Seasonal variation in male testosterone levels in a tropical bird with year-round territoriality

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ABSTRACT. Testosterone is important in mediating investment in competing activities such as territoriality, parental care, and maintenance behavior. Most studies of testosterone function have focused on temperate species and less is known about the role of testosterone in territoriality or variation in mating systems of tropical species. Results of studies of tropical species with year-round territoriality indicate that territorial aggression during the non-breeding season is maintained with low levels of testosterone, and increased levels of testosterone in males during the breeding season may increase mating opportunities or aid in competition for mates. We studied seasonal variation in testosterone levels of male Red-throated Ant-tanagers (*Habia fuscicauda*), a socially monogamous species with year-round territoriality and with high levels of extra-pair matings (41% of young), to determine if testosterone levels increased during the breeding season. We captured males during the non-breeding and breeding seasons and collected blood samples for hormone analysis. We found that mean testosterone concentrations were low during the non-breeding season (0.18 ± 0.05 [SD] ng/ml, range = 0.11–0.31 ng/ml), and significantly higher during the breeding season (2.37 ± 2.47 ng/ml, range = 0.14–6.28 ng/ml). Testosterone levels of breeding males were not related to aggression levels as measured by attack rates toward a stuffed decoy or singing rates during simulated territorial intrusions. These results suggest that the higher testosterone levels of breeding male Red-throated Ant-tanagers may be important in an extra-pair mating context, possibly in display behavior or mate attraction, but additional study is needed to clarify the role of testosterone during the breeding season.

RESUMEN. **Variación estacional en los niveles de testosterona en un ave tropical con defensa territorial todo el año**
La testosterona es importante en la mediación de la inversión en actividades de competencia como la defensa del territorio, cuidado parental y la conducta de mantenimiento. La mayoría de los estudios, sobre el funcionamiento de la testosterona, ha enfocado en especies de la zona templada y se sabe poco sobre el rol de esta en la territorialidad o la variación en los sistemas de apareamiento de especies tropicales. El resultado de estudios de especies tropicales, que defienden su territorio todo el año, indican que la agresión territorial, fuera de la época de reproducción, se mantiene con niveles bajos de testosterona y que el incremento en los niveles de esta (en machos), durante la época de reproducción puede incrementar las oportunidades de apareamiento o ayudar en la competencia por parejas. Estudiamos la variación estacional en los niveles de testosterona en *Habia fuscicauda*, una especie social y monógama que mantiene un territorio a través del año y con altos niveles de copulación extra-pareja (41% de los hijos), para determinar si los niveles en testosterona aumentaban durante la época de reproducción. Capturamos machos durante y fuera de la época de reproducción y le tomamos muestras de sangre para analizar los niveles de testosterona. Encontramos que la concentración promedio de testosterona fue baja durante la época no reproductiva ((0.18 ± 0.05 [SD] ng/ml, alcance = 0.11–0.31 ng/ml), y significativamente más alta durante el periodo de reproducción (2.37 ± 2.47 ng/ml, alcance = 0.14–6.28 ng/ml). Los niveles de testosterona de machos reproductivos no estuvieron relacionados con los niveles de agresión, medidos como la tasa de ataques a señuelos o tasas de cantos durante la simulación de intrusiones territoriales. Estos resultados sugieren que el incremento en los niveles de testosterona de los machos reproductivos de la tangara estudiada pudieran ser importantes en conseguir copular con otras hembras que no son sus parejas, posiblemente en la conducta exhibida o en la atracción de parejas prospectivas, pero se necesitan otros estudios para dejar claro el rol de la testosterona durante la época reproductiva.

Key words: aggressive behavior, extra-pair fertilizations, *Habia fuscicauda*, mating system, sexual selection

Avian life histories are characterized by trade-offs among competing activities, such as investment in reproduction versus maintenance behaviors (Ricklefs and Wikelski 2002, Hau et al. 2010). Testosterone has been of much interest in understanding life history diversification because of its role in mediating reproductive behavior, territory defense, and parental care (Wingfield et al. 1990, Hau 2007). Testosterone is important in courtship and territorial behavior that can increase reproductive success (Enstrom et al. 1997, Reed et al. 2006), but may interfere

