Patterns of Testosterone in White-tailed Deer and their Relationship to Reproductive Success

by

Monet Arianna Gomes

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Approved by

Stephen S. Ditchkoff, Chair, William R. and Fay Ireland Distinguished Professor, School of Forestry and Wildlife Sciences
William D. Gulsby, Assistant Professor, School of Forestry and Wildlife Sciences
Sarah Zohdy, Assistant Professor, School of Forestry and Wildlife Sciences and College of Veterinary Medicine

Abstract

Testosterone governs most facets of reproduction in vertebrates, and its effects on behavior and sexually selected traits have been documented in many species. We evaluated annual and lifetime patterns of circulating serum testosterone in a freely breeding population of white-tailed deer (*Odocoileus virginianus*) in Alabama across 10 years. Although this region experiences peak breeding roughly 2–3 months later than most white-tailed deer populations in the U.S., we found peak testosterone levels coincide with the height of the breeding season for our population. Testosterone was positively associated with antler and body size only until age 6.5. Antler size, but not testosterone, was associated with annual reproductive success. Additionally, there were differences in lifetime patterns of testosterone between individuals, which may relate to differences in lifetime reproductive strategies. This suggests testosterone plays an indirect role in reproductive success through its relationship with antler size and may relate to lifetime patterns of reproductive investment.

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Abstract:

White-tailed deer (*Odocoileus virginianus*) experience seasonal fluctuations of testosterone, coinciding with the antler cycle and peak breeding. We investigated patterns of testosterone in a freely breeding population of white-tailed deer in Alabama with a late (January) breeding period. Testosterone peaked during the height of the breeding season, despite this period occurring approximately three months later than in most white-tailed deer populations. Age-related differences in testosterone were only prevalent during the breeding season, with bucks ≥3.5 years old having greater testosterone concentrations (853 ng/dl ±96 SE; $P = 0.012$) than bucks 1.5–2.5 years old (364 ng/dl ±100 SE). Additionally, an individual's testosterone level as a yearling was positively associated with their lifetime maximum testosterone level ($P = 0.006$), and an individual's mean testosterone level was positively associated with lifetime testosterone variation ($P < 0.001$). We believe these data can provide insight into the hormonal patterns and lifetime reproductive strategies of white-tailed deer.

Introduction

In male vertebrates, androgenic hormones such as testosterone play a major role in all facets of reproduction. Testosterone may be generated in both males and females from the adrenal gland and gonads. Once it has entered the bloodstream, testosterone may circulate throughout the body where it may interact with androgen receptors and may be locally metabolized or converted into other hormones such as estrogens. In both males and females, testosterone is essential for the primary sexual characteristics directly involved in breeding, including reproductive organ development and spermatogenesis in males. Additionally,

testosterone facilitates the development of secondary sexual characteristics, which play a role in sexual selection and reproductive success without directly facilitating reproduction (Darwin 1871; Hiller-Sturmhöfel and Bartke 1998). Furthermore, the association between testosterone and dominance behaviors associated with copulation and reproduction has been well documented (Rose et al. 1971; Miller et al. 1987; Chunwang et al. 2004; Bartoš et al. 2012). In Cervids, such as white-tailed deer (*Odocoileus virginianus*), testosterone promotes antler growth, increased muscle mass, and increased scent marking behaviors. Testosterone secretion in deer follows an annual cycle, stimulated by changes in daylength (Bubenik et al., 1990). This cycle gives rise to the annual cycle of antler development and casting, where testosterone remains low during the period of antler growth, increases during antler calcification prior to the breeding season, then dramatically decreases following the breeding season, which results in antler casting (Morris and Bubenik 1982; Bubenik 1982; Bubenik et al. 1975, 1982; DeYoung and Miller 2011).

There have been a multitude of studies describing testosterone patterns in white-tailed deer, and the associations between testosterone and both primary and secondary sex characteristics (Mirarchi et al. 1978, Bubenik and Schams 1986, Bubenik et al. 1990). Such research was foundational in establishing testosterone's association with day length and reproductive state, and the major role it plays in the calcification of antlers and antler casting (Mirarchi et al. 1977*b*; Bubenik and Leatherland 1984; Killian et al. 2005; Stewart et al. 2018). However, much of our knowledge of testosterone patterns in white-tailed deer has been generated using captive populations and, as a result, our understanding of how testosterone influences ecological patterns in white-tailed deer and other ungulates is limited. However, research into factors such as inter-individual variation and how individuals differ in their endocrinological patterns across their lifetimes is limited (Williams, 2008). Without this

information, the implications that this inter-individual variation has on testosterone-mediated traits that play a role in sexual selection remain ambiguous. Furthermore, the relationship between early-life testosterone and maximum testosterone secretion has not been described in white-tailed deer. Since many characteristics in white-tailed deer can be limited by poor development early in life (Harmel 1982; Harmel et al. 1989), it is possible that testosterone concentrations during early reproductive years may also relate to testosterone secretion and reproductive effort later in life. By utilizing longitudinal data of a freely breeding population, our research controls for potential confounding variables, such as nutritional availability, like captive studies, while assessing factors that contribute to reproductive success over the course of a lifetime under more natural social and reproductive dynamics.

On the contrary, studies conducted in a more natural setting may provide better insight into the associations testosterone may have with behavior and reproductive success, and by collecting these data on a population of known-individuals we seek to investigate these trends across the lifespan of an individual. While testosterone trends have been described at the population-level, variation within individuals of the same age class is not often emphasized (Mirarchi et al. 1977*a*; Bubenik and Schams 1986; Ditchkoff et al. 2001*a*). Though testosterone varies with age, differences among individuals throughout their lifetime may indicate differences in life-history strategy (Hau, 2007). This becomes increasingly important when evaluating testosterone levels as they relate to sexual selection, as variation among individuals must exist for sexual selection to occur (Darwin 1871). Because of the influence of testosterone on sexually selected traits, we might expect that there are significant differences among individuals, and that patterns of secretion may vary throughout life.

This research seeks to characterize annual and lifetime patterns of testosterone production in a freely breeding captive white-tailed deer population exhibiting seasonally late breeding. Through annual sampling of individually identified and known-age white-tailed deer, we can describe annual patterns, patterns of testosterone relative to age, and whether these patterns differ over the course of a lifetime within and among individuals. Additionally, year-round ad-libitum supplemental feeding allows us to mitigate potential influences of nutritional limitations that might be present in wild populations (Bartoš et al. 2010; Fattorini et al. 2018). Because our population exhibited peak breeding in mid-January, nearly two months later than most populations in temperate North America (Newbolt et al., 2017), we expected that testosterone concentrations would also peak later than in other populations. Furthermore, we expected to see a positive relationship between testosterone and age, and sought to investigate whether those differences exist throughout the year (Bubenik and Schams 1986; Ditchkoff et al. 2001*b*). We also expected to see significant differences among individuals in the population throughout their lives, since testosterone influences traits under sexual selection, (Darwin 1871; Jašarević et al. 2012). By establishing a foundational knowledge of patterns of testosterone secretion we can further investigate the role that physiology plays in the lifetime behavioral and reproductive ecology of white-tailed deer.

Methods

Study Area

Located north of the town of Camp Hill, Alabama, the Auburn Captive Facility (ACF) was a part of Auburn University's Piedmont Agricultural Research Station. The facility maintained a population of between 100-120 white-tailed deer within a 174-ha area enclosed by a 2.6-m fence. The population consisted of wild deer that were present in the area when the fence

was erected in 2007, and their subsequent offspring. No outside deer were introduced into the population, and deer within the fence were not subject to hunting. The population was regulated through natural mortality, capture related mortality, and selective release of fawns outside the facility fence (Newbolt et al., 2017).

The facility consisted of 40% open fields and 60% mixed forest. The forested areas had a closed canopy with little understory growth. Primary tree species found within the forest included oak (*Quercus* spp.), hickory (*Carya* spp.), maple (*Acer* spp.), and pine (*Pinus* spp.) of varying age classes. Bermuda grass (*Cynodon* spp.) was the most prevalent grass species in the fields, but fescue (*Festuca* spp.), big bluestem (*Andropogon gerardii*), Johnson grass (*Sorghum halepense*), dallisgrass (*Paspalum dilatatum*), and bahia grass (*Paspalum notatum*) were also common. Food plots were also present within the enclosure and contained differing warm and cool season deer forages to provide supplemental nutrition (Waer et al., 1992). Additionally, three feeders containing 18% protein pellets ("Deer Feed," SouthFresh Feeds, Demopolis, Alabama; Record Rack®, Nutrena Feeds, Minneapolis, MN) were available to deer ad libitum throughout the year to supplement nutrition. To attract deer for capture-related purposes during fall and winter, four timed-released feeders deployed approximately 2 kg of corn (*Zea mays*) daily.

Field Methods

We captured and immobilized deer using a mixture of Telazol ® (Fort Dodge Animal Health, Fort Dodge, Iowa) and xylazine (Lloyd Laboratories, Shenandoah, Iowa) administered to the hindquarters with telemetry darts (2.0 cc, type C, Pneu-Dart Inc., Williamsport, PA). We administered Telazol® at a concentration of 125mg/ml and a rate of approximately 2.2mg/kg, while we administered Xylazine at a concentration of 100mg/ml given at a rate of approximately

2.2mg/kg. We loaded the immobilizing drug mixture into darts equipped with radio transmitters and fired using a .22 caliber blank (Kilpatrick et al., 1996). Using VHF telemetry, we located immobilized deer. If necessary, deer resistant to the tranquilizer mixture received additional mixture. After deer were moved to the necessary location for data collection and data collection was complete, we injected Tolazoline (1.5 mL/45.36 kg) in equal amounts into muscle in the shoulder and hindquarters to reverse sedation.

