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Original Article

Patterns of Fecal Hormones in a Fenced Population of White-Tailed Deer

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ABSTRACT Analysis of voided feces has become a popular method of monitoring physiological parameters of many wildlife species. During the breeding seasons of 2006–2007 and 2007–2008, we collected fecal samples to determine concentrations of fecal glucocorticoid (FG) and fecal testosterone (FT) metabolites in a fenced population of white-tailed deer (*Odocoileus virginianus*). The purpose of the study was to determine how concentrations of FG and FT metabolites fluctuated throughout the breeding season, whether there was a difference between males and females, and whether there was a relationship between the two hormones. FG and FT metabolites of males peaked during the rut, while female glucocorticoid levels remained relatively stable throughout the breeding season. We also observed a significant positive correlation between FG and FT metabolites. During the pre-rut and rut, males that exhibit high testosterone also had elevated glucocorticoid metabolite concentrations, but in the post-rut there was no relationship between FT and FG concentrations. We hypothesize that the effect of testosterone on glucocorticoid secretion is partially a result of testosterone-induced behavior, and not strictly a physiological link between the two hormones.

KEY WORDS corticosterone, fecal hormones, feces, glucocorticoids, *Odocoileus virginianus*, stress, testosterone, white-tailed deer.

Monitoring hormone levels of free-ranging wildlife can provide valuable insights into the sources and costs of behavior and the reproductive status of various species. Over the past decade, the method of obtaining hormone profiles has shifted toward non-invasive collection of samples, specifically through voided feces. Hormones have historically been measured through blood profiles, but blood collection in free-ranging wildlife can be stressful to the individual, time-consuming, and costly. Capture stress and subsequent blood collection can affect hormone concentrations, thus making measurements unreliable (Hamilton and Weeks 1985, Harper and Austad 2000). Additionally, fecal hormone analyses reflect an integrated level of hormone secretion over a period of time (dependent on gut passage and defecation rate) due to the pooling of metabolites, which may serve to give a better "picture" of hormone levels than point samples (Creel et al. 1997, Pelletier et al. 2003).

Measuring glucocorticoid secretion in feces has become a topic of particular interest due to the possible deleterious effects of stress on vertebrates. Glucocorticoid secretion occurs during the stress response, which enables an animal to cope with a stressful situation by mobilizing energy necessary for the response and minimizing energy expenditure in other tissues that are not needed for immediate survival (Munck et al. 1984, Sapolsky 2002). Although the effects of stress on wildlife are still not fully understood, research to date suggests that there can be substantial physiological and/or long-term effects (Ozoga and Verme 1982, Blanc and Thériez 1998, Stefanski and Engler 1998, Stefanski 2001, Stefanski et al. 2001).

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Testosterone can be used to indicate male aggression, dominance, and reproductive status (Sapolsky 1983, Creel et al. 1997, Li et al. 2001, Pelletier et al. 2003). Elevated levels of testosterone have been associated with the breeding season and aggressive behavior in many species (Ketterson et al. 1991, Johnsen 1998, Pelletier et al. 2003, Li et al. 2004). However, there are conflicting reports regarding the relationship between glucocorticoids and testosterone.

The general consensus is that glucocorticoid secretion suppresses androgen production as a result of the negative interactions between the hypothalamic–pituitary–adrenal axis and the hypothalamic–pituitary–gonadal axis (Greenberg and Wingfield 1987, Rivier and Rivest 1991). However, several studies have documented a positive relationship between testosterone and glucocorticoid levels (Ketterson et al. 1991, Johnsen 1998), and others have found no distinct trend (Bartoš et al. 2010) or trends that differ among individuals depending on dominance status (Sapolsky et al. 1986, Knapp and Moore 1997).

The aim of this study was to develop an understanding of the relationship between glucocorticoid secretion and testosterone levels in white-tailed deer (*Odocoileus virginianus*). We studied a fenced population of white-tailed deer held at an abnormally high density with a large proportion of mature

males in order to investigate the potential for elevated levels of social stress during the breeding season, and the possible effects on testosterone. The specific objectives were to 1) examine seasonal variation in levels of fecal glucocorticoid (FG) metabolites, 2) determine whether levels of FGs differ between males and females, and 3) examine the relationship between FG and fecal testosterone (FT) metabolites.

