The relationship between plasma steroid hormone concentrations and the reproductive cycle in the Northern Pacific rattlesnake, *Crotalus oreganus*

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A B S T R A C T

We describe the reproductive cycle of Northern Pacific rattlesnakes (*Crotalus oreganus*) by quantifying steroid hormone concentrations and observing reproductive behaviors in free ranging individuals. Addi tionally, we examined reproductive tissues from museum specimens. Plasma steroid hormone concentra tions were quantified for both male and female snakes throughout the active season (March October). We measured testosterone (T), 5α dihydrotestosterone (DHT), and corticosterone (B) concentrations in both sexes and 17β estradiol (E2) and progesterone (P) in females only. We observed reproductive behav iors (e.g., consortship, courtship, and copulation) in the field and measured testis and follicle size in male and female snakes from museum collections to relate steroid hormone concentrations to the timing of reproductive events. Our study revealed that *C. oreganus* in central California exhibits a bimodal pattern of breeding, with most mating behavior occurring in the spring and some incidences of mating behavior observed in late summer/fall. Each breeding period corresponded with elevated androgen (T or DHT) lev els in males. Testes were regressed in the spring when the majority of reproductive behavior was observed in this population, and they reached peak volume in August and September during spermato genesis. Although we did not detect seasonal variation in female hormone concentrations, some females had high E2 in the spring and fall, coincident with mating and with increased follicle size (indicating vitellogenesis) in museum specimens. Females with high E2 concentrations also had high T and DHT con centrations. Corticosterone concentrations in males and females were not related either to time of year or to concentrations of any other hormones quantified. Progesterone concentrations in females also did not vary seasonally, but this likely reflected sampling bias as females tended to be underground, and thus unobtainable, in summer months when P would be expected to be elevated during gestation. In females, P was positively correlated with T and DHT, and E2 was positively correlated with T.

1. Introduction

Hormones are major proximate factors mediating reproductive cycles and behaviors, and thus are targets of selection in the evolu tion of the diverse mating systems seen in vertebrates. It is there fore essential to gain an understanding of the diversity of hormonal profiles exhibited by vertebrate groups, because these patterns can be applied in future phylogenetic analyses, models, and other stud ies integrating ultimate and proximate causation in the evolution of mating systems in vertebrates (Duvall et al., 1992; Stamps, 1991). Snakes as a group exhibit a diversity of reproductive charac teristics: oviparity and viviparity, income and capital breeding, and mating tactics ranging from scramble competition and mating balls to searching for widely distributed mates (Duvall et al., 1992; Seigel and Ford, 1987). However, the roles of hormones in the diverse reproductive patterns observed in this group of ~3000 species are poorly understood and have been studied in a limited number of species (reviewed in Taylor and DeNardo, in press). The majority of research in this area has focused on one subspecies of garter snake, *Thamnophis sirtalis parietalis* (Krohmer, 2004; reviewed in Taylor and DeNardo, in press). Recently, researchers have investigated the relationships between reproduc tive events and steroid hormone concentrations in free ranging pitvipers (Viperidae, Crotalinae), primarily New World taxa, to bet ter understand the diverse reproductive cycles of snakes and the roles of hormones in vertebrate reproductive cycles. Because of their well studied phylogenies (e.g., Ashton and de Queiroz, 2001; Douglas et al., 2002; Murphy et al., 2002; Pook et al., 2000), diverse mating systems, and relative ease of study in the field (e.g., large bodied, abundant), certain species of pitvipers have emerged as models for studying the evolution of vertebrate

reproductive physiology (Almeida Santos et al., 2004; Beaupre and Duvall, 1998; Schuett et al., 2006). However, research on these organisms has focused chiefly on the role of androgens in male reproductive behavior with little work on females (but see Taylor et al., 2004).

This study examines the relationship between steroid hormone concentrations and events of the vertebrate reproductive cycle such as mating behavior, spermatogenesis, and vitellogenesis in the Northern Pacific rattlesnake, *Crotalus oreganus*. Temperate pitvipers, in general, display one of two patterns of mating and courtship behavior. Many species follow a bimodal pattern of breeding with mating occurring during late summer/fall and spring, whereas others show a unimodal pattern with a single breeding period in mid to late summer (reviewed in Aldridge and Duvall, 2002; Graham et al., 2008; Schuett, 1992). The timing of spermatogenesis in male temperate pitvipers appears to be fixed, with all species studied to date exhibiting peak spermatogenesis in fall (Aldridge and Duvall, 2002; Schuett, 1992). Species with a unimodal mating pattern are said to follow an "associated" breeding pattern, where reproductive behavior occurs concurrently with male gonadal activity (Crews, 1984; Saint Girons, 1982). However, in snakes displaying a bimodal pattern, the spring breeding period is "dissociated" from spermatogenesis. The dissociation of reproductive behaviors from spermatogenesis in bimodally breeding snakes has allowed examination of the role of hormones in male snakes during the spring breeding period independent of spermatogenesis (Crews, 1984; Saint Girons et al., 1993). Studies investigating this phenomenon in reptiles have yielded mixed results. In some species the spring breeding period coincides with elevated androgen levels and/or testicular hypertrophy (Krohmer et al., 1987; Schuett et al., 2005; Taylor et al., 2004). In others, testes are regressed and androgen concentrations are low or variable (Camazine et al., 1980; Licht, 1982). The timing of vitellogenesis is also variable in snakes. Most pitvipers initiate vitellogenesis in the fall, followed by a pause in follicular activity over winter, and a continuation and completion of vitellogenesis by the time of ovulation the following spring/summer (type II vitellogenesis, Aldridge, 1979). However, Taylor et al. (2004) showed that *Crotalus atrox* in the Sonoran Desert initiate and complete vitellogenesis in the spring of a reproductive year (type I vitellogenesis, Aldridge, 1979).

