

Seasonal, Condition-Dependent, and Individual Variation in Testosterone in a Natricine Snake

RICHARD B. KING^{1,2} AND RACHEL M. BOWDEN^{3,4}

¹Department of Biological Sciences, Northern Illinois University, DeKalb, Illinois 60115 USA

³School of Biological Sciences, Illinois State University, Normal, Illinois 61790 USA

ABSTRACT.—We collected plasma samples from adult males in a natural population of Plains Gartersnakes (*Thamnophis radix*) to characterize seasonal variation in testosterone. As in other New World temperate zone natricines, testosterone was high during spring emergence, declined as courtship and mating progressed, and then increased in late summer and fall. Based on patterns seen in this and other studies, we suggest that the rapidity with which testosterone decreases following spring emergence may be associated with the length of the mating season; and that it decreases rapidly in species and populations in which the mating season is brief and more slowly in species and populations in which mating occurs over an extended period. Testosterone showed a clear, positive association with body condition in adult male Plains Gartersnakes. Whether this association translates into greater reproductive success warrants further investigation. Because we collected blood samples multiple times from individual males within years, we were also able to demonstrate significant individual variation in seasonal testosterone profiles, although how this might relate to individual variation in reproductive behavior is unknown. Given their phylogenetic, ecological, and geographic diversity, New World natricine snakes represent a rich resource for studying hormonal associations with reproductive patterns.

In snakes, understanding of the relationship between circulating testosterone levels and courtship and mating continues to be refined (DeNardo and Taylor, 2011; Krohmer and Lutterschmidt, 2011). For example, among New World natricine snakes (Gartersnakes, Watersnakes, and their allies), initial analyses that focused on northern populations of the Common Garter-snake, *Thamnophis sirtalis*, suggested that both spermatogenesis and elevated testosterone levels occurred in summer and were dissociated from spring courtship and mating (Crews et al., 1984). More-detailed follow-up studies confirmed this pattern for spermatogenesis but demonstrated that testosterone levels remain elevated until spring emergence and drop rapidly as courtship and mating ensue (Krohmer et al., 1987; Krohmer and Lutterschmidt, 2011). In other species of New World natricines, testosterone levels remain elevated throughout the mating season with a protracted decline following emergence (Weil and Aldridge, 1981). Among rattlesnakes, sympatric species with contrasting mating systems show different patterns of seasonal variation in testosterone (Schuett et al., 2005). Together, these studies indicate that there is much variability in seasonal testosterone patterns across even closely related species.

In this study, we characterized seasonal variation in testosterone in the Plains Gartersnake, *Thamnophis radix*. New World natricine snakes represent a large radiation comprising about 55 species divided into three clades: the Watersnake clade (*Nerodia*, some *Regina*, *Tropidoclonion*), the Gartersnake clade (*Adelophis*, *Thamnophis*), and the semi-fossorial clade (*Clonophis*, *Seminatrix*, *Storeria*, some *Regina*, *Virginia*) (Alfaro and Arnold, 2001). The Gartersnake clade is further divided into the Mexican subclade, widespread subclade, and Ribbonsnake subclade (de Queiroz et al., 2002). To date, assessments of seasonal variation in circulating hormones have focused on members of the Watersnake clade and the Ribbonsnake and Mexican subclades within the Gartersnake clade. As a member of the widespread subclade within the Gartersnake clade, our study of the Plains Gartersnake expands the phylogenetic scope of such analyses. The Plains Gartersnake also has a broad geographic distribution (from Ohio in the east to Montana in the west and from New

Mexico in the south to Manitoba in the north (Rossman et al., 1996:map 21). Thus, the results presented here can serve as a basis for future analyses of geographic variation.

We also investigated several possible sources of variation in testosterone beyond a seasonal pattern. In particular we tested for effects of year and body condition. In addition, because plasma samples from a subset of animals included in our analyses were collected multiple times over the course of the year, we tested for individual differences in seasonal testosterone profiles. Such differences might be expected, given evidence of heritable variation in testosterone among captive-reared natricine snakes (King et al., 2000, 2004), but had not yet been investigated in free-ranging adult animals.

