MATING SYSTEM OF MALE MOJAVE RATTLESNAKES (*CROTALUS SCUTULATUS*): SEASONAL TIMING OF MATING, AGONISTIC BEHAVIOR, SPERMATOGENESIS, SEXUAL SEGMENT OF THE KIDNEY, AND PLASMA SEX STEROIDS

GORDON W. SCHUETT[1,2], SHARON L. CARLISLE[3], ANDREW T. HOLYCROSS[4], JACK K. O'LEILE[3], DAVID L. HARDY, SR.[5], EDWARD A. VAN KIRK[6], AND WILLIAM J. MURDOCH[6]

ABSTRACT: The mating system of free-ranging male Mojave Rattlesnakes (*Crotalus scutulatus*) from several populations in Arizona is described. Specifically, we report on seasonal timing of mating, male agonistic behavior, gross and cytological changes of the testis (including spermatogenesis) and kidney (including the sexual segment), and patterns of plasma sex steroids (testosterone, T; 5α-dihydrotestosterone, DHT; and17ß-estradiol, E2) during the active season (March–September). Mating and male-male agonistic behavior occurred in two discrete periods: from late summer to early autumn (July–September, the monsoon season), and early to mid-spring (March–May). Gross morphology of the testis and kidney showed seasonal trends coincident with periods of sexual activity. Histological analyses showed that the seasonal cycle of the testis and sexual segment of the kidney are similar to those of other temperate snakes, including pitvipers and other rattlesnakes. Spermatogenesis is the aestival pattern (Type I), and has been described for other pitvipers, including rattlesnakes. Changes in the sexual segment of the kidney were coincident with changes of the testis. Levels of plasma sex steroids (T, DHT, E2) showed seasonal patterns, and peak levels of all three steroids were associated with the timing of sexual activity and/or activities of the testis (spermatogenesis) and kidney (sexual segment). We present a heuristic phylogenetic analysis of mating patterns in rattlesnakes (*Crotalus* and *Sistrurus*), utilizing a newly-constructed mtDNA-based phylogeny (Murphy et al. (this volume) and recent syntheses of pitviper mating seasons (Schuett, 1992; Aldridge and Duvall, 2002).

INTRODUCTION

Our theoretical and empirical understanding of the evolution of animal mating systems has broadened considerably in the past 20 years, and recent trends show assurance of continued expansion and maturation of this field (Andersson, 1994; Arnold and Duvall, 1994; Reynolds, 1996; Birkhead and Møller, 1998; Gibbs and Weatherhead, 2001; Avise et al., 2002). Modern comparative studies of mating systems have integrated complex modeling (microevolutionary models), phylogeny (character correlation analyses), genetics (microsatellite DNA markers), and physiology (neuroendocrine analyses) to gain further insights into the origin, evolution, and maintenance of the various types of mating systems. In vertebrates, studies include microsatellite analysis of maternity assignment in syngnathid fishes (Jones and Avise 2001), female mating behavior in lek-breeding birds (Semple et al., 2001), mating seasons and mate-searching polygyny in male vipers (Duvall et al., 1992, 1993; Duvall and Beaupre, 1998; Aldridge and Duvall, 2002), and gene-hormone-behavior interactions in gartersnake families (King, 2002).

Field studies of reptilian mating systems, particularly in snakes, lag behind those of other vertebrates (see Duvall et al., 1992, 1993; Gans and Crews, 1992; Seigel, 1993; Shine and Bonnet, 2000). There is, however, a contingent of emerging studies resulting from recent technological advances in radiotelemetry and GIS analysis, as well as DNA-based techniques in parental assignment (Duvall et al., 1992, 1993; Höggren and Tegelström 1995, this volume; Gibbs and Weatherhead, 2001). From these new-generation field studies, it is apparent that pitvipers and true vipers have been selected as research subjects based on their desirable size, ease of location, population densities, and other factors (Duvall et al., 1992, 1993; Bonnet et al., this volume).

In several respects, our knowledge of reproduction in viperid snakes is limited when compared to taxa such as colubrid snakes (e.g., *Nerodia*, *Thamnophis*). Although information on seasonal patterns of plasma sex steroids is available for several colubrids (e.g., Aldridge et al., 1990; Moore and Lindzey, 1992), few comparable studies involve vipers (reviewed by Bonnet et al., this volume). Surprisingly, few studies of sex steroids involve pitvipers, and none concern

[1]Department of Herpetology, Zoo Atlanta, 800 Cherokee Avenue, S.E., Atlanta, Georgia 30315-1440, USA
E-mail: gschuett@zooatlanta.org
[2]Department of Biology, Center for Behavioral Neuroscience, Georgia State University, 33 Gilmer Street, S.E., Unit 8, Atlanta, Georgia 30303-3088, USA
[3]Department of Life Sciences, Arizona State University West, P. O. Box 37100, Phoenix, Arizona 85069-7100, USA
[4]Department of Biology, Arizona State University, Tempe, Arizona 85287-1501, USA
[5]P. O. Box 16176, Portal, Arizona 85632, USA
[6]Department of Animal Science, University of Wyoming, Laramie, Wyoming 82071, USA

rattlesnakes (see Schuett et al., 1997). In general, lack of information on proximate mechanisms (e.g., physiological processes) is a serious impediment to development of a robust understanding of the ecology and evolution of mating systems (for related concerns, see Drickamer and Gillie, 1998; Feder et al., 2000; Wood and Swann, 2000; Ricklefs and Wikelski, 2002). Thus, equipped with new concepts and technological advances, we are positioned to resolve these deficiencies.

Our primary goal in this study was to provide integrative information on several components (behavioral, morphological, physiological) of the mating system in male Mojave Rattlesnakes (*Crotalus scutulatus*). Despite the fact that this is a commonly encountered, wide-ranging species, we lack data on fundamental aspects of its reproductive biology, such as timing of sexual activity (see Wright and Wright, 1957; Fowlie, 1965; Klauber, 1972; Armstrong and Murphy, 1979; Lowe et al., 1982; Campbell and Lamar, 1989; Ernst, 1992; Degenhardt et al., 1996; Goldberg and Rosen, 2000; Werler and Dixon, 2000). Also, there are no published accounts of seasonal profiles of plasma sex steroids in *C. scutulatus* or any other rattlesnake species; this information is essential to the development of a comprehensive theory of mating systems in this group of snakes. Last, we present a preliminary (heuristic) phylogenetic analysis of seasonal mating patterns in rattlesnakes (*Crotalus* and *Sistrurus*), using a newly-constructed mtDNA-based phylogeny (Murphy et al., this volume; Fig. 6) and recent syntheses on the mating seasons of temperate New World pitvipers (Schuett, 1992; Aldridge and Duvall, 2002).

Although in this study we advance the knowledge of the reproductive biology of free-ranging *C. scutulatus*, we realize that it is an initial attempt to provide some level of synthesis. In view of insufficiencies herein, we suggest several directions for future research.

MATERIALS AND METHODS

Gross Morphology and Histological Analyses

Specimen acquisition.—A sample of 65 adult male *C. scutulatus* from central and southeastern Arizona were obtained either as recent road-kills (Cochise Co.) or museum specimens frome Arizona State University Tempe (Cochise, Maricopa, Pima, Pinal, Santa Cruz, and Yavapai counties; Appendix I).

