Long-term Sperm Storage and Plasma Steroid Profile of Pregnancy in a Western Diamond-backed Rattlesnake (Crotalus atrox)

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Sophisticated information on reproduction and mating systems of non-avian reptiles is growing rapidly, and important advancements span a wide range of disciplines (Campbell and Brodie, 1992; Duvall et al. 1992, 1993; Gans and Crews 1992; Gibbs and Weatherhead 2001; Schuett et al. 2002a; see Shime 2003). Notwithstanding this growth there are obvious and significant deficiencies in knowledge of certain taxa, especially in snakes. Studies of endocrine patterns in this group involve only a limited number of species, and most of them are New World natricines (Moore and Lindzey 1992; Whittier and Tokarz 1992). Consequently, diversity of proximate mechanisms is lacking which seriously impedes our ability to develop robust evolutionary models (Drickamer and Gillie 1998). Many important research questions regarding proximate regulation of reproduction need to be addressed, and these include seasonal patterns of plasma sex steroids, differential functions of steroids on development and activation of behavior, and the dynamics of free- and bound steroids (and their binding globulins) with respect to receptor function (Bonnet et al. 1994, 2001, 2002; Breuner and Orchinik 2002; Jennings et al. 2000; Moore and Lindzey 1992; Schuett et al. 2002a; Seigel and Ford 1987; Whittier and Tokarz 1992).

There are, for instance, few studies of steroid profiles of pregnancy in snakes (Whittier and Tokarz 1992), and no studies of sex steroid profiles in cases where females are unequivocally utilizing sperm from long-term (over winter) storage, referred to as long-term sperm storage (LTSS; Schuett 1992). In numerous species of vipers, LTSS is an important (or even obligate) component of reproduction (overviewed by Schuett 1992; Sever and Hamlett 2002). Although there are no a priori data to suggest that there are causal connections between LTSS, ovarian activity, and sex steroid profiles during pregnancy, we cannot assume they do not exist (see Ameida-Santos et al. in press). For example, if females that mate in spring ovulate earlier, perhaps due to the act of coitus itself (e.g., Mendonça and Crews 1990), development and birth of offspring might be accelerated compared to females with LTSS and no sexual activity in spring.

While conducting field behavioral endocrinological studies on the Western Diamond-backed Rattlesnake (Crotalus atrox) in the area of the White Tank Mountains (39 km W of central Phoenix, Maricopa County, Arizona), an adult female was collected by one of us (GWS) on 17 September 1999, 2110 h, on Sun Valley Parkway, 15.2 km W of the McMicken Dam Spillway. Due to her good state of health and high body mass, she had not likely produced a litter that summer. Thus, she was a good candidate for having been mated in late summer (cf. Bonnet et al. 1994, 2002; G. Schuett and R. Repp, unpubl. data) and to test for LTSS (Schuett 1992). It is not established that female C. atrox show LTSS, and it is important to know whether or not copulations restricted to the first mating season (late summer and autumn) are sufficient for the initiation of pregnancy the following spring (Schuett 1982, 1992).

In south-central and southern Arizona and other areas of its expansive distribution, C. atrox shows sexual behavior (i.e., courtship, coitus, male-male aggression) in two distinct calendar periods. Based on the events of the male’s reproductive cycle (e.g., peak spermatogenesis and levels of plasma sex steroids in late summer; meiotic regression in autumn) and timing of ovulation and fertilization in late spring, the first mating season is from late August to at least mid October, which includes the monsoon season (mid July to mid September). Following hibernation (mid November to late February), the second mating season is from early March to mid May, and sexual activity can occur at or near traditional communal dens during emergence (Repp 1998; Schuett et al. 2001). This bimodal pattern of mating seasons in C. atrox was established by long-term studies using radiotelemetry, observations in the field, published accounts, and personal communication (e.g., Klauber 1972; Schuett et al. 2001, in press; Taylor et al. 2004; G. Schuett and R. Repp, unpubl. data; S. Beaufre, pers. comm.; E. Nowak, pers. comm.). Detailed information on the tim-
ing of mating seasons of pitvipers is reviewed in Schuett (1992),
Aldridge and Duvall (2002), and Schuett et al. (2002a).

