

Relationship of plasma sex steroids to the mating season of copperheads at the north-eastern extreme of their range

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Abstract

A multi-year radio-telemetric study of the copperhead *Agkistrodon contortrix* (Serpentes: Viperidae) was conducted at the north-eastern extreme of its range to determine the relationship of plasma sex steroids of males to the mating season. Blood samples were collected *in situ* approximately every 2 weeks (repeat-test group) from radio-telemetered males during the 7-month active season (April–October) from 2001 to 2003 and assayed for concentrations of testosterone (T) and progesterone (P4). Blood samples were also obtained from a large number of incidental males (single-test group) for the analysis of seasonal levels of T and P4. The profiles of T and P4 showed a peak in August–September that corresponded to the single mating season (late July to late September). Both T and P4 had similar seasonal profiles, but absolute levels of these steroids were significantly different, with concentrations of T four- to fivefold greater. The mating season of the population we investigated differs from other (e.g. southern) populations, which show two mating seasons (late summer/early fall and spring) before the period of ovulation in mid- to late spring. When a mating season is absent in spring, inseminated females are obligated to store sperm over winter until ovulation in the spring. In studies of *A. contortrix* that document two mating seasons, peak levels of T in males are coincident with both of these periods. In contrast, we found that peak levels of T and P4 in males coincided with the occurrence of the single mating season, and levels were basal in spring. These results further support the idea that plasma sex steroids influence the expression of sexual behaviour in *A. contortrix* and other snakes. Nonetheless, confirmation of this view awaits experimental studies.

Introduction

In comparison with other vertebrates, even other reptile taxa, far less is known about endocrinological and reproductive events in snakes (Seigel & Ford, 1987; Moore & Lindzey, 1992; Schuett *et al.*, 1997, 2005, 2006; Bonnet, Naulleau & Lourdais, 2002; Taylor, DeNardo & Jennings, 2004), especially in females (Seigel & Ford, 1987; Whittier & Tokarz, 1992; Almeida-Santos *et al.*, 2004; Schuett *et al.*, 2004a; Taylor & DeNardo, in press). Lack of endocrinological information on reproduction in snakes is, moreover, most pronounced with respect to free-living individuals (Bonnet *et al.*, 2002; Schuett *et al.*, 2002, 2005; Zaidan, Kreider, & Beaupre, 2003; Taylor *et al.*, 2004; Graham *et al.*, 2008). Historically, knowledge of hormones and reproductive activities of snakes has been limited to a small number of taxa (Seigel & Ford, 1987; Moore & Lindzey, 1992), primarily New World natricines, for example, genera *Nerodia* and *Thamnophis* (Weil & Aldridge, 1981; Krohmer, Grassman & Crews, 1987; Mendonça & Crews, 2001) and Old World viperines, for example, *Vipera aspis* and *Vipera*

berus (Naulleau & Fleury, 1984; Saint Girons, Bradshaw & Bradshaw, 1993; Bonnet *et al.*, 2002; Shine, 2003, 2005). Recent field investigations of other various North American pitviper taxa, including the cottonmouth *Agkistrodon piscivorus* (e.g. Zaidan *et al.*, 2003; Graham *et al.*, 2008) and the rattlesnakes *Crotalus scutulatus* (Schuett *et al.*, 2002), *Crotalus atrox* (Schuett *et al.*, 2004a, 2005, 2006; Taylor *et al.*, 2004) and *Crotalus molossus* (Schuett *et al.*, 2005), have advanced our understanding of sex steroids and reproductive events of males and, to a far lesser extent, females (Taylor & DeNardo, in press).

The main goal of this study was to obtain profiles of plasma T and P4 of free-living male copperheads *Agkistrodon contortrix* in the period when they are active (April–October); specifically, during the single mating season, which occurs from late July through September (Smith *et al.*, 2009). We measured both T and P4 because they have known or suspected biological importance in male squamate reptiles (Young, Greenberg & Crews, 1991; Moore & Lindzey, 1992; Edwards & Jones, 2001; Norris, 2006). Testosterone, other androgens and estrogens have been

measured in males of viperid snakes, including North American pitvipers (Schuett *et al.*, 1997, 2002, 2005, 2006; Zaidan *et al.*, 2003; Taylor *et al.*, 2004; Graham *et al.*, 2008), but measurement of P4 in viperids has been measured in only a single species, for example, *V. aspis* (Saint Girons *et al.*, 1993). Based on findings of a previous study on *A. contortrix* (Schuett *et al.*, 1997), as well as studies on other Old- and New World temperate viperids (e.g. Saint Girons *et al.*, 1993; Bonnet *et al.*, 2002; Schuett *et al.*, 2002, 2005, 2006; Taylor *et al.*, 2004; Graham *et al.*, 2008), our central hypothesis was that levels of plasma T and P4 would be highest during the single mating season in late summer and basal in spring. We compared our hormone results to those of other studies of temperate vipers, especially in Schuett *et al.* (1997), who investigated annual levels of plasma T of captive male *A. contortrix* obtained from southern Texas.

Methods

Study site

The study site was located in a 485 ha parcel of basalt trap rock ridge ecosystem situated 4.75 km north-west of Meriden, CT. Topography is consistent with trap rock systems found throughout the Central Connecticut River Valley. North and south oriented ridges, ≥ 200 m in elevation, are bordered to the west by steep cliff faces and extensive talus slides, and to the east by gently sloping woodlands. Two prominent basalt ridges are located at this site. Details of the topography and climate of this region are presented elsewhere (Smith, 2007; Smith *et al.*, 2009).