Knowledge of the reproductive cycle of the organism in a study investigating seasonal hormone concentrations and their relationship to reproduction is crucial because we expect to observe a relationship between reproductive pattern (e.g., unimodal vs. bimodal, type I vs. type II vitellogenesis) and seasonal steroid hormone profiles (Schuett et al., 2005). Estrogens, such as 17β estradiol (E2), stimulate reproductive behaviors and vitellogenesis in females of some reptile species, including vipers, and are expected to be elevated during these events (Bonnet et al., 1994; Callard et al., 1990; Ho et al., 1982). Species that use type I vitellogenesis are predicted to have elevated concentrations of E2 in the spring only (Taylor et al., 2004). Species that use the type II pattern, in contrast, are expected to have elevated levels of E2 in the fall and spring, but no pitviper species exhibiting type II vitellogenesis have previously been studied. Androgens, such as testosterone (T) and 5α dihydrotestosterone (DHT), stimulate reproductive behaviors and spermatogenesis in male reptiles (Moore and Lindzey, 1992; Norris, 1997). Testosterone and DHT are elevated during periods of spermatogenesis and mating in all pitvipers studied to date (Graham et al., 2008; Schuett et al., 1997, 2002, 2005; Taylor et al., 2004). Snakes following a bimodal pattern of breeding show two annual peaks in the concentrations of the hormones associated with sexual activity (i.e., T and DHT in males; Schuett et al., 1997, 2002, 2005; Taylor et al., 2004). Snakes demonstrating the unimodal pattern show one annual peak in plasma concentrations of these hormones (Graham et al., 2008; Johnson et al., 1982; Schuett et al., 2002; Zaidan et al., 2003).

In addition to E2, T, and DHT, corticosterone (B) and progesterone (P) also play important roles in vertebrate reproductive cycles. Corticosterone is elevated during reproductive events in many reptile species in order to mobilize energy stores when resources are limited (Moore and Jessop, 2003; Romero, 2002), so species that experience strong energy limitations during reproduction may have elevated B concentrations associated with reproductive events. In viviparous taxa, P is produced by the corpus luteum and placenta and has a role in maintaining pregnancy (Bonnet et al., 2001; Custodia Lora and Callard, 2002). Also, P is produced by the adrenal glands in male and non reproductive female garter snakes, *Thamnophis elegans* (Highfield and Mead, 1975), but its function in this species is not known.

Here we describe the relationship between steroid hormone concentrations and reproductive events in *C. oreganus* using three methods: (1) quantification of steroid hormone concentrations (B, DHT, and T in males; B, DHT, T, E2, and P in females) throughout the active season in free ranging snakes, (2) observation of reproductive behaviors in free ranging snakes, and (3) examination of the reproductive anatomy of preserved museum specimens.

2. Methods

2.1. Study site

This study was conducted on the northernmost portion of the Chimineas Ranch unit of the Carrizo Plain Ecological Reserve in the foothills of the Caliente Mountain range in central California (35°N, 119°W, 750 m altitude). This area represents the southern limit of the range of *C. oreganus*. Habitat is primarily oak savannah and grazed grasslands with prevalent rocky outcrops.

2.2. Study species and field procedures

Like many crotaline snakes, *C. oreganus* has male biased sexual size dimorphism (Shine, 1993). Because male and female *C. oreganus* reach sexual maturity by about 60 cm SVL (Diller and Wallace, 2002), only snakes greater than this size were included in this study. Male snakes in this study averaged 88.13 ± 1.9 (standard error of mean, SEM) cm in snout vent length (SVL) and 640.8 ± 42.6 g in mass ($n = 39$). Female snakes had an average SVL of 74.0 ± 1.6 cm and an average mass of 338.1 ± 23.0 g ($n = 19$).

We used a combined approach of radiotelemetry and mark recapture to study the seasonal reproductive behavior and physiology of *C. oreganus*. All snakes caught at the site received a 12 mm passive integrated transponder (PIT) tag (AVID, Norco, CA, USA). Acrylic paint was injected into the three proximal rattle segments to create a unique three color code for each snake allowing individual recognition in the field. Twenty snakes (10 males and 10 females) were implanted with 13 g radiotransmitters (SI 2T, Holohil, Carp, Ontario, Canada). Male snakes were implanted in late fall of 2006 and females in early spring of 2007. Snakes were located via radiotelemetry 1 4 times per week throughout the active seasons (the period during which above ground activity was observed, March through October) in 2007 and 2008, and in 2008 snakes were bled monthly when possible. Randomly encountered snakes ($n = 42$) also were bled. Blood was collected from the caudal vein using a Na^+ heparinized 1 cc syringe with a 25 gauge needle within 5 min of capture. Samples were kept refrigerated for up to 24 h, brought to the lab, and centrifuged to separate plasma from blood cells. These procedures were employed to ensure that steroid hormone levels were not altered by methods used to handle snakes

or samples (Schuett et al., 2004; Taylor and Schuett, 2004). Plasma samples were stored at −20 °C until radioimmunoassay.

Snakes were frequently inaccessible for blood sampling because they remained underground for long periods of time, a behavior likely intensified by extremely dry conditions in 2007 2008. In to tal, 58 blood samples were collected from 39 male snakes (27 from radio tagged males and 31 from randomly encountered males), and 41 blood samples were collected from 19 females (30 from radio tagged females and 11 from randomly encountered females).

2.3. Behavior

Reproductive behaviors were classified as one of three types of male female accompaniment: consortship, courtship, or copula tion. Consortship was defined as two snakes of the opposite sex found within one meter of each other without courtship or copula tion taking place. Snakes in this population do not overwinter in large communal dens, so snakes in such proximity were almost al ways males and females during the breeding season, and consort ships were never observed outside of the breeding seasons. Courtship in rattlesnakes is characterized by chin rubbing and/or intertwining of the animals' tails. Copulation was only recorded if cloacal penetration was observed. Male male combat is an addi tional reproductive behavior that occurs in pitvipers as males com pete for access to females (Andren, 1986; Carpenter, 1977). However, combat was not observed in this study.