STUDY AREA

The property of Three Notch Wildlife Research Foundation (Three Notch) was located in east-central Alabama, USA, approximately 10 km east of Union Springs in Bullock County. The study area encompassed 258.2 ha, and had been enclosed by a 3-m deer-proof fence since 1997. Food plots and supplemental feeding provided the deer herd with a high-quality diet throughout the year. A high-protein commercial deer feed (20% protein; Purina Antlermax, St. Louis, MO) was provided *ad libitum*. Approximately 20% of the available habitat (48 ha) was in food plots, with the intent of providing deer with a wide array of food sources. Warm-season food plots generally consisted of iron and clay peas (*Vigna unguiculata*), corn (*Zea mays*), and various clovers (*Trifolium* spp.), whereas cool-season plots were usually made up of winter rye (*Secale cereale*) and white clover (*T. repens*).

Forest cover on the site varied from open, mature stands of loblolly pine (*Pinus taeda*) in upland areas to dense over stories of oaks (*Quercus* spp.) in creek drainages. Ridges were primarily dominated by loblolly pine or food plots, and lowland areas were planted in clover. Prescribed fire was used each year in upland areas to enhance natural browse for deer. Water sources on the site included the headwaters of the Pea River and a large, centrally located pond (approx. 20 ha) that provided the deer herd with an abundant year-round water source.

Hunting on the property was non-commercial and generally limited only to the landowner and family members. Archery was the primary method of harvest, and was limited to mature bucks (5 yrs or older) and does of any age class. Due to limited hunting success (archery equipment only), the selective harvest of the landowner, and an abundance of food sources, the enclosure had an extremely high population of deer with a sex ratio favoring males. A pre-hunting-season camera survey estimated a 2.64:1 (M:F) sex ratio, but after antlerless harvest, breeding-season sex ratios exceeded 4:1 (McCoy et al. 2011).

METHODS

Sampling Protocol

We collected fresh fecal samples within the study area throughout the calendar year. We defined fresh samples as those that were still soft, moist, and coated with mucous. According to Huber et al. (2003), red deer (*Cervus elaphus*) fecal samples only remained shiny and moist for the first 3 hrs after defecation. Additionally, Washburn and Millspaugh (2002) reported that FG concentrations remained stable in feces for $\geq$7 days in the absence of rainfall. Therefore, we are confident that samples collected under our protocol (shiny and moist) provided valid estimates of FG concentrations.

We collected fecal samples approximately three times per week during each of the 4 months surrounding the breeding season (1 November 2006 to 22 March 2007 and 1 November 2007 to 28 February 2008). Each day we randomly selected a starting point for our search effort, which allowed us to assume that each animal in the enclosure had an equal opportunity of being sampled throughout the study period. Once a location was selected, we searched for fresh fecal samples throughout the interconnecting network of game trails and bedding areas within approximately 50 ha–60 ha of our starting location, thus collecting 10–15 samples each day. In southeastern Alabama, breeding activity usually ranges from November to February, with peak breeding activity occurring in late January (Causey 1990). By collecting samples before and after the time of peak breeding activity, we were able to examine how stress levels fluctuate before, during, and following the breeding season.

FG Analysis

The methods for processing of fecal samples have been detailed previously by Millspaugh et al. (2001, 2002). Briefly, we froze fecal samples until they were processed, and we freeze-dried approximately 10 g of each sample and sifted it through a stainless-steel mesh (Wasser et al. 1994, 1996; Millspaugh et al. 2001, 2002). We extracted FGs using a modification of Schwarzenberger et al. (1991). Dried fecal samples (0.2 g) were mixed with 2.0 mL of 90% methanol and vortexed for 30 min, centrifuged at 2,200 revolutions per minute for 20 min, and the supernatant stored at -80° C until assayed. We used MP Biomedicals ^{125}I-corticosterone radioimmunoassay kits (MP No. 07-120103; MP Biomedicals, Orangeburg, NY) that have been previously validated to accurately measure FG concentrations in white-tailed deer (Millspaugh et al. 2002). We diluted fecal extracts 1:20 in assay buffer, and expressed concentrations on a dry-weight basis (ng/g). Antiserum had the following cross-reactivities (provided by the company): 100% corticosterone, 0.34% desoxycorticosterone, 0.1% testosterone, 0.05% cortisol, 0.03% aldosterone, 0.02% progesterone, 0.01% androstenedione, and 0.01% 5α-dihydrotestosterone.