The goal of this paper is to report for the first time unequivocal
LTSS in a female Western Diamond-backed Rattlesnake (Crotalus
atrox), and to describe the profile of several important circu-
lating steroid hormones (testosterone [T], 17β-estradiol [E2],
progesterone [P4], and corticosterone [CORT]) during her preg-
nancy.

METHODS

Subject.—At the time of capture, the subject was 740 mm SVL,
50 mm TL, and 435.0 g. Her identification number was CA-149.
She was maintained in strict isolation in a glass enclosure (91 cm
L x 30 cm W x 25 cm H) with a screen cover, supplied with news-
print as a floor covering and heat tape (8 cm wide) was situated
beneath and across the front end of the cage (35°C). Artificial light-
ing (eight 40 W fluorescent tubes) positioned 3 m above the cage
was electronic timer-controlled to simulate natural (Arizona time)
photoperiod year round. Laboratory rodents (hamsters and rats)
were offered food every 10 days until 15 November 1999, and
water was available in a glass bowl ad libitum. From 15 Novem-
ber 1999 to 1 March 2000, CA-149 was maintained under condi-
tions to simulate hibernation (Schuett et al. 1997b).

Collection of blood and plasma.—Sampling periods (N = 11)
occurred in September 1999, and from April to August 2000 (Fig.
1). In the laboratory, the subject was gently removed from her
individual enclosure using a hook and/or grabber tongs, and sec-
cured in a standard squeeze-box. This procedure was done quickly
(1-3 min) to avoid handling stress and possible effects on sex ste-
roid levels (Schuett et al. 2004). Once secured, 1.0 ml of blood
was collected from tail vessels, generally within 1 min, using a
1.0 ml sterile tuberculin syringe (25-G5/8”) treated with porcine-
derived heparin sodium (1,000 units/ml). The subject was returned
to her enclosure immediately following this procedure. Blood was
transferred to a 1.5 ml centrifuge tube and immediately placed on
ice for several minutes until it could be centrifuged (Taylor and
Schuett 2004). All blood samples were centrifuged for 4 min at
6000 rpm, and plasma was collected and transferred to 1.5 ml
Nalgene® centrifuge tubes for storage in an ultra-low freezer (-
80°C) until radioimmunoassays (RIAs) could be performed (<1
year).

Radioimmunoassay of plasma.—The general procedures for con-
ducting RIAs for measuring concentration of plasma T and CORT
(Schuett and Grober 2000; Schuett et al. 1996, 1997b, 2002a, in
press; Taylor and Schuett 2004) and E2 (Schuett et al., in press;
Taylor and Schuett 2004) are published, and those for P4 will be
discussed in detail below. Briefly, RIAs of T, CORT, and E2 in-
cluded validation (quantitative recovery and parallelism), and
samples were analyzed in duplicate (N = 22 for each steroid). Two
RIAs were performed for T; the intra-assay coefficients of varia-
tion (CV) were 9.1% and 11.1%, and the inter-assay CV was 11.9%.
One RIA was performed for CORT, and the intra-assay CV was
2.4%. Two RIAs were performed for E2; the intra-assay CVs were
7.9% and 12.5%, and the inter-assay CV was 11.9% Values for T,
CORT, and E2 are presented as arithmetic means ± 1 SE (ng/ml).

For RIAs of P4, antibody-coated tubes and radiolabeled P4 were
purchased from Diagnostic Products Corporation, Los Angeles,
CA (catalog numbers TPG1 and TPG2). Standards were prepared
be serial dilutions of a stock solution in phosphate buffered saline
(PBS) containing 0.1% gelatin. Snake plasma (50 µl) plus 350 µl
of PBS were extracted in 5.0 ml of diethyl ether (Fisher Science-
tific). After removing and drying the ether layer, the sample was
re-suspended in 200 µl of PBS-0.1% gelatin. Extraction recovery
of H3-progestesterone (New England Nuclear, Boston, MA, NET-
381) was 69%. The entire 200 µl of extract plus an additional 100
µl of PBS-0.1% gelatin was dispensed into the antibody tubes.
Following addition of 1.0 ml of tracer, the tubes were incubated at
room temperature (21°C) for 4 hr. Quantitative recovery of P4
added to C. atrax plasma was 100%. Parallelism was demonstrated
between the inhibition curve for the standards and dilutions of C.
atrox plasma for plasma volumes > 12.5 µl. A single RIA was
performed and assay samples were run in duplicate (N = 22). The
intra-assay coefficient of variation was 8.7%. Values for P4 are
presented as arithmetic means ± 1 SE (ng/ml).

