laid.

Determining onset of activity

Exhaustive nest searching was conducted during the breeding season (May 10–June 30, 2016). Dark-eyed juncos nest on the ground, making nests easy to find and monitor (Nolan et al. 2002). To determine onset of activity for individual females, Thermochron iButtons (Model DS1921G-F# with iButton Connectivity Kit Model SK-IB-R) were placed in the nests of incubating females to record nest temperature to the nearest 0.5°C every 3 min for four consecutive mornings (Graham et al. 2017a). Briefly, nest temperature from 03:00 to 08:00 was plotted and the first major dip in temperature was used to indicate the timing of the first off bout of the morning. All first daily departure times were calculated with respect to sunrise using the United State Naval Observatory data base (http://www.usno.navy.mil/USNO). We have previously shown onset of activity to be a highly repeatable behavior within this population. A subset of females in the current study was part of this previous study ($n = 24$ of 30) (Graham et al. 2017a).

Incubation trapping and blood collection

To attempt to assess sex steroid hormone levels at the time of behavioral activity data, females were captured from the nest using either a butterfly net or strategically placed mist nets later in the morning following the last recording period for iButton calculation of daily activity onset (Graham et al. 2017b). GnRH injections followed the same protocol used with pre-breeding females. A total of 30 females had complete activity data and a blood sample collected during incubation for measurement of hormones. Of the 30 females, 13 females also had pre-breeding GnRH-induced E_2 samples. Our goal with this protocol design was to test whether individual variation in maximum E_2 levels is related to female activity onset at a physiologically relevant time when females are preparing for reproduction (pre-breeding) and a temporally similar time to the behavior measured (incubation). However, due to the low number of detectable samples during incubation, we did not have sufficient sample size to calculate repeatability of E_2 between the two time points ($n = 3$ females with detectable E2 levels both pre- and post-egg laying). Blood samples were stored on ice until centrifugation to collect plasma. Plasma samples from incubation were assayed for plasma E_2 and T.

Hormone assays

Plasma E_2 levels were measured following the manufacturer's guidelines using a commercially available enzyme immunoassay kit (Enzo Life Sciences, Cat # ADI-900-174) that has previously been used in songbirds, including the dark-eyed junco (Gall et al. 2013; Wilcoxen et al. 2015; Needham et al. 2019). Briefly, hormones were extracted (3×) from 100 μL of plasma using diethyl ether, dried in nitrogen gas at 25°C, and reconstituted in assay buffer overnight (1:4.3 dilution). When 100 μL of plasma was not available, the concentration was adjusted ($n = 1$). Reconstituted samples were plated in duplicate (100 μL per well) and concentrations were calculated using a four-parameter logistic curve-fitting program (Microplate Manager; Bio-Rad Laboratories, Inc.).

The sensitivity of the assay is 14.0 pg/mL and this value was conservatively assigned to any individuals measuring below the detection limit ($n = 4$ of 13 pre-breeding, $n = 22$ of 30 incubating). Pre-breeding females were run as part of a larger set of samples ($n = 13$ of 94) across three plates. Inter-plate variation was 5.4% and intra-plate variation ranged from 3.0% to 8.8% (plates 1:4.2%, 2:3.0%, 3:8.8%) (Needham et al. 2019). All incubating female samples fit on a single plate and intra-plate variation was calculated at 3.71%.

Plasma T from the samples collected during incubation was also measured following the manufacturer's guidelines of a commercially available enzyme immunoassay kit on a single plate (Enzo Life Sciences, ADI-900-065). Hormones were extracted (2×) 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 after running the E_2 assay ($n = 7$ of 30). Reconstituted samples were plated in duplicate (100 μL per well) and concentrations determined using a five-parameter logistic curve-fitting program (Microplate Manager, Bio-Rad Laboratories, Inc.). Samples that were below detection limit ($n = 3$) were set at sensitivity of the assay (5.67 pg/mL). Intra-plate variation was calculated at 4.12%.