FT Analysis

To determine the sex of fecal samples collected in the field, we also measured FT metabolites using a commercially available testosterone enzyme immunoassay kit (DSL-10-4000; Diagnostic Systems Laboratories, Webster, TX), which had been used previously to monitor FT in male Pampas deer (*Ozotoceros bezoarticus bezoarticus*; Pereira et al. 2005). Fecal testosterone was extracted from feces following the same protocol as used for FGs. The particular kit that we used did not supply diluent, because the protocol for the assay did not call for any dilutions. To dilute our samples we used the wash solution provided in the kit (buffered saline with a non-ionic detergent, mixed with deionized water). To verify the use of the wash solution

as a diluent, we diluted five samples with both the wash solution and the 0 ng/g testosterone standard. A linear regression between samples diluted with the wash solution and those diluted with the 0 ng/g standard indicated a 1:1 relationship with a slope estimate of 0.986 and an $r^2 = 0.996$; thus, we assumed that the wash solution was a suitable diluent for use in our enzyme immunoassay. Fecal extracts were diluted 1:32 in saline buffer prior to assay, and concentrations were expressed as ng/g (dry wt). The antiserum had the following cross-reactivities (provided by the company): 100% testosterone; 6.6% 5α-dihydrotestosterone; 2.2% 5-androstane-3β, 17β-diol; 1.8% 11-oxotestosterone; 0.9% androstenedione; 0.6% 5β-dihydrotestosterone; 0.5% 5β-androstane-3β, 17β-diol; 0.4% estradiol-17β; and 0.2% 5α-androstane-3α-ol-17-one. We conducted parallelism tests with serially diluted fecal extracts (1:2–1:64) and the standard curve (0.1 ng/mL–25.0 ng/mL) to validate the assay for use with white-tailed deer. The sensitivity of the test was 0.04 ng/mL, and inter- and intra-assay coefficients of variation were <8%.

To assess physiological relevance of the FT measurements, we assayed fecal extracts of harvested deer (known sex), and determined a range of FT concentrations for males ($n = 41$, $\bar{x} = 645.84 \pm 264.54$, range = 78.42–3,736) and females ($n = 46$, $\bar{x} = 30.06 \pm 4.12$, range = 9.22–59.6). Male FT concentrations varied widely, but all were greater than females. One male fawn, not included in the above analysis, had a concentration of 69 ng/g, so it is possible that young-of-year males could have concentrations within the range of females. However, with a large sample size, we felt confident that the disparity in measured testosterone metabolites of breeding-aged males versus females was large enough to assign sex to field collected samples of unknown origin. It is important to note here that fecal samples pulled from harvested animals were not subjected to the same environmental conditions as samples collected in the field. However, FGs have been shown to be stable in feces for up to a week, so we assumed that FT measures were not affected by lack of environmental exposure. An extensive literature review did not reveal any studies of the stability of testosterone in feces or the possible effects of environmental exposure, so this could be an important avenue for future research.

Statistical Analysis

To satisfy the requirements of normality, all fecal data (FG and FT) were transformed using the natural log (Fichtel et al. 2007). Data were analyzed using a factorial analysis of variance (ANOVA; PROC GLM; SAS Institute 9.1, Cary, NC) with period, sex, and year as main effects, and year × sex, year × period, period × sex, and year × period × sex as interaction effects. We separated the breeding season into three periods: pre-rut (1 November–31 December), rut (1 January–8 February), and post-rut (9 February–22 March) to allow for comparison among periods prior to, during, and following the breeding season. Several pregnant females at Three Notch were harvested during the 2007 ($n = 13$) and 2008 ($n = 12$) seasons that provided information on timing of the breeding season via fetal aging. According to these data, peak of the breeding season occurred between the last week of January and the first week of February during both years of the study.