Research subjects

Individuals that are recaptured and re-sampled repeatedly can result in problems related to pseudoreplication when the samples are considered independent. In addition, using repeated measures designs in hormone studies may affect hormone levels as a result of handling stress (Schuett *et al.*, 2004b). Therefore, two blood-sampling regimes were used and compared: a repeat-test group and a single-test group (see Schuett *et al.*, 1997), and data from the repeat-test group were used to corroborate results from the single-test group. The repeat-test group consisted of adult males that were surgically fitted with internal (intra-coelomic) radio-transmitters and blood-sampled in the field every 2 weeks from April through October during the years 2001–2003. The single-test group consisted of males that lacked radio-transmitters, but were incidentally located during radio-tracking the repeat-test group and were sampled only once during the same 3-year study period. Eleven males were included in the repeat-test group, resulting in 224 plasma samples. From these 224 samples, radioimmunoassays (RIAs) for T and P4 were successfully assayed in 223 and 177 samples, respectively. In the single-test group, 60 males were sampled, and 56 of these samples were successfully assayed for T, and 42 were successfully assayed for P4.

Every individual encountered in the field was permanently marked for identification using passive integrated transponder (PIT) tags (125 kHz 12 mm, Biomark, Boise, ID, USA; Gibbons & Andrews, 2004). A total of 117 individuals were marked with PIT tags. Of these, 52.2% ($n = 60$) were males and 47.8% ($n = 55$) were females. Two marked subjects were juveniles (20 cm or less) and remained unsexed. As described below, 20 males were initially implanted with radio-transmitters (Holohil, Carp, Ontario, Canada, 5.5 g SB-2T). Because subjects were periodically lost to predation or premature radio-transmitter failure, all 20 males could not be sampled for the entire 3-year period. However, robust data were collected for statistical analysis from 11 males: eight were sampled for 2 years of the 3-year study, and three were sampled for a single field season.

Following initial capture and immediate blood collection in the field, snakes were transported to the laboratory. While there, measurements were taken from each subject, including body mass (BM: ± 0.5 g using a triple beam balance), snout–vent length (SVL: ± 0.2 cm using a non-stretchable cloth measuring tape) and tail length (TL: ± 0.2 cm using a non-stretchable cloth measuring tape). PIT tags were injected one-third of the body length anterior from the cloaca and the last three characters of the 10-character PIT code were used as an identification code for all records pertaining to an individual. Individuals that did not receive radio-transmitters were returned to the field within 24 h.

Implantation of radio-transmitters

For subjects selected for the repeat-test group, surgical implantation of transmitters followed up-to-date methods (Reinert, 1992; Hardy & Greene, 1999), described in detail in Smith *et al.* (2009). Implantation of the transmitters was performed within 24 h of capture, and subjects were held overnight and released at the site of capture within 24 h following surgery.

Radio-tracking

Subjects implanted with radio-telemeters were located every 48–72 h on foot using radio-tracking equipment described in detail in Smith *et al.* (2009). Ingress to dens for hibernation typically occurred in mid-October and from this time until egress in mid-March, individuals remained within their respective hibernacula (see Smith *et al.*, 2009). During this period of inactivity, subjects were monitored once every 14 days to assess location and transmitter function.

Collection of blood and plasma

Collection of blood samples to obtain plasma for sex steroid analysis was accomplished in the field from both repeat-test group and single-test group subjects by gently restraining subjects in a clear acrylic tube. A small volume of blood (0.5–1 mL; $< 4\%$ of total blood volume) was collected via cardiac centesis using a sterile disposable 1 mL tuberculin syringe (coated with sodium heparin), fitted with a sterile disposable 26 G needle. Sampling typically required < 60 s

from initial capture until the blood sample was obtained (see Schuett *et al.*, 2004b) and the individual released (or held for further processing). Blood was transported in individual 1.5 mL centrifuge tubes at ambient ($\sim 20^\circ\text{C}$) temperature. Steroid levels are unaffected when blood is maintained at ambient temperatures for short (<24 h) periods (Taylor & Schuett, 2004). In the laboratory, the blood samples were placed in disposable microcentrifuge tubes (1 mL) and centrifuged at 2.3 g. at room temperature ($\sim 21\text{--}23^\circ\text{C}$) for 5 min. Plasma was collected using a micropipette fitted with a sterile disposable tip and transferred to another microcentrifuge tube that was permanently labelled with the specimen identification code and date. Plasma samples were placed in an ultra-low freezer (-80°C) until RIAs could be performed.

RIA of plasma sex steroids

Procedures for conducting RIAs of T and P4 followed Schuett *et al.* (2004a,b, 2005, 2006). Briefly, commercial RIA kits were used with several appropriate modifications (e.g. use of snake plasma rather than rat plasma for validation procedures) to measure steroid concentrations from the collected plasma of subjects. Validation included both quantitative recovery and parallelism. Concentration values for both T and P4 are in ng mL^{-1} , and presented herein as arithmetic means 8 ± 1 SE.

Two RIAs were performed for T and all samples were run in duplicate (duplicate reactions from a single extraction) (repeat-test group: $n = 448$; single-test group: $n = 120$). The intra-assay coefficients of variation (CVs) were 9.1 and 11.1%, and the inter-assay CV was 11.9%. A single RIA was performed for P4 and assay samples were run in duplicate (repeat-test group: $n = 448$; single-test group: $n = 120$). The intra-assay CV was 8.7%.

Behavioural observations

Reproductive behaviour was determined by direct observation of bisexual pairings, courtship and coitus. Copulation events were pooled for the years 2001–2003. Because males may copulate repeatedly with the same individual over a period of 1 or 2 days, copulations were considered independent when one of the two following criteria were met: (1) one individual of the pair moved >100 m from the other between copulations, and (2) extended courtship of a pair was interrupted by a copulation with a third individual. If neither of these criteria were met, then repeated copulations by a pair were considered to be a single event.

Statistical analyses

All data were inspected for outliers, normality (skewness and kurtosis), and equality of variance before performing statistical tests (Zar, 1999). All statistical tests were performed using the software program Systat version 12 (Systat Software Inc., San Jose, CA, USA). The α -level of significance was set at $P \leq 0.05$. Both T and P4 (single-test group and repeat-test group) were natural-log transformed to

achieve normality, and variation in transformed T and P4 was distributed homogeneously between the months. Male body size has been shown to be largely unrelated to plasma steroid levels in other pitvipers (Taylor *et al.*, 2004; Schuett *et al.*, 2005). In this study, male copperheads were all approximately equivalent in SVL and mass (SVL: $\bar{x} = 74.51 \pm 1.75$ cm; mass: $\bar{x} = 211.45 \pm 15.56$ g); therefore, both of these factors were not included as potential covariates in the statistical analysis of steroid levels.