Statistical analyses

All statistical analyses were conducted in R version 3.2.2 (R Core Team 2015) using package lme4 (Bates et al. 2015). Hormone concentrations were non-normally distributed, thus these values were natural log transformed for all models which improved normality. We first tested for a correlation between T and E_2 from our samples collected during incubation; this relationship was previously reported from pooled plasma samples from multiple GnRH injected female juncos (Rosvall et al. 2013). A correlation between these values would allow future researchers to measure T as a proxy for E_2, which requires less than one third the amount of plasma to assay using commercially available enzyme immuno-assays. To confirm whether there was collinearity between E_2 and T, we ran a linear model to determine if E_2 and T were correlated in incubating females with detectable levels of both T and E_2 ($n = 8$). However, the lack of a relationship allowed us to include E_2 and T values as independent variables in a single model (see "Results" section).

To test for relationships between pre-laying GnRH-induced E_2 levels and onset of activity during incubation, we used a linear mixed-effects model with onset of activity recorded over 4 days as the dependent variable and pre-breeding E_2 levels during incubation as the independent variable. A second linear mixed-effects model was run comparing onset of activity with T and E_2 values in all 30 incubating females. This model additionally included the day of the year that final behavioral samples were measured (May 1 = 1). Sampling occurred over a 44 day period, thus we wanted to account for any potential changes in timing of behavior with respect to sunrise across the reproductive season (Graham et al. 2017a). In both models, female ID was included as a random effect to control for repeated measures in nest departure time. Effect sizes (r) for the mixed-effects model were calculated using the formula provided by Nakagawa and Cuthill (2007).

Results

Individuals with higher levels of GnRH-induced E_2 during both pre-breeding ($F_{1,11} = 5.27$, $P = 0.04$, $r = -0.27$, Fig. 1) and incubation ($F_{1,26} = 6.26$, $P = 0.02$, $r = -0.18$, Fig. 2) displayed earlier nest departure times during incubation. GnRH-induced T was not related to onset of daily activity ($F_{1,26} = 0.65$, $P = 0.43$, $r = 0.06$, Fig. 3). We additionally observed a trend for females sampled later in the season rising earlier relative to sunrise compared with females sampled earlier in the season ($F_{1,26} = 3.66$, $P = 0.07$, $r = -0.14$). There was not a significant correlation between GnRH-induced T and E_2 in incubating females ($F_{1,6} = 0.35$, $P = 0.57$, Supplementary Fig. S1). A summary of E_2 and T data from both time-points is reported in Table 1.

Discussion

We found that individual variation in pre-breeding levels of GnRH-induced E_2 is related to daily behavior activity patterns in free-living female songbirds. Stimulated-E_2 samples collected immediately following iButton behavioral data during incubation were undetectable in the majority of samples. In incubating females, maximal levels of T were not correlated with onset of activity. There was a trend for incubating females recorded later in the season to awaken earlier relative to sunrise compared with females recorded earlier in the season. We additionally did not find a relationship between GnRH-induced plasma T and E_2.

We observed that higher GnRH-induced E_2 levels in female dark-eyed juncos, particularly in samples

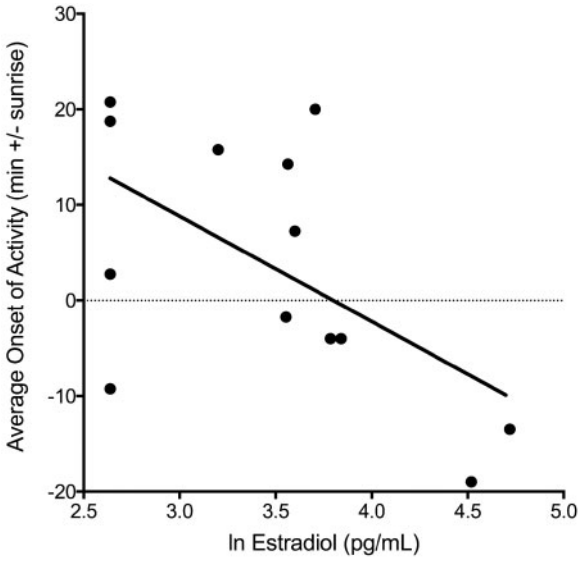

Fig. 1 Pre-breeding levels of GnRH-induced E_2 were negatively with onset of daily activity during incubation ($r = -0.27$, $P = 0.04$); females with higher post-GnRH E_2 during the pre-breeding period. (Note for visualization purposes the average of daily activity onset time relative to sunrise is plotted. The statistical linear mixed effects model included each day as a separate data point controlling for individual.)