Upon initial capture, we aged individuals aged using tooth replacement and wear, then assigned a unique 3-digit individual identification number visibly displayed on ear tags (Newbolt et al., 2017; Servinghaus and Moen, 1985). We collected 10 ml of blood for testosterone analysis via venipuncture of the jugular vein, centrifuged the samples to separate blood cells from serum, and stored them at -80° C in Cryule plastic cryogenic vials (Wheaton, Millville, NJ). All animal handling and research in this study was approved by the Auburn University Institutional Animal Care and Use Committee (PRN 2008-1421; PRN 2010-1785; PRN 2013-2372; PRN 2016-2964; PRN 2019-3599).

Testosterone Measurement

We analyzed serum testosterone concentrations using enzyme-linked immunosorbent assays (ELISAs; Gionfriddo et al. 2011). Prior to ELISAs, we performed hormone extraction based off the Steroid Liquid Sample Extraction Protocol from Arbor Assays Inc. (Ann Arbor, MI), with modifications made for available equipment and optimal sample concentrations, done by adding 1 ml ethyl acetate to 0.1 ml serum and vortexing the mixture. The mixture was then frozen at -20°C. We poured the top solvent layer off, then performed extraction once more, repeating the procedure. We then dried samples in glass test tubes via a hot water bath at 60-65°C and at room temperature in a fume hood for 12-24 hours.

To prepare extracted samples for ELISAs, we dissolved samples at room temperature to a concentration of 0.8µl using 250µl DetectX® Testosterone Enzyme Immunoassay Kit Assay Buffer. We ran samples in duplicates using the procedures and materials provided in the DetectX® Testosterone Enzyme Immunoassay Kit from Arbor Assays Inc. We read optical density from samples in the completed ELISA plate at 450nm using a Molecular Devices Spectra Max 190 plate reader and Molecular Devices SoftMax® Pro (Copyright © 1999-2009 MDS Analytical Technologies, US, Inc.) software. Using "Arbor Assays Testosterone EIA kit" online data analysis tool, (MyAssays Ltd., accessed 9 January 2019 through 21 August 2019, at https://www.myassays.com/arbor-assays-testostrone-eia-kit.assay), we calculated concentrations of testosterone from absorbance data. To do this, we compared absorbance of sample-filled wells to that of standardized samples prepared to concentrations of 10,000 pg/ml, 4,000 pg/ml, 1,600 pg/ml, 640 pg/ml, 256 pg/ml, 102.4 pg/ml, and 40.96 pg/ml. From the absorbance reading of these known standardized samples, we created a standardized curve. To calculate testosterone concentration of serum samples, we compared absorbance of serum to the absorbance of the standardized curve.

Statistical Analysis

We examined testosterone data for bucks older than ≥1.5 years old using program R (v3.6.1; R Core Development Team 2019) to understand trends in testosterone concentrations throughout the pre-breeding, breeding, and post-breeding seasons. We used samples collected during September–April of 2007–2017, and grouped together monthly population means. We grouped data by age class, with bucks 1.5–2.5 years old classified as "immature" and bucks ≥3.5 classified as "mature" (Strickland and Demarais 2000; Michel et al. 2018). For each month, we used T-tests to analyze differences between the average testosterone concentration by age class,

during each month. We evaluated the relative strength of support for models of the relationship between testosterone concentration and age, month, individual, and an interaction term between age and month, using Akaike's Informational Criteria (AIC). We considered models with ΔAIC_c values <2 as informative (Arnold, 2010).

To analyze lifetime patterns of testosterone for individuals, we first standardized testosterone with respect to time of capture. Specifically, we generated residuals for testosterone from the monthly average and standardized this value compared to the population mean for each respective month within our capture period. This method accounts for potential interactive effects of sample month on testosterone, generating a numeric value that can be compared across time (Ayatollahi, 1995). After generating testosterone standardized residuals, we calculated mean testosterone concentration for individuals captured ≥2 times, hereby referred to as mean testosterone level. We then ran a linear mixed-effects model to generate a value for the effect of age on testosterone, including random effects for mean testosterone level for each individual. From this model we generated residuals for the testosterone level of each capture, for every individual included in this subset. These residuals accounted for individual effects and effects of age on testosterone level. We then compared the range of an individual's age-adjusted testosterone levels to their mean testosterone levels using a linear mixed effects model.

Results

We measured testosterone of 151 bucks 1.5–2.5 years old and 77 bucks ≥3.5 years old (n = 228) from September – March, 2007–2017. Population monitoring efforts, as described in Newbolt et al. (2017), indicated that >90% of the adult deer population was captured during the study period. Testosterone concentrations ranged from 1.12–2,432 ng/dl. The best fit (and only competitive) model for serum testosterone included age, month, and the interaction between

those factors (Table 1.1). In general, testosterone increased from September to January, where it peaked, but was greater for mature bucks (853.17 ng/dl ±95.88 SE) than immature bucks (364.05 ng/dl ±100.37 SE; $P = 0.012$; Figure 1.1) during January. Outside of the breeding season, we found no age-related differences between young and mature bucks ($P > 0.136$).

We compared testosterone levels of 29 bucks captured at 1.5 years to the maximum testosterone level measured over their lifetime. We found that an individual's testosterone level at 1.5 years of age was correlated with maximum testosterone level later in life. Specifically, when a buck's testosterone level as a yearling was one standard deviation above the population average, that individual's maximum testosterone level during life was approximately 0.636 standard deviations above the population average (± 0.021 SE, $P = 0.006$; Figure 1.2). There were 50 individuals from which we were able to measure testosterone in two or more breeding seasons. When comparing an individual's mean testosterone level to the range of testosterone levels produced over that individual's lifetime (testosterone variation), we found a positive association ($P < 0.001$) between mean testosterone level and testosterone variation. When mean testosterone level increased by 1 standard deviation above the population average, testosterone variation increased by 1.135 standard deviations (± 0.186 SE; Figure 1.3).

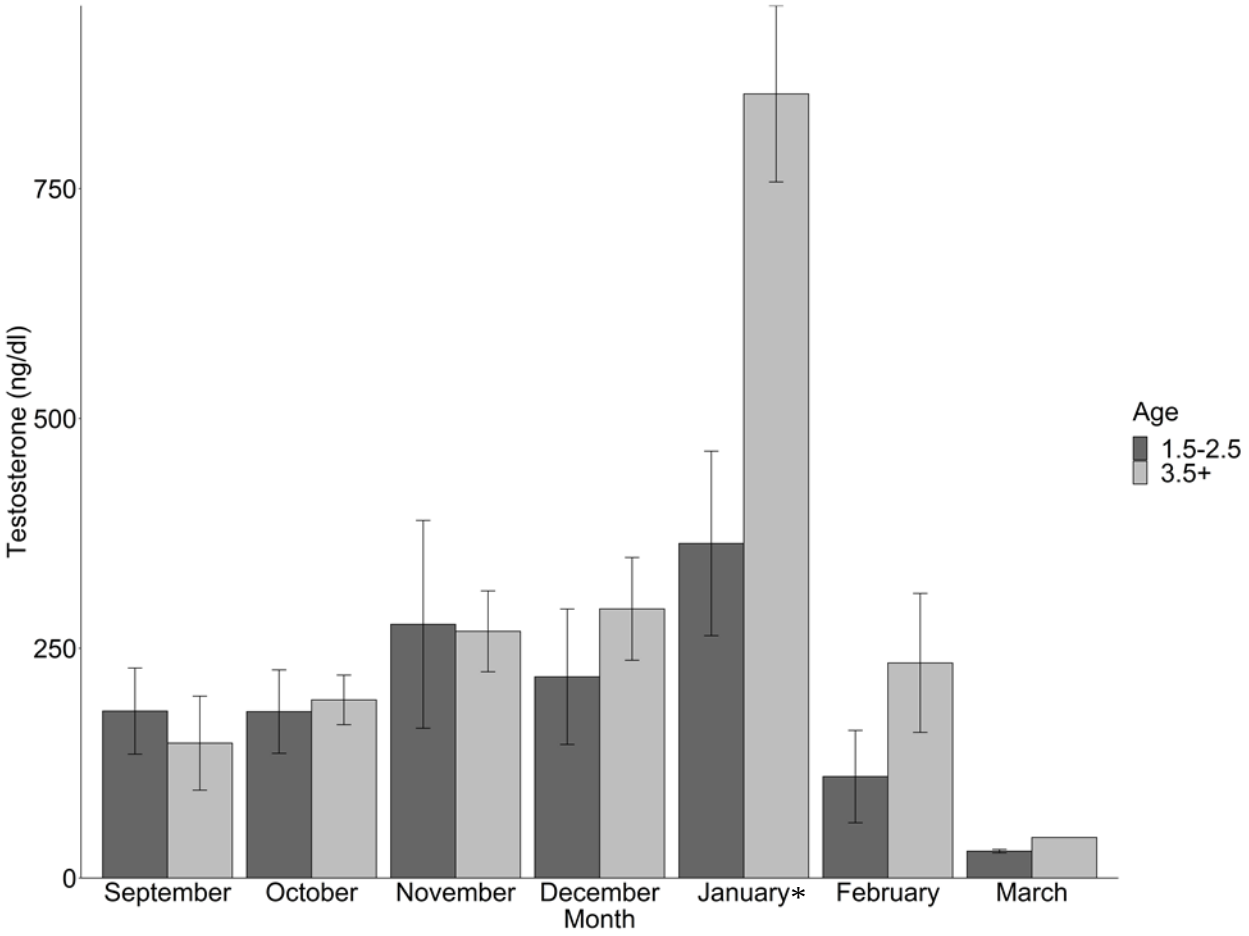

Figure 1.1. Monthly average testosterone concentrations of male white-tailed deer captured at the Auburn Captive Facility, September—March 2007—2017. Deer are sorted by age group. An asterisk (*) located next to a month indicates significant differences between age groups within a month. January represents the month of peak breeding at this facility.