Comparisons of mean monthly levels of T and P4 for the single-test group were performed using ANOVA. Monthly comparisons of the repeat-test group were performed using a repeated-measures model. Initial analyses were followed by Tukey's HSD tests to determine which months had significantly different mean steroid levels. Additionally, a Pearson's correlation was used to assess the relationship between T and P4 levels.

Results

Sex steroids: testosterone and progesterone

The T profiles of male *A. contortrix* in this study showed a single peak from August to September (Fig. 1a). At emergence from hibernation (mid-March to early April), plasma T levels were basal and remained at or near those levels throughout May and most of June. From June to August, T levels increased sharply, and following the peak in August–September, T declined up to the onset of hibernation in mid- to late October. Because samples were collected in the field and animals were not accessible during hibernation (late October through late March), T levels during winter could not be ascertained; however, it is assumed that they remained unchanged during the onset of hibernation as comparisons of steroid levels between October and April showed a significant difference in only one of four sampling groups (October vs. April: repeat-test group – testosterone $P = 0.49$; single-test group – testosterone $P = 0.03$; repeat-test group – progesterone $P = 0.47$; single-test group – progesterone $P = 0.29$) (see Schuett *et al.*, 1997).

Monthly levels of plasma T of both the single-test group and repeat-test group are presented in Table 1a, b. During the 7-month sampling period, the overall trend of plasma T levels was similar for both groups (Fig. 1a). With the exception of May and September (MAY: paired t -test: $t_{47} = 2.88$, $P = 0.006$; SEP: paired t -test: $t_{54} = 3.15$, $P = 0.002$), mean values for the sampling months between the two groups were not significantly different.

Statistical analyses of the single-test group showed that T levels varied significantly among months (ANOVA: $F_{6,48} = 18.12$, $P < 0.001$). Mean T levels in late summer (August and September) were significantly greater than mean levels in spring, early summer and fall (April, May, June, July and October; Tukey's HSD test: d.f. = 49, $P < 0.01$) (Table 2a). Statistical results were similar for the repeat-test group (ANOVA, $F_{6,216} = 22.335$, $P < 0.001$; Tukey's HSD test: d.f. = 216, $P < 0.01$) (Table 2b). The year of

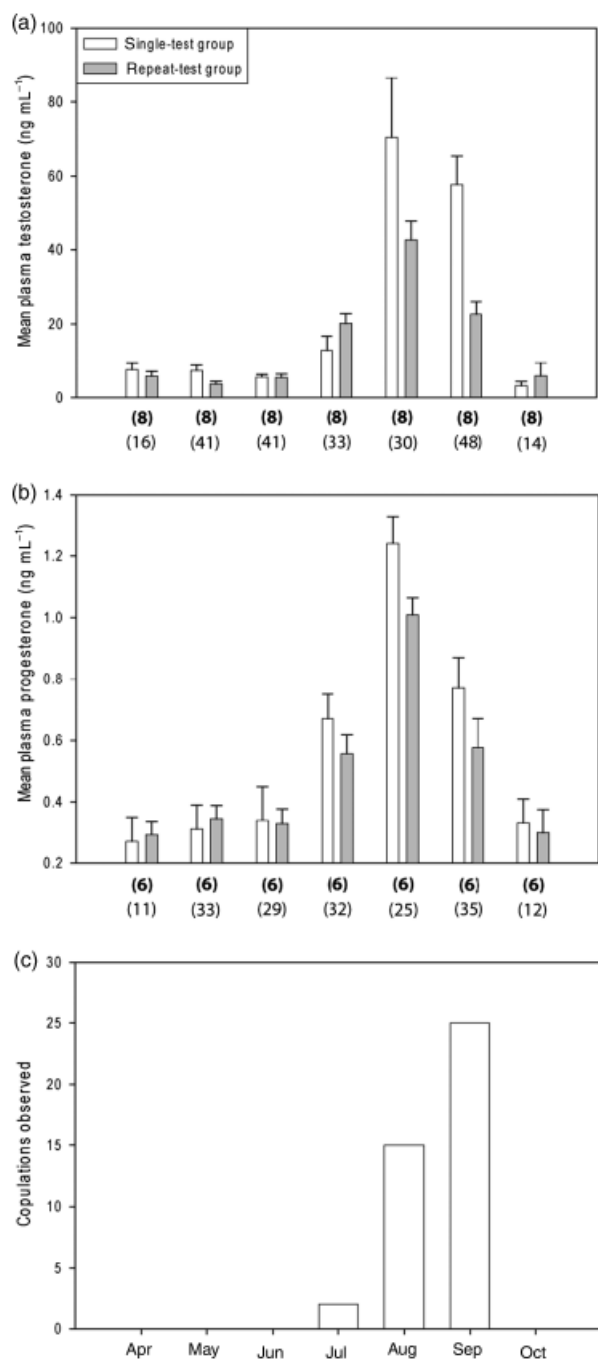


Figure 1 Profile of (a) mean (± 1 SEM) plasma testosterone (T) and (b) mean (± 1 SEM) plasma progesterone (P4) in male *Agkistrodon contortrix* under two sampling regimes (single-test group and repeat-test group). Sample size for each month is indicated in parentheses (bold text, single-test group; normal text, repeat-test group). Data were pooled across years (2001–2003). (c) Observed copulations in the field for years 2001–2003.

blood collection did not have a significant effect in either of the test groups (single-test group: $P = 0.51$; repeat-test group: $P = 0.31$).

The P4 profile showed a single August peak and followed the general trend of T (Fig. 1b). Levels of P4 were basal from April through June, increased in July, reached maximum levels in August, and decreased in the last 2 months of the active season (September and October). Because subjects were not accessible during hibernation (late October through late March), levels of P4 could not be ascertained but were assumed to remain basal during hibernation (see previous comments regarding T).