2.4. Radioimmunoassay

Concentrations of steroids were measured by standard radioim munoassay (RIA) techniques following extraction and chromato graphic separation (Husak et al., 2007; Moore et al., 2000; Wingfeld and Farner, 1975). Based on a study on another rattle snake species (Taylor et al., 2004), we used 20 μl of plasma when we predicted steroid concentrations to be high, and we used 50 μl when we predicted steroid concentrations to be low. Male and female samples were run in separate assays, and B of females was determined in a separate direct assay without chromato graphic separation. For individual extraction efficiency determina tion, we equilibrated each sample overnight with 2,000 cpm of tritiated steroid. Each sample was extracted with 5 ml of distilled dichloromethane with the dichloromethane phase removed and dried in a warm water bath, under a stream of nitrogen gas, and re suspended in 10% ethyl acetate in isooctane. To remove neutral lipids and to isolate individual steroids, all samples were trans ferred to diatomaceous earth (Celite, Sigma) columns for chro matographic separation. For females, P, DHT, T, and E2 were eluted with 2 ml of 2%, 1.5 ml of 10%, 2 ml of 20%, and 2.5 ml of 40% ethyl acetate in isooctane, respectively, and saved. For males, neutral lipids and other steroids were eluted with 2 ml of isooctane and discarded. Dihydrotestosterone, T, and B were eluted with 1.5 ml of 10%, 2 ml of 20%, and 2.5 ml of 50% ethyl acetate in isooc tane, respectively, and saved. After this, samples were dried in a 40 °C water bath under nitrogen gas, re suspended in 600 μl phos phate buffered saline, and maintained overnight at 4 °C. For the di rect B assay of female samples, samples were extracted with 5 ml of distilled dichloromethane and re suspended in 600 μl phos phate buffered saline, and maintained overnight at 4 °C. The remainder of all assays was similar. Individual extraction efficiency for each steroid (mean recoveries were 29% for P4; 50% and 43% for DHT of males and females, respectively; 68% and 66% for T of males and females, respectively; 53% for E2; 58% and 91% for B of males and females, respectively) was determined from 100 μl of the sam ple while 200 μl of the sample was allocated to each of two dupli cates for the assay. Serial dilutions for the standard curves were performed in triplicate (range of curves: P4, 1000 2 pg; DHT, T, and E2, 500 1 pg; B, 2000 4 pg). All samples were incubated over night with 100 μl of antiserum (P4: P 1604, Wien Laboratories, Flanders, NJ 07876; DHT and T: T 3003, Wien Laboratories, Suc casunna, NJ 07876; E2: Biogenesis, Poole, England; B: Esoterix Endocrinology, Calabasas Hills, CA 91301) and 100 μl of tritiated steroid. Unbound steroid was separated using dextran coated charcoal and the bound steroid decanted into scintillation vials. Samples were counted on a liquid scintillation counter and final concentrations corrected for individual extraction efficiency. Aver age intra assay coefficients of variation (CV) for females were 6% for P4, 16% for DHT, 23% for T, 17% for E2, and 23% for B. Average intra assay CVs for males were 11% for DHT, 3% for T, and 13% for B.

2.5. Data analysis: male hormones

Hormone concentrations in male snakes were analyzed in two ways: by collapsing the data into seasons and by comparing mean monthly hormone concentrations. For the former analysis, we par titioned the active period into three seasons, early/spring (March April), middle/summer (May June), and late/fall (July September) following Aldridge (2002). We did this to relate seasonal trends in hormones to the timing of spermatogenesis reported in Aldridge (2002), but these divisions also roughly reflect seasonal changes in behaviors (e.g., reproductive behaviors, habitat use, movement frequency) observed in this population of snakes. The mean con centration of each hormone (B, DHT, and T) in each season was compared with ANOVA or ANCOVA (see below) using all samples collected. Some males, especially radio tagged individuals, were represented in multiple seasons. However, the paucity of recap tures prevented the use of a repeated measures analysis (i.e., only two snakes were represented in all of the three seasons). This data analysis ("full seasonal data set") has the advantage of including all data points collected, but is problematic due to pseudoreplication introduced by the fact that some of the samples were from the same male and therefore were not independent. To address this potential problem, repeated measures on snakes sampled in multi ple seasons were discarded, reducing the sample size for statistical analysis from 58 to 39 samples. Data were discarded in the follow ing manner to maintain sufficient sample sizes in each of the three seasons. The fewest samples were obtained in fall due to the lack of surface activity, so fall samples were preferentially selected over summer and spring samples when snakes were repeatedly mea sured (e.g., we included the data from a snake sampled in fall and discarded any spring or summer data from that snake). Sum mer had the next lowest sample size, so spring samples were thrown out in favor of summer samples when the same snake was sampled in both seasons. Although this method of reducing data is not random, it does not bias results because samples were not selected based on hormone concentrations. By discarding data in this way, we achieved a data set that was smaller but did not suffer from pseudoreplication ("reduced seasonal data set"). The analysis on the full and reduced seasonal data sets resulted in the same conclusion for each hormone (see Results), indicating that the inclusion of pseudoreplicates did not dramatically affect the results.

Mean monthly hormone concentrations were compared using the full data set. It was not possible to throw out data to eliminate pseudoreplication in this analysis, as the sample sizes for each month would have been too low. Data used for this analysis are hereafter referred to as the "full monthly data set."

All analyses were conducted using Minitab statistical software (version 15) at an alpha level of 0.05. To satisfy the assumptions of normality and homogeneity of variance, B concentrations were log transformed, DHT concentrations were ln transformed, and T concentrations were square root transformed. Because B and T

concentrations were positively correlated with SVL (B: $r = 0.371$, $p = 0.004$; T: $r = 0.370$, $p = 0.004$), SVL was used as a covariate in the analyses. For B and T, data were analyzed with ANCOVA using a general linear model with season or month as a fixed factor, SVL as a covariate, and transformed hormone concentration as the dependent variable. For all ANCOVAs, linear relationships existed between the covariate and dependent variable (hormone concentration). DHT was not correlated with SVL ($r = 0.197$, $p = 0.138$), and results were analyzed by one way ANOVA. All pair wise comparisons were run using a Tukey honestly significant difference test (HSD). In addition, Pearson correlation analysis was used to evaluate correlations among concentrations of the three hormones.

2.6. Data analysis: female hormones

For females, there were too few samples to make monthly comparisons, or to use a reduced data set lacking pseudoreplicates. For these reasons, only the full seasonal female data set was analyzed. Testosterone, DHT, and P were correlated with SVL (T: $r = -0.474$, $p = 0.002$; DHT: $r = -0.559$, $p < 0.001$; P: $r = -0.559$, $p = 0.001$) and were analyzed using ANCOVA with SVL as the covariate. For all ANCOVAs, linear relationships existed between the covariate and dependent variable (hormone concentration). Testosterone concentrations were log transformed, and DHT and P concentrations were square root transformed to satisfy ANCOVA assumptions. 17β estradiol and B were not correlated with SVL and were therefore analyzed without SVL as a covariate. Corticosterone concentrations were square root transformed to meet ANOVA assumptions, and were analyzed by one way ANOVA. We were unable to meet the assumption of normality for E2 data, so seasonal E2 concentrations were analyzed using a Kruskal Wallis test. In addition, Pearson correlation analysis was used to evaluate correlations among female hormone concentrations.