MATERIALS AND METHODS

Study Animals.—Plains Gartersnakes were captured by hand at a 2.65-ha study site along the banks of the Kishwaukee River adjacent to Northern Illinois University, DeKalb County, Illinois as part of a larger study of population ecology (Stanford and King, 2004; King and Stanford, 2006). Upon capture, snakes were placed in cloth bags and brought into the laboratory where they were classified by sex, measured to obtain snout–vent length (SVL) and mass, and marked individually (Stanford and King, 2004). Blood samples (0.1–0.5 ml) were drawn from caudal vessels using heparinized, disposable tuberculin syringes, separated into plasma and red blood cell fractions, and frozen (–20°C) for later analysis. Data collection and blood sampling occurred on the day of capture or the following day, after which snakes were released where found. Snakes that were recaptured were processed as described above provided that 2 weeks had elapsed between captures. Estimated dates of first emergence from hibernation (earliest date on which Plains Gartersnakes were observed) and of courtship and mating were obtained from observations made from 1995–2004.

Analyses described here include 76 plasma samples collected from 40 adult males (≥ 350 mm SVL; Stanford and King, 2004) collected between 30 March (Julian Date = 89) and 20 September (Julian Date = 264). At the time of sample collection, males averaged 429 mm SVL (range = 351–508) and 40 g (23–70). Most samples were collected in 1999 ($N = 32$ samples from

²Corresponding Author. E-mail: rbking@niu.edu

⁴E-mail: rmbowde@ilstu.edu

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19 males) and 2000 (38 samples from 23 males) but, because we sought to characterize individual patterns of seasonal variation, samples collected in 2001 (5 samples from 2 males) and 2002 (2 samples from 1 male) were also analyzed. Only a single plasma sample was collected from 24 males during our study. For another 16 males, 2–5 plasma samples were collected within a given year, including three males from which multiple samples were collected in two ($N = 2$ males) or three ($N = 1$ male) years.

Testosterone Assays.—Plasma samples were transferred to Illinois State University for radioimmunoassay (RIA) (Wingfield and Farner, 1975; Bowden et al., 2001; Hausmann et al., 2012). Two assays were conducted, one consisting of plasma from males that were sampled just once (assay 1 conducted in March 2007) and the other consisting of plasma from males that were sampled multiple times (assay 2 conducted in September 2007). Samples were prepared by diluting 100 μ l of plasma with water to a total volume of 400 μ l. Tritiated tracer (2000 cpm; NET 553, Perkin Elmer, Boston, MA) was added to each sample for calculation of recovery values. Plasma was extracted with 4 ml diethyl ether and resuspended in 550 μ l phosphate buffered saline gelatin and allowed to sit overnight. Plasma samples were run in a competitive-binding RIA using a specific antibody for testosterone (T3003, Research Diagnostics, Flanders, NJ). Steroid concentrations were calculated and compared to a standard curve that ranged from 1.95 to 500 pg for plasma. Recovery values averaged 70% for assay 1 and 68% for assay 2. Intra-assay coefficient of variation (CV) was 3.9% for assay 1 and 3.8% for assay 2, while the interassay CV was 9.1%.

Statistical Analysis.—The fact that only a single plasma sample was collected from some animals, and that multiple samples were collected from other animals, complicated statistical analysis. We proceeded by first using all data to characterize seasonal variation in testosterone. Testosterone values were log transformed to meet the assumption of homoscedasticity (variances of untransformed data were correlated with testosterone) and Julian Date was the independent variable in quadratic regression analysis. Residuals from this quadratic regression were then used to test for differences between assays and between years (1999 and 2000) via two-sample t -tests and for associations with SVL and body condition via correlation analysis. For animals from which multiple samples were collected within a year, one sample was selected at random for these analyses. SVL was log transformed prior to analysis. As a measure of body condition, we generated residuals from the regression of $\log(\text{mass})$ on $\log(\text{SVL})$. Although we did not systematically palpate animals to detect recently consumed prey, we rarely recovered prey during the long-term study of this population (Stanford and King, 2004) and assumed that condition is largely unaffected by recent feeding history. To test for differences in testosterone levels among individuals from which multiple plasma samples were collected, we used analysis of variance with residual testosterone as the dependent variable and snake as a random factor. Repeated-measures analysis was not used because of variation in the number and date of observations per male (2 to 5) within a given year. In this analysis, observations on the same individual in different years were treated as independent.