Measurements.—Snout-vent length (SVL) and body mass (specimens drained of ethanol) were collected prior to removing the right side of the reproductive tract (i.e., testis, kidney, and ductus deferens up to the cloaca). We dissected the right testis, kidney, and ductus deferens of each specimen, and when these organs were present and intact they were removed as a single unit. We measured the total length of the right side of the reproductive tract, as well as the total length, mid-width, mid-height, and mass of the testis and kidney. Measurements were taken using a Denver Instruments Balance and Mitutoyo digital calipers. Tissues were individually housed and stored in 70% ethanol.

From each specimen, a section was obtained from the mid-region of the testis, the anterior region of the kidney, and the region of the ductus deferens between the testis and the kidney. After processing, tissues were embedded in paraffin, sectioned at 10 µm, and stained with Erlich's hematoxylin, followed by phloxine eosin counterstain. Histological measurements (µm) were obtained using Scion Image, ver. 1.62, and calibrated with an ocular micrometer. Measurements of the testis included diameter of the seminiferous tubule (STD) and diameter of the lumen of the ST (STLD), and those of the kidney were diameter of the sexual segment of the kidney (SSKD) and cell height of the SSK (SSKCH).

Reproductive Behavior

Field studies.—Because published accounts of seasonal timing of mating and other sexually-related activities (e.g., male-male fighting) of Arizona populations of *C. scutulatus* are not available, we (or our colleagues) initiated field observations. Certain aspects of reproduction in *C. scutulatus* are found in Wright and Wright (1957), Klauber (1972), Lowe et al. (1989), and Goldberg and Rosen (2000), and others, but only Jacob et al. (1987) and Reiserer (2001) provide direct evidence of sexual behavior (e.g., courtship, mating, and/or male-male fighting). Jacob et al. (1987) briefly described courtship-like activities in *C. scutulatus* from northern Chihuahua, Mexico. Reiserer (2001) documented courtship and male-male fighting in fall in a population of *C. scutulatus* from the Mojave Desert. Despite these observations, the nature of the mating season in this species remains uncertain. Does *C. scutulatus* show a bimodal mating pattern (summer and spring) as described by Schuett (1992)? Accordingly, data in spring are needed to address this question. Although Lowe et al. (1989) stated that *C. scutulatus* mates in spring (February–May), no primary reference is provided.

Thus, the main objective for field observations was to determine whether *C. scutulatus* shows sexual behavior in spring.

Plasma Sex Steroid Analyses

Subjects.—From 1998 to 2000, 41 adult male *C. scutulatus* were collected for hormonal analyses. Subjects were obtained from Maricopa and Cochise counties, Arizona. Months of sampling were in the active period (March–September); because of dry conditions, we were unable to locate animals in April and May. Localities selected were based on those from which specimens for histological analysis were derived.

Collection of blood and plasma.—Subjects were captured humanely and bled within several minutes to several hours. Procedures follow Schuett et al. (1997), except that subjects were secured in a clear plastic tube of appropriate size, and 1.0 ml of blood was harvested from the ventral caudal region without the use of anesthesia (Halothane or Isoflurane).

Radioimmunoassay of plasma sex steroids.—Radioimmunoassays (RIAs) of sex steroids examined in this study [testosterone (T), 5α-dihydrotestosterone (DHT), and 17ß-estradiol (E2)] were performed on plasma that was stored in an ultra-low (– 80°C) freezer. Procedures for RIA measurements followed commercial kits with slight modifications (e.g., rat plasma replaced by snake plasma). The above three steroids have known influences in reptiles (Norris, 1997; Bentley, 1998).

Radiolabeled T and antibody were purchased from Research Products International (Mount Prospect, Illinois; catalog number TMM-210). The primary antibody detected T and not other androgens. Samples were extracted in anhydrous diethyl ether prior to RIA. The extraction efficiency of radioactive T was > 95%. Recovery of unlabeled T added to samples was 93.3%. Parallelism existed between inhibition curves obtained with standards and serial dilutions of ether-extracted plasma. Validation of the RIA involved parallelism and quantitative recovery of exogenous steroid, and was performed using *C. scutulatus* plasma from adult males and females. Assay samples were run in duplicate (N = 82) in two assays. The intra-assay coefficient of variation (CV) was 9.1% and 11.1%, and the inter-assay CV was 11.9%. All T values are presented as means ± SE (nanograms per milliliter, ng/ml).

Radiolabeled DHT and antibody were purchased from Diagnostic Systems Laboratories (Webster, Texas; catalog number DSL 9600). Protocol for extraction and RIA provided by the manufacturer was used, except that 0.20 ml of snake plasma and 0.20 ml of phosphate buffered saline (with 0.1% gelatin) were used for extractions. Where concentrations of steroid were predicted to be high, the extract was diluted before assaying the sample. Parallelism was demonstrated between inhibition curves for the standards provided with the kit, as well as serial dilutions of *Crotalus* plasma. Extraction efficiency could not be determined based on the kit. Assay samples were run in duplicate (N = 82) in a single assay. The intra-assay CV was 8.9 %. All DHT values are presented as arithmetic means ± SE (picograms per milliliter, pg/ml).

Radiolabeled E2, antibody, and a precipitating solution were purchased from Diagnostic Products Corporation (Los Angeles, California; catalog numbers E2D1, E2D2, and N6). Standards were prepared by serial dilutions in methanol of a stock solution. The anti-estradiol antibody was diluted 1:3 in phosphate buffered saline (PBS) containing 1:400 rabbit sera. One hundred microliters of snake plasma (with 300 µl of PBS) was extracted in 5.0 ml diethyl ether (Fisher Scientific). After removing and saving the ether layer, the sample was heated to 90°C for 5 min, extracted with an additional 5.0 ml of diethyl ether, and 200 µl of PBS-0.1% gelatin was added to the extract following evaporation of the ether. Extraction recovery of H^3-estradiol (New England Nuclear, Boston, Massachusetts; NET-381) was 78%. For the RIA, 100 µl of diluted antibody, 100 µl of I^{125} E2, and 1.0 ml of precipitating solution were used. A 24-h incubation (4°C) period followed each step. Antibody-bound I^{125} was separated by centrifugation at 1600 g. Validation involved quantitative recovery and parallelism. Quantitative recovery of E2 added to snake plasma was 100%, and parallelism was demonstrated between the inhibition curve for the standards and dilutions. Assay samples were run in duplicate (N = 82) in two different assays. The intra-assay CVs were 7.9% and 12.5%, and the inter-assay CV was 11.9%. All E2 values are presented as arithmetic means ± SE (picograms per milliliter, pg/ml)

Statistical analysis.—Statistical methods follow Zar (1999) and tests were performed using StatView 5.02 (SAS, Inc.) and Stat 2000 (Bonett, 1994). Data were subjected to inspection for outliers, normality (skweness and kurtosis), and equality of variance prior to performing statistical tests. Outliers were not detected, and conditions of normality and equality of variance were met. Associations between body size (snout-vent length, body mass) and other measure-