2.7. Reproductive tissues

Reproductive tissues (testes and follicles) were examined in museum specimens ($N = 57$ males; $N = 45$ females) and in four road killed specimens collected near the field site (0 males, four females). Museum specimens examined are listed in Appendix A. Some museum specimens were road killed, while some were collected alive and preserved. Any specimens labeled as having been kept in captivity were not included in the study. All museum specimens examined were from California, and almost half (49%) of the snakes sampled were from San Luis Obispo County. An incision was made on the ventral surface of each snake from mid body to the region just posterior to the gonads. For males, left testis length, width, and height were measured with digital calipers (the left testis was chosen because many of the specimens were missing the right testis, presumably removed for another study) to calculate left testis volume. Females were evaluated for reproductive state by measuring the length of the largest follicle present. Follicles smaller than 5 mm in length (pre vitellogenic) were grouped as <5 mm.

2.8. Data analysis: museum specimens

Left testis volume (LTV) was square root transformed to meet the assumptions of the ANCOVA. Because LTV was strongly correlated with SVL, data were analyzed by ANCOVA with season or month as a fixed factor, SVL as a covariate, and LTV as the dependent variable. There was a linear relationship between SVL and LTV. Secondary vitellogenesis was identified by the presence of enlarged (>10 mm) follicles in female ovaries. Rahn (1942) described follicles during primary vitellogenesis (non reproductive) of a closely related rattlesnake species, *C. viridis*, as 4 6 mm in length. Follicles greater than 6 mm in length are likely to have entered secondary vitellogenesis (Aldridge, 1979). In order to ensure a correct diagnosis of fall vitellogenesis, we considered follicles over 10 mm in length to have entered secondary vitellogenesis.

3. Results

3.1. Seasonal behavior

Consortships were observed on 27 occasions, all in spring or fall, with 67% of accompaniments observed in April (Fig. 1). Courtship behaviors were observed on two occasions in April, and copulation was observed once in September. Appendix B lists the dates of all observed accompaniments.

3.2. Male hormone data

Mean seasonal hormone levels (T, DHT, and B) are presented in Fig. 2. Descriptive statistics are presented in Table 1. Statistical results for each hormone are in the following sections.

3.2.1. Testosterone

There was a significant effect of season on T concentrations both in the full seasonal data set ($F_{2,56} = 13.06$, $p < 0.001$) and in the reduced seasonal data set ($F_{2,37} = 9.86$, $p < 0.001$). Tukey HSD tests on both the full and reduced seasonal data sets revealed that fall T concentrations were significantly higher than both spring and summer, which were not significantly different from each other. The full monthly analysis revealed a significant effect of month ($F_{6,52} = 5.15$, $p < 0.001$). Post hoc analyses showed that T concentrations in August were significantly higher than in April, May, and June. All other pair wise comparisons were non significant.

3.2.2. Dihydrotestosterone

There was a significant effect of season on DHT concentrations both in the full seasonal data set ($F_{2,56} = 6.14$, $p = 0.004$) and in the reduced seasonal data set ($F_{2,37} = 4.32$, $p = 0.021$). Tukey HSD tests on both the full and reduced seasonal data sets showed that DHT concentrations were significantly lower in summer than in fall or spring, which were not significantly different from each other. There was also an effect of month on DHT concentrations ($F_{6,52} = 2.47$, $p = 0.036$). However, a Tukey HSD test did not indicate any

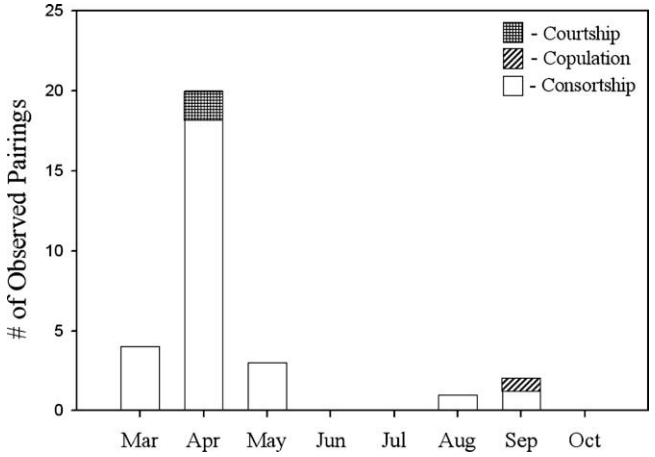

Fig. 1. Observed pairings of male and female *Crotalus oreganus* during the 2007 and 2008 active seasons. Sixty seven percentage of observed pairings occurred in April. Two courtship events were observed in April, and one copulation was observed in September.