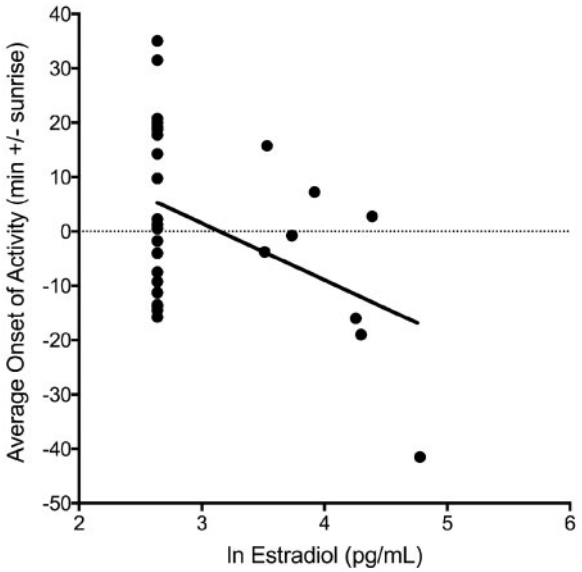

Fig. 2 GnRH-induced levels of E_2 are correlated with onset of activity in incubating female dark-eyed juncos ($r = -0.18$, $P = 0.02$). (Note for visualization purposes the average of daily activity onset time relative to sunrise is plotted. The statistical linear mixed effects model included each day as a separate data point controlling for individual.)

collected prior to laying, were correlated with earlier onset of daily activity (i.e., first nest departure). Samples from pre-breeding females offered a time when individuals were preparing for reproduction

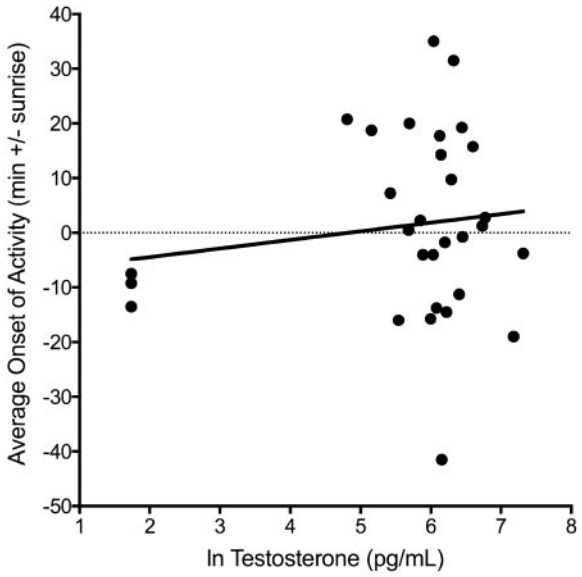

Fig. 3. While most females had detectable levels of GnRH-induced T, there was no relationship with onset of activity ($r = 0.06$, $P = 0.43$). (Note for visualization purposes the average of daily activity onset time relative to sunrise is plotted. The statistical linear mixed effects model included each day as a separate data point controlling for individual.)

and had an active and sensitive hypothalamic–pituitary–gonadal (HPG) axis. One drawback to sampling at this pre-breeding time point is that it is not possible to directly temporally relate hormone response and behavior since hormone sampling was temporally distinct from behavioral sampling that was obtained during incubation. Thus, we also collected hormone samples from incubating female dark-eyed juncos to compare hormone levels to behavioral observations taken at the same time-period. While E_2 levels were, in general, lower during the incubation period and many samples were below the detection limit of the assay, we observed the same pattern as with the females sampled just prior to breeding; females with higher levels of E_2 following GnRH-injection initiated daily activity earlier than females with lower stimulated E_2 levels. Thus, we cautiously suggest a functional link between an individual's reproductive endocrine axis and patterns of daily activity, though the direction of this link is as of yet unknown. Experimental captive studies in mammals and birds indicate that manipulation of sex-steroid hormones alters aspects of the circadian system or daily timing of behavioral rhythms (Gwinner 1974, 1975; Daan et al. 1975; Morin et al. 1977; Takahashi and Menaker 1980; Albers 1981). The current data collected from free living birds are also in agreement with an observational study in humans that found a correlation between