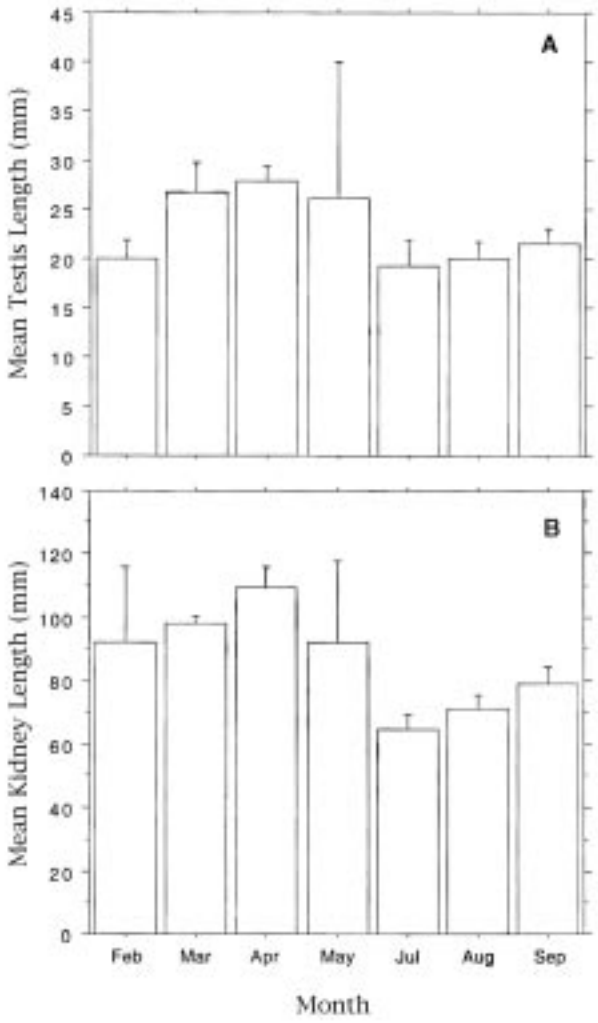

Fig. 1. Mean ± SE length of the (A) testis and (B) kidney in *Crotalus scutulatus* from Arizona during the active season (February–September). Data in June were not available.

ments (plasma steroid levels) were tested using regression analysis. Seasonal differences of means were tested using ANOVA or ANCOVA. For histological data, due to unbalanced sampling over seven months (February–September; June excluded for lack of data), samples were pooled to create a spring (February–May) category, and one for summer (July–September). Plasma sex steroids were analyzed on a monthly basis (March, and July–September). All tests were two-tailed. Data are presented as arithmetic means ± SE, and the α-level of significance was set at $P \leq 0.05$. Post hoc tests (pairwise comparisons) were Fisher's PLSD.

RESULTS

Reproductive Behavior

We documented sexual activity (e.g., accompaniment, courtship, coitus, male-male fighting) in populations of *C. scutulatus* from south-central Arizona and southern California. From 21 to 28 March (1994 to 1996), in Maricopa Co., Arizona, we observed courtship on four occasions and male-male fighting on one occasion. Also, on 19 March 1994, courtship was observed between two individuals near the city of Marana, Pinal Co., Arizona (R. Repp, pers. comm.). Further, courtship, coitus, and male-male fighting were documented in late September and early October in two populations from the Mojave Desert (San Bernardino Co., California; M. Cardwell, pers. comm.; R. Reiserer, 2001, pers. comm.). Thus, sexual activity occurs in late summer and early fall, and in spring, in several northern populations of *C. scutulatus*.

Table 1. Seasonal occurrence of spermatogenic stages (as per Goldberg and Parker, 1975) in *Crotalus scutulatus* from Arizona.

	Spermatogenic stage						
Month	**I**	**II**	**III**	**IV**	**V**	**VI**	**Total**
Jan						1	1
Feb						1	1
Mar	1					2	3
Apr	6						6
May	2					1	3
Jun							0
Jul			1	2	3		6
Aug				3	9	2	14
Sep	2				6	7	15
Total	11	0	1	5	18	14	49

Morphological Analyses

Gross morphological measurements.—Mean snout-vent length (SVL) was 712.92 ± 15.81 mm (range 444.0–1022.0 mm, N = 64), and ANOVA revealed that monthly mean values were significantly different ($F_{6, 57} = 2.80$, $P = 0.019$). Pairwise comparisons (Fisher's PLSD) showed that April vs July ($P = 0006$), April vs August ($P = 0.0015$), and April vs September ($P = 0.0178$) were significantly different. Comparison of pooled SVL data by season, spring (February–May) vs summer (July–September) was significant ($P = 0.002$), with the spring mean ($\bar{x} = 795.87 \pm 32.00$ mm) significantly greater than the summer mean ($\bar{x} = 685.27 \pm 16.51$ mm).

Mean body mass was 289.68 ± 18.30 g (range 89.0–698.6 g, N = 60), and ANOVA revealed that monthly mean values were not significantly different ($F_{6, 56} = 1.45$, $P = 0.211$). Pairwise comparisons (Fisher's PLSD) showed that only April vs July ($P = 0.0073$) and April vs August ($P = 0.035$) were signifi-

cantly different. Comparison of pooled data by season, spring (February–May) vs summer (July–September), was not significant ($P = 0.105$); thus, mean body mass in spring ($\bar{x} = 338.96 \pm 37.47$ g) and mean body mass in summer ($\bar{x} = 271.75 \pm 20.511$ g) were approximately equivalent. Snout-vent length (mm), as predicted, was positively correlated with body mass ($r^2 = 0.80$, $F_{1, 58} = 232.32$, $P < 0.0001$, N = 60).

Mean testis length (N = 58; Fig. 1a) and mean kidney length (N = 60; Fig. 1b) varied across months (February–September). Simple regression showed that testis length and kidney length were positively correlated with SVL ($r^2 = 0.37$, $P < 0.0001$, and $r^2 = 0.64$, $P < 0.0001$, respectively); therefore, analysis of covariance (ANCOVA) was employed to compare seasonal means, and SVL was used as the covariate. Because sample sizes in spring (February–May) for both testis and kidney data were too small for parametric manipulations, data were collapsed into two categories: spring (N = 16) and summer (N = 41).

Comparison (ANCOVA) of mean testis length in spring vs summer revealed a significant difference ($P = 0.002$), with the spring mean ($\bar{x} = 26.35 \pm 2.46$ mm, N = 16) greater than the mean in summer ($\bar{x} = 20.31 \pm 1.05$ mm, N = 16). Testis length was positively correlated with testis mass ($r^2 = 0.266$, $F_{1, 56} = 20.28$, $P < 0.0001$); mean testis mass was identical to testis length with respect to seasonal trends. Mean testis mass in spring ($\bar{x} = 0.315 \pm 0.042$ g) was significantly greater than the mean mass in summer ($\bar{x} = 0.202 \pm 0.020$ g) ($P = 0.008$).