Monthly plasma P4 levels of both sampling regimes (single-test group and repeat-test group) are presented in Table 1c, d. During the 7-month sampling period (April–October), the overall trend of plasma P4 levels was similar in both groups (Fig. 1b). With the exception of August (AUG $t = -2.35$, d.f. = 11, $P = 0.038$), mean values for the sampling months between the two groups did not differ. Statistical analyses of the single-test group showed that P4 varied significantly among months ($n = 42$, $F_{6,34} = 11.938$, $P < 0.001$). Also, mean P4 levels in mid- and late summer (July, August and September) were significantly greater than mean levels in spring and early summer [(April, May and June) Tukey's HSD test: d.f. = 35, $P < 0.01$] (Table 2c). The mean P4 peak in August was significantly greater than all other months (Tukey's HSD test: d.f. = 35, $P < 0.01$). Statistical results were similar for the repeat-test group ($n = 177$, $F_{6,170} = 13.893$, $P < 0.001$; Tukey's HSD test: d.f. = 170, $P < 0.01$) (Table 2d), with the August mean P4 level greater than all other months (Tukey's HSD test: d.f. = 170, $P < 0.001$). The year of blood collection did not have a significant effect on mean P4 levels in either of the test groups (single-test group: $P = 0.11$; repeat-test group: $P = 0.16$). Differences in P4 between the single-test group and repeat-test groups follow the trend seen in T. Although between-groups statistical comparisons of monthly P4 levels in the present study were significantly different in August only, the overall trend across all months was that P4 levels were lower in the repeat-test group.

There was a significant correlation between T and P4 levels within the single-test group [$r(41) = 0.68$, $P < 0.05$]. Compared with T levels measured during the same period, circulating levels of P4 were four to five times lower (e.g. August single-test group: progesterone $\bar{x} = 1.24 \pm 0.09$ ng mL⁻¹; testosterone $\bar{x} = 70.41 \pm 16.12$ ng mL⁻¹).

Sexual behaviour

A large number ($n = 42$) of copulations were recorded (Fig. 1c), which coincided with elevated levels of T and P4. Most copulations (95.2%) occurred in August (35.7%) and September (59.5%), with far fewer in late July (0.04%). Copulations, bisexual pairing and courtship were not observed outside this period. Thus, in combination with our results on sex steroids, there was no evidence for a mating season in spring.

Discussion

Here, we show that circulating levels of T and P4 in male *A. contortrix* at the north-eastern extreme of their

Table 1 Descriptive statistics of levels (ng mL⁻¹) of plasma testosterone (T) and progesterone (P4) for adult male *Agkistrodon contortrix*

Month	Minimum–Maximum	Mean ± se	Transformed mean ± se
<i>(a) Testosterone single-test group</i>			
APR (n=8)	0.56–13.53	7.55 ± 1.79	0.90 ± 0.47
MAY (n=8)	2.60–15.89	7.30 ± 1.49	1.16 ± 0.19
JUN (n=8)	2.49–8.68	5.51 ± 0.78	1.53 ± 0.15
JUL (n=8)	1.81–32.87	12.91 ± 3.80	2.18 ± 0.36
AUG (n=8)	23.13–130.97	70.41 ± 16.12	4.04 ± 0.25
SEP (n=8)	25.20–82.03	57.70 ± 7.66	3.52 ± 0.16
OCT (n=8)	0.97–10.75	3.21 ± 1.15	0.83 ± 0.29
<i>(b) Testosterone repeat-test group</i>			
APR (n=16)	0.20–16.21	5.80 ± 1.31	0.85 ± 0.32
MAY (n=41)	0.20–22.98	3.67 ± 0.70	0.52 ± 0.16
JUN (n=41)	0.20–29.64	5.43 ± 0.98	1.01 ± 0.20
JUL (n=33)	0.55–55.28	20.13 ± 2.64	2.61 ± 0.18
AUG (n=30)	0.23–130.97	42.62 ± 5.05	3.39 ± 0.22
SEP (n=48)	0.50–82.03	22.62 ± 3.39	2.36 ± 0.21
OCT (n=14)	0.22–51.67	5.86 ± 3.60	0.56 ± 0.39
<i>(c) Progesterone single-test group</i>			
APR (n=6)	0.00–0.61	0.27 ± 0.08	0.23 ± 0.06
MAY (n=6)	0.00–0.58	0.31 ± 0.08	0.26 ± 0.06
JUN (n=6)	0.00–0.70	0.34 ± 0.11	0.27 ± 0.08
JUL (n=6)	0.31–0.81	0.67 ± 0.08	0.41 ± 0.05
AUG (n=6)	1.00–1.52	1.24 ± 0.09	0.80 ± 0.04
SEP (n=6)	0.37–1.11	0.77 ± 0.10	0.56 ± 0.06
OCT (n=6)	0.15–0.62	0.33 ± 0.08	0.28 ± 0.06
<i>(d) Progesterone repeat-test group</i>			
APR (n=11)	0.12–0.61	0.29 ± 0.04	0.25 ± 0.03
MAY (n=33)	0.00–1.10	0.35 ± 0.04	0.28 ± 0.03
JUN (n=29)	0.00–1.20	0.33 ± 0.05	0.27 ± 0.03
JUL (n=32)	0.00–1.73	0.56 ± 0.06	0.35 ± 0.04
AUG (n=25)	0.43–1.53	1.01 ± 0.05	0.69 ± 0.03
SEP (n=35)	0.00–2.75	0.58 ± 0.09	0.41 ± 0.05
OCT (n=12)	0.00–0.96	0.30 ± 0.08	0.24 ± 0.05

Data were pooled across all years (2001–2003). Transformed means represent natural-log transformation.