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with parental care and could be detrimental to immune function and survival (Dufty 1989, Reed et al. 2006, also see Roberts et al. 2004). Most studies of the function of testosterone have focused on temperate-zone birds, where territory establishment and mating behavior overlap during a short breeding season (Stutchbury and Morton 2001, 2008, Goymann et al. 2004, Hau 2007). In most species, breeding males have high levels of testosterone in the plasma and, in the past decade, a number of studies have focused on better understanding the extent to which these high levels of testosterone are associated with territory defense or competition for and courtship of mates (Goymann et al. 2007, Goymann and Landys 2010). More recent studies have focused on testosterone function in tropical species because, in these species, males often defend territories year-round, but only breed during part of the year. This allows comparison of the link between testosterone and territoriality in non-breeding and breeding contexts (Stutchbury and Morton 2001, 2008, Hau et al. 2008, Goymann 2009).

To date, studies of tropical species with year-round territoriality indicate that testosterone concentrations are typically low (<1 ng/ml) during the non-breeding season even though males display aggressive behavior during simulated territorial intrusions (Levin and Wingfield 1992, Wikelski et al. 1999, Fedy and Stutchbury 2006, Busch et al. 2008, Gill et al. 2008, Addis et al. 2010). In most species studied, circulating testosterone levels remain low (<1 ng/ml) throughout the year (Levin and Wingfield 1992, Wikelski et al. 1999, Fedy and Stutchbury 2006, Busch et al. 2008, Gill et al. 2008), and the results of several studies indicate that males in tropical species have, on average, lower testosterone levels than males in temperate species (Levin and Wingfield 1992, Stutchbury and Morton 2001, 2008, Goymann et al. 2004, Hau et al. 2008). Stutchbury and Morton (2001, 2008) proposed that different ecological conditions for lowland tropical species, namely less seasonality associated with long-term monogamous pair bonds, are likely associated with reduced competition for mates, particularly extra-pair mates, which is an important factor influencing peak levels of circulating testosterone for males. Several studies of species across a wide range of latitudes have revealed that breeding testosterone levels are negatively correlated with the length of breeding seasons, likely because shorter breeding seasons are associated with synchronous breeding and increased opportunities for extra-pair matings (Stutchbury and Morton 2001, 2008, Goymann et al. 2004, Garamszegi et al. 2008). In Dark-eyed Juncos (*Junco hyemalis*), which have an extra-pair mating system, increased levels of testosterone have been shown to be beneficial in male–male competition for mates or in enhancing courtship behavior and attractiveness to females (Chandler et al. 1994, 1997, Enstrom et al. 1997). Such increases in testosterone levels (>2 ng/ml) during breeding may be an adaptation to the presence of extra-pair mating opportunities. Studies of tropical resident species with year-round territoriality and where the frequency of extra-pair matings is known may be useful in determining if increased opportunities to compete for and attract multiple females is associated with increased testosterone levels in males.

We examined seasonal variation in testosterone levels of male Red-throated Ant-tanagers (*Habia fuscicauda*), a tropical species with year-round territoriality. Pairs defend territories year-round using calls and, during the breeding season, males also use song (Willis 1961, 1972). Neighboring pairs interact at territorial boundaries throughout the year, more frequently during the breeding season, but territorial disputes with non-territorial males and territory takeovers take place during the non-breeding season, indicating that aggressive territory defense during the non-breeding season is highly adaptive (Chiver 2011). Red-throated Ant-tanagers have a distinct breeding season characterized by the onset of singing by males from mid-February through late March, with females initiating first clutches on average 23 d following the start of their mate's singing and coinciding with the start of the rainy season (Chiver 2011). Rates of nest predation are high and clutch replacement and care of young, if successful, takes place over a period of 5–6 mo (Willis 1960). Extra-pair matings are common in this population, with 41% of young (17 of 41 nestlings in 10 of 19 broods) resulting from extra-pair fertilizations (Chiver 2011). We predicted that, as in other tropical species with year-round territoriality, territory defense during the non-breeding season would not be associated with increased testosterone levels. If testosterone

is important in a mating context, either in sustaining aggression in male–male competition or in mate attraction, then we predicted that plasma testosterone levels would be significantly higher during the breeding season.