Similarly, comparison (ANCOVA) of mean kidney length in spring vs summer showed that there was significant differences ($P < 0.0001$), with the spring mean ($\bar{x} = 102.13 \pm 5.94$ mm, N = 16) greater than the summer mean ($\bar{x} = 72.78, \pm 2.90$, N = 43). Kidney length was positively correlated with kidney mass ($r^2 = 0.651$, $F_{1, 58} = 108.26$, P < 0.0001); mean kidney mass was identical to kidney length with respect to seasonal trends. Mean kidney mass in spring ($\bar{x} = 2.142 \pm 0.274$ g) was significantly greater than the mean mass in summer ($\bar{x} = 1.53 \pm 0.156$ g) ($P = 0.004$).

Testis length was positively correlated with kidney length ($r^2 = 0.41$, $F_{1, 55} = 37.86$, $P < 0.0001$).

Histological analyses.—We followed Goldberg and Parker (1975) in delineating spermatogenic stages (stages I–VI) in snakes. Our analyses show that *C. scutulatus* (N = 49) followed the aestival (Type I; Schuett, 1992) cycle (Table 1). Initiation (early recrudesence) of spermatogenesis (stage II) was not detected, but its occurrence is in early summer

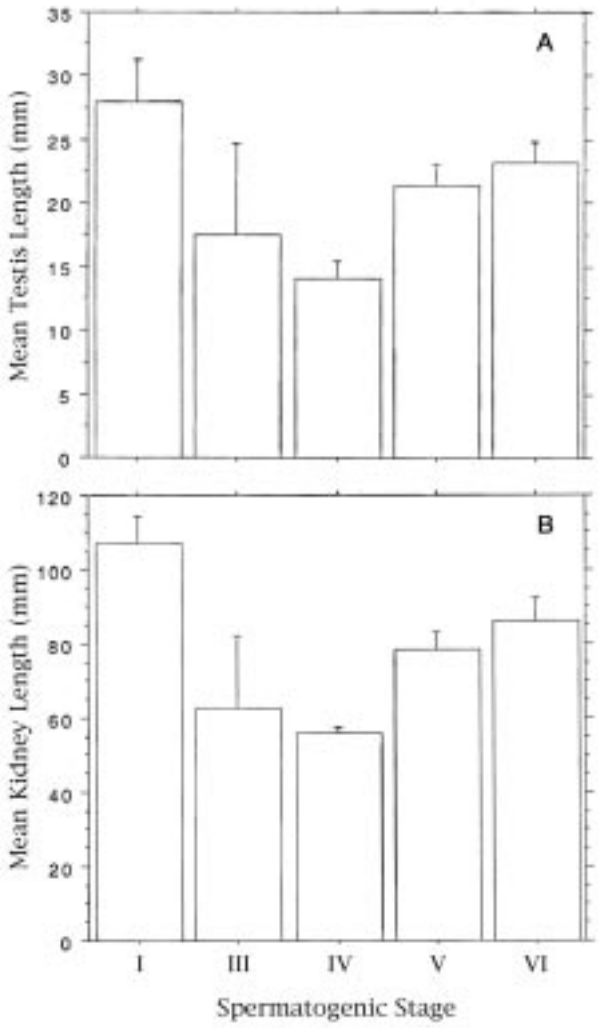

Fig. 2. Mean ± SE length of the (A) testis and (B) kidney in *Crotalus scutulatus* from Arizona compared to spermatogenic stages (I–VI; Table 1). Stage II is not included due to lack of data.

(Goldberg and Rosen, 2000). Late recrudescence (stage III) was detected in July, followed by early spermiogenesis (stage IV) in July–August. Peak spermatogenesis (stage V) occurred in August–September. Early regression (stage VI) was first detected in August, and continued through fall, winter, and spring. Completion of regression (stage I) was detected as early as September, but primarily from March–May.

Of the histological measurement made on the testis (seminiferous tubule diameter, STD; seminiferous tubule lumen diameter, STLD) and anterior kidney (sexual segment diameter, SSKD; sexual segment cell height, SSKCH), none were correlated with SVL (STD: $r^2 = 0.021$, $F_{1, 45} = 0.969$, $P = 0.330$, NS. STLD: $r^2 = 0.001$, $F_{1, 45} = 0.06$, $P = 0.809$, NS. SSKD: $r^2 = 0.001$, $F_{1, 51} = 0.056$, $P = 0.814$, NS. SSKCH: $r^2 =$

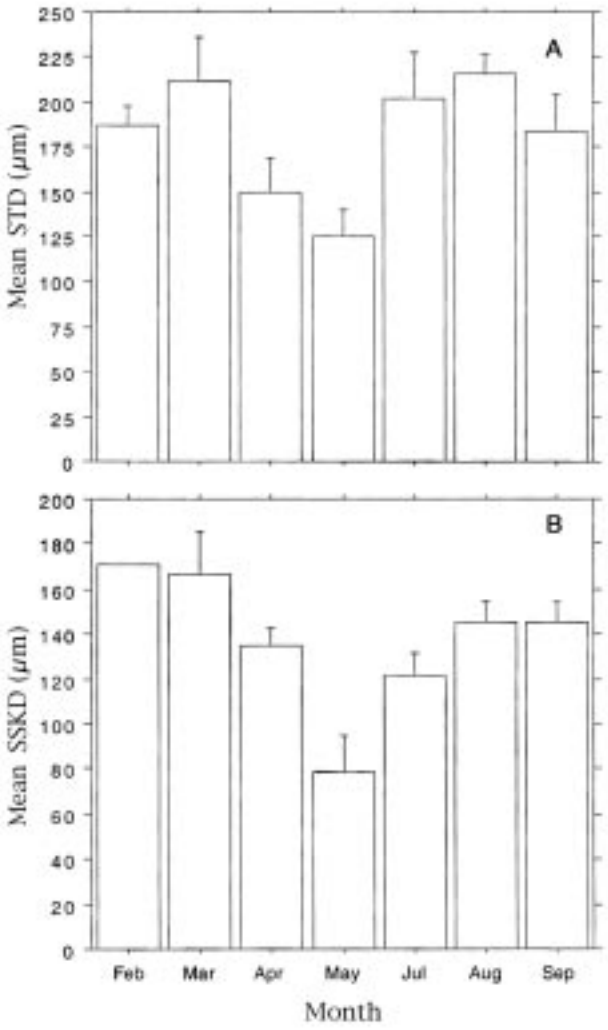

Fig. 3. Mean ± SE diameter of (A) seminiferous tubules (STD) and (B) sexual segment of the kidney (SSKD during the active season (February–September). June is not included due to lack of data.