expansive geographic range (Gloyd & Conant, 1990) were elevated and peaked in late summer, which preceded and coincided with the mating season that occurs from late July through September. Although P4 has not been reported in other North American pitvipers, similar profiles of T have been reported in males of the closely related cottonmouth, *A. piscivorus* (Zaidan *et al.*, 2003; Graham *et al.*, 2008), as well as profiles of T, 5 α -dihydrotestosterone and 17 β -estradiol in males of black-tailed rattlesnakes *C. molossus* (Schuett *et al.*, 2005). This steroid pattern is in contrast to annual T profiles presented for southern populations of *A. contortrix* (Schuett *et al.*, 1997), as well as other temperate North American pitvipers, such as the Mohave rattlesnake *C. scutulatus* (Schuett *et al.*, 2002) and western diamond-backed rattlesnake *C. atrox* (Taylor *et al.*, 2004; Schuett *et al.*, 2005, 2006). Moreover, it is different from the seasonal pattern of plasma T of other species of North American snakes, such as the natricines *Nerodia sipedon* (Weil & Aldridge, 1981) and *Thamnophis sirtalis* (Krohmer *et al.*, 1987), as well as the European viperine *V. aspis* (Naulleau, Fleury &

Boissin, 1987; Saint Girons *et al.*, 1993; Bonnet *et al.*, 2002). In these species, the profile of T during the active season shows two peaks (bimodal), with an initial peak occurring from mid- to late summer and a second peak in the following spring, shortly after emergence from hibernation, and during the second mating season. However, unlike the population of copperheads we studied, the above-mentioned taxa show mating behaviour: (1) in spring only (e.g. *Nerodia*) or (2) in both late summer/fall and in spring (e.g. *C. atrox*) (for a review, see Graham *et al.*, 2008).

Elevation of T in males during the mating season is commonly observed in a range of vertebrates and has been repeatedly demonstrated to influence sexual behaviours, including male–male aggression, mate-searching, courtship and coitus (Lindzey & Crews, 1992; Schuett, 1997; Schuett *et al.*, 2002; Norris, 2006). In male snakes from temperate regions that (1) exhibit two mating seasons (bimodal-type), the first occurs in late summer/fall and the second occurs in spring (e.g. *C. atrox*) or (2) a spring-only mating season (e.g. *N. sipedon*), elevation of plasma T occurs in spring shortly

Table 2 Matrices of pair-wise comparison probabilities (Tukey's HSD multiple comparisons) of levels (ng mL^{-1}) of mean plasma testosterone (T) and progesterone (P4) for adult male *Agkistrodon contortrix*

	APR	MAY	JUN	JUL	AUG	SEP	OCT
<i>(a) Testosterone single-test group</i>							
APR	1.000						
MAY	0.987	1.000					
JUN	1.000	0.989	1.000				
JUL	0.715	0.986	0.835	1.000			
AUG	0.000	0.000	0.000	0.001	1.000		
SEP	0.000	0.000	0.000	0.001	1.000	1.000	
OCT	0.596	0.181	0.456	0.029	0.000	0.000	1.000
<i>(b) Testosterone repeat-test group</i>							
APR	1.000						
MAY	0.298	1.000					
JUN	0.637	0.448	1.000				
JUL	0.000	0.000	0.000	1.000			
AUG	0.000	0.000	0.000	0.014	1.000		
SEP	0.001	0.000	0.000	0.385	0.001	1.000	
OCT	0.178	0.546	0.252	0.000	0.000	0.000	1.000
<i>(c) Progesterone single-test group</i>							
APR	1.000						
MAY	0.691	1.000					
JUN	0.599	0.898	1.000				
JUL	0.003	0.008	0.001	1.000			
AUG	0.000	0.000	0.000	0.002	1.000		
SEP	0.009	0.002	0.002	0.530	0.009	1.000	
OCT	0.559	0.852	0.954	0.013	0.000	0.003	1.000
<i>(d) Progesterone repeat-test group</i>							
APR	1.000						
MAY	0.666	1.000					
JUN	0.837	0.761	1.000				
JUL	0.021	0.008	0.004	1.000			
AUG	0.000	0.000	0.000	0.000	1.000		
SEP	0.029	0.013	0.007	0.821	0.000	1.000	
OCT	0.931	0.580	0.751	0.013	0.000	0.018	1.000

Data were pooled across all years (2001–2003). Significant values ($P < 0.05$) are denoted in bold.

following hibernation, and it corresponds with breeding activity (see Krohmer *et al.*, 1987; Naulleau *et al.*, 1987; Saint Girons *et al.*, 1993; Taylor *et al.*, 2004; Schuett *et al.*, 2006). The source of T contributing to elevated levels in spring is presumed to be testicular, despite the fact that meiotic activity is regressed (Schuett *et al.*, 2002; see Benner & Woodley, 2007). However, extra-gonadal sources of T are possible and include adrenocortical tissues (e.g. zona reticularis; Schuett *et al.*, 2002).

In all North American snake species studied to date, high levels of T in late summer parallel peak spermatogenesis (Graham *et al.*, 2008). Histological examination of a limited number ($n = 10$) of testis samples obtained from our study population suggests that spermatogenesis follows an aestival cycle as well, with peak spermatogenesis occurring in late summer (Smith, 2007). But unlike other populations of *A. contortrix* studied (e.g. Fitch, 1960), males ($n = 5$) of the present population did not have sperm present in their ductus deferens in spring (Smith, 2007), a feature which coincides with the lack of a mating season in spring. Thus,

sperm produced in late summer/fall are used entirely for the single mating season.

In contrast to the anecdotal reports of Finneran (1948) and Petersen & Fritsch (1986), that reported that *A. contortrix* from Connecticut exhibit a mating season in spring, our 3-year study revealed that all sexual behaviours (e.g. bisexual pairings, courtship and copulations) occurred from late July through September; moreover, routine cloacal swabs taken from females in the spring (see Fitch, 1960) failed to show the presence of sperm (Smith, 2007; Smith *et al.*, 2009). Thus, based on multiple lines of evidence, a mating season in spring is absent in the population of *A. contortrix* we studied. When a mating season is absent in spring, inseminated females are obligated to store sperm over winter until ovulation in the spring (Schuett, 1992). Similarly, McDuffie (1960) reported that a population of *A. contortrix* from southern Ohio showed no evidence of a mating season in spring. Accordingly, further phenological analyses of mating seasons in copperheads throughout their range are warranted.