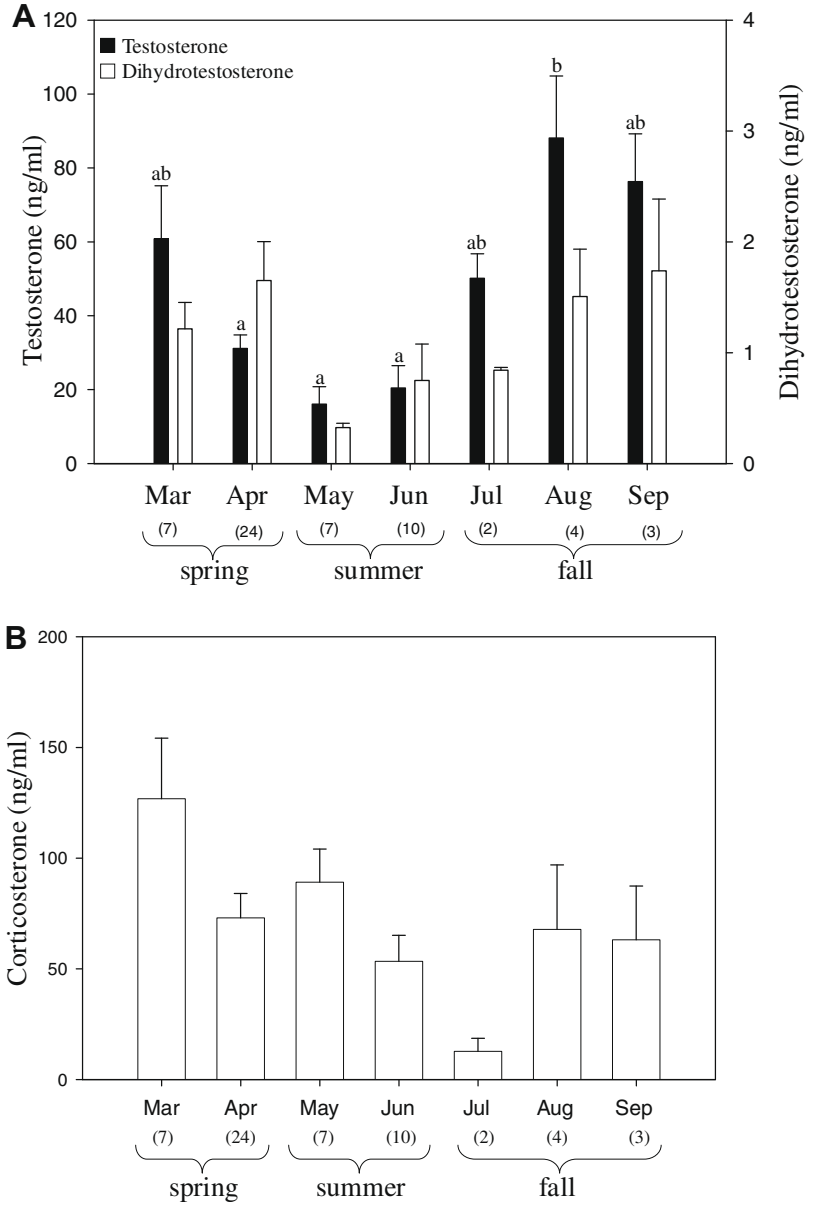

Fig. 2. Mean monthly plasma hormone concentrations in male *Crotalus oreganus*. Sample sizes are in parentheses. (A) Plasma T and DHT concentrations. Plasma T concentrations were significantly higher in fall than in summer or spring. Plasma DHT concentrations were significantly higher in spring and fall than in summer. Only T concentrations were significantly different when analyzed by month. Months with significantly different T concentrations are assigned different letters. (B) Plasma B concentrations. There were no significant seasonal or monthly differences in B concentrations.

Table 1
Mean plasma hormone levels of *Crotalus oreganus* in ng/ml (±SEM). Months where no snakes were sampled or hormones were not quantified are marked with "–."

	Sex	*n*	Testosterone	DHT	Corticosterone	17β-estradiol	Progesterone
March	Male	9	60.8 ± 14.4	1.2 ± 0.2	126.8 ± 27.4	–	–
	Female	5	0.2 ± 0.02	0.3 ± 0.03	55.7 ± 20.3	0.2 ± 0.03	0.6 ± 0.1
April	Male	24	31.1 ± 3.7	1.7 ± 0.4	73.0 ± 11.0	–	–
	Female	10	0.6 ± 0.1	0.7 ± 0.1	76.8 ± 19.6	10.0 ± 8.3	1.2 ± 0.1
May	Male	6	16.1 ± 4.7	0.3 ± 0.04	89.1 ± 15.0	–	–
	Female	11	0.9 ± 0.3	0.7 ± 0.03	78.8 ± 19.5	8.1 ± 6.1	1.2 ± 0.1
June	Male	10	20.5 ± 6.0	0.8 ± 0.3	53.4 ± 11.7	–	–
	Female	5	0.2 ± 0.03	0.3 ± 0.04	59.3 ± 39.9	0.2 ± 0.02	0.6 ± 0.04
July	Male	2	50.1 ± 6.6	0.8 ± 0.03	12.7 ± 5.9	–	–
	Female	2	0.2 ± 0.002	0.3 ± 0.02	50.7 ± 11.5	0.2 ± 0.1	0.7 ± 0.04
August	Male	4	88.1 ± 16.8	1.5 ± 0.4	67.8 ± 29.2	–	–
	Female	2	0.2 ± 0.02	0.3 ± 0.03	19.4 ± 5.0	0.2 ± 0.01	0.7 ± 0.03
September	Male	3	76.3 ± 12.9	1.7 ± 0.7	63.1 ± 24.2	–	–
	Female	0	–	–	–	–	–
October	Male	0	–	–	–	–	–
	Female	4	0.8 ± 0.5	1.2 ± 1.0	28.2 ± 12.3	17.6 ± 11.7	2.0 ± 1.4

significant difference between any pair of months. There was a positive correlation between T and DHT (Table 2).

3.2.3. Corticosterone

There was no significant effect of season on B concentrations (full seasonal data set: $F_{2,56} = 0.75$, $p = 0.48$; reduced seasonal data set: $F_{2,37} = 0.71$, $p = 0.49$). The full monthly analysis also showed no significant variation ($F_{6,52} = 1.33$, $p = 0.26$). There was no significant correlation between B and T or DHT (Table 2).

3.3. Male testis volume

ANCOVA results indicate a significant effect of season ($F_{2,57} = 3.47$, $p = 0.038$) and month ($F_{7,52} = 2.51$, $p = 0.028$) on LTV (Fig. 3). Seasonal post hoc analysis showed that fall LTV was significantly higher than in summer and spring, which were not significantly different from each other. Tukey HSD tests revealed that LTV in September was significantly higher than in April or May. All other pair wise comparisons were not significant.

3.4. Female hormones

We did not detect seasonal variation in concentrations of any of the sampled hormones in females (DHT: $F_{2,37} = 0.56$, $p = 0.58$; T: $F_{2,37} = 1.0$, $p = 0.78$; P: $F_{2,37} = 0.51$, $p = 0.61$; B: $F_{2,37} = 1.27$, $p = 0.29$; E2: H = 1.05, $p = 0.59$, Fig. 4). It is important to note that these analyses likely included both reproductive and non reproductive females, and there was large variation in E2, T, DHT, and P concentrations within each season. Results of pair wise correlations between each hormone indicated positive correlations between T and DHT ($r = 0.7833$, $p < 0.001$), T and E2 ($r = 0.51$, $p < 0.001$), T and P ($r = 0.72$, $p < 0.001$), and DHT and P ($r = 0.97$, $p < 0.001$). All other correlations were non significant (Table 2).

Table 2
Correlation tables for all pair-wise comparisons of hormone concentrations in (A) male and (B) female *Crotalus oreganus*. Significant correlations are highlighted in bold.