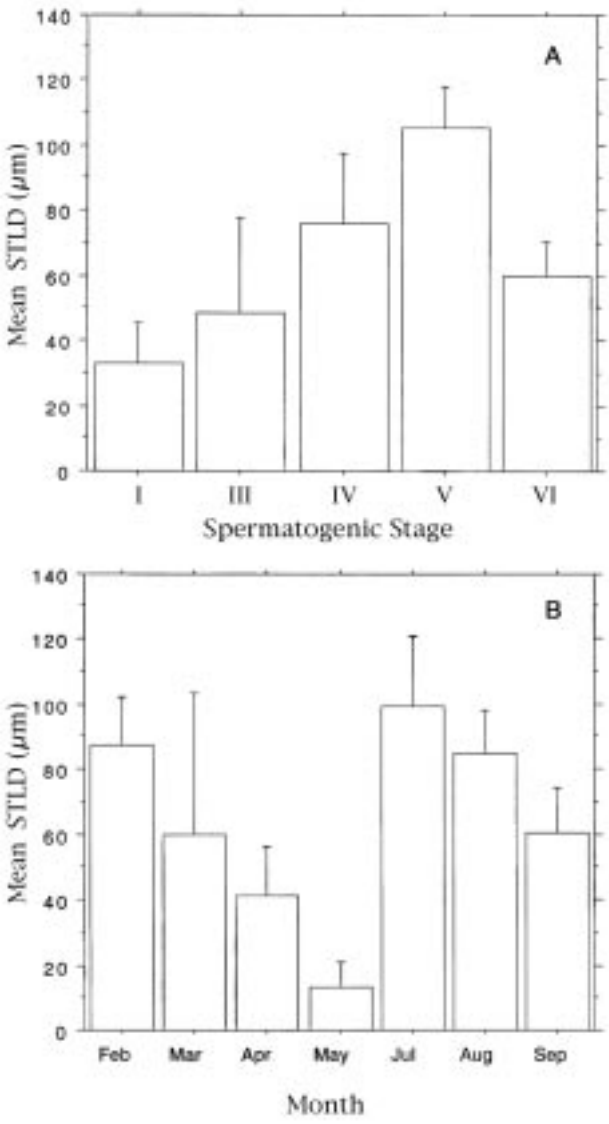

Fig. 4. Mean ± SE lumen diameter of the seminiferous tubules (STLD) compared to (A) spermatogenic stage and (B) month.

0.002, $F_{1, 51} = 0.089$, $P = 0.767$, NS), testis length (STD: $r^2 = 0.019$, $F_{1, 45} = 0.884$, $P = 0.352$, NS. STLD: $r^2 = 0.015$, $F_{1, 45} = 0.698$, $P = 0.408$, NS), or kidney length (SSKD: $r^2 = 0.001$, $F_{1, 50} = 0.01$, $P = 0.920$, NS. SSKCH: $r^2 = 0.001$, $F_{1, 50} = 0.006$, $P = 0.941$, NS). Therefore, ANOVA was appropriate to compare seasonal differences of means.

A seasonal pattern in mean size of STD was detected (Fig. 3a), with highest mean values in summer (July–August) and early spring (February–March), and the lowest mean value in late spring (May). Spring (February–May) vs summer (July–September) means were marginally not significant ($F_{1, 44} = 3.57$, $P = 0.066$, NS). A seasonal pattern in mean size of the SSKD was detected (Fig. 3b), and was congruent with the pattern of the STD. Spring (February–May) vs summer (July–September) means were not significantly different ($F_{1, 51} = 0.477$, $P = 0.493$). The same pattern was obtained for SSKCH ($F_{1, 51} = 0.517$, $P = 0.0475$; no figure), but the spring mean was significantly greater than the summer mean. A seasonal pattern in mean size of the STLD was detected (Fig. 4b), and was congruent with the pattern of the STD, SSKD, and SSKCH. Spring (February–May) vs summer (July–September) means were marginally not significant ($F_{1, 44} = 3.29$, $P = 0.077$).

The relationship of mean STD (Fig. 5a), SSKD (Fig. 5b), and STLD (Fig. 4a) to spermatogenic stage (Table 1) was inspected and showed that maximal mean STD, SSKD, and STLD were associated with peak spermiogenesis (stage V) and minimal during

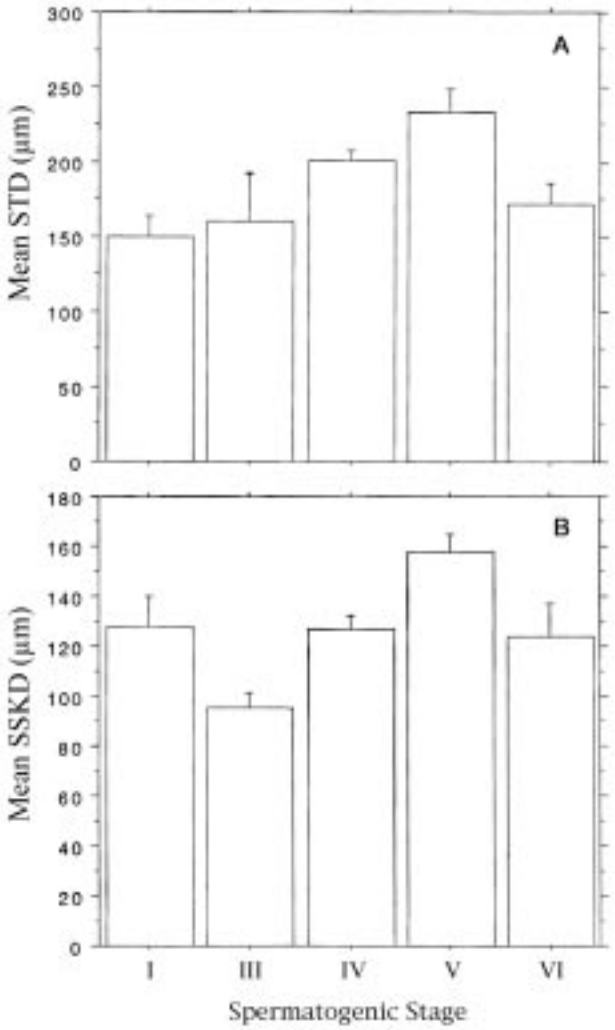

Fig. 5. Mean ± SE diameter of the (A) seminiferous tubules (STD) and (B) sexual segment of the kidney (SSKD) compared to spermatogenic stages (stages I–VI; Table 1). Stage II is not included due to lack of data.

late regression (stage I). The results obtained for mean SSKCH were similar to SSKD. Pairwise comparisons showed significant trends for STD (stage: I vs IV, P = 0.0003; III vs V, P = 0.050; V vs VI, P = 0.0022), SSKD (stage: I vs V, P = 0.035; III vs V, P = 0.0191; V vs VI, P = 0.0171), and STLD (stage: I vs V, P = 0.0003; V vs VI, P = 0.0074).

Sperm was present in the ductus deferens in all months; however, not all individuals had sperm present.

Relationships of gross morphology to histology.— Mean testis length (Fig. 2a) and mean kidney length (Fig. 2b) changed significantly (ANCOVA: $P < 0.05$) with respect to spermatogenic stage (Table 1). Mean testis length was greatest at stage I ($\bar{x}$ = 28.04 ± 3.25 mm, N= 11), during the time of complete regression,