Table 1 Summary of GnRH-induced E_2 and T data for 30 females with behavioral recordings of activity onset during incubation

Time – Hormone	*n*	Mean(pg/mL)	Range (pg/mL)
Pre-breeding E_2	13	40.20 ± 8.37	14.0–112.11
Incubation E_2	30	27.03 ± 4.78	14.0–118.69
Incubation T	30	479.83 ± 61.47	5.67–1503.11

Undetectable levels are reported as the lowest detection limit of the assay (E_2 = 14 pg/mL, T = 5.67 pg/mL). Mean values reported ± SEM.

early awakening and higher levels of E_2 in women (Bracci et al. 2014).

While we observed relationships between post-GnRH E_2 and onset of daily activity during both pre-breeding and incubation, we found many individuals with undetectable hormone levels from samples obtained during incubation. Indeed, while we were able to detect circulating concentrations of E_2 in nearly 70% of samples in the period leading up to laying, we were only capable of detecting E_2 in roughly one quarter of our samples obtained from incubating females. Regulatory mechanisms during incubation may dampen the responsiveness of the pituitary and/or ovary to GnRH stimulation. In mammals and birds, baseline E_2 levels fluctuate drastically throughout the breeding cycle (Elias et al. 1984; Williams et al. 2004) and baseline levels have been observed to quickly drop back to non-breeding levels following laying in another songbird, the European starling (*Sturnus vulgaris*) (Williams et al. 2004). GnRH-induced levels of T in females are also significantly reduced during incubation compared to pre-breeding (George and Rosvall 2018), suggesting a significant decline in sensitivity of the pituitary and/or ovary to upstream (i.e., GnRH) stimulation after laying has ended. Elevated levels of prolactin may act as one mechanism that may be responsible for the significant decline in sensitivity of the axis during incubation; high levels of prolactin may suppress the response of the gonads to a surge in LH (Bailey 1950; Zadworny et al. 1989; Sharp and Blache 2003), which could result in lower E_2 levels that our assay was unable to detect. This would result in the increase in the number of undetectable hormone samples from incubating females versus pre-breeding females.

Our finding that GnRH-induced E_2 levels measured during pre-breeding are correlated with onset of activity during incubation suggests that between-individual relationships linking E_2 and activity onset persist across different reproductive phases; those individuals with the highest maximal levels of E_2 during the pre-breeding stage are likely always awakening earliest. We do note that our field study was only able to find nests of 13 individuals that were sampled prior to breeding, thus our statistical power may be low with increased leverage held by single points. Female awakening and nest departure time has been found to be repeatable across the winter months (Stuber et al. 2015). Similar relationships between E_2 and onset of activity at two time-points also suggest onset of activity is repeatable within individuals across the breeding season, but further work with a larger sample size is needed to address this relationship. Furthermore, steroid hormones which classically act via intracellular receptors that alter transcription (e.g., E_2) often influence biological responses over prolonged time-periods (Nelson and Kriegsfeld 2016). Thus, although the pre-breeding hormone samples were temporally distinct from the activity data collection, when considering that both pre-breeding and incubation relationships with nest departure time showed similar patterns in the predicted direction suggest a biologically relevant relationship.

The lack of a correlation between T and E_2 from samples obtained during the incubation phase was unexpected. Previously, a positive correlation between pooled E_2 and T samples after a GnRH-challenge was reported in photo-stimulated, captive female dark-eyed juncos (Rosvall et al. 2013). Sampling during incubation may be a contributing factor to the lack of a correlation between T and E_2 in our study. Our sample size was greatly reduced due to few detectable E2 samples during incubation compared with pre-breeding, reducing the likelihood of detecting a correlation. Unfortunately, we were unable to assay T during the pre-breeding period due to limitations on plasma volume. Future work is needed to understand the relationships between these two steroid hormones throughout the breeding season.