Hormone 1	Hormone 2	*R*	*P*
A			
T	**DHT**	**0.748**	**<0.001**
T	B	0.036	0.791
DHT	B	−0.107	0.423
B			
E2	DHT	0.14	0.40
T	**DHT**	**0.78**	**<0.001**
T	**E2**	**0.51**	**0.001**
P	DHT	0.97	<0.001
P	E2	0.11	0.51
P	**T**	**0.72**	**<0.001**
B	DHT	0.002	0.99
B	E2	0.11	0.49
B	T	0.07	0.65
B	P	−0.05	0.79

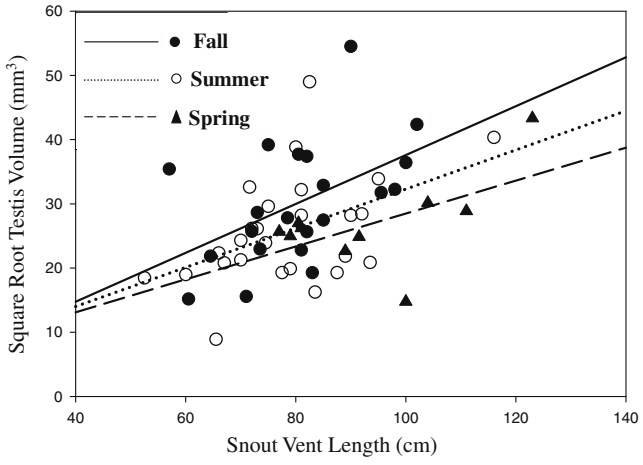

Fig. 3. Left testis volume (LTV) plotted against snout-vent length (SVL) in each season in *Crotalus oreganus* museum specimens. Fall LTV was significantly greater than both summer and spring LTV, which were not significantly different from each other.

3.5. Female follicle size

Vitellogenic follicles or embryos (>10 mm) were present in specimens collected in the months of March, April, May, June, July, September, and October (Fig. 5). The presence of follicles that had entered secondary vitellogenesis in the late summer and fall is indicative of Type II vitellogenesis.

4. Discussion

Crotalus oreganus in central California show a bimodal pattern of mating, with reproductive behaviors and elevated androgen levels occurring in both late summer/fall and spring, a pattern concurrent with other bimodally breeding pitvipers (*Agkistrodon contortrix*, Schuett et al., 1997; *C. scutulatus*, Schuett et al., 2002; *C. atrox*, Taylor et al., 2004). In general, the two androgens studied show similar patterns, although T concentrations were always much higher than DHT concentrations in male snakes. In *C. oreganus*, T and DHT are elevated in the fall, coinciding with spermatogenesis and mating behavior (Aldridge, 2002; Fitch, 1949; Hersek et al., 1992). In basking *C. atrox* in Arizona during winter, T concentrations were lower than fall or spring but remained elevated above summer baseline concentrations (Schuett et al., 2006). It is possible that the same phenomenon occurs in *C. oreganus*, but we were unable to sample the snakes because they remained underground throughout the winter. The majority of observed reproductive behavior in this population occurred in spring, when T concentrations were not significantly elevated above levels quantified outside the breeding season. It is noteworthy that four of the seven males sampled in March had very high T concentrations (>80 ng/ml). Testosterone concentrations in this month were highly variable among individuals, and this variation likely led to a lack of a statistically significant difference in T concentration between spring and summer months. Highly variable T concentrations in March could have been the result of many factors, as there are numerous potential causes of variation in plasma T concentration in vertebrates, including time of day, genetic effects, and social context (reviewed in Kempenaers et al., 2008). These factors were not controlled for in this study and might have been responsible for the individual variation in T concentrations measured in spring. Interestingly, T did not approach peak concentrations in any males sampled during the peak month of breeding (April). Concentrations in April were elevated, but were significantly lower than concentrations during spermatogenesis in August. This supports the "conditioning" role of testosterone (Crews, 1991; Naulleau et al., 1987; Saint Girons et al., 1993), in which T prepares a snake for the act of mating, but is not necessarily circulating at peak levels at the time that mating occurs. Dihydrotestosterone concentrations, in contrast, were elevated in both spring and fall, and peak concentrations coincided with the month of peak reproductive behavior (April). Dihydrotestosterone is elevated during the breeding season in all pitvipers (rattlesnakes) in which levels of this hormone have been measured (*C. scutulatus,* Schuett et al., 2002; *C. atrox,* Schuett et al., 2005; *C. molossus,* Schuett et al.,

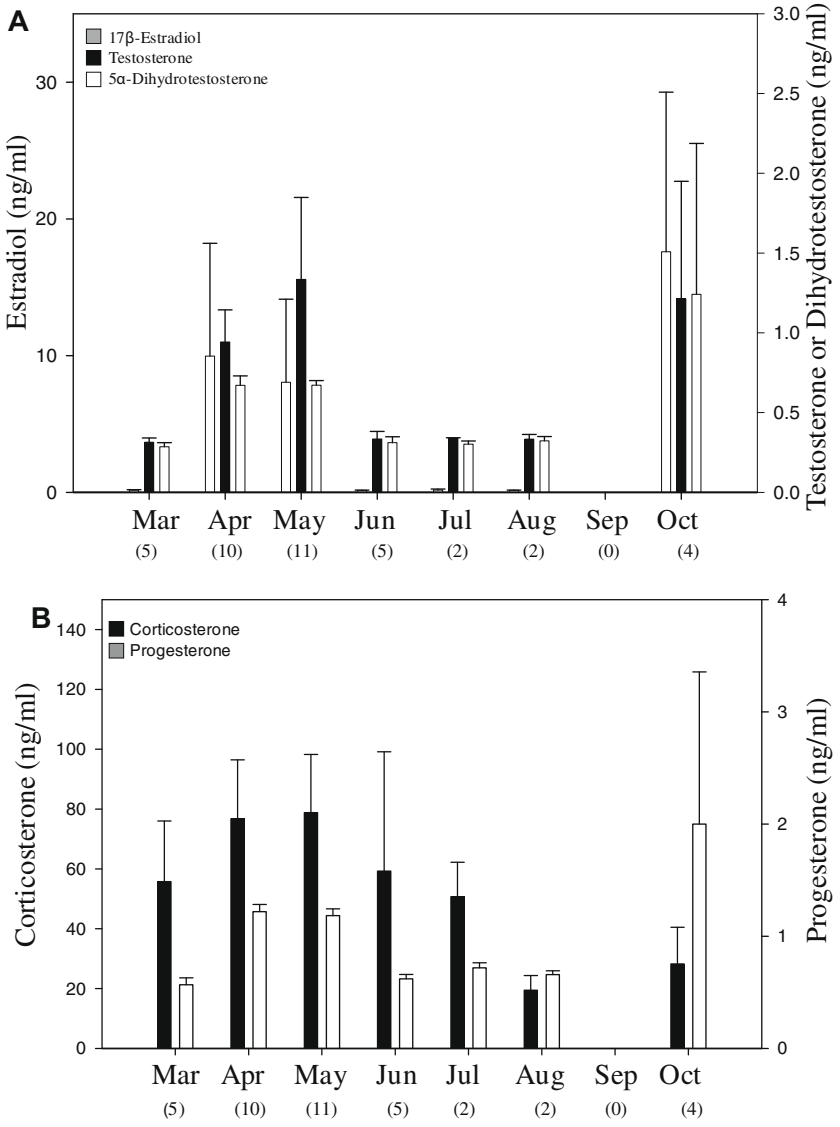

Fig. 4. Mean monthly plasma hormone concentrations in female *Crotalus oreganus*. There were no diagnosable seasonal trends in the concentrations of these hormones. (A) Plasma E2, T, and DHT concentrations. (B) B and P concentrations.