Ultrasoundography.—To determine size of follicles, the approxi-
mate time of ovulation, and presence of embryos, ultrasound imag-
ing (Dynamic Imaging, Concept/MC) was performed multiple
times from the time of capture through July 2000. Size of follicles
was determined as best as possible to the nearest millimeter, and
numbers were estimated. Embryos were counted but not measured.

RESULTS

The reproductive pattern and plasma steroid profiles of CA-149
at the time of her capture to 5 days post-partum are shown in Fig.
1.

On 17 September 1999, at the time of capture, the concentra-
tion of T and P4 was relatively higher than E2; CORT was rela-
tively high (67.28 ng/ml). Ultrasound revealed multiple follicles
(> 5) that were ≥ 3 mm in width, and thus not in the process of
primary follicular growth, i.e., yolk deposition (Aldridge 1979;

On 3 April 2000, the first post-hibernation measurement, E2
was elevated (highest level measured), CORT levels were similar
to the value obtained on 17 September, and both T and P4 were
relatively low. Ultrasound revealed growth of the follicles since
17 September, presumably occurring from 1 March to 3 April.
Multiple clustered follicles of ≥ 35 mm in width were detected.
On 10 May 2000, E2 had decreased, and on 10 June E2 was at its
lowest concentration remained near (or at) that 1.0 ng/ml through-
out gestation and five days following parturition. From 10 May
and following parturition, the profile of T tracked that of E2. Ul-
trasound revealed that ovulation had occurred by 10 May (i.e.,
multiple ova ≥ 55 mm were arranged linearly), and seven em-
byros were detectable by 1 July. The concentration of P4 increased
sharply following 10 May, and its highest concentration was on 1
July. Subsequently, P4 decreased sharply up to and several days
beyond the date of parturition (22 August). CORT increased after
3 April, and reached its highest spring concentration on 10 May
(263.89 ng/ml). Following ovulation, CORT sharply declined to
baseline by 10 June, and levels subsequently increased a second
time, peaking from 29 July to 12 August, and decreasing by 18
August. On 22 August, levels of CORT rose to the highest con-
centration at the time of parturition. Seven offspring (4 males, 3
females) were of healthy appearance and normal size, and no in-
fertile masses were present.
DISCUSSION

This is the first account to unequivocally demonstrate LTSS in female *C. atrox* (Schuett 1992), and among the few descriptions of steroid patterns of pregnancy in a viperid snake (Bonnet et al. 2001, 2002; Saint Girons et al. 1993; Taylor et al. 2004; Tsai and Tu 2002). We suggest that the present female mated in late summer or fall of 1999, showed LTSS during winter, ovulated in May, and the stored sperm had normal fertilizing capacity. Because there are no reliable data to support the view that LTSS extends beyond a single reproductive season, mating in late summer or autumn is the most parsimonious conclusion. Moreover, there is little evidence to support a spontaneous parthenogenetic event because such rare cases of reproduction in captive snakes have never resulted in healthy litters composed of both sexes (Schuett et al. 1997a). In the present case, timing of reproductive events, such as follicular growth and ovulation, as well as patterns of plasma sex steroids, were indistinguishable from wild female *C. atrox* that have the opportunity to mate in spring (Taylor et al. 2004). In most North American viperids, vitellogenesis and follicular development appear to be largely confined to summer and autumn, and females thus enter hibernation with large, yolked follicles and subsequent growth of follicles may occur in spring (Aldridge 1979; Aldridge and Duvall 2002; Schuett 1992). In low elevation regions of south-central and southern Arizona, these follicular events in *C. atrox* appear to be confined to spring on a biennial basis (Taylor et al. 2004; G. Schuett and R. Repp, unpubl. data; S. Beaupre, pers. comm.; but see Rosen and Goldberg 2002). Although Tinkle (1962) reported that female *C. atrox* from northwestern Texas had enlarged follicles in autumn, the observations by Fitch and Pisani (1993) of *C. atrox* from Oklahoma are similar to our findings and indicate that the majority of follicular yolkling occurs in spring. Based on these incongruent data, further studies will be required to better understand the role of environmental factors and geographic location in the timing of follicular development in this species (Rosen and Goldberg 2002).