Given the close association of androgens with the expression of reproductive and aggressive behaviour in males of many vertebrate species (Baum, 1992), it might be expected that most copulations would be observed when levels of T are highest. In our Connecticut population of *A. contortrix*, the highest levels of mean T occurred in August, with declines occurring thereafter. However, the greatest number of copulations was observed in September. Several possible explanations might account for this pattern. The null hypothesis assumes no functional association between the presence of circulating T in males and the display of courtship, copulatory and aggressive behaviours. Elevated levels of plasma T during summer would therefore be related exclusively to spermatogenesis, which typically peaks in late summer in temperate pitvipers (e.g. Schuett, 1992; Schuett *et al.*, 2002). However, given the current knowledge regarding the relationship of androgens to male reproductive behaviour in snakes, this view is not strongly supported (see Graham *et al.*, 2008).

An alternative hypothesis is that elevated levels of T in male snakes are involved in a behavioural cascade, and that high levels: (1) are necessary only to initiate/modulate reproductive behaviour and are not required for long-term maintenance (e.g. weeks) and/or (2) decay at a greater rate than does sexual behaviour. A range of studies have established that male reproductive behaviour in squamate reptiles can persist for varying amounts of time following natural declines or experimental removal of androgens, for example, mechanical or chemical castration (e.g. Crews, 1991).

In the present population of *A. contortrix*, we suggest that basal levels of T and the absence of a mating season in spring reflect a deep phylogenetic influence of the hypothalamo-pituitary-gonadal (HPG) axis (Schuett *et al.*, 2002, 2005). A recent phylogeographic analysis of *A. contortrix* provides evidence that the most-recent common ancestor likely occurred in western North America (north-eastern Mexico) and populations dispersed eastward (Douglas *et al.*, 2009). Accordingly, based on reproductive information on western and southern populations of *A. contortrix* (reviewed in Aldridge & Duvall, 2002; Graham *et al.*, 2008), we suggest that the ancestral (pleisiomorphic) condition of seasonal peaks of T (and other steroids) is bimodal, that is, late summer/fall and spring (Schuett *et al.*, 1997), whereas the derived (apomorphic) condition is a single peak in late summer, such as in the present study. It is possible ancient temperature regimes – and other environmental factors – initially shaped the current pattern of unimodal steroidogenesis in the population we studied, but the resultant modification of the HPG axis presently precludes the expression of significant levels of T in the spring. As such, a trait that was ancestrally phenotypically plastic became fixed, possibly through a process of genetic assimilation (e.g. West-Eberhard, 2003).

The summer peak of plasma T in this study strongly agrees with results of a laboratory population, although values were somewhat lower (Schuett *et al.*, 1997). Specifically, in the colony of *A. contortrix* (Texas, USA) studied by Schuett *et al.* (1997), mean T values (August) for the single-

test group ranged from 65.72 to 118.68 ng mL⁻¹ (\bar{x} = 103.88 ± 12.48 ng mL⁻¹). The mean T value (August) of the single-test group in this study was lower (range = 23.13–130.97 ng mL⁻¹; \bar{x} = 70.41 ± 16.12 ng mL⁻¹). In both studies, the same individuals, using the same methods and laboratory, performed the RIAs; hence, we feel that procedural differences are minimal and our comparisons reasonable. Ultimately, beyond the noise resulting from RIA procedures, understanding individual variation in sex steroid levels in males will likely be informative with respect to understanding differential reproductive success.

Differences between our single- and repeat-test groups followed trends seen in previous studies (Schuett *et al.*, 1997; Zaidan *et al.*, 2003). Studies that incorporate both single-point (i.e. each subject is measured once only) and repeat sampling designs typically show lower steroid levels among repeat-test groups (Schuett *et al.*, 1997; Zaidan *et al.*, 2003). This result has been attributed to the effects of processing, for example, loss of blood (Schuett *et al.*, 1997) and/or handling stress (Schuett *et al.*, 2004b). Statistical comparisons of monthly T levels between the single- and repeat-test groups within the present study were significantly different only in May and September; however, the overall trend across all months was that of slightly lower T levels in subjects that were sampled repeatedly. Our study corroborates results of previous studies concerning repeat-sampling designs in measuring sex steroids in snakes (e.g. Schuett *et al.*, 1997), and thus provides additional caution when interpreting seasonal levels and trends.

The profile of P4 during the active season (April–October) followed the general trend observed for T. Given the corresponding patterns of T and P4, and the relationship of T to male mating behaviour, a functional role of P4 in controlling and/or modulating male sexual behaviour in *A. contortrix* is suggested. Regulation of male mating behaviour by P4, however, remains unclear in a range of reptilian taxa (Young *et al.*, 1991; Lindzey & Crews, 1992; Crews & Moore, 2005), including the viperid snake *V. aspis* (Saint Girons *et al.*, 1993).

Owing to the fact that stress reduces circulating levels of T, which subsequently inhibits sexual behaviour in males (e.g. Lance *et al.*, 2004), Wagner (2006) has suggested that P4 functions to offset depressed levels of T in the presence of normal environmental stress, and thus permit reproductive behaviour under such conditions. This view is a potentially productive framework for future studies of P4 in male copperheads and other snakes. Alternatively, prolonged elevated levels of P4 might inhibit male sexual behaviour when conditions for reproduction are not favourable, such as during periods of extended environmental stress (see Wingfield, Lynn & Soma, 2001).

