



Sympatric rattlesnakes with contrasting mating systems show differences in seasonal patterns of plasma sex steroids

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Long-term field studies conducted in Arizona show that two species of sympatric rattlesnakes differ in the structure of their mating systems, primarily in frequency and timing of mating seasons, despite exposure to identical environmental conditions. The western diamond-backed rattlesnake, *Crotalus atrox*, has two distinct mating seasons within a single annual spermatogenic cycle. The first mating season occurs from late August to early October. Following a hibernation period of 4 months, the second mating season occurs from mid-March to early May. Because there is a mating season in spring, long-term sperm storage by females during winter is facultative. In contrast, the black-tailed rattlesnake, *Crotalus molossus*, has a single mating season (mid-July to early September) within a single annual spermatogenic cycle. Due to the absence of a mating season in spring, long-term sperm storage by females during winter is obligatory. In both species, ovulation and fertilization occur in spring, and offspring are produced from mid-July to early September. Based on these robust data, we tested the hypotheses that seasonal patterns of plasma sex steroids (testosterone, 5 α -dihydrotestosterone, and 17 β -estradiol) differ between males in wild populations of *C. atrox* and *C. molossus*, and that peak levels would be coincident with the mating seasons. Specifically, we predicted that there would be two peaks of sex steroids in *C. atrox* and one peak in *C. molossus*, and that baseline levels would be detected outside the periods of mating and spermatogenesis. Our results supported these predictions. Furthermore, absolute concentrations of plasma testosterone and 5 α -dihydrotestosterone, but not 17 β -estradiol, were higher in *C. atrox* than in *C. molossus*. We discuss a possible scenario for the evolution of the different mating seasons in these sympatric rattlesnakes, and advocate that comparative approaches to address such questions should integrate proximate and ultimate causation to increase explanatory power.

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Notwithstanding exposure to similar ecological conditions, closely related species living in sympatry can show significant variation in the structure of their mating systems, particularly with regard to mode of reproduction, seasonal timing and frequency of sexual activity, mate choice and parental care, and male aggression (Andersson 1994; Whitfield & Tomkovitch 1996; Sun 1997; Paton &

Crouch 2002; Shuster & Wade 2003). In addition to behavioural differences, sympatric taxa can show significant physiological differences such as those related to endocrine function (Gorman et al. 1981; Wingfield et al. 1990). Numerous theoretical models explain the origin and maintenance of the diversity of mating systems (Adkins-Regan 1998; Shuster & Wade 2003), but those that integrate ultimate and proximate causation show the most promise in providing robust explanatory power and predictability (Stamps 1991; Drickamer & Gillie 1998; Feder et al. 2000). Because proximate mechanisms are targets of selection, a broad knowledge of their variation is

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important to the development of integrative models of mating systems (Real 1994; Shuster & Wade 2003).

In terrestrial vertebrates, these types of integrative studies on sympatric taxa have concentrated primarily on mammals and birds, with far less emphasis on squamate reptiles. The few studies on snakes, for instance, show that sympatric species can vary substantially with regard to timing of reproductive events such as spermatogenesis (Aldridge 1979; Gorman et al. 1981; Saint Girons 1982; Aldridge & Duvall 2002) and seasonal patterns of

a permanent identification number using injectable PIT-tags (AVID, Inc., Norco, California, U.S.A.). After processing, all snakes were returned to their original capture sites.

Radioimmunoassay of Plasma Sex Steroids

We measured testosterone (T), 5 α -dihydrotestosterone (DHT) and 17 β -estradiol (E2) for all collected samples. Procedures for radioimmunoassay (RIA) measurements of T, DHT and E2 followed commercial kits with appropriate modifications (e.g. use of snake plasma in place of rat plasma). Plasma samples from 65 *C. atrox* from the WTM site and 20 from Portal were run in duplicate in RIAs for T, but only WTM samples were also assayed for DHT and E2. We similarly assayed 58 *C. molossus* samples from Portal and 11 from the WTMs. The T and DHT values are presented as arithmetic means \pm 1 SE (ng/ml), and E2 values are presented as arithmetic means \pm 1 SE (pg/ml).