There are few studies on female snakes regarding patterns of circulating steroids related to sexual activity and pregnancy to make robust comparisons (Chan et al. 1973; Highfill and Mead 1975a, b; Kleis-San Francisco and Callard 1986; Whittier et al. 1987); data on vipers are very limited (Bonnet et al. 1994, 2001, 2002; Saint Girons et al. 1993; Taylor et al. 2004; Tsai and Tu 2001). Taylor et al. (2004) investigated plasma steroids in free-ranging pregnant *C. atrox* in south-central Arizona, and plasma steroid profiles of the present female did not deviate from free-ranging individuals that have a second opportunity to mate in spring. Furthermore, the steroid data of the present female are, in general, similar to those reported for other viperids (Bonnet et al. 2001, 2002; Tsai and Tu 2001), as well as other viviparous squamates (Edwards and Jones 2001; Martínez-Torres et al. 2003; Xavier 1987).

Most authors report elevated E2 levels during follicular growth, and subsequent declines during gestation (Bonnet et al. 2001; Taylor et al. 2002), and it is suggested that high post-ovulatory P4 levels suppress E2 levels during pregnancy (Bonnet et al. 1994; Edward and Jones 2001). The high P4 levels during autumn in the present female may have been associated with sexual receptivity.
and/or ovarian function (Whittier and Tokarz 1992). There was, however, no evidence of follicular growth at that time based on ultrasound results. It is doubtful that yolk deposition occurred from 15 Nov.–1 Mar.; nonetheless, it is a possibility because the next ultrasound measurement was not taken until 3 April. Although few authors have reported on androgen levels in female snakes (Saint Girons et al. 1993), these are also generally low during gestation. Comparison of CORT levels at the time of birth to other vipers is species is not possibly due to lack of information; the pattern of CORT levels in C. atrox reported by Taylor et al. (2004) were essentially identical to those we report here.

The functional roles of estrogens (e.g., 17β--estradiol), progesterone, and androgens (e.g., testosterone) in female reptiles have been reviewed (Whittier and Tokarz 1992). Despite the fact that a robust understanding of the functions of steroids is lacking for female snakes, estrogens and progestins can have relatively straightforward effects on behavioral processes related to sexual receptivity (McNicol and Crews 1979; Whittier and Tokarz 1992; Whittier et al. 1987; Wu et al. 1985), vitellogenesis and yolk deposition (Garstka et al. 1985; Ho 1987; Ho et al. 1982; Wilson and Wingfield 1992), and maintenance of pregnancy (Bonnet et al. 1994, 2001; Chan et al. 1973; Custodia-Lora and Callard 2002; Edward and Jones 2001; Tsai and Tu 2001; Xavier 1987).

Reproductive tissues of females can produce androgens (testosterone, 5α-dihydrotestosterone), but little information concerns their function(s) (Edwards and Jones 2001). There is, however, increasing evidence that androgens might play a role in follicular maturation and oviductal maintenance (Staub and DeBeer 1997). Androgens in females (and males) can be converted in the central nervous system (and other regions) to estrogens (i.e., aromatization) and other steroids (Callard et al. 1977, 1978); nonetheless, they also might have direct involvement in normal organization-activation processes (Staub and DeBeer 1997).

The significance of plasma corticosterone (CORT) in reproduction is not well known in female reptiles, and results are equivocal (Girling and Cree 1995; Wilson and Wingfield 1992). But several studies show its positive association with seasonal timing of sexual behavior, vitellogenesis and yolk deposition, mid-pregnancy anorexia, and extreme (i.e., peak) levels during parturition (Taylor et al. 2004; Wilson and Wingfield 1992; this study). The function of CORT and corticotropin-releasing hormone (CRH) during pregnancy and parturition has been studied rather extensively in humans and sheep models; the sharp spike in plasma CORT at parturition in humans, sheep, and snakes (this study) may derive from multiple origins (Bell et al. 1997; Petershack et al. 1999). Because the source of plasma CORT in pregnant snakes is not understood (i.e., it is probably not limited to strict adrenal involvement), fetal, placental, and other sources should be considered based on results of mammalian systems (Smith 1999; Weiss 2000). In C. atrox, plasma CORT levels are highly elevated during parturition (see Fig. 1), exceeding by two-fold the levels observed in male C. atrox that have been exposed to moderate handling stress (Schuett et al. 2004). Nonetheless, unlike stressed males which appear agitated and assume defensive postures, female C. atrox are unremarkable and calm prior to and during parturition. Therefore, the role of CORT in both physiological and behavioral processes in snakes, as in other vertebrates, appears to be complex and multi-functional.