METHODS

We studied 25 pairs of Red-throated Ant-tanagers in Soberania National Park, located ~50 km southeast of Panama City, Panama (9°8′N 79°43′W), during the 2008 and 2009 field seasons. Most pairs ($N = 19$) were part of a long-term study of breeding behavior and, during the breeding season, we sampled an additional six pairs observed prior to capture (~30 min/pair) to confirm that the focal male was the territory owner and was paired (females present), and that the pair was breeding (males singing). Following nest initiation in late March and April (average first egg date = 10 April, range = 26 March–1 May for 23 first clutches in 2008 and 2009), pairs typically lose nests to predation and re-nest several times over a 5-mo breeding period (Chiver 2011).

Blood sampling. We collected blood samples for hormone analysis during the non-breeding (18 February–17 March 2008 and 2009) and breeding (12 May–1 June 2009) seasons. The non-breeding period, as defined here, includes the time prior to the onset of nest initiation in the population, but some birds may have been undergoing physiological changes related to breeding readiness at this time, which is typically associated with increased testosterone production (Wikelski et al. 2000). However, the non-breeding period is when territorial defense is most likely to be important because studies of other tropical year-round territorial species have revealed that this is when juveniles and non-territorial individuals prospect for and establish territories (Greenberg and Gradwohl 1983, Gill and Stutchbury 2006). Similarly, the prebreeding period was the only time when territorial takeovers were observed in Red-throated Ant-tanagers, and this occurred when non-territorial males living as helpers in nearby territories aggressively challenged territorial males (Chiver 2011). Our sampling in May and June for breeding levels of testosterone followed nest initiation in the population we monitored (for pairs where nests were not located, we observed behaviors indicating that nests had been initiated such as

nest building, copulations, or female absence for prolonged periods from pair foraging, Chiver 2011). Due to high predation rates (90% before hatching, Chiver 2011), we expected that pairs were unlikely to have young and, during behavioral observations prior to capture (30 min), we did not observe males or females feeding young on territories where we sampled.

We captured males using two 12-m-long mist-nets placed perpendicular to one another and playback of male song and female calls. Territories were mapped as part of a previous study by following pairs while foraging and noting boundary interactions with neighboring pairs (Chiver 2011). Pairs defend 2–5-ha territories and we placed nets in areas near the center of territories. Playback tracks consisted of 3–5 min recordings of one of three different pairs from the population and were played continuously until capture. Recordings consisted of males singing at a rate of 10–15 songs/min, with the same song repeated at ~5–7 s intervals. We used a taxidermic mount of an adult male placed 1 m above the speaker that was placed on the ground within 3 m of the intersection of the two nets. We used a decoy during all except the first five capture attempts during the non-breeding season when one was not available. We observed the response of males from ~5 m from the end of one of the nets and recorded the time of first response (when the male was first observed to approach the speaker) until they hit the net and, during the breeding season, we also recorded the number of songs uttered and the number of flights within 1 m of the decoy (attacks).

Following extraction from the net, we took a blood sample (maximum = 100 μl) from the brachial vein, stored it on ice for 2–6 h in the field, then, in the lab, centrifuged it and stored the plasma frozen until analysis. During the breeding season, we sampled each individual twice: within 5.5 min of capture (range = 3–5.5 min) and 15–20 min after capture. The two samples were intended to determine if the timing of sampling after capture affected testosterone concentrations and to allow comparison of breeding values to non-breeding values that, except for one sample taken at 5 min following capture, were taken 15–20 min after capture. During the non-breeding season, we collected blood samples after banding and recording standard morphometric measurements. During

the breeding season, following blood sampling within 5.5 min after extraction from the net, we banded the birds, took standard measurements, and returned the bird to a cloth bag until taking the second blood sample. We obtained blood samples from 16 males during the non-breeding season and nine males during the breeding season (including eight with samples collected both within 5.5 min and 15–20 min after capture).