Radiolabelled T and antibody were purchased from Research Products International (Mount Prospect, Illinois, U.S.A.; catalogue number TMM-210). The primary antibody detected T, and the percentage of cross-reactivity with other androgens (and other steroids) was minimal or not detectable. Sensitivity of the T assay was 4 pg/ml. All plasma samples (0.2 ml) were extracted in anhydrous diethyl ether prior to RIA. Extraction efficiency was determined by adding H³-testosterone (25 000 cpm) to 10 μ l (twice the volume used for the assay) of snake plasma and phosphate-buffered saline, incubating overnight at 4°C, and then conducting an ether extraction. The quantity of H³-testosterone extracted was compared to the amount added to the sample. The extraction efficiency of radioactive T was 93.3%. Cold recovery of unlabelled T that was added to the plasma samples and previously determined to be low in T was 95.0%. Parallelism occurred between inhibition curves obtained with standards and serial dilutions of ether-extracted plasma. Validation of the RIA also involved quantitative recovery of exogenous steroid using snake plasma from adults not used in this study. Two RIAs were performed. The intra-assay coefficients of variation (CVs) were 9.1% and 11.1%, and the interassay CV was 11.9%.

Radiolabelled DHT and antibody were purchased from Diagnostic Systems Laboratories, Inc. (Webster, Texas, U.S.A.; catalogue number DSL 9600). The protocol for extraction and RIA provided by the manufacturer was followed, except that 0.20 ml of snake plasma and 0.20 ml of phosphate-buffered saline (with 0.1% gelatin) were used for extractions. Chromatographic steps were not required. In cases where concentrations of steroid were predicted to be high, the extract was diluted before assaying the sample. The oxidation-extraction step reduced cross-activity with testosterone to 0.02%, and cross-reactivity with other androgens (and other steroids) was minimal or not detectable. The RIA analytical sensitivity for DHT was 4 pg/ml. Validation involved showing parallelism between inhibition curves for the standards provided with the kit and serial dilutions of snake plasma. Cold recovery was not performed. Concentrations of DHT were determined in a single RIA, and the intra-assay CV was 8.9%.

Radiolabelled E2, antibody and a precipitating solution were purchased from Diagnostic Products Corporation (Los Angeles, California, U.S.A.; catalogue numbers E2D1, E2D2 and N6, respectively). Standards were prepared by serial dilutions in a stock solution of methanol. The anti-estradiol antibody was diluted 1:3 in phosphate-buffered saline (PBS) containing 1:400 rabbit sera. One hundred microlitres of snake plasma (with 300 μ l of PBS) was extracted in 5.0 ml of diethyl ether (Fisher Scientific, Chicago, Illinois, U.S.A.). After removing and saving the ether layer, the sample was heated to 90°C for 5 min and then extracted with an additional 5.0 ml of diethyl ether. Two hundred microlitres of PBS-0.1% gelatin were added to the extract following evaporation of the ether. Extraction efficiency was determined by adding H³-estradiol (25 000 cpm) to 10 μ l (twice the volume used for the assay) of snake plasma and phosphate-buffered saline, incubated overnight at 4°C, and followed by ether extraction. The quantity of H³-estradiol extracted was compared to the amount added to the sample. Extraction recovery of H³-estradiol (New England Nuclear, Boston, Massachusetts, U.S.A.; NET-381) was 78%. For the RIA, 100 μ l of diluted antibody, 100 μ l of I¹²⁵ E2 and 1.0 ml of precipitating solution were used. A 24-h incubation (4°C) period followed each step. Antibody-bound I¹²⁵ was separated by centrifugation at 1600 G. The RIA sensitivity for E2 was 4 pg/ml. Validation involved showing that quantitative recovery of E2 added to snake plasma was 100%, and that parallelism occurred between the inhibition curves for standards and dilutions of snake plasma. Two RIAs were performed. The intra-assay CVs were 7.9% and 12.5%, and the interassay CV was 11.9%.

Statistical Analyses

Statistical methods followed Zar (1999) and tests were performed using SAS (SAS Institute 1999). Data were inspected for outliers, normality (skewness and kurtosis) and homogeneity of variance prior to performing statistical tests. Plasma sex steroids were analysed on a monthly basis (February–September). Different subjects were sampled at each monthly interval for each species (*C. atrox* and *C. molossus*) during each year; thus, repeated measures were not necessary. Seasonal and yearly differences in plasma sex steroids were assessed using ANCOVA with month and year as the fixed main effects and SVL as the covariate. Regression analysis showed a significant positive correlation between SVL and body mass for both *C. atrox* ($F_{1,63} = 162.03$, $P < 0.0001$, $R^2 = 0.72$) and *C. molossus* ($F_{1,56} = 158.47$, $P < 0.0001$, $R^2 = 0.74$). Because SVL is a more stable character than body mass, it was used as the covariate in ANCOVAs. All steroid data (T, DHT and E2) were natural-log transformed to achieve normality. Sequential Dunn–Sidak adjustments were used to control for compounding type I error in the three ANCOVA models for each species ($\alpha_{\text{adj}} = 1 - (1 - \alpha)^{1/k} = 0.017$ for the most significant model) due to non-independence of the steroid profiles (see regression of nontransformed steroid levels in Results). Student–Newman–Keuls (SNK) multiple comparisons determined