The relationship between FG and FT was examined with a correlation (PROC CORR; SAS Institute 9.1), using Pearson's correlation coefficient. To further examine this relationship, we used a linear model (R Version 2.11.1, www.r-project.org) to test the effect of testosterone, period, and testosterone × period on FG concentrations. The level of significance was set at 0.05 for all statistical tests.

RESULTS

Fecal Glucocorticoids

We collected and assayed 529 fecal samples (432 M, 97 F) from the population. Male FG concentrations ranged from 7.33 ng/g to 355.48 ng/g ($\bar{x} = 33.74 \pm 1.38$), and females ranged from 7.06 ng/g to 47.95 ng/g ($\bar{x} = 17.75 \pm 0.63$). We found a significant period × sex interaction (factorial ANOVA: $F_{2,517} = 6.48$, $P = 0.002$), where FG levels of males were greater during the rut ($n = 220$, $\bar{x} = 43.06 \pm 1.89$) than during the pre-rut ($n = 192$, $\bar{x} = 28.3 \pm 2.09$; $P < 0.001$; Fig. 1) and post-rut ($n = 63$, $\bar{x} = 22.2 \pm 1.28$; $P < 0.001$), while female FG levels remained relatively stable throughout the breeding season. Male FG concentrations were greater than females during the pre-rut (F: $n = 77$, $\bar{x} = 18.57 \pm 0.84$; $P < 0.001$) and rut (F: $n = 50$, $\bar{x} = 19.24 \pm 0.98$; $P < 0.001$). We also found a significant year effect (factorial ANOVA: $F_{2,517} = 2.94$, $P = 0.05$), where males during the rut had greater FG levels in 2008 ($n = 101$, $\bar{x} = 49.9 \pm 5.16$) than in 2007 ($n = 116$, $\bar{x} = 37.9 \pm 5.2$). Though not statistically significant (*t*-test: $t_{20} = 1.181$, $P = 0.126$), harvest data suggested that male body weights were greater when supplemental feed was available in 2007 ($n = 14$, $\bar{x} = 86.28$ kg) than when the feeding program was suspended in 2008 ($n = 8$, $\bar{x} = 81.65$ kg).

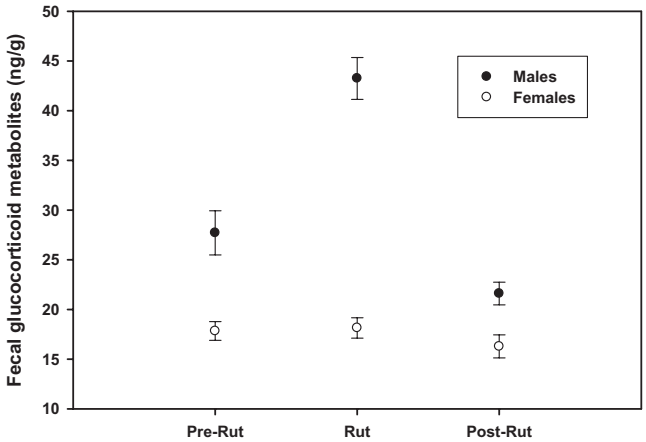

Figure 1. Mean FG concentrations for a fenced population of white-tailed deer during the pre-rut (1 November–31 December), rut (1 January–8 February), and post-rut (9 February–22 March) periods of the breeding season of 2006–2007 and 2007–2008 at Three Notch in east-central Alabama, USA.

Fecal Testosterone

Mean FT concentrations for females and males were 39.47 ± 1.81 ng/g and 747.45 ± 51.70 ng/g, respectively. FT concentrations varied widely for males (range = 78.42 ng/g–5,750 ng/g), but were fairly consistent for females (range = 9.28 ng/g–69.17 ng/g). Male FT peaked during the rut (factorial ANOVA: $F_{2,517} = 5.28$, $P = 0.005$; Fig. 2), more than doubling in concentration from pre-rut levels, and then returned to pre-rut levels during the post-rut.

