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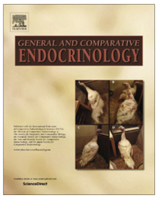

Research paper

Consistent individual variation in day, night, and GnRH-induced testosterone concentrations in house sparrows (*Passer domesticus*)

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ABSTRACT

The hypothalamic-pituitary-gonadal (HPG) axis, with gonadotropin-releasing hormone (GnRH) initiating the endocrine cascade, regulates testosterone secretion. Testosterone, through its pleiotropic effects, plays a crucial role in coordinating morphology, physiology and behavior in a reproductive context. The concentration of circulating testosterone, however, varies over the course of the day and in response to other internal or external stimuli, potentially making it difficult to relate testosterone sampled at one time point with traits of interest. Many researchers now utilize the administration of exogenous GnRH to elicit a standardized stimulation of testosterone secretion. However, it has remained unclear if and how this exogenously stimulated activation of the HPG axis is related with endogenously regulated testosterone that is capable of influencing testosterone related traits. Repeated measures of a hormone can uncover consistent individual variation in hormonal differences at the HPG axis level, variation that potentially stems from underlying genetic variation in a population experiencing identical environmental cues. Thus, we asked, using the house sparrow (*Passer domesticus*), how daily endogenous variation in testosterone profiles relates to GnRH-induced testosterone secretion. Further, we explore the relationship between endogenous daily testosterone peaks and GnRH-induced testosterone with badge size, a morphological trait related with status within a social group. We found that GnRH-induced testosterone levels reflect a highly repeatable hormonal phenotype that is strongly correlated with nighttime testosterone levels. The results demonstrate the usefulness of GnRH-induced testosterone in studies aimed at understanding individual variation and selection on endogenously regulated testosterone levels and the potential importance of nighttime testosterone levels to physiology and behavior.

1. Introduction

Researchers aiming to understand the role hormones may play in evolutionary processes have often attempted to relate circulating hormone levels with discreet phenotypic traits and direct fitness-related traits (Balthazart, 1983; Comendant et al., 2003; Dixson and Anderson, 2004; Harding, 1983; Hau et al., 2010; Hutchison, 1978; Kempenaers et al., 2008; Moore, 1984; Ouyang et al., 2011b; Solís and Penna, 1997). Testosterone-mediated traits have often been related with morphological, physiological and behavioral traits such as dominance rank, plumage characteristics, immune function and genetic quality (Griffith et al., 1999; Jensen et al., 2004; Veiga, 1993). For example, morphological 'ornaments' have been observed to be related with testosterone. However, hormone levels in circulation are highly variable and actively respond to the environment and individual experiences (reviewed in: Ketterson et al., 2005; Wingfield et al., 1990) and also vary as a result of underlying endogenous rhythms (Bell-Pedersen et al., 2005; Daan et al., 1975; Gwinner, 1975, 1974; Morin et al., 1977; Takahashi and Menaker, 1980). Thus, relating an individual's circulating hormone levels sampled at a single time point poses challenges for interpretation and investigation of relationships with other traits of interest (Fusani, 2008).

Classic manipulation experiments have enabled investigators to research the broad effects of hormones such as testosterone at the population level. However, these types of manipulations do not easily allow for natural temporal changes in these levels or individual variation in endogenously produced levels (Balthazart et al., 1984; Desjardins and Turek, 1977; Lofts et al., 1973; Stetson, 1972).

An additional approach being utilized by evolutionary endocrinologists is a hormone 'challenge' to probe the capability of endocrine systems to respond to upstream stimulation or feedback. This approach attempts to generate a *standardized* physiological response produced by an individual, while allowing for individual variation in maximal capacity of the system to remain. These

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values can then be related with individual variation in other traits of interest that may influence individual variation in fitness. For example, injections of the glucocorticoid receptor agonist dexamethasone or with adrenocorticotropic hormone (ACTH) allow researchers to assess negative feedback and maximal stimulation components, respectively, of the endocrine stress response (Bauer et al., 2015; Brown et al., 2001; Romero et al., 2008, 1998; Romero and Wikelski, 2010, 2006). Similarly, 'gonadotropin-releasing hormone (GnRH) challenges' (GnRH is endogenously released from the hypothalamus and activates the reproductive endocrine axis) have been employed to relate maximum sex steroid secretion with reproductive success (McGlothlin et al., 2010), parental care behavior (Goymann and Wingfield, 2004) and sexually selected traits (McGlothlin et al., 2008; McGlothlin and Ketterson, 2008)

While these 'challenges' are providing important insight into relationships between to daytime baseline levels and magnitude of responsiveness (Apfelbeck and Goymann, 2011; Goymann et al., 2015, 2007; Greives et al., 2006; McGlothlin et al., 2008; Moore et al., 2002), further research is needed to better understand how these exogenously stimulated short-term increases in testosterone relate to endogenous levels experienced by an individual at other key points in time. Said another way, given the temporal fluctuations in hormone titers exhibited within an individual throughout the day, does the 'challenge' approach provide insight into the levels that an individual endogenously produces at a given point throughout the day? Specifically, in this investigation we attempt to better understand the relationship between endogenous daily variations in testosterone and levels observed following a 'GnRH challenge.'