RESULTS

Testosterone levels ranged from 0.3 to 68.1 ng/ml, averaged 3.9 ng/ml (following back-transformation), and showed a strong seasonal pattern (Fig. 1). Levels were high in spring

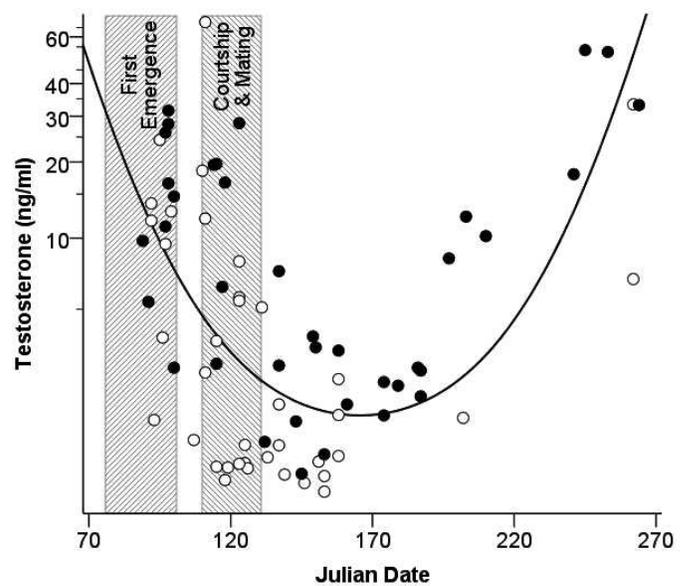


FIG. 1. Seasonal variation in testosterone among adult male Plains Gartersnakes, *Thamnophis radix*. Solid line represents the best-fit quadratic regression. Filled and open symbols represent individuals with body condition above or below the median, respectively. Cross-hatching indicates observed range of dates of first emergence from hibernation and of courtship and mating from 1995–2004 (though not necessarily of males in which testosterone was assayed).

and fall and low in summer (quadratic regression: $\log(T) = 4.729447 - 0.055274 \cdot \text{Julian Date} + 0.000167 \cdot \text{Julian Date}^2$; $F_{2,73} = 24.25$, $P < 0.001$, $r^2 = 0.40$). High testosterone levels in spring overlapped with dates of first emergence from hibernation, which ranged from 17 March (Julian Date = 75) to 11 April (Julian Date = 101) and with observed incidences of courtship and mating ($N = 12$), which fell between 21 April (Julian Date = 111) and 11 May (Julian Date = 131).

After removing the effects of season by computing residuals and including just one observation per male per year, testosterone levels did not differ between assays ($t = -0.73$, $df = 42$, $P = 0.469$) or between years ($t = -0.30$, $df = 39$, $P = 0.764$) and were not correlated with $\log(\text{SVL})$ ($r = 0.26$, $df = 42$, $P = 0.085$). Residual testosterone levels did show a significant positive correlation with body condition ($r = 0.42$, $df = 42$, $P = 0.004$; Fig. 2). Residual testosterone levels also differed significantly from male to male among those males from which multiple plasma samples were collected in a given year, ($F_{1,19} = 2.92$, $P = 0.004$; Fig. 3).