Testosterone analysis. To determine circulating levels of testosterone, we used a Coat-a-count Total Testosterone kit along with a tri-level control set (Catalogue number TKTT1, CON6, Siemens Diagnostic Products, Los Angeles, CA). The kit uses a radioimmunoassay procedure where ^{125}I-labeled testosterone competes for a fixed period of time with testosterone in the sample to bind to testosterone antibodies immobilized in the tube coating. In brief, blood samples and labeled testosterone are added to the coated tubes and, after the standard incubation period of 3 h, the reactants were discarded, the tubes air dried, and the labeled testosterone bound to the antibodies in the tube wall was quantified with a gamma counter. The concentration of testosterone in the sample was determined using a calibration curve generated during the same run using a range of known concentrations to determine corresponding radioactivity levels. The antibody used is highly sensitive to testosterone with little cross-reactivity with other hormones in the blood (<3.5% for DHT). The detection limit was 0.04 ng/ml. We followed the kit procedure except that we halved all reagents and diluted samples in a ratio of 1:1.5 using the kit buffer. To calculate final concentrations, we multiplied the measured concentration value by 2.5 to account for the dilution. For eight individuals where sample volume allowed, we ran duplicate samples to assess variation within the assay. Most samples ($N = 25$) were run without replicates. Variation within assays (assessed as variation between duplicates) was 9%. Variation from known control samples was 6% for a medium concentration (4.03 ng/ml) sample and 27% for low (1.45 ng/ml) and high (7.50 ng/ml) concentration samples. The error range for low and high concentration samples was substantial, but we assumed error to be random and, because breeding and non-breeding samples were analyzed at the same time, we expected that

the results of our comparison between seasons would not be affected.

Statistical analyses. We first tested variables for normality and, because none of the variables had a normal distribution, we transformed the data (using logarithmic, inverse, or square root transformations). No transformations of the numbers of flight attacks (flights above the decoy), flight attack rate (number of flight attacks divided by playback duration from first response to capture), and singing rate (number of songs uttered divided by playback duration from first response to capture) resulted in normal distributions so we used non-parametric tests for these variables. To examine seasonal differences in time to capture (playback duration) and testosterone levels, we used two-sample tests. We used paired-sample *t*-tests to determine if testosterone varied when sampled within 5.5 min versus 15–20 min postcapture. We used correlation tests to examine the relationship between testosterone concentration and flight attack and song rates during the breeding season. We report means ± 1 SD. All statistical analyses were conducted in R 2.14.0 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

We found no significant difference between seasons in the duration of playback before capture of Red-throated Ant-tanagers (two sample *t*-test, $t = -1.8$, $P = 0.11$). Mean time of playback before capture was 17.0 ± 14.7 min ($N = 9$ trials) during the breeding season and 24.6 ± 11.3 min ($N = 16$ trials) during the non-breeding season. Five trials during the non-breeding season involved playback with no decoy, but capture times for trials with and without decoys were similar (no decoy $= 24.0 \pm 8.2$ min and with decoy $= 27.3 \pm 12.6$ min, two sample *t*-test, $t = 0.5$, $P = 0.63$).

Testosterone levels were significantly higher (two sample *t*-test, $t = -4.3$, $P = 0.001$ for samples measured 15–20 min after capture) during the breeding season (mean $= 2.37 \pm 2.47$ ng/ml, range $= 0.14–6.28$ ng/ml, $N = 8$) than during the non-breeding season (mean $= 0.18 \pm 0.05$ g/ml, range $= 0.11–0.31$ ng/ml, $N = 16$; Fig. 1). During the breeding season, testosterone levels in blood samples collected 15–20 min after capture (mean $= 2.37 \pm 2.47$ ng/ml) were significantly lower (paired
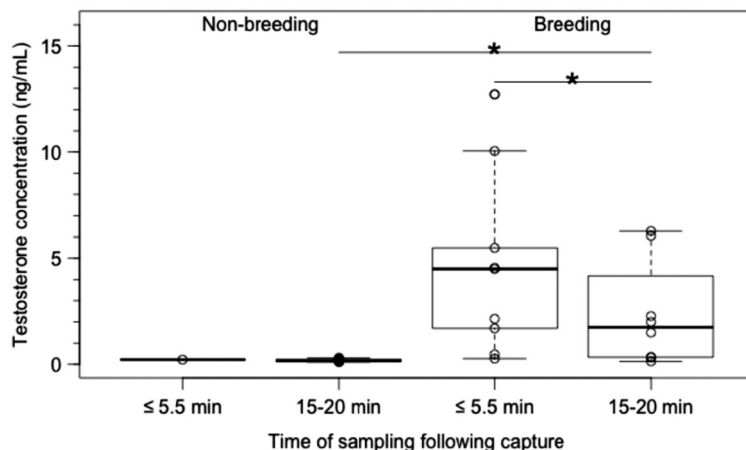
Fig. 1. Testosterone concentration (raw data points superimposed over ranges) following playback of conspecific song during the non-breeding and breeding periods. Male Red-throated Ant-tangers were sampled either within 5.5 min or 15–20 min following capture in a mist net. Non-breeding period: $N = 1$ sampled within 5.5 min of capture, $N = 15$ sampled 15–20 min after capture. Breeding period: $N = 9$ sampled within 5.5 min, $N = 8$ sampled 15–20 min after capture. Asterisks above horizontal lines denote significant differences between the groups connected by the lines.