In conclusion, we demonstrated that LTSS operates effectively in C. atrox resulting in pregnancy and normal offspring. In a laboratory setting, LTSS and lack of spring mating did not modify timing of reproductive events such as vitellogenesis, yolk deposition, ovulation and fertilization, and parturition. Furthermore, levels of plasma sex steroids in the present female were comparable to those of free-living pregnant female C. atrox that had the opportunity to mate in spring (Taylor et al. 2004).

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Radio Telemetry and Post-emergent Habitat Selection of Neonate Box Turtles (Emydidae: Terrapene carolina) in Central Illinois

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Although factors influencing turtle offspring prior to nest emergence have received considerable attention by researchers (Gutzke and Crews 1985; 1988; Janzen et al. 2000; Packard and Packard 1987), the activity of neonates following their emergence from the nest is poorly understood (but see Burger 1976; Butler and Graham 1995; Keller et al. 1997). Previous field research has produced valuable information on several aspects of neonate ecology for several species (Brewster and Brewster 1991; Butler and Sowell 1996; Janzen 1993). However, a thorough understanding of life history patterns for many species is absent, and some existing information is conflicting (e.g., Congdon et al. 1999; Janzen et al. 2000). The lack of knowledge is primarily due to the cryptic nature of neonates and various logistical problems associated with studying small animals in the field. Recent advances in radio telemetry technology such as decreased transmitter size and increased battery life facilitate tracking small neonate turtles for a longer duration.

We studied nest dispersal and habitat use in neonate box turtles using a relatively new, very small radio transmitter. Few studies have been conducted using telemetry on neonate turtles (e.g., Butler et al. 1995), and none has focused on nest dispersal and habitat use of neonate box turtles.

The study was conducted at Rhodes-France Boy Scout Reservation (RFBSR) located in western Shelby County, Illinois, USA (39°19′N; 89°02′W), from March to April 2002. Two nests were located by radio tracking gravid female turtles during summer 2001 (Flitz 2003). The nests were sited in relatively open areas next to a tree stump in a grassy field and at the edge of a fire trail (see Flitz 2003 for more description). Nest disturbance was prevented by using excluder devices, made of hardware cloth of 0.6 cm\(^2\) mesh and 30 cm diameter with walls buried 15 cm into the ground, around the nest until the end of the 2001 activity season. Upon hatching and emergence, neonate turtles from both nests (clutch sizes were 4 and 5, respectively) were collected, brought to our laboratory and allowed to overwinter in an outdoor enclosure (1.5 x 1.5 m) under ambient conditions. Each turtle was marked with a unique series of notches in the marginal scutes. This facilitated identification and placement back at the proper nest site the following spring.

After overwintering, single-stage radio transmitters (model LTM, Titley Electronics, Australia; 0.95 g) were attached to the carapace of six randomly chosen neonates (three from each clutch) using a non-toxic silicon adhesive (Fig. 1). Each transmitter cost approximately US $170, had an average lifespan of 28 days (pers. obs.; D. Titley, pers. comm.), and did not contain a thermistor. We relocated the subjects using a Telonics TR2 receiver (159,000-160,000 MHz) and a 6-element Yagi antenna. On average, the transmitter represented 13.4% of individual body mass (mean ± 1 S.E. mass of neonates = 7.11 ± 0.10 g). At the time we designed this study, the LTM model was the smallest transmitter of this longevity being manufactured for attachment on turtles. We concede that this mass exceeds normal guidelines for relative mass of transmitters (usually 5–8%, and rarely up to 10%; Cochran 1980; Richards et al. 1994); however, we did not observe differences in the mobility of neonates outfitted with these transmitters (discussed below).

On 30 March 2002, all neonates were returned to their respective nest sites at RFBSR and allowed to disperse. Each neonate was located 15 times between 0900 and 1700 h on an alternate day cycle (study duration = 32 days), and locations were marked with forestry flags. Upon relocation, air temperature at 1 m above ground (±1° C), and distance (±1 cm) and compass bearing from the previous location were recorded. Many movements were of small magnitude and were within the margin of error of most handheld Global Positioning System units, so we did not take GPS

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