DISCUSSION

Testosterone showed a clear seasonal pattern in adult male Plains Gartersnakes. Circulating levels were high during spring emergence, declined as courtship and mating progressed, and then increased in late summer and fall (Fig. 1). This pattern is generally similar to that seen in other New World temperate zone natricines (*Nerodia sipedon*, Weil and Aldridge, 1981; Krohmer, 2004; *Thamnophis sirtalis*, Weil, 1985; Krohmer et al., 1987; Moore et al., 2000b; Clesson et al., 2002; Cease et al., 2007; Lutterschmidt and Mason, 2009), but the rapidity with which testosterone decreases following spring emergence apparently varies among species and populations (Table 1). Testosterone decreases rapidly in species or populations in which the mating season is brief (*Thamnophis sirtalis parietalis* and *Thamnophis*

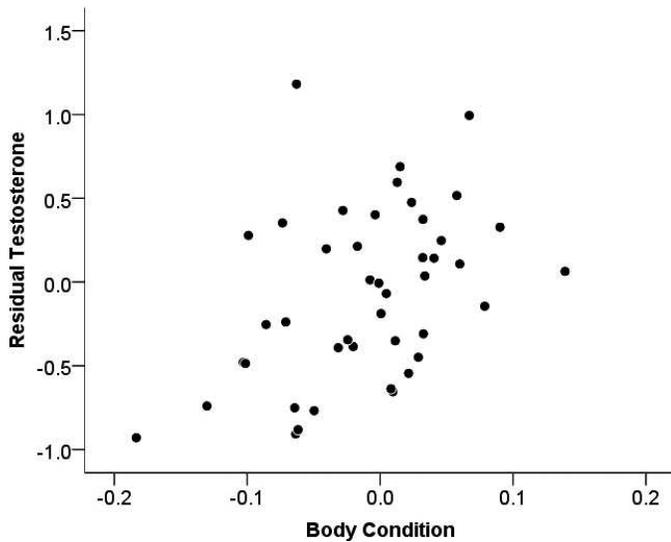


FIG. 2. Association between testosterone (residuals from the regression of $\log(T)$ on Julian Date) and body condition (residuals from the regression of $\log(\text{mass})$ on $\log(\text{SVL})$) ($r = 0.42$, $df = 42$, $P = 0.004$) among adult male Plains Gartersnakes.

sirtalis sirtalis, Weil, 1985; Krohmer et al. 1987; Clesson et al., 2002; Cease et al., 2007; Lutterschmidt and Mason, 2009) and more slowly in species or populations with relatively extended mating seasons (*N. sipedon*, *T. radix*, and *Thamnophis sirtalis coccinus*, Weil and Aldridge, 1981; Moore et al., 2000b; Krohmer, 2004; this study) (Table 1). *Nerodia fasciata* from Louisiana exhibit a somewhat different pattern. Peak testosterone concentrations occur in early spring (March and April), decrease during over the mating season and remain low over summer, increase again in fall, but are low in winter (Lorenz et al., 2011). Hibernation is brief in *N. fasciata* (3–5 months) and interrupted by emergence to bask and feed (Lorenz et al., 2011). In contrast, male *Thamnophis melanogaster* from Mexico show no fixed seasonal pattern of changes in testosterone concentrations. Instead, testosterone is apparently cued by circulating estrogen concentrations in females, and mating occurs during multiple brief synchronous periods each year (Garstka and Crews, 1982).

Testosterone showed a clear, positive association with body condition in adult male Plains Gartersnakes (Fig. 2). Such an association is also evident in the Asp viper, *Vipera aspis* (Aubret et al., 2002:fig. 2; Bonnet et al., 2002:fig. 3). Furthermore, male Vipers with higher fat reserves travel further, court more intensely, and obtain more matings, suggesting that condition and testosterone contribute positively to male reproductive success (Aubret et al. 2002; Bonnet et al., 2002). Body condition (along with size) contributes positively to mating success in the Red-sided Gartersnake, *T. sirtalis parietalis* (Shine et al., 2000) but not in the Northern Watersnake, *Nerodia sipedon* (Weatherhead et al., 2002). No association between body condition and testosterone is evident in the Red-spotted Gartersnake, *T. sirtalis concinnus* (Moore et al., 2000b) or the Brown Treesnake, *Boiga irregularis* (Mathies et al., 2010). We have too few observations to test directly for an association between condition or testosterone and mating in *T. radix*. However, copulation duration increases in *T. radix* as the ratio of male to female mass increases toward one (King et al., 2009), suggesting that male condition might contribute to reproductive success.