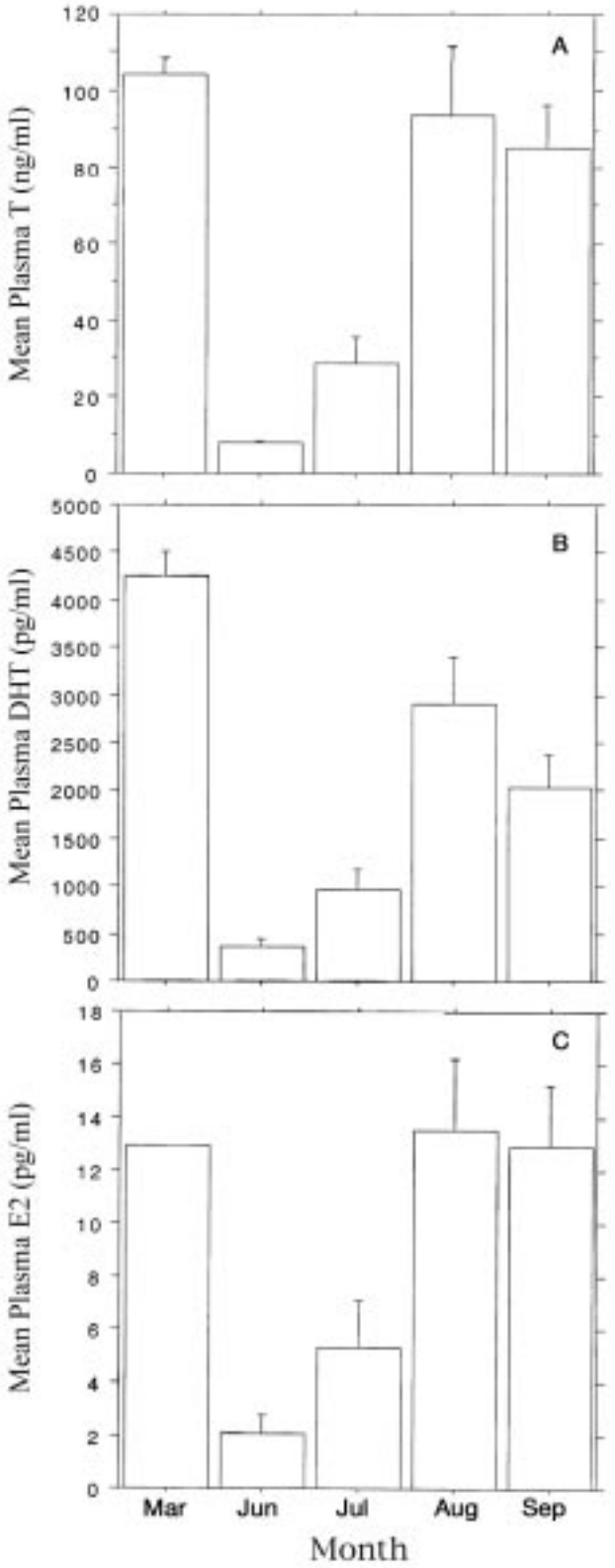

Fig. 6. Mean ± SE levels of plasma (A) testosterone (T), (B) 5α-dihydrotestosterone (DHT), and (C) 17ß-estradiol (E2) in male *Crotalus scutulatus* from Arizona during the active season (March–September). Data from April–May were not available.

and lowest at stage IV ($\bar{x}$ = 14.01 ± 1.36 mm, N = 4), during early spermiogenesis. Pairwise comparisons (N = 10) revealed that five cases were significant (stage: I vs III, P = 0.043; I vs IV, P = 0.0008; I vs V, P = 0.012; IV vs V, P = 0.048; and IV vs VI, P = 0.017). Mean kidney length followed the same pattern as the testis with respect to spermatogenic stage, and mean kidney length was greatest at stage I ($\bar{x}$ = 106.93 ± 7.45 mm), lowest at stage IV ($\bar{x}$ = 55.95 ± 1.38 mm). Pairwise comparisons (N = 10) revealed that seven cases were significant (stage: I vs III, P = 0.0003; I vs IV, P = 0.0001; I vs V, P = 0.0001; I vs VI, P = 0.0018; III vs VI, P = 0.040; IV vs V, P = 0.0090; IV vs. VI, P = 0.0010).

Sex Steroid Analyses

Gross morphological measurements.—Mean SVL was 720.39 ± 18.74 mm (range 539.0–1012 mm, N = 41), and body mass was 303.51 ± 26.35 g (range 77.3–852.0 g). ANOVA tests for both SVL and body mass did not reveal significant differences with respect to month with the exception of March (in all tests, P < 0.001). As predicted, body mass was highly correlated with SVL (r^2 = 0.823, $F_{1, 39}$ = 181.32, P < 0.0001). Plasma levels of T, DHT, and E2 were not significantly correlated with SVL, but T and DHT were with body mass (T: r^2 = 0.106, $F_{1, 39}$ = 4.64, P = 0.037. DHT: r^2 = 0.141, $F_{1, 37}$ = 6.05, P = 0.019. E2: r^2 = 0.021, $F_{1, 39}$ = 0.852, P = 0.362, NS). Accordingly, body mass was used as the covariate in ANCOVA tests comparing seasonal differences in plasma levels of T and DHT.

Plasma sex steroids.—Levels of the plasma testosterone (T), 5α-dihydrotestosterone (DHT), and 17β-estradiol (E2) showed seasonal differences (Fig. 6) that were significantly different (ANCOVA), with lowest levels (presumably baseline) in June, and highest levels in late summer (August–September) and in spring (March) (Fisher's PLSD. T: March vs June, P = 0.021; March vs July, P = 0.047; June vs August, P = 0.007; June vs September, P = 0.004; July vs August, P = 0.015; July vs September, P = 0.007. DHT: March vs June, P = 0.0058; March vs July, P = 0.009; March vs September, P = 0.045; June vs August, P = 0.018; July vs August, P = 0.027. E2: March vs September, P = 0.041).

Although concentrations of plasma T, DHT, and E2 were greatly different (T > DHT > E2), all were congruent with respect to overall pattern (Fig. 6). Regression showed that all steroids were positively and significantly correlated with each other (T x DHT: r^2 = 0.391, $F_{1, 37}$ = 23.76, P < 0.0001; T x E2: r^2 = 0.426, $F_{1, 39}$ = 28.92, P < 0.0001; DHT x E2: r^2 = 0.144, $F_{1, 37}$ = 6.20, P = 0.017).

Peak levels (summer and spring) of all three plasma sex steroids corresponded with the seasonal timing of sexual behavior (e.g., courtship, coitus, and male-male fighting) in late summer and spring, timing of spermatogenesis in summer, and hypertophy of the SSK in summer and spring.

DISCUSSION

Seasonal Timing of Mating

Field observations of Mojave Rattlesnakes (*C. scutulatus*) in Arizona and California indicate that there is a mating season in spring; thus, as described by Schuett (1992), the mating pattern is bimodal. In northern populations of *C. scutulatus*, we propose that the first mating period is from mid July to early September, corresponding to the monsoon season, and the second one is from late March to early May. More work, however, needs to be accomplished to verify the length of these mating periods. This pattern has been described for several other species of North American pitvipers, including rattlesnakes (Schuett, 1992; Aldridge and Duvall, 2002), and is also known in several species of viperines (e.g., *Vipera aspis*; Saint Girons, 1982; Saint Girons et al., 1993; Bonnet et al., this volume).

Morphological Analyses

Gross morphology.—The gross morphological and histological seasonal changes of the testis and kidney are remarkably similar, and both show greatest mean length and mass in summer and spring during periods of sexual activity. Mean length and mass of the testis and kidney were greater in spring than in summer.