We found a significant correlation between male FT concentrations and corresponding FG levels ($n = 432$, $r = 0.607$, $P < 0.001$). FGs and FT were strongly correlated during pre-rut ($n = 178$, $r = 0.438$, $P < 0.001$) and rut ($n = 192$, $r = 0.663$, $P < 0.001$), but post-rut analysis revealed no correlation ($n = 62$, $r = 0.005$, $P = 0.97$). Linear regression analysis revealed a significant testosterone × period interaction ($F_{2,426} = 11.08$, $P < 0.001$; Fig. 3), where the slope estimates were greater during both pre-rut and rut than during post-rut (difference in slopes = 0.227 ± 0.151 and 0.324 ± 0.142, 95% CI; $P = 0.003$ and $P < 0.001$, respectively). The slope was also greater during the rut compared with the pre-rut (difference in slopes = 0.097 ± 0.089, 95% CI; $P = 0.029$). Note that the relationship between FT and FG concentrations during the post-rut was not significant (slope = 0.002 ± 0.134, $P = 0.973$).

DISCUSSION

Seasonal Variation

Our data revealed that there was a period effect, where the intensity of rutting activity apparently drives FG levels in male white-tailed deer. Pre-rut levels were elevated in males, which we attribute to rising testosterone levels and subsequent increases in male–male aggressive interactions, sparring matches, and dominance displays that serve to firmly establish social status. FG levels of males increased by an average of 66% during the rutting period, when sparring matches would have increased in intensity and frequency, and males would have been continually searching for females to secure breeding opportunities (Marchinton and Hirth 1984). Our measures of FG metabolites fell below baseline measures reported for captive white-tailed deer (40 ng/g–90 ng/g; Millspaugh et al. 2002), but were similar to those found in free-ranging elk (17.41 ng/g–34.21 ng/g; Millspaugh et al. 2001). We hypothesized that the unique characteristics of the Three Notch population (high density of mature males leading to intense breeding competition) would cause increased and prolonged secretion of glucocorticoids. Though FG levels did increase during the breeding season, it does not appear that the social stressors present in this particular population were strong enough to elicit excessive or long-term glucocorticoid secretion. Therefore, breeding competition that results in severe social stress is unlikely to occur in free-ranging populations of white-tailed deer, because mature males are seldom found at such high densities, and more balanced or female-skewed sex ratios allow for less intense breeding competition.

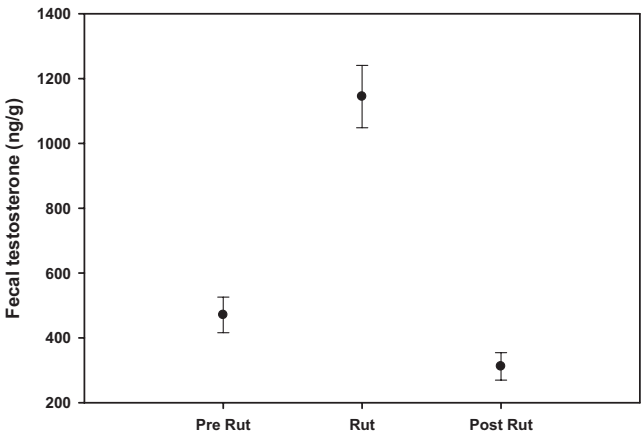

Figure 2. Mean FT concentrations for a fenced population of male white-tailed deer during the pre-rut (1 November–31 December), rut (1 January–8 February), and post-rut (9 February–22 March) periods of the breeding season of 2006–2007 and 2007–2008 at Three Notch in east-central Alabama, USA.

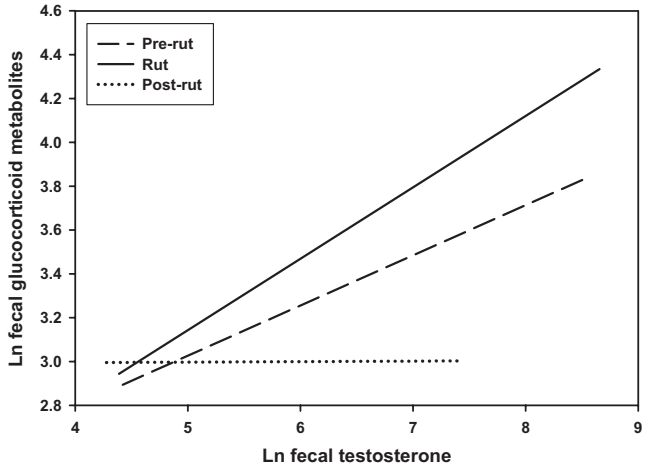

Figure 3. Linear regression showing the relationship between FT and FG metabolites of a fenced population of male white-tailed deer during the pre-rut (1 November–31 December), rut (1 January–8 February), and post-rut (9 February–22 March) periods of the breeding season of 2006–2007 and 2007–2008 at Three Notch in east-central Alabama, USA.