Because we collected blood samples multiple times from individual males within years, we were also able to demonstrate

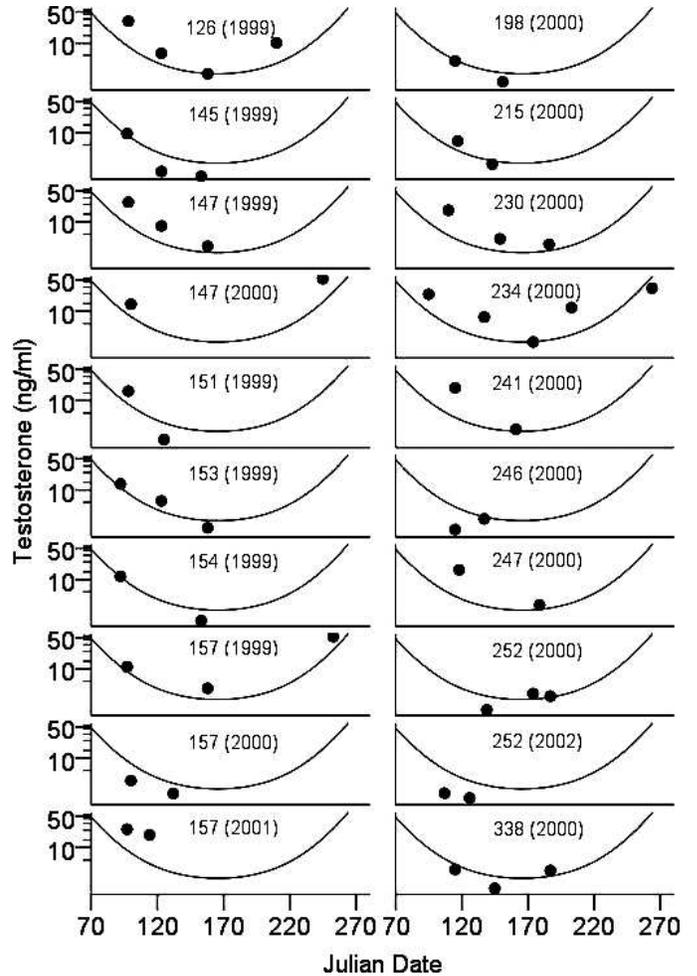


FIG. 3. Individual variation in testosterone among adult male Plains Gartersnakes. Each panel represents a single individual in a single year. Solid lines represent the best-fit quadratic regression from Fig. 1.

significant individual variation in seasonal testosterone profiles (Fig. 3). Some males showed consistently high testosterone levels, relative to the seasonal pattern derived from Fig. 1, while others showed consistently low levels. Admittedly, our analysis of individual variation is inexact; individual males were sampled different numbers of times and on different dates and, as a consequence, individual seasonal profiles are frequently incomplete (Fig. 3). However, individual differences might be expected given evidence of heritable variation in testosterone among captive-reared natricine snakes (King et al., 2000, 2004). To our knowledge, the present study represents the first such demonstration among free-ranging adults. How this individual variation might relate to reproductive behavior (e.g., the timing, duration, and intensity of courtship and mating) is unknown. It is noteworthy that radiotelemetry has sometimes been used to locate individual snakes for repeated blood samples and hormone analysis (Taylor et al., 2004; Lind et al., 2010). However, assay results from such studies have not been analyzed so as to test for individual-level variation.

The analysis of steroid hormones in free-ranging animals is not without problems. Stress associated with capture, handling, and temporary captivity can affect circulating levels of testosterone and other hormones (Moore et al., 2000a), potentially masking natural patterns of variation. That we were able to detect significant seasonal, condition-dependent, and

TABLE 1. Extent of mating season and patterns of seasonal variation in testosterone among male New World natricine snakes based on field-collected blood samples.