differences in steroid hormone levels across sampled months and years. Changes in SVL or body mass across months and years were examined with one-way ANOVA. Body condition was determined as the residual value from the regression between SVL and body mass, following Moore et al. (2000). The effect of body condition on natural-log transformed plasma steroid levels was assessed using an ANCOVA with species, month and year as fixed main effects and residuals as the covariate. The α level of significance was set at $P < 0.05$.

RESULTS

Western Diamond-backed Rattlesnake

Body size

Mean SVL was 788.35 ± 20.61 mm (range 405–1077) and mean body mass was 417.32 ± 32.59 g (range 60.0–1153.5). The SVL of subjects sampled in March was significantly greater than that of subjects sampled in August and April (ANOVA: $F_{7,50} = 2.88$, $P = 0.013$; SNK: $P < 0.05$). Body mass in subjects sampled in February and March was significantly greater than that sampled in April and August (ANOVA: $F_{7,50} = 3.54$, $P = 0.004$; SNK: $P < 0.05$). All other monthly comparisons of SVL and body mass were not significant. There were no differences in SVL or body mass of subjects collected in 1998 versus 1999 (SVL: $F_{1,50} = 1.97$, $P = 0.16$; body mass: $F_{1,50} = 0.55$, $P = 0.46$).

Plasma sex steroids

Regression of nontransformed steroid values showed highly significant relationships among the three plasma hormones (T \times DHT: $R^2 = 0.481$, $F_{1,56} = 51.69$, $P < 0.0001$; T \times E2: $R^2 = 0.685$, $F_{1,63} = 137.27$, $P < 0.0001$; DHT \times E2: $R^2 = 0.326$, $F_{1,56} = 27.06$, $P < 0.0001$). Neither SVL (covariate) alone nor its interaction with month or year was significant in the three steroid models (Table 1). Seasonal patterns of T, DHT and E2 were similar, and concentrations of all steroids revealed significant variation among months (Table 1, Fig. 1). Seasonal variation in plasma T in this species was very similar between the two sites. Compare the values from the WTMs in Fig. 1 with the following results for *C. atrox* from Portal: March (mean = 88.74 ± 11.50 ng/ml, $N = 4$), April (mean = 42.79 ± 3.94 ng/ml, $N = 4$), May (mean = 11.076 ± 2.02 ng/ml, $N = 3$), July (mean = 15.56 ± 4.65 ng/ml, $N = 3$), August (mean = 72.41 ± 8.65 ng/ml, $N = 4$) and September (mean = 92.63 ± 7.42 ng/ml, $N = 2$).

Inspection of mean values in Fig. 1 showed two distinctive seasonal peaks (August–September and February–March) of T, DHT and E2 that were coincident with the first (late August–early October) and second mating season (mid-March–early May). Post hoc SNK multiple comparisons showed that T, DHT and E2 showed significant bimodality (Fig. 1). There were significant differences in absolute T and DHT levels between sampling years, but the seasonal pattern was the same in both years for all three steroids (Table 1). The significant month by year interaction for DHT could be traced, using linear contrasts, to several

significant monthly and/or yearly differences but not to differences in the overall seasonal trend between years.

Black-tailed Rattlesnake

Body size

Mean SVL was 958.81 ± 14.786 mm (range 708–1150) and mean body mass was 647.35 ± 35.29 g (range 190–1322). Neither SVL nor body mass varied significantly across the months of sampling (SVL: $F_{5,48} = 0.76$, $P = 0.58$; body mass: $F_{5,48} = 1.48$, $P = 0.21$). Individuals sampled in 1999 had significantly greater mass, but not greater SVL, than those sampled in 1998 (SVL: $F_{1,48} = 3.04$, $P = 0.09$; body mass: $F_{1,48} = 6.07$, $P = 0.017$).