Table 1.1 AIC model selection for factors that influence testosterone concentration in male white-tailed deer captured at the Auburn Captive Facility, September—March 2007—2017. Factors in candidate models included: age (Age), month during the sample period (Month), an interactive effect between age and month (Age x Month), and individual male (Individual).

Model	Parameters	df	ΔAIC_c	w_i
15	*Age+Month+Age x Month*	16	0	1
7	*Age+Month*	8	25.562	0.00
5	*Month*	6	40.665	0.00
3	*Age*	4	73.501	0.00
1	*1 (Null)*	2	85.51	0.00
16	*Individual+Age+Month+Age x Month*	98	241.36	0.00
6	*Individual+Month*	88	243.84	0.00
8	*Individual+Age+Month*	90	244.23	0.00
2	*Individual*	84	271.2	0.00
4	*Individual+Age*	86	275.7	0.00

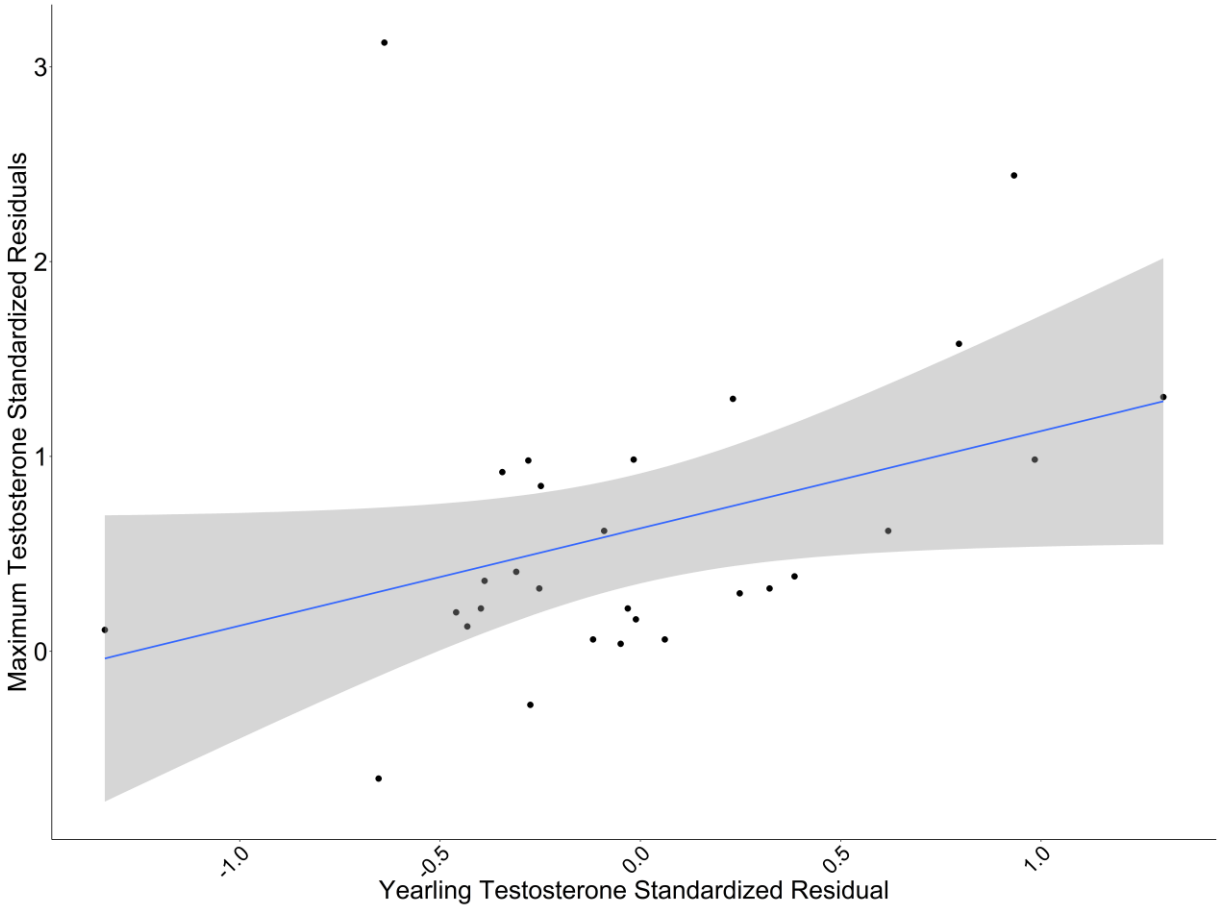

Figure 1.2. Yearling testosterone standardized residual compared to maximum testosterone standardized residual for individuals captured at the age of 1.5 years and at least one other time throughout the study period (2007 through 2017), at the Auburn Captive Facility, Auburn, AL.

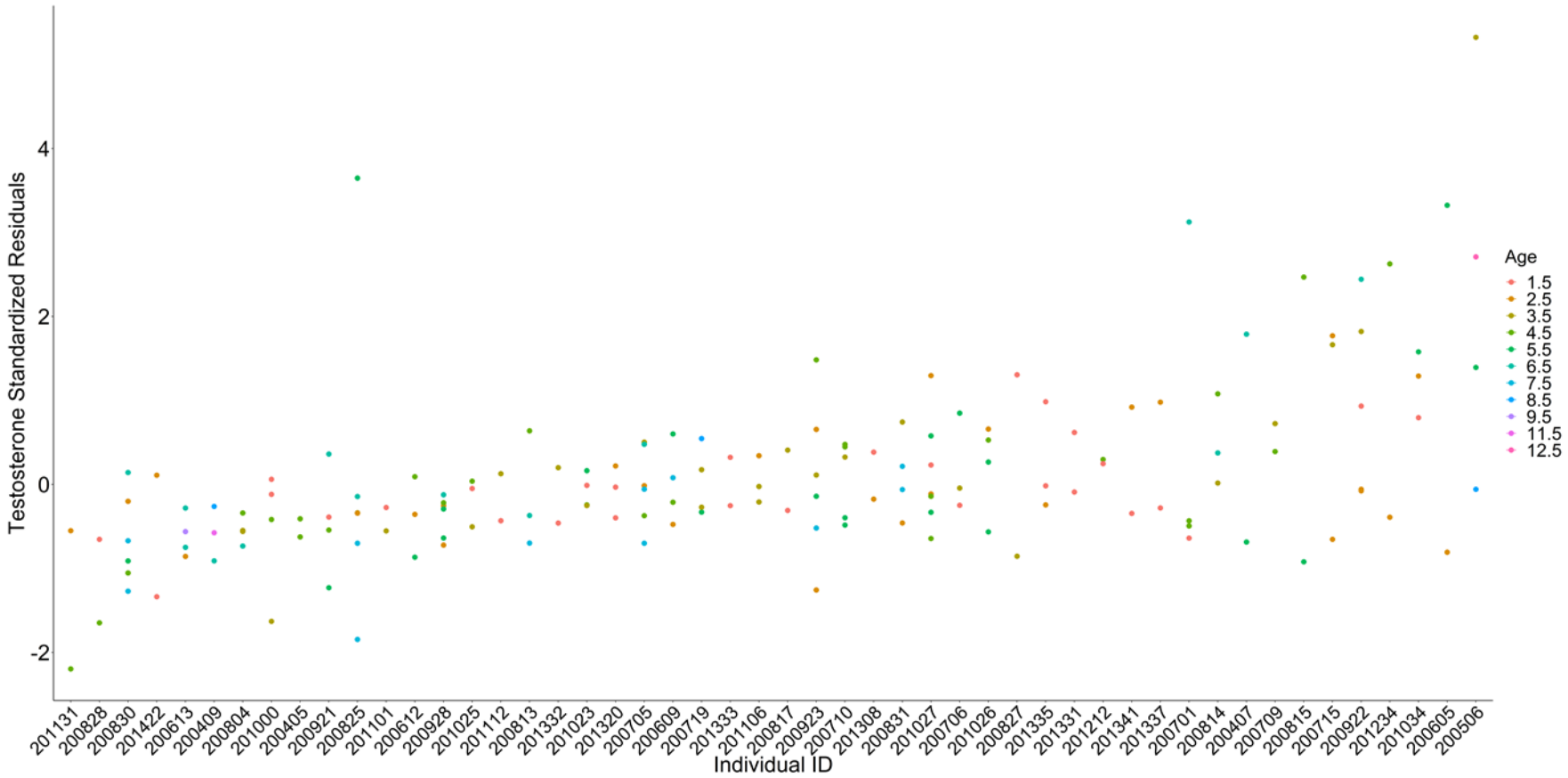

Figure 1.3. Individual effects related to variance of lifetime testosterone. Individual ID represents males at the Auburn Captive Facility, Auburn, AL, captured multiple times over the course of the study (2007—2017). Individuals are ordered least to greatest for average lifetime testosterone. Testosterone concentrations were standardized by assigning standardized residuals by comparing testosterone concentration to the population mean for a given capture month. The number of times individuals were captured ranged from 2—7 times. Colors represent the different ages at which individuals were captured. With greater average testosterone, comes greater lifetime variation in testosterone.