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References

- Aldridge, R.D. & Duvall, D. (2002). Evolution of the mating season in the pitvipers of North America. *Herpetol. Monogr.* **16**, 1–25.
- Almeida-Santos, S.M., Abdalla, F.M.F., Silveira, P.F., Yamanouye, N.Y., Breno, M.C. & Salomão, M.G. (2004). Reproductive cycle of the Neotropical *Crotalus durissus terrificus*: I. Seasonal levels and interplay between steroid hormones and vasotocinase. *Gen. Comp. Endocrinol.* **139**, 143–150.
- Baum, M.J. (1992). Neuroendocrinology of sexual behavior in the male. In *Behavioral endocrinology*: 97–130. Becker, J.B., Breedlove, M.S. & Crews, D. (Eds). Cambridge, MA: MIT Press.
- Benner, S.L. & Woodley, S.K. (2007). The reproductive pattern of male dusky salamanders (genus *Desmognathus*) is neither associated nor dissociated. *Horm. Behav.* **51**, 542–547.
- Bonnet, X., Naulleau, G. & Lourdaux, O. (2002). Benefits of complementary techniques: using mark–recapture and physiological approaches to understand costs of reproduction in the Asp viper (*Vipera aspis*). In *Biology of the vipers*: 483–495. Schuett, G.W., Höggren, M., Douglas, M.E. & Greene, H.W. (Eds). Eagle Mountain, UT: Eagle Mountain Publishing, L.C.
- Crews, D. (1991). Trans-seasonal action of androgen in the control of spring courtship behavior in male red-sided garter snakes. *Proc. Natl. Acad. Sci. USA* **88**, 3545–3548.
- Crews, D. & Moore, M.C. (2005). Historical contributions of research on reptiles to behavioral endocrinology. *Horm. Behav.* **48**, 384–394.
- Douglas, M.E., Douglas, M.R., Schuett, G.W. & Porras, L.W. (2009). Climate change and evolution of the New World pitviper genus *Agkistrodon* (Viperidae). *J. Biogeogr.* **36**, 1164–1180.
- Edwards, A. & Jones, S.M. (2001). Changes in plasma testosterone, estrogen, and progesterone concentration throughout the annual reproductive cycle in male viviparous blue-tongue skinks, *Tiliqua nigrolutea*, in Tasmania. *J. Herpetol.* **35**, 293–299.
- Finneran, L.C. (1948). Reptiles at Branford, Connecticut. *Herpetologica* **4**, 123–126.
- Fitch, H.S. (1960). Autecology of the copperhead. *Univ. Kans. Publ. Mus. Nat. Hist.* **13**, 85–288.
- Gibbons, J.W. & Andrews, K.M. (2004). PIT tagging: simple technology at its best. *BioScience* **54**, 447–454.
- Gloyd, H.K. & Conant, R. (1990). *Snakes of the Agkistrodon complex: a monographic review. Contributions in Herpetology, No. 6*. Oxford, OH: Society for the Study of Amphibians and Reptiles.
- Graham, S.P., Earley, R.L., Hoss, S.K., Schuett, G.W. & Grober, M.S. (2008). The reproductive biology of male cottonmouths (*Agkistrodon piscivorus*): do plasma steroid hormones predict the mating season? *Gen. Comp. Endocrinol.* **159**, 226–235.
- Hardy, D.L. Sr & Greene, H.W. (1999). Surgery on rattlesnakes in the field for implantation of transmitters. *Sonoran Herpetol.* **12**, 25–27.
- Krohmer, R.W., Grassman, M. & Crews, D. (1987). Annual reproductive cycle in the male red-sided garter snake, *Thamnophis sirtalis parietalis*: field and laboratory studies. *Gen. Comp. Endocrinol.* **68**, 64–75.
- Lance, V.A., Elsey, R.M., Butterstein, G. & Trosclair, P.L. III (2004). Rapid suppression of testosterone secretion after capture in male American alligators (*Alligator mississippiensis*). *Gen. Comp. Endocrinol.* **135**, 217–222.
- Lindzey, J. & Crews, D. (1992). Interactions between progesterones and androgens in the stimulation of sex behaviors in male little striped whiptail lizards, *Cnemidophorus inornatus*. *Gen. Comp. Endocrinol.* **86**, 52–58.
- McDuffie, G. (1960). *Studies on the ecology and life history of the copperhead, Agkistrodon contortrix mokeson (Daudin), in Ohio*. Unpublished dissertation, University of Cincinnati, Cincinnati, OH.
- Mendonça, M.T. & Crews, D. (2001). Control of attractivity and receptivity in female red-sided garter snakes. *Horm. Behav.* **40**, 43–50.
- Moore, M.C. & Lindzey, J. (1992). The physiological basis of sexual behavior in male reptiles. In *Biology of the reptilia*, Vol. 18: 70–113. Gans, C. & Crews, D. (Eds). Chicago, IL: The University of Chicago Press.
- Naulleau, G. & Fleury, F. (1984). Relations entre la testostéronémie, la thyroïdémie et le cycle sexuel chez les mâles de *Vipera aspis* et *Vipera berus*. *Bull. Soc. Herpetol. France* **32**, 45–52.
- Naulleau, G., Fleury, F. & Boissin, J. (1987). Annual cycles in plasma testosterone and thyroxine in the male Asp viper *Vipera aspis* L., (Reptilia, Viperidae), in relation to the sexual cycle and hibernation. *Gen. Comp. Endocrinol.* **65**, 254–263.
- Norris, D.O. (2006). *Vertebrate endocrinology*, 4th edn. San Diego, CA: Academic Press.
- Petersen, R.C. & Fritsch, R.W. (1986). *Connecticut's venomous snakes: the timber rattlesnake and northern*