We did not detect a relationship between GnRH-induced T and activity onset during incubation. One explanation for the lack of a relationship is the lack of a correlation between T and E_2 during incubation. Alternatively, there may be sex-specific differences in AR and ER abundance on the SCN. Previous work in mammals has found that males have higher expression of AR (Iwahana et al. 2008) but females have higher expression of ER in the SCN (Vida et al. 2008). Finally, behavioral timing in females may be less sensitive to T and more responsive to E_2 as captive studies reporting an effect of sex-steroid implants on female circadian behavior have also solely used E_2 and not T (Morin et al. 1977; Takahashi and Menaker 1980).

While timing of nest initiation was not the focus in this study, recent evidence points to a relationship between daily rhythms and reproductive timing in the wild where those who awaken earliest also breed at earlier dates (Graham et al. 2017a). In wild great tits (*Parus major*), implants that provide prolonged nighttime-like levels of melatonin and weaken circadian rhythms in captive birds (Turek et al. 1976; Beldhuis et al. 1988) delayed activity onset in free-living males (Greives et al. 2015), but also led to delayed onset of clutch initiation in free-living females (Greives et al. 2012), further suggesting a relationship between daily rhythms and seasonal timing. As seasonal timing has been shown to influence annual reproductive success (Perrins 1970; Bourdon and Brinks 1982; Olsson and Shine 1997; Dawson and Clark 2000; Lepage et al. 2000; Doody et al. 2004; Low et al. 2015), a better understanding of the mechanisms, including sex-steroid hormones, that may link endogenous daily rhythms and seasonal timing is warranted. Interestingly, while many studies have documented the clear advantages of earlier seasonal breeding, few individuals breed during this optimal time, thus causing continued exploration of the mechanisms controlling timing of seasonal breeding (Verhulst and Nilsson 2008; Wilson and Nussey 2010; Low et al. 2015). It is possible that individuals with higher maximal levels of sex-steroid hormones (likely occurring at night) (Laucht et al. 2011) are able to prepare for breeding sooner. For example, evidence suggests that spermatogenesis occurs at night and that the diel peak of T at night helps regulate this process (Riley 1937; Suresh and Moudgal 1995). The ability of high maximal levels of E_2 to influence not only variation in daily rhythms, but also its potential relationship with seasonal timing of breeding at the individual level, requires further research in free-living individuals.

We observed substantial (>1 h) variation between the onset of daily activity during the incubation period. In additional to interactions with seasonal timing systems (see above), this variation in onset of daily behavioral activity may also be influenced by additional selective pressures. For example, early risers may have increased time for foraging, but could experience sleep debt (Ouyang et al. 2017) and perhaps generate a greater number of reactive oxygen species and elevated oxidative stress (McEwen 2006). Furthermore, cooler early morning temperatures may impact embryonic development of the eggs of early morning rising mothers (DuRant et al. 2013). Future work will be needed to more fully understand both the mechanistic basis giving rise to this variation in daily timing, but also the likely selective pressures acting to shape these endogenously-driven patterns of behavior.

Conclusion

Our study provides evidence for a relationship between maximum-induced levels of the sex-steroid hormone E_2 and daily timing of activity onset in the wild. This is an important first step in understanding how hormones may interact with or regulate daily timing behaviors. However, future work is needed to determine if these findings hold in other species and are related to timing during other life history traits. We suggest future studies with access to technology that is capable of remotely monitoring activity patterns outside of the incubation period address whether similar relationships between sex steroid hormones and daily behavioral patterns occur during the pre-breeding and egg laying stages in free-living female birds. Our current understanding of how maximal production of E_2 from the HPG axis relates to individual variation in behavior is limited and future experimental work is needed to better understand the regulatory effects of E_2 on timing of behavior in free-living organisms.

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Supplementary data

Supplementary data available at *ICB* online.

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