Here, we ask if elevated nighttime levels of endogenous testosterone secreted by individuals are related to maximum testosterone secretion following stimulation with exogenous GnRH. To address this, we measured daytime baseline and GnRH-induced testosterone titers as well as circulating baseline testosterone levels at night from captive house sparrows (*Passer domesticus*). Badge size in house sparrows has been related with nighttime testosterone levels (Laucht et al., 2011) and amount of white in tail feathers in dark-eyed juncos (*Junco hyemalis*) is related with GnRH-induced testosterone (McGlothlin et al., 2008). Therefore, we related testosterone levels with badge size, a well-studied phenotypic trait in the house sparrow (Cordero et al., 1999; Laucht et al., 2011, 2010; Liker and Barta, 2001; Veiga, 1993; Whitekiller et al., 2000).

Further, we explored the repeatability of these endogenous hormones levels as well as GnRH-induced levels by sampling once per week for four weeks. Previous research evaluating diurnal changes in testosterone have observed a significant testosterone peak during the nighttime in house sparrows (Laucht et al., 2011). Such studies have begun to explore if and how these night levels were related to phenotypic traits and behaviors (Balthazart, 1976; Foerster et al., 2002; Laucht et al., 2011). However, in many species, obtaining circulating testosterone levels during the night would be very difficult in the wild. Thus, if peak night testosterone is correlated with GnRH-induced levels and both are repeatable, the use of a GnRH 'challenge' approach may provide additional opportunities to address research questions aimed at understanding links between the endogenous activity of the reproductive endocrine system and phenotypic traits of interest (e.g. ornamentation, behavior).

2. Materials and methods

2.1. Study individuals

Seventeen male house sparrows were captured in baited potter traps in Fargo, ND during October 2014. All individuals were held in captivity for a total of six months. Each house sparrow was individually housed at the North Dakota State University animal housing facility in 59.7 × 39.4 × 30.5 cm wire cages. All cages were visually, but not acoustically isolated. The birds had *ad libitum* access to food (canary seed), drinking water, bath water, and grit. Vitamin water was provided one week out of every month (eCOTRITION Pro Ultra-Care Vita-Sol for caged birds). After capture, individuals were maintained on a light-dark cycle of 8L: 16D for eight weeks to ensure a photosensitive state (Farner et al., 1966; King and Farner, 1963). The population was then photostimulated with a long day light-dark cycle of 16L:8D (light on: 8:00 am, light off: 12:00am) to trigger gonadal recrudescence and a reproductive state (King and Farner, 1963; Small et al., 2007). The longest day length experienced by house sparrows in Fargo, ND is 15 h and 52 min of light (US Naval Observatory, http://aa.usno.navy.mil/). Temperature was held between 22.2 and 23.9 °C. Animal care guidelines were followed and approved by our institutional IACUC (#A14044).

2.2. Individual sampling and measuring

Four weeks following the light transition to 16L:8D, all 17 individuals were blood sampled. Birds were determined to be in reproductive state based on presence of an enlarged cloacal protuberance and an ability to produce an ejaculate. All birds were subjected to four weeks of sampling, with blood taken each week at three time points: daytime, post-GnRH, and nighttime. Blood samples to measure baseline day and night hormone levels were always collected within thirty minutes of entering the animal room. For the daytime blood sampling, we captured all males two hours after lights on, a time chosen to mimic a common morning sampling time obtained in field studies. Males then received an intramuscular injection of chicken GnRH-I (1.25 μg dissolved in 50 μL PBS for a final concentration of 25 ng/μL; American Peptide product #54-8-23, Sunnyvale, CA, USA). A dose of 2 mg/kg GnRH-I was administered (Jawor et al., 2007, 2006). Individuals were then held in cloth bags and bled a second time 30 min after the GnRH injection. A GnRH challenge induces a standardized response of the pituitary and gonads to secrete testosterone (Jawor et al., 2007, 2006). For nighttime sampling, all males were captured 2.5 h after lights off (2:30am), approximately 40 h after the daytime sampling. Testosterone levels in juncos return to baseline levels within 2 h following GnRH injection (Jawor et al., 2006), thus the daytime sampling 40 h prior to nighttime sampling likely had minimal effect on circulating testosterone levels. All three bleeding sample time points (daytime, GnRH-induced, and nighttime) were repeated for 4 weeks to assess individual repeatability.

Approximately 50 μL of blood collected in a heparinized microhematocrit capillary tube was taken from the wing vein during each sampling event. Blood was kept on ice until centrifugation, and plasma was aspirated and stored at −80 °C until assayed for testosterone. The order in which birds were sampled was random across all sampling events. There was no effect of time passed from entering the room to end of blood sampling on baseline daytime testosterone levels ($p = 0.719$, $df = 58$, $t = 0.361$) or nighttime testosterone levels ($p = 0.063$, $df = 55$, $t = 1.901$; package lme4: testosterone as dependent variable, time since entering room as fixed effect and ID as random variable).

To relate testosterone levels with a morphological trait known to be linked with reproductive behaviors and fitness in house sparrows (Buchanan et al., 2001; Laucht et al., 2011), we calculated badge length: each bird was held ventrally with his throat and bib stretched out and a ruler was used to measure length of the badge to the nearest millimeter (Griffith et al., 1999).

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