Plasma sex steroids

Regression of nontransformed steroid values showed highly significant relationships among the three plasma hormones (T \times DHT: $R^2 = 0.544$, $F_{1,53} = 63.28$, $P < 0.0001$; T \times E2: $R^2 = 0.441$, $F_{1,56} = 44.2$, $P < 0.0001$; DHT \times E2: $R^2 = 0.428$, $F_{1,53} = 39.6$, $P < 0.0001$). Neither the covariate (SVL) nor its interaction with month or year was significant in the three steroid models (Table 1). Seasonal patterns of T, DHT and E2 were similar. Plasma concentrations of T, DHT and E2 revealed significant variation among months (Table 1, Fig. 2). Seasonal variation in plasma T in *C. molossus* was very similar between the two sites. Compare the values from the Portal area in Fig. 2 with the following results obtained for *C. molossus* from the WTMs: March (mean = 9.48 ± 0.52 ng/ml, $N = 2$), April (mean = 12.61 ± 3.21 ng/ml, $N = 2$), June (mean = 38.32 ± 2.65 ng/ml, $N = 2$), July (mean = 92.19 ng/ml, $N = 1$), August (mean = 82.19 ± 3.78 ng/ml, $N = 2$) and September (mean = 13.60 ± 4.49 ng/ml, $N = 2$). Mean concentrations of T and DHT in April and September were significantly lower than concentrations in May through August (SNK: $P < 0.05$); E2 showed a similar trend but had a distinct peak in June and July (Fig. 2). Unlike *C. atrox*, the seasonal pattern of peak steroid levels was unimodal and coincident with the single period of mating (mid-July to mid-September). There were significant overall differences across the two sampling years for T only, but the seasonal pattern was similar in both years for all three steroids (Table 1).

Species Differences in Levels of Sex Steroids

An analysis was conducted to examine differences in absolute levels of T, DHT and E2 between males of *C. atrox* and *C. molossus*. All plasma steroid levels were natural-log transformed to achieve normality. Significant between-species differences were found for DHT and E2 ($F_{1,100} > 5.14$, $P < 0.026$), whereas differences for T approached significance ($F_{1,100} = 3.47$, $P = 0.06$). Nontransformed mean levels of T (55.95 ± 7.79 ng/ml) and DHT (3.14 ± 0.52 ng/ml) in *C. atrox* were higher than in *C. molossus* (T: 42.60 ± 5.92 ng/ml; DHT: 1.08 ± 0.15 ng/ml), whereas the opposite was the case for

Table 1. Summary of the effects of month, year and snout-vent length (SVL, covariate) on levels of plasma testosterone (T), 5 α -dihydrotestosterone (DHT) and 17 β -estradiol (E2) in male *Crotalus atrox* and *C. molossus*

Species	Sex steroid	Effect	F	df	P	Interpretation
<i>Crotalus atrox</i>	T	Month	25.69	7, 41	<0.0001	Elevated: Feb–Mar, Aug–Sep; Baseline: Apr–Jul 1998 > 1999
		Year	13.02	1, 41	0.0008	
		SVL	1.45	1, 41	0.2361	
		SVL*month	1.88	7, 41	0.0974	
		SVL*year	0.16	1, 41	0.6884	
		Month*year	1.85	6, 41	0.1132	
	DHT	Month	18.57	7, 34	<0.0001	Elevated: Feb–Mar, Aug–Sep; Baseline: Apr–Jul 1998 > 1999
		Year	16.49	1, 34	0.0003	
		SVL	0.06	1, 34	0.8135	
		SVL*month	1.81	7, 34	0.1164	
		SVL*year	0.13	1, 34	0.7208	
		Month*year	4.38	6, 34	0.0022	See Results
	E2	Month	10.06	7, 41	<0.0001	Elevated: Feb–Apr, Sep; Baseline: Feb, May–Aug
		Year	0.63	1, 41	0.4302	
		SVL	2.33	1, 41	0.1342	
SVL*month		1.16	7, 41	0.3457		
SVL*year		0.27	1, 41	0.6088		
Month*year		1.69	6, 41	0.1468		
<i>Crotalus molossus</i>	T	Month	9.92	5, 41	<0.0001	Elevated: May–Aug; Baseline: Mar–Apr, Sep 1999 > 1998
		Year	5.36	1, 41	0.0241*	
		SVL	3.41	1, 41	0.0722	
		SVL*month	1.26	5, 41	0.2983	
		SVL*year	1.99	1, 41	0.1659	
		Month*year	2.25	3, 41	0.0972	
	DHT	Month	14.09	5, 38	<0.0001	Elevated: May–Aug; Baseline: Mar–Apr, Sep
		Year	3.37	1, 38	0.0743	
		SVL	2.53	1, 38	0.1203	
		SVL*month	1.02	5, 38	0.4208	
		SVL*year	0.05	1, 38	0.8249	
		Month*year	1.20	3, 38	0.3231	
	E2	Month	3.73	5, 41	0.0071	Elevated: May–Aug; Baseline: Mar–Apr, Sep
		Year	3.57	1, 41	0.0659	
		SVL	0.59	1, 41	0.4485	
SVL*month		2.18	5, 41	0.0746		
SVL*year		3.72	1, 41	0.0608		
Month*year		0.50	3, 41	0.6860		

See Figs 1 and 2 for graphical representation of the sex steroid data.