- copperhead*, 2nd edn. State Geological and Natural History Survey of Connecticut, Bulletin 111.
- Reinert, H.K. (1992). Radiotelemetric field studies of pitvipers: data acquisition and analysis. In *Biology of the pitvipers*: 185–198. Campbell, J.A. & Brodie, E.D. Jr (Eds). Tyler, TX: Selva.
- Saint Girons, H., Bradshaw, S.D. & Bradshaw, F.J. (1993). Sexual activity and plasma levels of sex steroids in the asp viper *Vipera aspis* L., (Reptilia, Viperidae). *Gen. Comp. Endocrinol.* **91**, 287–297.
- Schuett, G.W. (1992). Is long-term sperm storage an important component of the reproductive biology of temperate pitvipers? In *Biology of the pitvipers*: 169–184. Campbell, J.A. & Brodie, E.D. Jr (Eds). Tyler, TX: Selva.
- Schuett, G.W. (1997). Body size and agonistic experience affect dominance and mating success in male copperheads. *Anim. Behav.* **54**, 213–224.
- Schuett, G.W., Carlisle, S.L., Holycross, A.T., O'Leile, J.K., Hardy, D.L. Sr., Van Kirk, E.A. & Murdoch, W.J. (2002). Mating system of male Mojave rattlesnakes (*Crotalus scutulatus*): seasonal timing of mating, agonistic behavior, spermatogenesis, sexual segment of the kidney, and plasma sex steroids. In *Biology of the vipers*: 515–532. Schuett, G.W., Höggren, M., Douglas, M.E. & Greene, H.W. (Eds). Eagle Mountain, UT: Eagle Mountain Publishing, LC.
- Schuett, G.W., Grober, M.S., Van Kirk, E.A. & Murdoch, W.J. (2004a). Long-term sperm storage and plasma steroid profile of pregnancy in a western diamond-backed rattlesnake (*Crotalus atrox*). *Herpetol. Rev.* **35**, 328–333.
- Schuett, G.W., Hardy, D.L. Sr., Greene, H.W., Earley, R.L., Grober, M.S., Van Kirk, E.A. & Murdoch, W.J. (2005). Sympatric rattlesnakes with contrasting mating systems show differences in seasonal patterns of plasma sex steroids. *Anim. Behav.* **70**, 257–266.
- Schuett, G.W., Harlow, H.J., Rose, J.D., Van Kirk, E.A. & Murdoch, W.J. (1997). Annual cycle of plasma testosterone in male copperheads, *Agkistrodon contortrix* (Serpentes, Viperidae): relationship to timing of spermatogenesis, mating, and agonistic behavior. *Gen. Comp. Endocrinol.* **105**, 417–424.
- Schuett, G.W., Repp, R.A., Taylor, E.N., DeNardo, D.F., Earley, R.L., Van Kirk, E.A. & Murdoch, W.J. (2006). Winter profile of plasma sex steroid levels in free-living male western diamond-backed rattlesnakes, *Crotalus atrox* (Serpentes: Viperidae). *Gen. Comp. Endocrinol.* **149**, 72–80.
- Schuett, G.W., Taylor, E.N., Van Kirk, E.A. & Murdoch, W.J. (2004b). Handling stress and plasma corticosterone levels in captive male western diamond-backed rattlesnakes (*Crotalus atrox*). *Herpetol. Rev.* **35**, 229–233.
- Seigel, R.A. & Ford, N.B. (1987). Reproductive ecology. In *Snakes: ecology and evolutionary biology*: 210–252. Seigel, R.A., Collins, J.T. & Novak, S.S. (Eds). New York: Macmillan.
- Shine, R. (2003). Reproductive strategies in snakes. *Proc. Roy. Soc. Lond. Ser. B* **270**, 995–1004.
- Shine, R. (2005). Life-history evolution in reptiles. *Annu. Rev. Ecol. Evol. Syst.* **36**, 23–46.
- Smith, C.F. (2007). *Sexual dimorphism, and the spatial and reproductive ecology of the copperhead snake, Agkistrodon contortrix*. Unpublished doctoral dissertation. University of Connecticut, Storrs, CT, USA.
- Smith, C.F., Schuett, G.W., Earley, R.L. & Schwenk, K. (2009). The spatial and reproductive ecology of the copperhead (*Agkistrodon contortrix*) at the northeastern extreme of its range. *Herpetol. Monogr.*, in press.
- Taylor, E.N. & DeNardo, D.F. (in press). Hormone and reproductive cycles in snakes. In *Hormones and reproduction in vertebrates, Vol. 2 reptiles*. Norris, D.O. & Lopez, K.H. (Eds). New York: Academic Press.
- Taylor, E.N., DeNardo, D.F. & Jennings, D.H. (2004). Seasonal steroid hormone levels and their relation to reproduction in the western diamond-backed rattlesnake, *Crotalus atrox* (Serpentes: Viperidae). *Gen. Comp. Endocrinol.* **136**, 328–337.
- Taylor, E.N. & Schuett, G.W. (2004). Effect of temperature and storage duration on the stability of steroid hormones in blood samples from western diamond-backed rattlesnakes (*Crotalus atrox*). *Herpetol. Rev.* **35**, 14–17.
- Wagner, C.K. (2006). The many faces of progesterone: a role in adult and developing male brain. *Front. Neuroendocrinol.* **27**, 340–359.
- Weil, M.R. & Aldridge, R.D. (1981). Seasonal androgenesis in the male water snake *Nerodia sipedon*. *Gen. Comp. Endocrinol.* **44**, 44–53.
- West-Eberhard, M.J. (2003). *Developmental plasticity and evolution*. Oxford: Oxford University Press.
- Whittier, J.M. & Tokarz, R.R. (1992). Physiological regulation of sexual behavior in female reptiles. In *Biology of the reptilia*, Vol. 18: 24–69. Gans, C. & Crews, D. (Eds). Chicago, IL: The University of Chicago Press.
- Wingfield, J.C., Lynn, S. & Soma, K.K. (2001). Avoiding the 'costs' of testosterone: ecological bases of hormone-behavior interactions. *Brain Behav. Evol.* **57**, 239–251.
- Young, L.J., Greenberg, N. & Crews, D. (1991). The effects of progesterone on sexual behavior in male green anole lizards (*Anolis carolinensis*). *Horm. Behav.* **25**, 477–488.
- Zaidan, F. III, Kreider, D.L. & Beaupre, S.J. (2003). Testosterone cycles and reproductive energetics: implications for northern range limits of the cottonmouth (*Agkistrodon piscivorus leucostoma*). *Copeia* **2003**, 231–240.
- Zar, J.H. (1999). *Biostatistical analysis*, 4th edn. Upper Saddle River, NJ: Prentice-Hall.