Discussion

We observed concentrations of testosterone near the range of 50-2000 ng/dl reported for white-tailed deer (Seal et al. 1981). The range of concentrations often differ between studies conducted on wild versus captive individuals (Mirarchi et al. 1977*b*). Although these differences persist throughout the year, they are accentuated during the breeding season (Mirarchi et al., 1978). During the peak of the breeding season, when testosterone levels peak, captive deer produce testosterone concentrations of 1,330-1,540 ng/dl (Mirarchi et al. 1978, Bubenik and Bubenik 1985), compared to 2,370 ng/dl in wild deer (Mirarchi et al., 1978). Outside of the breeding season, wild deer have slightly greater testosterone, however both wild and captive deer have testosterone concentrations less than 300 ng/dl (Mirarchi et al., 1978). The peak concentrations for our population reached 2,432 ng/dl during the peak of the breeding season, similar to the peak reported in wild populations (Mirarchi et al., 1978). We observed testosterone concentrations as low as 1.12 ng/dl outside of the breeding season, similar to the low values seen in captive herds (Bubenik and Bubenik 1985, Stewart et al. 2018), and slightly lower than what has been reported as low concentrations in wild populations (McMillin et al. 1974).

We observed the typical pattern of low testosterone during antler growth, greater concentrations leading up to peak breeding, and a dramatic decrease following the breeding season. While peak concentrations of testosterone in our study occurred during the month of January, up to 3 months later than other studies conducted in temperate regions (McMillin et al. 1974, Mirarchi et al. 1978, Bubenik et al. 1983, Bubenik and Bubenik 1985, Bubenik and Schams 1986, Miller et al. 1987, Ditchkoff et al. 2001*b*, Stewart et al. 2018), peak testosterone levels still coincided with the peak of conceptions in this population (Newbolt et al. 2017). This is consistent with previous data reported by Bubenik et al. (1990), that documented testosterone

peaks associated with peak breeding season in both southern Texas (latitude 27°N) and southern Ontario (latitude 42°N). Deer in Texas experienced both peak breeding and peak testosterone concentrations in December, one month later than deer in Ontario. Similarly, many populations in Alabama, including our study area (latitude 33 °N), exhibit a later breeding period than other temperate regions, occurring in January rather than October or November. This is thought to be a product of restocking efforts from populations (i.e., southwestern Alabama) that historically exhibited later breeding (Leuth 1967). It is hypothesized that differences in reproductive timing throughout the range of white-tailed deer has evolved through genetic differences that exist with regards to photoperiod response (Bronson 1988). In regions of coastal Alabama, this decrease in photoperiodicity has been favored under natural selection. This later breeding period results in later fawning, and mortality due to seasonal springtime flooding is reduced. As deer were restocked from this region, this breeding timing persisted, and mild winters in the region allowed the persistence of this late breeding and fawning season. The differences in breeding timing that exist today are well-documented through behavioral observations, later parturition timing, and genetic analyses. Marked genetic differences due to source populations from restocking still manifest today, often despite a lack of geographical distance or barriers (DeYoung et al., 2003). Furthermore, deer with genetics from restocked populations tend to experience breeding timing that coincides largely with respective origin populations (Sumners et al., 2015), demonstrating that this breeding timing is heritable. Since testosterone cycles drive reproduction in males, it was unsurprising to find that the peak of the serum testosterone concentrations we observed coincided with the peak of the breeding season, despite a seasonally late breeding season (McCoy and Ditchkoff, 2012). Our data offer further evidence of the close relationship between the peak of the breeding season and peak in testosterone.

In our population, we observed a shift in the concise peak of testosterone correlating with a concise peak of the breeding season, rather than a prolonged period of elevated testosterone. While other regions of the white-tailed deer's range also experience much later breeding seasons, physiological patterns in these populations differ from our temperate-region population. For example, in equatorial regions with later breeding seasons, testosterone concentrations and antler growth patterns are weakly associated with daylight shifts (McMillin et al., 1974). Furthermore, the breeding season in these regions are often prolonged in comparison to our study population and most temperate North American populations (Richter and Labisky, 1985). Despite a late breeding season, our population experiences a concise breeding period, and strong associations between shifts in daylength, antler growth, and testosterone patterns (Newbolt et al., 2017). The drastic differences observed between testosterone concentrations in January, the month encompassing the peak of conceptions in this population, and the surrounding months differ from patterns in late-breeding populations at lower latitudes. Rather, the hormonal patterns observed are most similar to those of other North American populations, only at a later time period. While populations such as ours exhibit later breeding periods, the concise nature of the breeding season, and concise peak in testosterone surrounding it suggest that late-breeding deer in temperate regions may still face the selective forces of seasonality.

Testosterone in our population generally increased with age, consistent with previous research in white-tailed deer (Bubenik and Schams 1986, Miller et al. 1987, Ditchkoff et al. 2001*b*). In the context of the reproductive ecology of this species, increased testosterone at these ages correlates with greater reproductive investment. Ages at which deer have greater testosterone (≥3.5 years) are also the ages typically associated with greater antler size (Hewitt et al., 2014), body size (Sauer 1984, Strickland and Demarais 2000), and reproductive output

(Newbolt et al., 2017). However, bucks in these age classes also experience greater breeding season-related mortality (Ditchkoff et al. 2001*c*). Altogether, these findings affirm that testosterone is positively associated with the age classes at which deer invest more reproductive effort and incur the most breeding-related stress.

While age-related differences in testosterone occurred during the breeding season, they were not present during other parts of our sampling period. A lack of age-related differences outside of the breeding season suggests there is little benefit gained from maintaining elevated testosterone throughout the year. Although testosterone contributes to factors associated with reproductive success, testosterone-mediated behavior during the breeding season imposes physical and immunological handicaps by increasing physical exertion and injury risk, while decreasing immunity during a season of decreased resource availability (Folstad and Karter, 1992). Additionally, bucks often forgo nutritional resource acquisition to invest in intrasexual competition, mate chasing, copulation, and tending during the limited timeframe that does are in estrus (DeYoung and Miller 2011, Ditchkoff 2011). These activities often lead to injury or death during and after the breeding season (Ditchkoff et al. 2001*c*). Decreasing testosterone concentrations outside of the breeding season may serve as a 'compensatory trait', a mechanism evolved to cope with potential stressors imposed with sexually selected traits (Kirkpatrick, 1987). This pattern may indicate that the physiological burdens of elevated testosterone are beneficial only in the context of preparation for and participation in the breeding season.

Our ability to obtain repeated samples from known individuals throughout their life allowed us to describe lifetime patterns of testosterone, something not often done in wild populations (Festa-Bianchet, 2012). We found that yearlings with testosterone above the population average had greater lifetime maximum testosterone. Testosterone does not peak until

later in life, and this later peak can be attributed to the Principle of Allocation (Levins 1968). By this principle, younger individuals invest more heavily in somatic growth as opposed to reproductive effort. However, as deer age, and somatic growth consumes proportionally less energy, individuals may invest more heavily in reproductive efforts. Increasing the age of peak reproduction and maturation may increase lifetime reproductive capacity. However, in species that experience maturation later in life, individuals face a greater risk of dying prior to peak reproductive age, negatively impacting lifetime reproductive success (Stearns 1992). While the greatest levels of testosterone and reproductive output may occur later in life in deer, this pattern suggests that physiological characteristics early in a buck's reproductive tenure may indicate future effort put into breeding. Yearling deer do not invest as heavily in reproduction as older deer, as indicated by smaller sexually selected characteristics (Sauer 1984, Strickland and Demarais 2000, Hewitt et al. 2014) and fewer offspring produced (Newbolt et al., 2017). However, yearlings with greater testosterone concentrations early in life might adopt a different reproductive strategy, with greater reproductive investment earlier in life that remains consistent through peak reproductive ages. This relationship was similar to that seen in red deer (*Cervus elaphus*), where greater yearling antler size was positively associated to prime-age body and antler size (Lemaître et al., 2018). Previous work assessing individual differences as ungulates age has focused on traits such as neonatal mass compared to juvenile survival or mass, often not extending much later in life (Jorgenson et al. 1993, Sæther and Heim 1993, Festa-Bianchet et al. 1996). Although previous work has shown that neonatal testosterone in red deer correlate with yearling survival (Pavitt et al., 2014), our study is one of the first of our knowledge to assesss how testosterone early in life might relate to individual testosterone later in life in a semi-wild population.

Building upon these findings, we found that differences in lifetime patterns of testosterone exist between individuals. Individuals with low mean testosterone exhibit little testosterone variation throughout life, whereas others with greater mean lifelong testosterone exhibit greater testosterone variation throughout life. These results differ from those found in red deer, where differences in lifetime patterns among individuals exist with regards to reproductive senescence (Nussey et al., 2017), but were not documented with testosterone levels (Pavitt et al., 2015). However, in bighorn sheep (*Ovis canadensis*), Martin et al. (2013) observed individual differences in testosterone, when accounting for age, and suggest that these differences influence reproductive effort and success. The variation in testosterone patterns that we observed is consistent with patterns of variation in sexually selected traits (Darwin 1871, Jašarević et al. 2012). The premise of sexual selection is contingent on significant differences existing between individuals, often through traits that indicate quality of an individual. It follows that testosterone, which is affected by an individual's condition (Pérez-Rodríguez et al., 2006) and influences many facets of breeding (Gomes and VanDenmark, 1974), differs among individuals within a species (Kempenaers et al., 2008).