Perhaps the most interesting finding was the degree of congruence between the monthly patterns of the dimensions (length and mass) of the testis and kidney. Moreover, in contradistinction to several previous studies in snakes and other reptiles, both length and mass of the testis and kidney were maximal (i.e., not significantly different from summer values) during spring, the time when spermatogenic regression was in process. Thus, at this time the gonad is presumably hypertrophied as a result of endocrine activity.

Histology.—As we predicted, the histological cycle of the testis in *C. scutulatus* closely conforms to previous descriptions in other species of snakes (Goldberg and Parker, 1975; Fox, 1977; Saint Girons, 1982). Importantly, it is the same cycle as documented in other species of rattlesnakes (e.g., Aldridge, 1993;

Aldridge and Brown, 1995; Aldridge, 2002), including two previous studies of *C. scutulatus* (Jacob et al., 1987; Goldberg and Rosen, 2000). Briefly, *C. scutulatus* shows the aestival cycle (Type I; Schuett, 1992); in temperate species recrudesence of spermatogenic stages begins in late spring/early summer and reaches peak activity (stages IV–V) in late summer and early fall. Regression is initiated in fall, is likely in stasis during winter, and is completed in early spring. Although more research on other temperate species is needed, work on tropical rattlesnake taxa seems more likely to reveal different spermatogenic patterns, though this may not necessarily be the case (see Salomão and Almeida-Santos, this volume).

Burtner et al. (1956) were first to report histological analyses of the anterior kidney (sexual segment, SSK) in rattlesnakes, but none have been performed in *C. scutulatus* (Fox 1977; Aldridge, 2002). In this study, we found that the seasonal pattern of the sexual segment in *C. scutulatus* was similar to that described for other rattlesnakes (Aldridge, 2002). For example, both SSKD and SSKCH were hypertrophied in summer (probably in winter) and early spring, and thus coincided with the seasons of mating (summer and spring), spermatogenesis (summer), and peak levels of plasma sex steroids. Lowest mean values for SSKD and SSKCH were in late May, which correspond with baseline conditions of sexual activity in *C. scutulatus* (i.e., spermatogenic regression is completed, all plasma sex steroids are at their lowest levels, and sexual behavior is presumably in quiescence).

Sever et al. (2002) presented new data on the SSK in the colubrid snake *Seminatrix pygaea*, and reviewed the importance of the SSK in male squamate reproduction. Importantly, they discussed deficiencies in our knowledge of this organ in male snakes, and proposed future directions for research. To add to their list of techniques for future studies, we propose the use of immunocytochemistry (ICC) and in situ hybridization (ISH) for sex steroids (T, DHT, E2, P4) and neuropeptides, such as arginine vasotocin (Goodson and Bass, 2001), associated with reproduction. We agree with Sever et al. (2002:253) that "Activity of the sexual segment is as essential to mating activity as spermatogenesis or testosterone production...one cannot truly understand the reproductive biology of any male squamate without consideration of the secretory cycle of the sexual segment." Accordingly, we propose that the hypothalamic-pituitary-gonadal (HPG) axis be referred to as the HPGK-axis in male squamates and other taxa (e.g., male elasmobranchs; Bishop, 1959) to accomodate the important role of the kidney (SSK).

Plasma Sex Steroids

Seasonal profiles of plasma sex steroids in vipers.—Compared to the diversity of studies of other vertebrates, we know very little about the endocrinology of reproduction in snakes, especially in wild populations (e.g., Lance, 1984; Moore and Lindzey, 1992; Schuett et al., 1997; see Bonnet et al., this volume). Analyses of seasonal levels of circulating sex steroids in free-living male vipers are limited to several Old World species, such *V. aspis* and *V. berus* (Naulleau and Fleury, 1984; Naulleau et al., 1987; Saint Girons et al., 1993) and the pitviper *Agkistrodon piscivorus* (Johnson et al., 1982). No study, to the best of our knowledge, has investigated endocrine patterns in male rattlesnakes. Schuett et al. (1997) is the only published laboratory study on seasonal levels of plasma T in males of the Copperhead (*Agkistrodon contortrix*), a pitviper species from temperate North America (Gloyd and Conant, 1990). Most of what we know about the endocrinology of reproduction in snakes (both sexes) is based on New World natricines, primarily in several species of *Nerodia* and *Thamnophis* (Weil and Aldridge, 1981; Moore and Lindzey, 1992; Whittier and Tokarz, 1992; Moore et al., 2000a, b; Mendonça and Crews, 2001; Krohmer and Balthazart 2001; Krohmer et al., 2002). We are thus at a pivotal point with respect to developing studies to investigate the role of circulating sex steroids in wild populations of vipers and other lineages of snakes.

In the laboratory study of Schuett et al. (1997), it was demonstrated that seasonal reproduction in *A. contortrix* showed remarkable similarity to the mating season pattern (e.g., bimodal) in wild populations (Fitch, 1960; reviewed by Schuett, 1992). Peak levels of plasma T were coincident with sexual behavior (i.e., courtship, coitus, and male-male fighting) in late summer, early fall, and early spring, and were associated with gonadal activity (e.g., spermatogenesis) in summer. In contrast to the findings involving *A. piscivorus* (Johnson et al., 1982), the seasonal pattern of plasma T in *A. contortrix* was strongly bimodal (i.e., peaks in late summer and spring). Further, T levels were many fold higher (e.g., 103 ng/ml vs 3 ng/ml) in male *A. contortrix* than the levels of total androgen measured in male *A. piscivorus*.

The seasonal pattern and mean plasma T values we report here for free-living male *C. scutulatus* are similar to those reported in captive male *A. contortrix*

(Schuett et al., 1997). Because plasma levels of DHT and E2 in males have not been published for any pitviper, comparison of values is restricted to other taxa of snakes, as well as lizards. Saint Girons et al. (1993) provide a thorough analysis of seasonal sex steroid patterns in the Aspic Viper (*Vipera aspis*). In their analysis of populations from western France, they measured plasma T, DHT, E2, and progesterone (P4). Seasonal elevation of T, DHT, E2, and P4 for male *V. aspis* roughly corresponded to the mating season. Highest levels of T occurred in the spring mating period, mean levels of P4 were highest in the fall and spring mating periods, and DHT and E2 showed minor changes seasonally. Interestingly, males had low E2 levels in spring, the most active period of mating, compared to fall. Saint Girons et al. (1993) concluded that plasma levels of sex steroid in sexually active males varied substantially, but in general showed high levels of T, P, and DHT, and low levels of E2.

Compared to our analysis of *C. scutulatus*, mean peak levels of plasma T in *V. aspis* were lower (> 100 ng/ml vs < 55 ng/ml). Also, mean peak levels of plasma DHT were higher in *C. scutulatus* (> 4.0 ng/ml vs 1.47ng/ml). Mean levels of plasma E2, however, were similar in both species. Unfortunately, we do not have data on P4 levels. Moreover, although we found that timing of sexual activity was coincident with peak levels of plasma T, DHT, and E2, unlike Saint Girons et al. (1993) we did not track individual hormone profiles over seasons.

Future studies of plasma sex steroid profiles in *C. scutulatus* (and other species of vipers) should employ several conceptual approaches, in the field and laboratory, to better understand both population- and individual-level phenomena (e.g., radiotracking individuals) (see Bonnet et al., this volume). Currently, descriptive endocrinological analyses predominate the field, and certainly more are needed, but experimental ones are required before we can determine causal factors (see below).