*Student–Newman–Keuls post hoc multiple comparisons indicated no difference between years.

E2 (*C. atrox*: 13.83 ± 2.22 pg/ml; *C. molossus*: 14.96 ± 2.09 pg/ml).

Single-species seasonal steroid analyses indicated that *C. atrox* shows a bimodal pattern and *C. molossus* shows a unimodal pattern. When both species were included in the analysis, there was a significant species by month interaction for T, DHT and E2, confirming both qualitatively and statistically different monthly steroid profiles ($F_{5,100} > 8.42$, $P < 0.0001$).

There were significant differences between *C. atrox* and *C. molossus* in the effects of body condition on T ($F_{1,91} = 4.11$, $P = 0.046$) and DHT ($F_{1,81} = 7.39$, $P = 0.008$), and results for E2 were positive but did not achieve significance ($F_{1,91} = 3.47$, $P = 0.066$). The correlation between

body condition and sex steroid levels showed a positive tendency in *C. molossus* (T: $F_{1,32} = 3.01$, $P = 0.09$, $R^2 < 0.11$; DHT: $F_{1,29} = 2.01$, $P = 0.17$, $R^2 = 0.09$; E2: $F_{1,32} = 0.65$, $P = 0.43$, $R^2 = 0.05$), but not in *C. atrox* (T: $F_{1,41} = 0.33$, $P = 0.57$, $R^2 = 0.01$; DHT: $F_{1,34} = 0.18$, $P = 0.67$, $R^2 = 0.002$; E2: $F_{1,41} = 0.03$, $P = 0.87$, $R^2 = 0.003$). There was no robust indication in either species that body condition influenced levels of plasma steroids differentially across months or years (body condition \times month: *C. atrox*, T: $F_{7,41} = 0.53$, $P = 0.81$; DHT: $F_{7,34} = 1.23$, $P = 0.32$; E2: $F_{7,41} = 0.94$, $P = 0.49$; *C. molossus*, T: $F_{5,32} = 0.46$, $P = 0.80$; DHT: $F_{5,29} = 0.52$, $P = 0.76$; E2: $F_{5,32} = 0.22$, $P = 0.96$; body condition \times year: *C. atrox*, T: $F_{1,41} = 0.07$, $P = 0.80$; DHT: $F_{1,34} = 3.86$,