In our population, individuals of different quality may seek to maximize reproductive effort through different reproductive strategies (Kokko, 1998) in the form of different lifetime testosterone patterns. Two competing lifetime reproductive strategies observed in long-lived species like white-tailed deer are often referred to as 'live fast, die young' and 'slow and steady' (Bonduriansky et al., 2008). The individuals in our population that consistently produce lower testosterone levels over their whole lives might exhibit tendencies of the 'slow and steady' strategy. When sexually selected traits develop with age, as is the case with antlers in white-tailed deer (Hewitt et al., 2014), sexual selection may favor a strategy that prolongs the lifespan

of an individual. On the contrary, those individuals exhibiting a wide range of testosterone, and greater overall average testosterone level might exhibit a ‘live fast, die young’ reproductive strategy, where individuals exhibit a shorter, but more physically stressful breeding lifespan (Rolff 2002, Lemaître et al. 2018). Individual differences in body growth rate early in life have been documented for white-tailed deer, and it is hypothesized that these different growth rates may influence lifetime reproductive success (Michel et al., 2018). Based upon our data and previous research, we believe that further exploration of lifetime reproductive strategies in white-tailed deer should be investigated, and that including hormonal patterns may provide insight into such patterns.

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Chapter 2: Does Testosterone Influence Reproductive Success and Associated Physical Characteristics in Ungulates?

Abstract:

While hormones such as testosterone drive reproductive efforts and sexually selected traits in many species, research directly relating testosterone levels to annual and lifetime reproductive success is sparse. We sought to directly measure how testosterone levels relate to sexually selected characteristics and reproductive success in a freely breeding population of white-tailed deer (*Odocoileus virginanus*). We captured individuals during September–March from 2007–2017. We compared testosterone to antler size, body size, and annual reproductive success for each individual, and assessed lifetime patterns of individuals captured multiple years. We found a positive relationship between testosterone, body size ($P = 0.035$), and antler size ($P = 0.013$) for ages 1.5–5.5. However, we found a negative association between testosterone, body size ($P = 0.028$) and antler size ($P = 0.019$) for animals $\geq$6.5 years of age. Annual reproductive success was positively associated with antler size ($P < 0.001$), but not testosterone ($P = 0.080$), age ($P = 0.686$), or body size ($P = 0.096$). Despite individuals with greater variation in testosterone during their life having greater average lifetime testosterone (discussed in Chapter 1), these individuals did not have greater ($P = 0.25$) lifetime reproductive success. While we saw no association between testosterone and reproductive success directly, it may be possible that testosterone is indirectly related to reproductive success through its positive association with antler size. Additionally, we believe different lifetime testosterone patterns among individuals may relate to differences in lifetime reproductive strategies.

Introduction

Across the animal kingdom, species display a myriad of characteristics to attract and acquire mates (i.e. antlers, horns, bright colored plumage, etc.). These secondary sex characteristics, while not utilized directly in copulation, often come at a substantial cost and can inform mates of individual quality (Zahavi and Zahavi 1999). Variation in secondary sex characteristics among individuals can come from innate differences in genetic quality (Kruuk et al. 2002, Michel et al. 2016*a*), differences in immunological state (Folstad et al. 1996, Lagesen and Folstad 1998, Hill 2011), and differences in the ability to acquire nutrients (Jones et al. 2018). Through these advertised differences, females can differentiate among males and select fitter mates. White-tailed deer (*Odocoileus virginianus*) males may enhance their reproductive success through development of secondary sex traits that communicate overall quality (Zahavi and Zahavi 1999). Antlers, the most outwardly visible secondary sex trait in deer, are argued to be the fastest growing tissue in the animal kingdom (Goss 1983), are grown and shed annually, and come at a high physiological cost (French et al. 1956, Landete-Castillejos et al. 2007, Landete-Castillejos et al. 2012). Consequently, females may use antlers as an indicator of fitness (French et al. 1956, Malo et al. 2009, Michel et al. 2016) and overall quality (Ditchkoff et al. 2001*a*). When mates favor a large-antlered, fitter male, they increase the opportunity to improve offspring viability and fitness later in life (Morina et al. 2018).

The breeding system of a species consists of the behaviors and mechanisms by which individuals successfully acquire mates and produce offspring. Strategies that males use to increase reproductive success differ depending on the breeding system of a species. For harem-holding species such as red deer (*Cervus elaphus*) and elk (*Cervus canadensis;* Clutton-Brock et al. 1979, Clutton-Brock et al. 1988) paternity is monopolized by a small number of dominant individuals. However, white-tailed deer utilize a tending-bond breeding system, where during a

short estrous period (~3 weeks) males pursue females, copulate, and guard them for 24–48 hours post-copulation. This tending period helps to ensure that a mate is the sole sire of the litter, and minimizes potential breeding from competitors and multiple paternities within a litter (Sorin 2004; Newbolt et al. 2017). However, this system does not allow males to monopolize females and results in breeding opportunities for males of more varied age and size classes than is found in harem breeding systems (Sorin 2004, DeYoung et al. 2009, Newbolt et al. 2017). Because of this, population dynamics such as population density, age structure, and sex ratio (Newbolt et al. 2017) affect the relative importance of sexually selected characteristics on reproductive success. For white-tailed deer in herds with a high degree of competition among dominant, mature males, factors such as antler size, body size, and age are associated with reproductive success (Lindstedt and Boyce 1985, Forsyth et al. 2005, DeYoung et al. 2006, DeYoung et al. 2009, Ditchkoff 2011, Newbolt et al. 2017, Morina et al. 2018).

Androgens such as testosterone play a dynamic role in all facets of male breeding. While directly aiding in spermatogenesis and copulation, testosterone also indirectly acts on reproductive efforts. In general, testosterone in deer aids the development of sexually selected traits (Perez-Rodriguez et al. 2006) and increases muscle mass (Griggs et al. 1989, Ditchkoff 2011), social dominance (Chunwang et al. 2004), and signpost communications to potential mates (Miller et al. 1987, Miller et al. 1998). Furthermore, increasing testosterone facilitates the final stages of antler development, inducing antler bone matrix synthesis and velvet shedding (Bubenik et al. 1975, Morris and Bubenik 1982, Bubenik et al. 2005). For white-tailed deer specifically, elevated testosterone also increases vocalizations, neck swelling, mate chasing, and mate tending (Miller et al. 1987, Pereira et al. 2005, Ditchkoff 2011). During the breeding

period, increasing levels of testosterone prepare a male white-tailed deer for the multitude of physiological changes necessary to successfully compete for breeding opportunities.

Most research on reproductive success in cervids and other ungulates has examined how androgen-mediated characteristics like antler size and body size directly affect reproductive success, but research directly relating testosterone to reproductive success is sparse. Research directly measuring this relationship in Cervids has been limited to red deer (Malo et al. 2005) and Père David's deer (Chunwang et al. 2004). Testosterone levels themselves may be indicators of reproductive capability, as testosterone is associated positively with sperm concentrations per ejaculate and overall sperm motility, and are negatively associated with sperm malformation (Stewart et al. 2018). Additionally, several studies suggest that the composition of bodily excretions such as testosterone metabolites in urine (Miller et al. 1998), glandular secretions used for scent-marking (Quay and Müller-Schwarze 1970, Johnson 1976), or darker pelage (Bubenik and Bubenik 1985) provide potential mates with information regarding testosterone levels. Characteristics that advertise testosterone levels signal dominance and physiological capability to reproduce, despite testosterone's immunosuppressive properties, and are favored under sexual selection (Weinstein et al. 1984, Folstad and Karter 1992, Hillgarth and Ramenofsky 1997, Perez-Rodriguez et al. 2006, Buczek et al. 2016). Research assessing the relationship between testosterone concentrations and reproductive success, in addition to the effects of testosterone on sexually selected traits, may provide insight into how testosterone directly influences both annual and lifetime reproductive success.

Building upon previous research, we sought to directly measure how testosterone levels relate to sexually selected characteristics and reproductive success in a freely breeding population of white-tailed deer. Our study is unique in that we monitored a population of

individually marked and annually captured group of captive individuals over a period of ten years. Using these data we were able to assess how testosterone relates to sexually selected characteristics known to increase reproductive success, and measure the number of offspring sired by individual males. Furthermore, while lifetime reproductive strategies are often studied at a population-level, these are not often done at an individual level (although, for examples, see Hogg 1987, McElligott and Hayden 2000, Festa-Bianchet 2012, and Markussen et al. 2018), making it difficult to detect intraspecific differences in lifetime reproductive strategies. Through long-term monitoring, we could assess how this relationship between testosterone and reproductive success may differ between individuals throughout the course of a lifetime.

Methods

Study Area

We conducted our study at the Auburn Captive Facility a part of Auburn University's Piedmont Agricultural Research Station, located north of the town of Camp Hill, Alabama. During the time of the study, we maintained a population of 100-120 white-tailed deer within a 174-ha area enclosed by a 2.6-m fence erected in 2007. Deer within the fence consisted of wild deer present in the area and their subsequent offspring. The area experienced no hunting pressure, and no outside deer were introduced into the population during this time. Population numbers were regulated through natural mortality, capture related mortality, and selective release of 6-month-old fawns outside the facility fence (Newbolt et al. 2017).