t-test, $t = 5.0$, $P = 0.002$) than those in blood samples collected within 5.5 min of capture (mean = 5.0 ± 4.4 ng/ml, $N = 8$).

During the breeding season, males remained responsive to simulated intrusion throughout the experiments, so the cumulative number of songs and flights increased with playback duration (songs, Pearson correlation test: $N = 9$, $r = 0.86$, $P = 0.002$; flight attacks, Spearman correlation test: $N = 9$, $r_s = 0.90$, $P = 0.001$). We found no relationship between male testosterone levels and either singing or flight attack rate (Spearman correlation test: singing rate $N = 9$, $r = 0.13$, $P = 0.70$, flight attack rate $N = 9$, $r = -0.24$, $P = 0.50$; Fig. 2).

DISCUSSION

We found that male Red-throated Ant-tangers responded to simulated territorial intrusions during both breeding and non-breeding seasons and that testosterone levels were significantly higher during the breeding season than during the non-breeding season. Although we did not quantify aggressive behavior during the non-breeding season, males did sing in response to playback of conspecific songs (though at lower rates than during the breeding season) and attacked the decoy (I. Chiver, pers. obs.). In

Rufous-collared Sparrows (*Zonotrichia capensis*), aggressive responses (approach to and flights towards a decoy) to simulated territorial intrusion were similar throughout the year except that singing rates were lower when birds were not breeding and, in addition, testosterone levels were lower when males were not breeding (Addis et al. 2010). Such results indicate that different aspects of aggression may be controlled via various pathways and that testosterone may influence singing rates and other song characteristics in some species (Smith et al. 1997). Studies to date indicate that territorial defense by tropical species of birds during the non-breeding period, including singing rates, is maintained with low levels of testosterone (Levin and Wingfield 1992, Hau et al. 2000, Wiley and Goldizen 2003, Fedy and Stutchbury 2006, Addis et al. 2010). During the non-breeding season, low levels of testosterone or steroid precursors potentially produced at sites other than the testes, such as the adrenal glands or at sites in the brain, have been found to be linked to aggressive territorial behavior in some species (Soma et al. 2000, 2008, Hau et al. 2000). In addition, physiological changes, such as an increased number of androgen receptors at brain sites known to be involved in the control of aggression (e.g., the preoptic area and nucleus
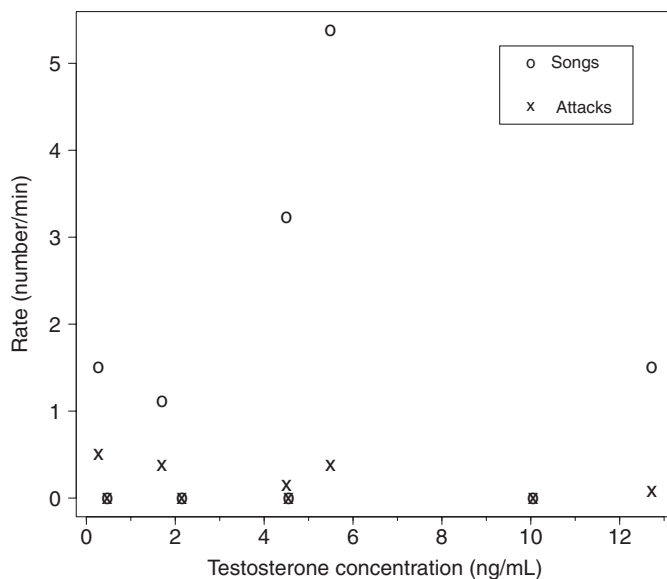
Fig. 2. Aggressive responses of male Red-throated Ant-tanagers to playback of conspecific songs and calls, including both singing rate and rate at which a taxidermic mount of a conspecific male was attacked, were not influenced by testosterone levels (sampled within 5.5 min of capture during the breeding season).