We observed a difference in FG concentrations between the 2 years of our study. It has been well-documented that male ungulates reduce feeding effort during the breeding season, which is most often attributed to the conflicting time constraints of finding food and/or participating in rutting activities (e.g., fighting, dominance displays, chasing; Espmark 1964, Coblentz 1976, Lincoln and Short 1980, Geist 1982). In 2007, males in our fenced population were surrounded by high-quality forages (e.g., food plots and supplemental feed) at concentrations greater than what is normally available for most free-ranging deer, and, thus could have obtained adequate nutrition without sacrificing time that could be spent searching for potential mates. In 2008, supplemental feed was not available during the rut, and males at Three Notch experienced greater FG levels than during 2007. Though not statistically significant, harvest data suggested that male body weights were greater in

2007 (when supplemental feeding was available) than in 2008. Though speculative, this situation hints at the possibility that a high-density, fenced population of white-tailed deer may need to have access to high-quality supplemental feed during the rut to alleviate the stresses of breeding and lack of winter forage.

Male FT also peaked during the rut, and followed a periodic pattern similar to FGs. This seasonal fluctuation was expected and has been reported elsewhere in reindeer (*Rangifer tarandus*; Leader-Williams 1988), axis deer (*Axis axis*; Bubenik et al. 1991), Père David's deer (*Elaphurus davidianus*; Li et al. 2001), and white-tailed deer (Mirarchi et al. 1978, Bubenik et al. 1983, Ditchkoff et al. 2001). Mean FT metabolite concentrations were comparable to those found in other studies. Though our measure of male FT (747.45 $\pm$ 51.70 ng/g) was more than twice that of the Pampas deer stags (343.9 $\pm$ 211.4 ng/g; Pereira et al. 2005), we only measured FT throughout the breeding season when FT should be at a maximum, whereas Pereira et al. (2005) measured FT throughout an entire year. Measures of female FT were similar between the two studies (white-tailed deer: 39.47 $\pm$ 1.81 ng/g, Pampas deer: 47 $\pm$ 22.9 ng/g). Additionally, our measures of male FT were similar to those found in Père David's deer during the rut (577.15 $\pm$ 144.08 ng/g; Li et al. 2001).

FGs and FT

During the pre-rut and rut periods, greater FT levels were generally accompanied by increasing FG concentrations. However, this relationship was not evident in the post-rut period. The time of actual conception occurs within a 2-week period in this high-fenced population (J. C. McCoy, unpublished data) because the abundance of males assures that most, if not all, females are bred when they enter estrous. Given this information, the sharp decline in FT and FG concentrations following the breeding season is likely due to the almost instantaneous cessation of breeding activity. Males with elevated FT concentrations in the post-rut did not exhibit the same elevated levels of FGs as in the pre-rut and rut. This may be evidence that the effect of testosterone on glucocorticoid secretion is partially a result of testosterone-induced behavior and not strictly a physiological link between two hormones.

There are some conflicting reports regarding the nature of the relationship between testosterone and glucocorticoid secretion (Rivier and Vale 1984, Johnsen 1998, Bartoš et al. 2010). Although there is evidence that stress inhibits testosterone (Rivier and Vale 1984), Bartoš et al. (2010) found no clear trend, and other studies have shown that glucocorticoid levels are positively correlated with testosterone (Ketterson et al. 1991). Our study revealed a positive association between the two hormones. However, our data suggest that the relationship was at least partially dependent upon stress-inducing behaviors that result from increased testosterone production during the breeding season. Our study provides important insight into the interaction between these two hormones and serves as an important reference for future work.

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