Within the Auburn Captive Facility, vegetation consisted of 40% open grass fields and 60% mixed forest. Forested areas had thick closed canopy with little understory growth. The primary tree species within this area included oak (*Quercus* spp.), hickory (*Carya* spp.), maple (*Acer* spp.), and pine (*Pinus* spp.) of varying age classes. The most prevalent grass species was

bermuda grass (*Cynodon* spp.). However, fescue (*Festuca* spp.), big bluestem (*Andropogon gerardii*), Johnson grass (*Sorghum halepense*), dallisgrass (*Paspalum dilatatum*), and bahia grass (*Paspalum notatum*) were also present. To supplement herd nutrition, we planted various food plots throughout the facility containing different warm and cool season forages (Waer et al. 1992). The herd also received year-round supplemental nutrition ad libitum from three protein feeders containing 18% protein pellets ("Deer Feed," SouthFresh Feeds, Demopolis, Alabama, USA Record Rack®, Nutrena Feeds, Minneapolis, MN). Additionally four timed-released feeders each deployed approximately 2 kg corn (*Zea mays*) daily in the fall and winter (Newbolt et al. 2017) to aid in attracting deer for population monitoring and capture purposes.

Field Methods

We captured and immobilized deer using a mixture of Telazol ® (Fort Dodge Animal Health, Fort Dodge, Iowa) and xylazine (Lloyd Laboratories, Shenandoah, Iowa) administered with telemetry darts (2.0 cc, type C, Pneu-Dart Inc., Williamsport, PA) aimed at the hindquarters. We administered a mixture of Telazol ® at a concentration of 125mg/ml and Xylazine was at a concentration of 100mg/ml with each drug given at a rate of approximately 2.2mg/kg. This mixture was loaded into the darts equipped with radio transmitters and fired using a .22 caliber blank (Kilpatrick et al. 1996). Upon successful sedation, we located deer using VHF telemetry and injected deer with additional tranquilizer mixture if necessary. We then moved deer to an open space appropriate for data collection, collected data, and injected Tolazoline (1.5 mL/45.36 kg) in equal amounts into muscle in the shoulder and hindquarters to reverse sedation. We then monitored deer at a distance until the individuals demonstrated the ability to move by their own will. All animal handling and research in this study was approved

by the Auburn University Institutional Animal Care and Use Committee (PRN 2008-1421; PRN 2010-1785; PRN 2013-2372; PRN 2016-2964; PRN 2019-3599).

Upon initial capture, we aged individuals using tooth replacement and wear (Severinghaus 1949). Based upon the individual's birth year, we assigned a unique 3-digit individual identification number and visibly displayed this number on ear tags (Servinghaus & Moen, 1985, Newbolt et al., 2017). Additionally, we collected a 2-cm^2 tissue sample from individuals using an ear notch tool and stored at -80° C in Cryule plastic cryogenic vials (Wheaton, Millville, NJ) later to be used for genetic analyses and determination of parentage. During captures, we also collected 10 ml of blood for testosterone analysis via jugular venipuncture. Blood was then centrifuged at the end of the capture to separate blood cells from serum and stored at -80° C in Cryule plastic cryogenic vials.

Testosterone Measurement

We determined serum testosterone concentrations using enzyme-linked immunosorbent assays (ELISAs). This method has been used to evaluate testosterone concentrations in white-tailed deer by Gionfriddo et al. (2011). Prior to conducting ELISAs, we extracted hormones from serum based off the Steroid Liquid Sample Extraction Protocol from Arbor Assays Inc. (Ann Arbor, MI), with modifications made for available equipment and optimal sample concentrations. We extracted hormones by adding 1 ml ethyl acetate to 0.1 ml serum, vortexing the mixture, then letting the mixture sit for 5 minutes to allow solvent layers to separate. We them moved samples to a -20°C freezer, and once the bottom layer of the solution froze, we poured the top solvent layer into another glass test tube. We then repeated this procedure and performed a total of two extractions on samples. We dried extracted samples in a fume hood by

submerging glass test tubes in a 60-65°C water bath in addition to let the solvent evaporate at room temperature for 12-24 hours.

Immediately prior to conducting ELISAs, we dissolved samples at room temperature to a concentration of 0.8µl using 250µl DetectX® Testosterone Enzyme Immunoassay Kit Assay Buffer. Using materials provided in the DetectX® Testosterone Enzyme Immunoassay Kit from Arbor Assays Inc., we ran samples through ELISA in duplicates. We read optical density from samples in the completed ELISA plate at 450nm using Molecular Devices Spectra Max 190 plate reader and Molecular Devices SoftMax® Pro (Copyright © 1999-2009 MDS Analytical Technologies, US, Inc.) software. Using observed optical densities, we calculated testosterone concentrations using MyAssays Ltd. online data analysis tool for the "Arbor Assays Testosterone EIA kit" (https://www.myassays.com/arbor-assays-testostrone-eia-kit.assay). We did these calculations were done by comparing the absorbances of sample-filled wells to that of standardized samples prepared at known concentrations (10,000 pg/ml, 4,000 pg/ml, 1,600 pg/ml, 640 pg/ml, 256 pg/ml, 102.4 pg/ml, and 40.96 pg/ml). From the absorbance reading of these standardized samples, we created a standardized curve and calculated serum concentrations by comparing serum absorbances to this standardized curve.

Genetic Analysis and Parentage Assignment

We sent tissue samples to DNA Solutions (Oklahoma City, OK) for microsatellite analysis of 18 loci (i.e., Cervid1, L, BM6506, N, INRA011, BM6438, O, BL25, K, Q, D, OarFCB193, P, S, RT5, RT7, RT13, BL42; Anderson et al. 2002, Meredith et al. 2005). Parentage was determined using software Parentage Version 1.1d (Huang et al. 2018). Based upon these data, we compiled a list of candidate parents, and considered an individual to be the parent at the 95% confidence limit. From parentage estimates, we determined annual

reproductive success to be the number of offspring produced per individual per year, in accordance with previous work done at this facility by Newbolt et al. (2017).

Statistical Analysis

We examined testosterone data for bucks older than ≥1.5 years old using program R (v3.6.1; R Core Development Team 2019) to understand the dynamics of testosterone and reproductive success. We used samples collected from September to April for the years 2007 – 2017 for individuals ≥1.5 years old.

Because testosterone concentrations vary temporally during the pre-breeding and breeding seasons (Mirarchi et al. 1978), our sample period, we standardized testosterone with respect to time of capture. To do this, we generated the residual of each individual testosterone concentration compared to the population mean of that month. Similar methodology is used frequently in the field of psychology (Ayatollahi 1995), and accounts for potential interactive effects of sample month on testosterone, generating a numeric value comparable across our sampling period. We refer to this standardized residual as testosterone value.

We assessed the relationship between testosterone, body size, antler size, and reproductive success using a variety of approaches. To evaluate testosterone's effects on body size, we first generated a single term for body size using principal component analysis (PCA) that included measurements of chest girth, hind foot length, and body length from tip of the nose to the base of the tail (Newbolt et al. 2017). Henceforth, we refer to the term generated from PCA as body size. We used measurements for gross Boone and Crockett antler score (Wright et al. 1997) to assess the relationship between testosterone and antler size. We used linear mixed effects models to examine the effects of our predictors, testosterone, age, and a testosterone*age interaction, on response variables of interest, including body size, antler score, and reproductive

success. In each model, individual ID was included as a random effect to account for heterogeneity in responses among individuals. Furthermore, we ran a generalized mixed effects (GLME) model to assess factors that potentially influence reproductive success. We included testosterone, testosterone*age, body size, antler size, and age as a quadratic variable in this model, based upon previous research on factors that influence reproductive success (Newbolt et al. 2017; Morina et al. 2018).

To analyze the potential effects of individuality on lifetime patterns of testosterone, antler size, and reproductive success, we subset our data to include only individuals that had been captured at least twice in their life, from the ages of 1.5 and older. We calculated each individuals average testosterone standardized residual (referred to as mean testosterone level) and ran a linear mixed-effects model to generate a value for the effect of age on testosterone with random effects for mean testosterone level for each individual. From this model we generated residuals for the testosterone level of each individual, for every capture of that individual over the course of their lifetime. These residuals accounted for individual effects and effects of age on testosterone level. We then ran a linear model to test if an individual's mean testosterone level related to testosterone variation, which we define as the range of testosterone levels from that individual.

Results

We determined testosterone concentrations for 82 deer over 228 captures from the months of October through March, 2007-2017. The greatest number of captures for an individual animal was 6 captures. Ages of captured individuals ranged from 1.5 years to 12.5 years, and these captures represent over 90% of the adult population (Newbolt et al. 2017). Of these captures, we used 197 to model the relationship between testosterone, body size, age, and

age*testosterone interaction (Figure 4). Body size was positively associated with both testosterone and age. As testosterone level increased 1 standard deviation above the population mean, body size increased by 3.71 principal components (± 1.74 SE; P = 0.035). Additionally, for every 1 year increase in age, body size increased by 3.66 principal components (± 0.34 SE; P < 0.001). However, we saw a negative relationship with our age*testosterone interactive term (-0.70 ± 0.31 SE; P = 0.028; Table 2.1). This negative interactive term indicates that with each year increase in age, the positive relationship between testosterone and body size decreased, and by age 5.5, the positive association between testosterone and body size is counteracted by the negative relationship between the interactive term and body size (Figure 2.1).

Our linear model assessed the relationship between antler score and three variables: testosterone, age, and an interaction between age*testosterone (Figure 2.2). There was a positive relationship between testosterone level and antler size. As testosterone level increased by 1 standard deviation above the population mean, antler size increased by 10.76 cm (± 3.99 SE; P = 0.013). Furthermore, with every 1 year increase in age, antler size increased by 11.34 cm (± 0.86 SE; P < 0.001). However, we found a negative relationship between age*testosterone and antler size (-1.78 ± 0.75 SE; P = 0.019; Table 2.2). This negative association indicates that as age increases, the positive relationship between testosterone and antler size diminishes. Consequently, by the age of 5.5, we see that testosterone has a negative relationship with antler score (Figure 5).