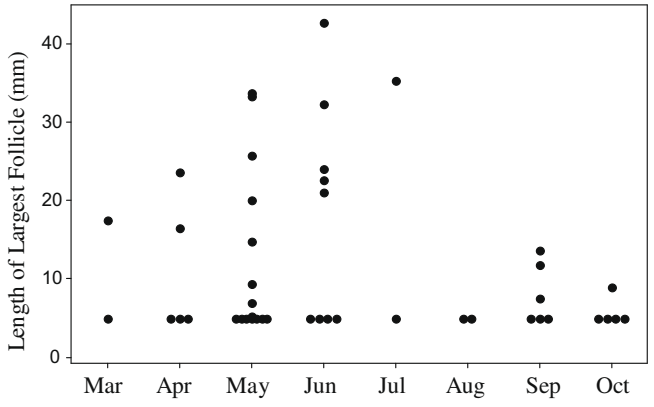

Fig. 5. Length of the largest follicle measured in female *Crotalus oreganus* museum specimens. All follicles <5 mm in length are shown as 5 mm. Vitellogenesis begins in fall and continues through the following spring.

2005). It is possible that DHT plays a more direct role in the initiation and/or control of reproductive behaviors than T.

Our results for plasma B concentrations in male snakes are similar to those reported for *C. atrox* in the Sonoran Desert (Taylor et al., 2004). There was no correlation between B and androgens (T and DHT), and there were no seasonal or monthly patterns in B concentrations. In contrast, in many reptiles B follows a seasonal cycle, with elevated concentrations occurring at the time of breeding Romero, 2002). The energy mobilization hypothesis (EMH) suggests that peak B levels coincide with the most energy limited (i.e., low availability of prey and/or high energy expenditure) time of year. Because reproduction requires a significant investment of energy, the breeding season is often the most energy limited time of the year and thus is characterized by elevated B concentrations in many reptiles (Moore and Jessop, 2003; Romero, 2002). Corticosterone levels in male *C. oreganus* were not elevated during either breeding season, corroborating studies by Taylor et al. (2004) and Graham et al. (2008) suggesting that male temperate pitvipers may not be subject to the energy limitations experienced by many reptile species during reproduction. Temperate pitviper species generally have low standard metabolic rates compared to most other reptiles and have large fat reserves, which may allow them to fuel the events of reproduction without initiating an adrenal

response (Andrews and Pough, 1985; Taylor et al., 2004; Tinkle, 1962).

No significant seasonal differences in hormone concentrations were detected in females. We expected to follow radio tagged females through their reproductive cycles to observe changes in steroid hormones; however, most of the females retreated into underground burrows early in the summer and remained there for most of the duration of the study, a behavior perhaps exacerbated by the extremely dry conditions. We therefore obtained samples unevenly from radio tagged females and from randomly encountered females, and had a very low sample size especially in the summer and fall. We were unable to determine the reproductive condition of females sampled during the year of the study, so were therefore unable to separate females into reproductive or non reproductive groups (as in Taylor et al., 2004). Because females were lumped into a single group, we observed high interindividual variation in the concentrations of several hormones. In particular, several females showed very high levels of E2 during the spring and fall breeding seasons, also coincident with the months in which vitellogenesis occurs in this population (as evidenced by our examination of female museum specimens). These females likely proceeded to reproduce but we were unable to confirm this. 17β estradiol directly stimulates the synthesis of vitellogenin in the reptilian liver and is most likely elevated to perform its role in vitellogenesis (Callard et al., 1990). However, E2 may also play a role in female receptivity and estrous behavior (Rhen and Crews, 2000; Whittier and Tokarz, 1992).

The same females that showed elevated E2 in spring and fall also showed elevated DHT and T at these times. This study is the first to quantify DHT in addition to T concentrations in female free ranging pitvipers. Our results indicate a possible role of T and DHT in reproductive behavior and/or vitellogenesis, as peak androgen levels were recorded during these events. However, because these events occur at the same time in this population of *C. oreganus*, it is not possible to ascertain the function of these hormones without experiments. The roles of T and DHT in the female cycle of snakes, and female vertebrates in general, are poorly understood (Staub and De Beer, 1997). Saint Girons et al. (1993) recorded elevated DHT concentrations in estrous aspic vipers (*V. aspis*), suggesting that DHT may be involved in stimulating female reproductive behavior. Testosterone is elevated during vitellogenesis in some snake species, but its role as a precursor to E2 is a confounding factor (Bona Gallo et al., 1980; Taylor et al., 2004; Whittier et al., 1987). Dihydrotestosterone, however, is a non aromatizable androgen, eliminating the confounding effect of a precursor product relationship with E2. A review of the literature on the role of androgens in vertebrate reproduction suggests that androgens indeed play a role in both the development and regulation of the female reproductive system (Staub and De Beer, 1997), but uncovering the exact role of these hormones in *C. oreganus* requires further study.

Most female snakes sampled had very low concentrations of P and B. The only other study to measure these hormones in free ranging female pitvipers showed that these hormones are elevated during gestation in summer (Taylor et al., 2004). In this study, we likely did not obtain samples from pregnant females at this time because they were in underground burrows throughout gestation. However, the female samples (mostly from spring) revealed several interesting relationships between P and other hormones. Progesterone concentrations were correlated with T and DHT concentrations. This correlation might have resulted from the fact that P is an intermediate in the synthesis of androgens in vertebrates (Slaunwhite and Samuels, 1956). The correlation between DHT and P in particular was highly significant, and warrants further investigation of any functional significance of this correlation. 17β estradiol is positively correlated with T, which could be due to a precursor product relationship, as E2 is derived from T via aromatization.