taeniae) may contribute to higher sensitivity to low levels of hormones and maintenance of similar levels of aggressive behaviors during the non-breeding season (Canoine et al. 2007). It has also been suggested that corticosterone, not testosterone, may be important for sustaining aggression during acute territorial encounters during both the breeding and non-breeding seasons (Landys et al. 2010). These alternative pathways may allow for rapid and aggressive responses during social interactions and may be common in species that defend territories year-round, including during the non-breeding season when gonads are regressed (Soma et al. 2000, 2008, Hau 2007, Goymann and Landys 2011).

Much remains to be learned about the environmental or social conditions associated with elevated testosterone levels (Hau 2007, Goymann 2009). Among tropical species, studies to date indicate that individuals maintain low testosterone levels year-round, associated with long breeding seasons (6–9 mo), low breeding synchrony, and lack of competition for extra-pair matings (Goymann et al. 2004, Fedy and Stutchbury 2006, Gill et al. 2008, Hau et al. 2008). Shorter breeding seasons (<6 mo) are

associated with higher levels of testosterone in males, possibly because these are predictive of synchronous nesting and increased competition for extra-pair fertilizations (Goymann et al. 2004, Garamszegi et al. 2008). Stutchbury and Morton (2001, 2008) proposed that, regardless of latitude, the main driver of high testosterone levels in socially monogamous species during the breeding season is increased competition for extra-pair mating opportunities. Several studies of monogamous tropical residents have revealed high testosterone levels during the breeding season and these may be associated with increased competition for extra-pair matings (Stutchbury et al. 1998, Moore et al. 2004, Addis et al. 2010). However, to date, little information is available concerning testosterone levels and the genetic mating systems of tropical bird species. We found that in Red-throated Ant-tanagers, with breeding seasons 5–6-mo long (Willis 1960) and frequent extra-pair matings (41% of young, Chiver 2011), males had elevated testosterone levels during the breeding season. Our results also indicate that testosterone levels were not linked to aggression level as measured by attacks of a stuffed decoy or singing rates during simulated territory intrusions. Previous

work with Dark-eyed Juncos (*Junco hyemalis*) indicates that increased androgen levels can benefit males in an extra-pair mating context via increased frequency or quality of courtship displays (Enstrom et al. 1997). Testosterone may similarly be important in display behavior or mate attraction in Red-throated Ant-tangers, but experimental work is needed to determine how increased levels of testosterone may specifically affect male behavior and female mate choice.

We also observed between-male variation in testosterone levels of Red-throated Ant-tangers during the breeding season and this could be a result of differences in mating opportunities at the time sampled. We calculated overall synchrony in the population (the proportion of fertile females on a given day, Kempenaers et al. 1993) as $14 \pm 7.1\%$, but this was averaged for the 13 territories where we located first nests and it varied among males from 0% to 22% (Chiver 2011). Thus, at any one time, a male may experience variation in the number of potential mates and this variation may be related to their testosterone level. In support of this, a study of male Rufous-collared Sparrows in Costa Rica revealed that testosterone concentrations varied during the year and was related to the proportion of the population in breeding condition (Addis et al. 2010).

Our results also indicate that time of sampling after a bird is captured influences measures of circulating levels of testosterone. During the breeding season, testosterone levels of male Red-throated Ant-tangers were significantly lower in blood samples obtained 15–20 min after capture than in samples collected within 5.5 min of capture, possibly because of increased levels of circulating corticosterone. Handling birds induces stress, resulting in activation of the fight-or-flight response and release of corticosterone into the blood (Wingfield et al. 1998, Angelier et al. 2010). Other investigators have also noted stress-induced decreases in plasma testosterone levels of male songbirds (Deviche et al. 2010, 2012, McGuire et al. 2013), but the physiological mechanism responsible for such declines remains unclear (Li et al. 2012). However, our results and those of other investigators suggest that sampling blood as soon as possible after capture may provide more accurate measures of plasma testosterone levels and improve our understanding of variation in absolute levels of testosterone across seasons and species.

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