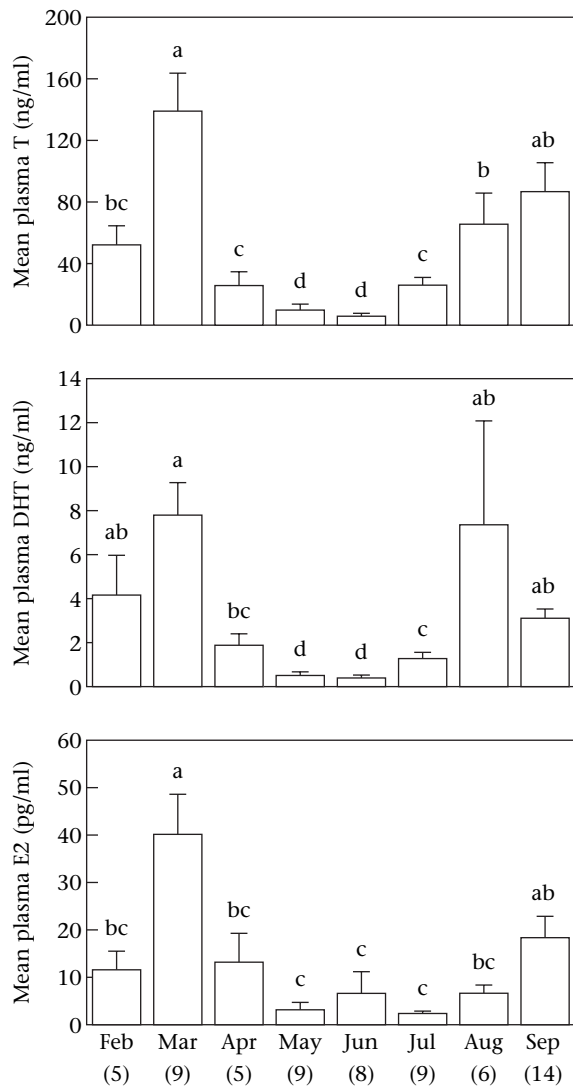


Figure 1. Monthly (February–September) mean \pm 1 SE values of the plasma sex steroids testosterone (T), 5 α -dihydrotestosterone (DHT) and 17 β -estradiol (E2) in adult male western diamond-backed rattlesnakes, *Crotalus atrox*, from the White Tank Mountain site in the vicinity of Phoenix, Arizona (Maricopa County) in 1998–1999. Histogram bars with the same letter were not significantly different (Table 1). The thick black lines beneath the abscissa denote the two mating seasons. Parenthetical values denote the monthly sample sizes but exceptions for E2 are in March ($N = 8$), June ($N = 7$), July ($N = 7$) and August ($N = 5$).

$P = 0.06$; E2: $F_{1,41} = 2.1$, $P = 0.16$; *C. molossus*, T: $F_{1,32} = 1.57$, $P = 0.22$; DHT: $F_{1,29} = 2.43$, $P = 0.13$; E2: $F_{1,32} = 0$, $P = 0.98$).

DISCUSSION

Plasma Sex Steroids and Mating Seasons

We have shown that annual cycles of the plasma sex steroids T, DHT and E2 were significantly different in males of two species of sympatric rattlesnakes with

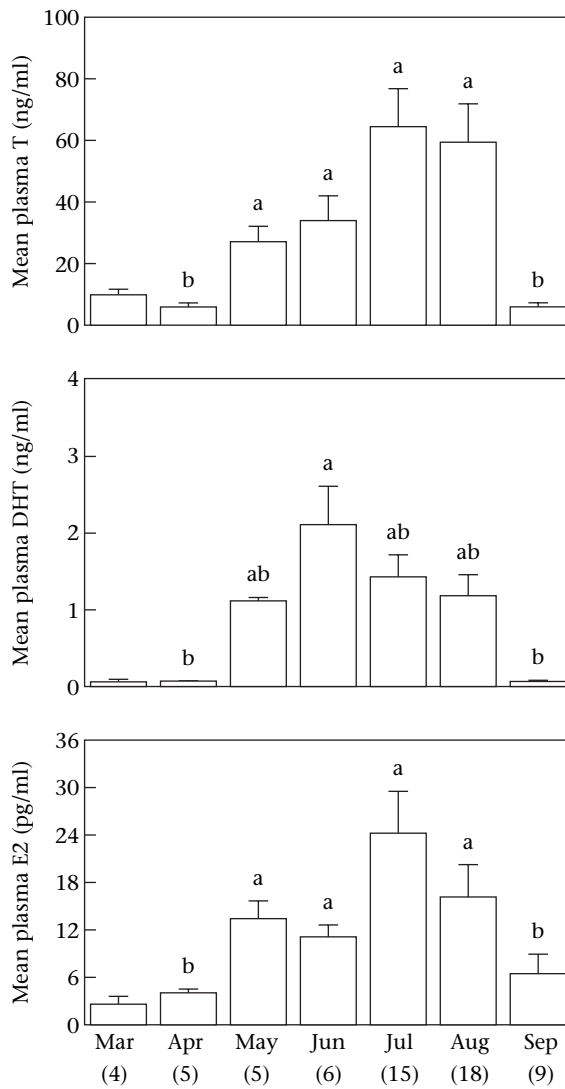


Figure 2. Monthly (March–September) mean \pm 1 SE values of the plasma sex steroids testosterone (T), 5 α -dihydrotestosterone (DHT) and 17 β -estradiol (E2) in adult male black-tailed rattlesnakes, *Crotalus molossus*, from the vicinity of Portal, Arizona (Cochise County) in 1998–1999. Data in March were derived from four males collected in 2000, and were not included in the statistical analyses (Table 1). Histogram bars with the same letter were not significantly different (Table 1). The thick black line beneath the abscissa denotes the single mating season. Parenthetical values denote the monthly sample sizes but exceptions for DHT are in July ($N = 14$), August ($N = 17$) and September ($N = 8$).

contrasting mating systems, and that peak levels were largely coincident with the mating seasons. Consistent with previous studies on male rattlesnakes (Schuett et al. 2002) and males of other viper species (Saint Girons et al. 1993), we found in *C. atrox* and *C. molossus* that T had the highest concentrations, followed by DHT and E2. In male *C. atrox*, peak levels of all three steroids showed a bimodal pattern coinciding with the first (late August to early October) and the second (mid-March to early May) mating seasons. During the active season, baseline levels of sex steroids occurred during May–July, the period when sexual

activity is quiescent. This pattern of sexual activity and sex steroid levels (T only) in males has also been described in free-living *C. atrox* from populations near Tucson, Arizona (Taylor et al. 2004), and in other New World pitvipers (Schuett et al. 1997).