We assessed the relationship between annual reproductive success and testosterone, age, antler score, body size, age*testosterone, and quadratic effects for age. We found a positive association with antler score and reproductive success (P < 0.001). For every 1 cm increase in antler score, we saw 0.011 more offspring sired annually (± 0.003 SE), or for every 88.25 cm

increase in antler score, an individual sired 1 more offspring per year (± 0.273 SE). We found no relationship between annual reproductive success and testosterone (P = 0.080), age (P = 0.686), or body size (P = 0.096). Additionally, we found no relationship between an individual's lifetime reproductive success and testosterone variation (P = 0.25; Table 2.3).

Table 2.1. Linear mixed effects model results estimating the relationship between skeletal body size (utilizing a single term generated from principal component analysis), and testosterone (T), age (Age), and a testosterone and age interactive effect (T x Age) in male white-tailed deer captured at the Auburn Captive Facility, September—March 2007—2017.

Parameter	Value	*df*	T-value	P-value
(Intercept)	3.162 ± 1.622	114	1.950	0.054
T	3.705 ± 1.737	114	2.133	0.035
Age	3.659 ± 0.338	114	10.830	<0.001
T x Age	-0.700 ± 0.314	114	-2.233	0.026

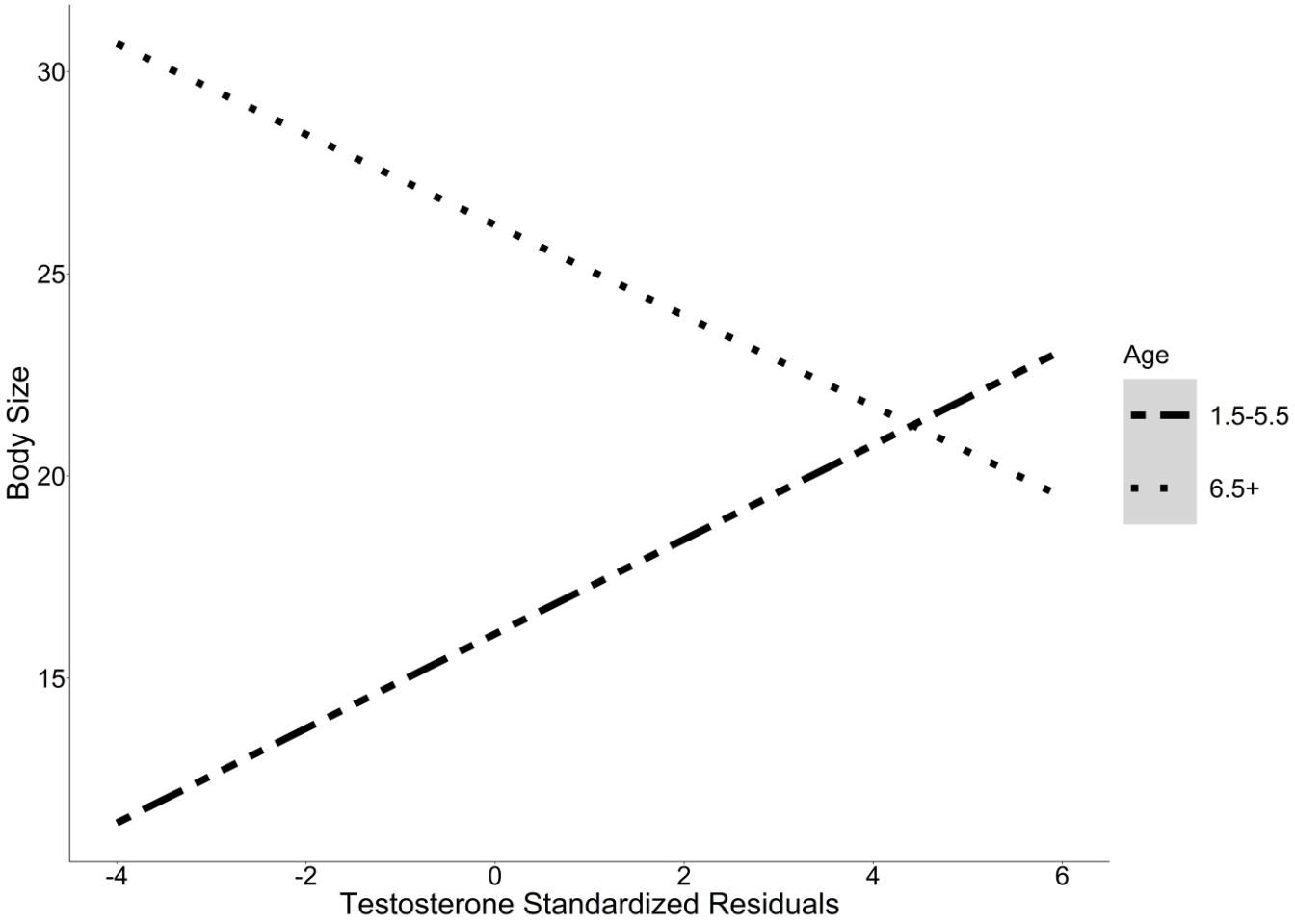

Figure 2.1. Testosterone compared to body size (generated from PCA) for male white tailed deer, grouped by ages of 1.5-5.5 and 6.5+. Data came from males captured at the Auburn University Captive Facility, Auburn, AL from September to March, 2007-2017. Testosterone standardized residual refers to testosterone concentrations from these captures, standardized to account for differences in testosterone concentrations due to temporal variation.

Table 2.2. Linear mixed effects model results estimating the relationship between antler size (gross Boone and Crocket score, converted to cm), and testosterone (T), age (Age), and a testosterone and age interactive effect (T x Age) in male white-tailed deer captured at the Auburn Captive Facility, September—March 2007—2017.

Parameter	Value	df	T-value	P-value
(Intercept)	50.643 ± 3.887	113	13.030	<0.001
T	10.076 ± 3.984	113	2.528	0.013
Age	11.338 ± 0.860	113	13.191	<0.001
T x Age	-1.776 ± 0.745	113	-2.383	0.019

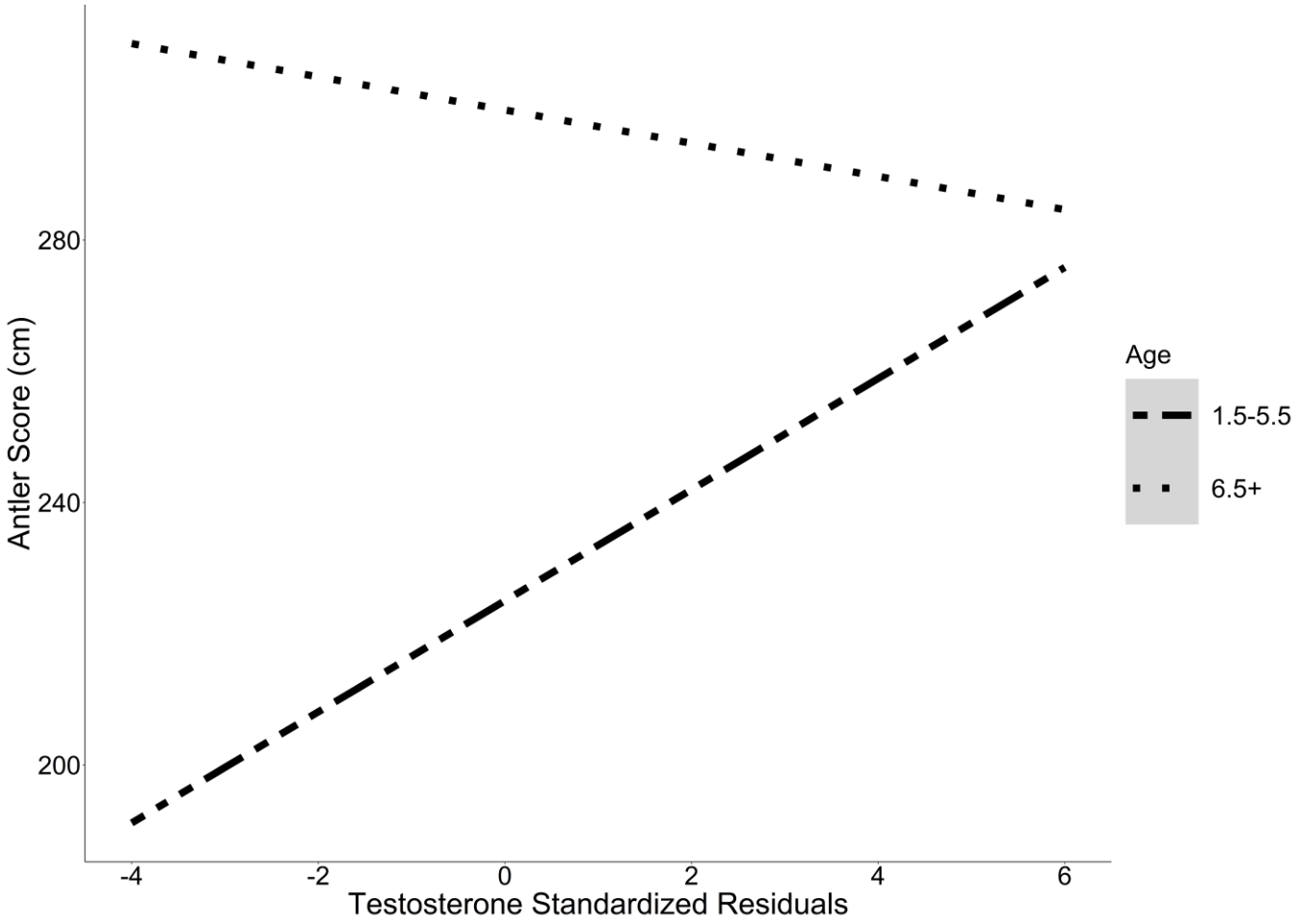

Figure 2.2. Testosterone compared to antler score (gross Boone and Crockett score, in cm) for ages 1.5-5.5 and 6.5+. Data were obtained from male white-tailed deer captured from the Auburn Captive Facility, Auburn AL, from September—March 2007—2017. Testosterone concentrations were standardized to account for time of capture within the sampling period.