Aldridge (2002) showed that *C. oreganus* at the southern end of their range undergo spermatogenesis in the late summer and fall, a pattern observed in all other pitvipers studied (Aldridge and Duvall, 2002). Examination of male museum specimens revealed that *C. oreganus* exhibit hypertrophy of the testes coincident with spermatogenesis in the late summer and fall. Testes were not hypertrophied during the spring breeding season. Left testis volume was at its lowest in the spring, when some males have high levels of circulating androgens and when the majority of reproductive behavior was observed. This result is in contrast to a study on another rattlesnake species with a bimodal mating system and androgen profile, *C. scutulatus* (Schuett et al., 2002), which reported increased testis length and mass in both spring and late summer/fall coincident with elevated androgen concentrations (Schuett et al., 2002). Studies on other reptiles including snakes, turtles, and the American alligator have shown that elevated androgen concentrations and reproductive behavior do not always coincide with testicular hypertrophy. In the American alligator, *Alligator mississipiensis*, testicular mass is not related to plasma T concentrations in the late summer breeding season (Lance, 1989). In the softshell turtle, *Trionyx sinensis*, and the red sided garter snake, *Thamnophis sirtalis parietalis*, spring breeding is coincident with low or variable T concentrations and regressed testes (Camazine et al., 1980; Licht, 1982). These studies suggest that the breeding season and androgen production can be temporally separated from testicular recrudescence. However, in snakes with a bimodal mating season, too few studies on testis size with known steroid hormone profiles are available for further evaluation of this phenomenon.

In conclusion, *C. oreganus* at the southern limit of their range demonstrate a bimodal breeding pattern and type II vitellogenesis. In the fall, males have hypertrophied testes and undergo spermatogenesis (Aldridge, 2002), and exhibit reproductive behaviors and elevated concentrations of both major androgens. Another study observed agonistic behavior between males during the fall breeding season in *C. oreganus* (Hersek et al., 1992). During spring, the testes are regressed, DHT concentrations are elevated, and T concentrations are variable, with several snakes displaying extremely high T concentrations in March. Male *C. oreganus* thus exhibit "associated" reproduction (Crews, 1984) in the fall breeding season because reproductive behaviors occur concurrently with elevated spermatogenic activity and circulating androgen concentrations. In spring, reproductive behaviors also occur concurrently with elevated androgen concentrations but are "dissociated" from peak spermatogenesis. It is apparent that many temperate pitviper species, including *C. oreganus*, may not fall neatly into the associated dissociated paradigm (Schuett et al., 2006). Spermatogenesis occurs throughout the active season, peaking in fall along with testis volume and androgen concentrations, but peak reproductive behaviors are nonetheless observed in the spring. Corticosterone concentrations were not elevated in males or females during either breeding season, suggesting that an adrenal response is not necessary during the breeding seasons of this population. Female *C. oreganus* exhibit reproductive behaviors and initiate vitellogenesis in late summer/fall and spring. During these seasons, several females had high E2, T, and DHT concentrations, and these females likely went on to reproduce. These hormones have all been implicated in the stimulation of reproductive behavior and/or vitellogenesis in female reptiles. Although our descriptive study does not identify causal factors underlying the observed relationships, it adds to the growing body of literature on the interplay between steroid hormones and the events of reproduction in wild populations of vertebrate animals. Experiments will identify the specific roles that these hormones play as proximate mediators of the events of the reproductive cycle in vertebrates.

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Appendix A

List of catalogue numbers of museum specimens of *Crotalus oreganus* examined in this study. Museum names are abbreviated as follows: Museum of Vertebrate Zoology at the University of Cal ifornia at Berkeley (MVZ), University of California at Santa Barbara Museum (UCSB), and Santa Barbara Natural History Museum (SBNHM). (A) Male snakes examined. (B) Female snakes examined.

MVZ: (A) 193428, 28218, 28214, 56723, 35466, 24838, 29281, 21917, 18539, 17585, 5329, 28772, 29335, 62064, 62068, 81066, 24254, 12364, 92685, 228714, 18407, 21381, 24252, 2777, 2775, 14597, 3799, 3800, 16462, 31841, 25321, 16464, 191378, 249868, 215726, 191407. (B) 57076, 58265, 2781, 41172, 24860, 229507, 170801, 249869, 179969, 215727, 191390, 191413, 191406, 24253, 21380, 21382, 83653, 17955, 16855, 62067, 193429.

UCSB: (A) 206223, 30722, 23178, 23170, 11370, 11500, 11502, 11501, 15564, 15563, 15547, 11368, 15779, 20064, 20062, 19951, 15561, 13504, 13502, 14735, 14319, 14318, 16022. (B) 2777, 2772, 34111, 147963, 192218, 79235, 17572, 18945, 64147, 56724, 44905, 6845, 6841, 56722, 19365, 12181, 14317, 15546, 13501, 14737, 14734.

SBNHM: (A) 2346. (B) 983, 910, 1351.

Appendix B

Dates of all observed pairings for the 2007 and 2008 active sea son of *Crotalus oreganus*. Copulations and courtships are high lighted in bold. The third column, titled "Contact", indicates whether the pairing involved physical contact between the pair. The consortship marked with a * represents an occasion where a male was pulled out of a burrow with a hemipenis fully everted. A radio tagged female was tracked to the same burrow.

Date	Behavior	Contact
3/11/2007	Consortship	No
3/24/2007	Consortship	Yes
4/6/2007	**Courtship**	Yes
4/7/2007	Consortship	No
5/4/2007	Consortship	Yes
4/7/2007	**Courtship**	Yes
4/1/2007	Consortship	Yes
3/23/2008	Consortship	Yes
3/24/2008	Consortship	No
4/5/2008	Consortship	No
4/5/2008	Consortship	Yes
4/5/2008	Consortship	No
4/5/2008	Consortship	No
4/6/2008	Consortship	Yes
4/6/2008	Consortship	No
4/12/2008	Consortship	No
4/12/2008	Consortship	Yes
4/12/2008	Consortship	Yes
4/19/2008	Consortship	No
4/19/2008	Consortship	No
4/19/2008	Consortship	Yes
4/26/2008	Consortship	No
4/26/2008	Consortship	Yes
4/26/2008	Consortship	Yes
5/2/2008	Consortship	Yes*
5/3/2008	Consortship	Yes
8/24/2008	Consortship	No
9/15/2008	Consortship	No
9/15/2008	**Copulation**	Yes

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