In male *C. molossus*, elevated levels of all three steroids occurred during May–August, and peak levels of T and E2 occurred during July–August, which coincided with the single mating season (Greene et al. 2002). In contrast to *C. atrox*, male *C. molossus* did not show elevated levels of plasma T, DHT and E2 during early spring (March–April), which coincides with the absence of sexual activity (Greene et al. 2002). Baseline levels of all three steroids occurred in September, and sexual activity in *C. molossus* is waning during this time (Greene et al. 2002). This pattern of sexual activity and sex steroid levels (T only) in males has been described in the pitviper *Agkistrodon piscivorus* (Zaidan et al. 2003).

Because our observations on the reproductive ecology of populations of *C. atrox* and *C. molossus* in Arizona have been long term (Repp 1998; Greene et al. 2002; Schuett et al. 2004), they strongly suggest that the seasonal timing of sexual activity in the populations we report on here is a stable feature of their mating systems (Schuett 1992; Aldridge & Duvall 2002; Schuett et al. 2002).

To the best of our knowledge, the present study is only the second one concerning snakes that has investigated mating seasons and annual patterns of sex steroids in closely related, sympatric congeners. Results on the Old World vipers *Vipera aspis* and *V. berus* show some similarities to the present study. These two viperid taxa are sympatric in areas of France (Arnold & Burton 1978). *Vipera aspis* mates in autumn and spring, and peak levels of sex steroids coincide with these mating seasons (Saint Girons et al. 1993). In contrast, *V. berus* mates only in spring, at which time it shows its only peak of sex steroid levels (Naulleau & Fleury 1984).

Between-year Differences in Levels of Plasma Sex Steroids

Although the seasonal patterns of plasma sex steroids were stable across 2 years in both *C. atrox* and *C. molossus*, we did detect significant between-year differences in absolute concentrations of T and DHT, but not E2, in *C. atrox* (Table 1). Significant between-year differences in steroid levels were not found in *C. molossus* (Table 1). We do not have a definitive explanation for these findings in *C. atrox*, but are confident that experimental factors can be excluded as causes for this variation. No methods of handling and processing snakes changed between years of the study, and all RIAs were performed by a single researcher (E.V.K.) under constant laboratory conditions. Biotic and abiotic factors also can influence levels of sex steroids in squamate reptiles (Krohmer et al. 1987; Bonnet & Naulleau 1996; Knapp et al. 2003). Knapp et al. (2003), for example, reported that above-average ambient temperatures and below-average precipitation negatively influenced peak levels of plasma T in non-territorial male tree lizards, *Urosaurus ornatus*. Annual

weather patterns might have also influenced steroid levels in male *C. atrox*. In 1998 and 1999, the periods during which we sampled *C. atrox* and *C. molossus*, El Niño and La Niña phenomena occurred, respectively (NOAA, National Weather Service, <http://www.nws.noaa.gov>). As is typical for El Niño, wetter-than-average and cooler-than-average conditions prevailed. During the following year, La Niña brought severe drought and above-average temperatures, especially in the areas of low-lying desert habitats surrounding Phoenix. This weather pattern might have affected the population of *C. atrox* we studied (in a low-elevation desert), due to the severity of environmental conditions and lack of resources (e.g. low-density populations of mammalian prey, lack of water). During La Niña, mean levels of sex steroids in male *C. molossus* were higher than in the previous year, but this difference was not significant (Table 1).

Weather patterns can influence the general health and well being of terrestrial vertebrates, which in turn can affect year-to-year variation of steroid levels (Knapp et al. 2003). As a consequence, body condition (residual values from the regression of SVL and body mass) might have influenced between-year differences in levels of plasma sex steroids in *C. atrox*. In several studies, there was a tendency for male vipers with greater mass to have higher concentrations of plasma sex steroids (Bonnet & Naulleau 1996; Aubret et al. 2002; Schuett et al. 2002). In the present study, however, we found no significant correlations between body condition and levels of sex steroids of *C. atrox* or *C. molossus*. Similar to our results, Moore et al. (2000) reported that body condition and plasma T levels in male garter snakes (*Thamnophis* sp.) were not significantly correlated.