Species (location)	Mating season	Sample dates (reference)	Seasonal pattern of variation in testosterone
<i>Nerodia fasciata</i> (Louisiana, USA)	Brief (3 weeks)	Monthly for entire year ($N = 12$) with 4–12 individuals sampled each month (Lorenz et al., 2011) Weekly during spring ($N = 7$) (Lorenz et al., 2011)	Elevated in spring and fall, low in summer and winter; decreases as mating season progresses
<i>Nerodia sipedon</i> (Missouri, USA)	Extended (Apr–Jun)	Throughout the active season (Apr–Sep) in each of 3 yr ($N = 114$) (Weil and Aldridge, 1981) Throughout the active season (May–Sep) ($N = 55$) (Krohmer, 2004)	Elevated in spring and fall, low in summer; elevated throughout mating season; elevated among courting and mating males; remained elevated if recently emerged males were maintained at low temperatures
<i>Thamnophis melanogaster</i> (Michoacan, Mexico)	Multiple brief synchronous periods each year	Prior to, during, or following exposure to estrogen-treated females ($N = 15$) (Garstka and Crews, 1982)	No fixed seasonal pattern; low prior to exposure to estrogen-treated females; elevated during and following exposure to estrogen-treated females
<i>Thamnophis radix</i> (Illinois, USA)	Extended (Mar–May)	Throughout the active season (Apr–Oct) during each of 2 yr ($N = 76$) (this study)	Elevated in spring and fall, low in summer; elevated throughout mating season; variable among courting and mating males
<i>Thamnophis sirtalis concinnus</i> (Oregon, USA)	Extended (Feb–May)	Throughout the active season (Feb–Nov) ($N = 78$) (Moore et al., 2000b)	Elevated in spring and fall, low in summer; elevated during the beginning of mating season
<i>Thamnophis sirtalis parietalis</i> (Manitoba, Canada)	Brief (3 weeks)	Three dates at 14-day intervals in spring, three dates at 5- to 6-day intervals in fall ($N = 32$) (Krohmer et al., 1987) 24 dates over a 40-day period in spring ($N = 204$) (Krohmer et al., 1987) Five dates at 3-day intervals ($N = 70$; 14 per date) during spring courtship (Cease et al., 2007) Fall ($N = 20$) and during and following cold (5°C) or warm (10°C) simulated hibernation ($N = 24$) (Lutterschmidt and Mason, 2009)	Elevated in spring and fall, low in summer; elevated during the beginning of mating season; decreased rapidly (within 14 days) following emergence from hibernation; decreased more slowly in males experiencing cold versus warm simulated hibernation; courtship occurred sooner among males experiencing cold versus warm simulated hibernation
<i>Thamnophis sirtalis sirtalis</i> (Wisconsin, USA)	Brief (3–5 days)	Spring (Apr), summer (Jul), fall (Sep, Oct) ($N = 28$) (Weil, 1985) Fall, during spring emergence, following courtship, summer ($N = 35$) (Clesson et al., 2002)	Elevated in spring and fall, low in summer; decreased rapidly following emergence from hibernation; low among subadult males ($N = 4$) during summer

individual patterns despite variation in the timing of blood sampling following capture suggests that such patterns are relatively robust in *T. radix*. However, future studies, especially those aimed at clarifying individual variation, would likely benefit from greater consistency in the timing of blood collection (e.g., by collecting blood samples in the field immediately after capture).

The diversity represented among New World natricines, including broadly versus narrowly distributed taxa in both temperate and tropical regions (Rossman et al., 1996), provides a rich resource for conducting population- and species-level investigations of hormonal associations with reproductive patterns in a geographic (cf. Lutterschmidt and Mason, 2008) and phylogenetic context. Furthermore, the widespread sympatry exhibited by many New World natricines (e.g., the occurrence of multiple species of *Nerodia*, *Regina*, *Storeria*, and *Thamnophis* across portions of the southeast and midwestern U.S.) facilitates comparative analyses across species experiencing similar climatic conditions but, perhaps, differing in the timing of key reproductive events.

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