Table 2.3. Generalized linear mixed effects model results estimating the factors that influence annual reproductive success in male white-tailed deer captured at the Auburn Captive Facility, September—March 2007—2017. Parameters included are antler size (gross Boone and Crocket score, converted to cm), body size (utilizing a single term generated from principal component analysis), testosterone (T), age (Age), and a testosterone and age interactive effect (T x Age).

Parameter	Value	Z-value	P-value
(Intercept)	-4.038 ± 0.636	-6.352	<0.001
Antler Size	0.012 ± 0.003	4.054	<0.001
Body Size	0.022 ± 0.013	1.664	0.096
T	0.523 ± 0.299	1.75	0.082
Age	-0.101 ± 0.251	-0.404	0.686
T x Age	-0.101 ± 0.056	-1.808	0.071

Discussion

We found that antler size was the factor that influenced reproductive success the greatest in our population of white-tailed deer, while testosterone, age, and body size did not have a direct relationship with reproductive success. Previous work has shown that females actively select for antler size (Morina et al. 2018), and that antler size is a factor that influences reproductive success in this species (Newbolt et al. 2017), so this finding was unsurprising. However, our results differed from studies showing an association between body size and reproductive success (Newbolt et al. 2017). Furthermore, although dominance behaviors shown to increase breeding success (DeYoung et al. 2006) often correlate with testosterone concentrations (Lincoln et al. 1972, Miller et al. 1987, Chunwang et al. 2004), we did not see a direct relationship between testosterone and reproduction. The aforementioned relationship between testosterone and antler size, combined with the positive association between antler size and reproductive success may suggest only an indirect impact of testosterone levels on reproductive success. These data are supported by the 'evolutionary potential hypothesis', where testosterone levels themselves may not be directly under selection, but downstream effects of testosterone on characteristics such as behavioral traits and secondary sex characteristics are under direct selection (Hau 2007). Furthermore, while testosterone is positively related to sperm counts and motility, deer are still capable of producing viable sperm during periods of low testosterone (Stewart et al. 2018). Although testosterone facilitates and enhances reproductive efforts, testosterone alone does not guarantee establishment of dominance, survival during the physiologically stressful breeding period, successful mate acquisition and copulation, or offspring recruitment. On the contrary, the direct relationship between antlers and reproductive success seen in our data suggests that factors such as advertising quality through sexually selected traits may be more important in this system.

We observed a continuum of lifetime testosterone patterns, ranging from individuals with consistent testosterone levels that were near the population mean, to individuals with great year-to-year variation in testosterone levels. Although lifetime patterns of testosterone have not been described previously in white-tailed deer, the variability in patterns we found is similar to what has been described in bighorn sheep (*Ovis canadensis*; Martin et al. 2013). However, long-term research conducted with red deer did not detect individual differences in lifetime testosterone patterns (Pavitt et al. 2015). Differences in lifetime testosterone patterns may arise from different lifetime reproductive strategies. Intraspecific variation in reproductive strategy has been described previously (e.g. roe deer, *Capreolus capreolus*, Vanpé et al. 2007; white-tailed deer, Lemaître et al. 2018; bighorn sheep, Martin et al. 2013), and is believed to be driven by individual quality (genetics, body size, antler size, dominance, etc.; Zahavi 1975). However, while we found individual variation in lifetime testosterone patterns, we found no relationship between this variation and lifetime reproductive success. This potentially suggests a diverse array of hormonal patterns may be suitable for successful reproduction. Because of testosterone's vital role in shaping breeding behaviors, the observed variety of lifetime testosterone patterns in our data suggests that the age and intensity of peak reproductive investment may differ among individuals. These data, in addition to previous research showing that paternity in white-tailed deer may be more widespread among males than originally believed (DeYoung et al. 2002; Sorin 2004; Newbolt et al. 2017), suggest that male white-tailed deer may utilize multiple mating strategies to secure reproductive success.

We observed a positive relationship between testosterone and body size for individuals aged 5.5 years and younger. Given previous literature (Griggs et al. 1989), this relationship was unsurprising. During this age period, individuals allocate resources to somatic growth, during

which, testosterone plays a major role. Testosterone facilitates skeletal growth (Young et al. 1989, Phillip et al. 2001), and this is especially important as individuals mature. Similarly, we found a positive relationship between testosterone and age for deer 1.5 to 5.5 years of age. Although the relationship between testosterone and antler size in cervids has been debated (Goss 1968, Price and Allen 2004, Bartoš et al. 2009, Demarais and Strickland 2011), as testosterone remains low during the period of antler growth, previous literature generally shows a positive association between testosterone and antler size (Bartoš et al. 2009, Bartoš et al. 2012). One study looking at both captive and wild red deer (*Cervus elaphus*) found a negative relationship between testosterone and antler size, however, testosterone was positively associated with antler strength (Malo et al. 2009). Additionally, previous research with white-tailed deer has found associations with testosterone and insulin-like growth factor (IGF-I), and IGF-I and antler size (Ditchkoff et al. 2001). IGF-I promotes testosterone production leading up to the breeding season, and also is positively associated with antler growth in multiple cervid species (Schams et al. 1992, Bartoš et al. 2009), and it may be through this relationship that we see the correlation between testosterone and antler size.

Contrary to the patterns we found for deer ≤5.5 years of age, deer ≥6.5 years had a negative relationship between testosterone and body and antler size. Previous studies with cervids and other large ungulates have documented declines in body mass and muscle mass associated with aging (Yoccoz et al. 2002; Reimers et al. 2005; Nussey et al. 2011). However, since our measure for body size was based upon skeletal measurements, we would not expect a senescent decline in that variable. As a result, our data simply suggest that testosterone level is negatively associated with skeletal size in males ≥6.5 years, where old individuals that have greater testosterone tend to be those with smaller bodies. While we could find no mention of

similar testosterone patterns in the literature, Ditchkoff et al. (2001*b*) found a similar pattern with respect to antler size and fluctuating asymmetry. They reported that up to age 5.5, there was a negative relationship between antler size and fluctuating asymmetry: however, at 6.5 and older, antler size and asymmetry were positively associated. They suggested that at these older ages, deer may be investing in reproduction so heavily that the associated stress may lead to increased levels of fluctuating asymmetry in antlers. It is possible a similar phenomenon may be occurring in older individuals in our population. Furthermore, older bucks with smaller body size and antler size relative to others within their age classes may need to compensate with increased dominance behaviors to successfully compete for breeding opportunities. These efforts may mitigate a male's inability to invest heavily in somatic growth towards body size earlier in life and inability to allocate significant resources to antler growth during the summer.

It is possible that older males with large antlers and body size have low testosterone because of elevated stress associated with maintenance and development of these tissues (Dmitriew 2011) in combination with behavioral stressors of the breeding season (Bubenik and Leatherland 1984). These stresses could theoretically increase secretion of glucocorticoids that trigger the hypothalamic-pituitary adrenal (HPA) axis to decrease testosterone production (Cumming et al. 1983). However, we believe it's more likely that larger individuals are capable of obtaining breeding opportunities because of their greater antler size (Morina et al. 2018), body size (McElligott et al. 2001), and associated dominance (Chunwang et al. 2004), despite having lower testosterone levels. These older age classes may represent a point where deer invest in reproduction at a tremendous physiological cost, as senescence is rapidly approaching. These efforts may be the last, and best, opportunity for a male to improve his lifetime fitness prior to senescence (Ditchkoff et al. 2001*b*). Patterns of hormonal secretion reported in previous studies

support this theory. Ditchkoff et al. (2001*d*) found that IGF-I in male white-tailed deer declined as early as 6.5 years of age, and Bubenik and Schams (1986) reported declines in testosterone after roughly 7 years of age. Reproductive senescence has been described in longer-lived cervids such as red deer (Nussey et al. 2017), with males showing sharp declines in rutting behavior and breeding success, and slight decreases in antler size and number of tines after the age of 9.

Pioneering research in white-tailed deer once stipulated that very few individuals monopolized breeding opportunities within a population (Hirth 1977, McCullough 1979, Marchinton and Hirth 1984, Miller and Ozoga 1997), however as research techniques advanced, data showed that reproductive success can be more widely distributed among males in a population, despite large amounts of variation in sexually selected traits such as antlers (Sorin 2004; DeYoung et al. 2006, 2009; Newbolt et al. 2017). This research builds upon this foundation, evaluating these breeding systems at a molecular and hormonal level, and these data suggest that just as deer of different phenotypes successfully reproduce, so may deer with different hormonal patterns. As this study is one of only a few to document these hormonal patterns in a freely breeding ungulate population, we believe further investigation is warranted. Similar to research with other long-lived ungulates (Martin et al. 2013; Pavitt et al. 2015; Nussey et al. 2017), we believe that research aiming to monitor populations at an individual-level while collecting in-depth data on hormonal patterns will aid in efforts to distinguish among reproductive strategies. As research investigating sexual selection shifts to include genetic and hormonal parameters, evaluating breeding systems and accounting for differences among individuals may provide more insight into the physiological and behavioral dynamics of reproductive strategies in highly competitive breeding systems.

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