Species Differences in Levels and Seasonal Patterns of Sex Steroids

Accumulating research on snakes shows that species vary in their absolute levels of plasma sex steroids (Gorman et al. 1981), and perhaps most interesting are those differences between closely related congeners. For example, during the late summer mating season, peak levels of plasma T in males of the closely related North American pitvipers *Agkistrodon contortrix* (copperhead) and *A. piscivorus* (cottonmouth) differ significantly, with copperheads having mean levels that are up to three times greater (Schuett et al. 1997; Zaidan et al. 2003). Similarly, males of *C. atrox* and *C. molossus* differed in their levels of DHT and E2 ($P = 0.026$), and differences between T levels approached significance ($P = 0.060$). In all cases, levels of plasma sex steroids were higher in *C. atrox* than in *C. molossus*, and these differences might be attributable to experimental and/or biotic/abiotic factors. All *C. molossus* subjects represented in Fig. 2 were anaesthetized prior to blood collection, and the *C. atrox* represented in Fig. 1 were not anaesthetized. However, in a prior study that followed the same handling protocol as that used for *C. molossus* in the present study, there was no indication that levels of plasma T in male copperheads were compromised (Schuett et al. 1997).

Alternatively, differences in absolute levels of plasma steroids in *C. atrox* and *C. molossus* might result from some aspect(s) of the mating systems that are not equivalent, such as relative testis size (Andersson 1994). In a wide range of vertebrates, interspecific differences in mating systems are often correlated with differences in relative testis size (Dunn et al. 2001; Byrne et al. 2002; Gage & Freckleton 2003). Because levels of plasma steroids can positively covary with testis size in some species of vertebrates (Emerson 1997), this relationship should be explored in *C. atrox* and *C. molossus*, as well as other species of rattlesnakes.

Proximate and Ultimate Causes for Seasonal Patterns of Plasma Sex Steroids

Differences in the annual patterns of plasma sex steroids in male *C. atrox* and *C. molossus* do not appear to be caused by variation in their annual gametogenic cycle. Both *C. atrox* (Jacob et al. 1987) and *C. molossus* (Goldberg 1999) show aestival spermatogenesis (Goldberg & Parker 1975; Schuett 1992). With few exceptions, this pattern of spermatogenesis is the most prevalent in snake species from temperate regions (Saint Girons 1982), and the only one described for rattlesnakes and other species of pitvipers inhabiting temperate North America (Schuett 1992; Aldridge & Duvall 2002; Schuett et al. 2002). Unlike the sympatric rattlesnakes we studied, which have identical spermatogenic cycles, the sympatric vipers *V. aspis* and *V. berus* have contrasting ones. Whereas the annual spermatogenic cycle in *V. aspis* is very similar to the cycle in *C. atrox* and *C. molossus*, *V. berus* shows major spermatogenesis in spring several weeks prior to the mating season (Naulleau & Fleury 1984).

In both *C. atrox* and *C. molossus*, elevated levels of plasma sex steroids are congruent with peak spermatogenesis and sexual activity in late summer. In *C. atrox*, but not in *C. molossus*, elevated levels of sex steroids also occur in early spring during the second mating season, when meiotic activity is in its regressive stages. Because *C. atrox* and *C. molossus* are sympatric at our study sites, it is unlikely that ambient environmental factors (e.g. photoperiod, temperature, precipitation) drive the large differences in the timing and frequency of their mating seasons and sex steroid patterns. Alternatively, we suggest that the periods of sexual activity (behavioural, physiological) that we have observed in these species reflect the retention of historic patterns that had their origins and evolved in areas other than those reported in this study (Aldridge et al. 1990; Aldridge & Duvall 2002). Unravelling this complex problem will require comparative methods that explicitly incorporate phylogenetic hypotheses with information on ancestral areas, centres of origin and ancient ecosystems (Brooks & McLennan 1991; Martins 1996; Ebach 1999; Donoghue & Moore 2003).

Comparatively little has been published regarding the proximate control of male sexual behaviour in snakes, and most studies involve northern populations of garter snakes, *Thamnophis sirtalis* (Moore & Lindzey 1992). Unlike most other vertebrates, dependence on hormones to

elicit male sexual behaviour in *T. sirtalis* has not been unequivocally shown (Crews et al. 1984; Moore & Lindzey 1992; Krohmer 2004). Because we found a strong positive association between the expression of male sexual behaviour and elevated levels of sex steroids in *C. atrox* and *C. molossus*, our study provides initial evidence that the expression of sexual behaviour (courtship, coitus and male–male fighting) is hormonally dependent in these two species (Moore & Lindzey 1992). However, experimental protocols such as those outlined by Moore & Lindzey (1992) will be required to determine the exact roles of these steroids in mediating sexual behaviour.

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