Function of circulating sex steroids in snakes.—Several androgen-dependent activities have been demonstrated experimentally in male snakes and other reptiles (Moore and Lindzey, 1992), which include activation of male aggression and sexual behavior, as well as control of spermatogenesis. But the functions of other androgens (e.g., DHT), estrogens (e.g., E2), progestogens (e.g., P4), and others messenger chemicals (e.g., neurohormones, neurotransmitters) in male reproduction are not well understood. Based on concentration, plasma T was the dominant sex steroid in our analyses (T > DHT > E2) and likely the most important with respect to its influence on sexual behavior, as well as gonad and kidney physiology (Moore and Lindzey, 1992). Nonetheless, correlation does not equate causation, and carefully planned experiments on snakes are necessary to understand the function(s) of circulating steroids.

Early experimental studies of sexual behavior involving male lizards of the genus *Anolis*, for example, were not able to show that DHT had a robust (positive) affect (e.g., Crews et al., 1978). There are, however, newer studies in which DHT had a positive effect on, or an association with, male sexual behavior (Tokarz, 1986; Moore and Lindzey, 1992; Moore et al, 1998; Edwards and Jones, 2001; Rosen and Wade, 2001). Although T has nearly always been shown to be a potent steroid in males, androgen precursors of T (e.g., DHEA) and non-aromatizable metabolites of T (e.g., DHT) also need to be studied for their effects in snakes.

The exact role and importance of estrogens (e.g., plasma E2) on the male sexual behavior in snakes are also unknown, but the concentrations we report are not likely to be incidental by-products of the metabolism of T (i.e., conversion of T to E2 via aromatase enzymes). In the natricine snake *Thamnophis sirtalis*, aromatase enzymes have recently been identified and located in specific brain regions associated with sexual activity (Krohmer and Balthazart, 2001; Krohmer et al., 2002), which suggests that estrogens (e.g., 17ß-estradiol) produced in the brain may be involved in male sexual behavior. In species that show male-male fighting, estrogens also might be involved in its expression. Not too surprising, there is growing evidence that estrogens (e.g., E2) are involved in the other aspects of reproduction in male reptiles, such as spermatogenesis (e.g., Chieffi et al., 2002).

Based on the study by Saint Giron et al. (1993) and research on lizards (Young et al., 1991; Witt et al., 1994; Moore et al., 1998), the functional role of P4 in male snakes is also in need of study.

Knowledge of the plasma steroid-binding proteins (sex hormone-binding globulin, SHBG; corticosteroid-binding globulin, CBG) and sex steroid receptors (SSR) in snakes and other squamate reptiles is limited (see Callard and Callard, 1987; Riley et al., 1988; Jennings et al., 2000). Although the function of SHBGs and CBGs in transporting steroids in circulation is understood, other functions are emerging (Hammond, 1995). Seasonal changes in SHBGs, for example, have been characterized in several species

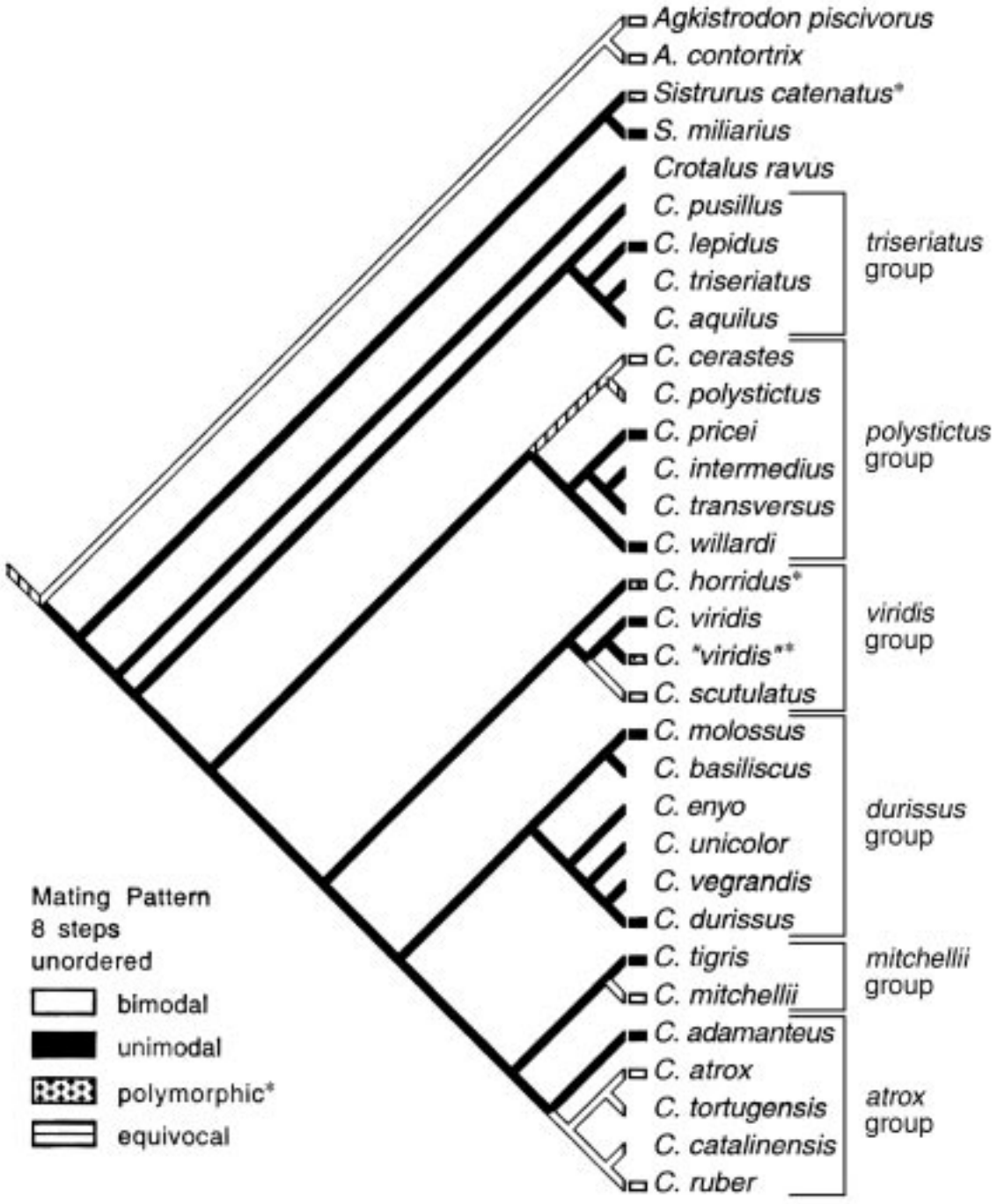

Fig. 7. Phylogenetic reconstruction of seasonal mating pattern in rattlesnakes (*Crotalus* and *Sistrurus*). *Agkistrodon* (*A. contortrix* and *A. piscivorus*) was used as the outgroup. Mating pattern is a two-state character (0 = bimodal; 1 = unimodal) and was treated as unordered (Maddison and Maddison, 2001). The ingroup consisted of 30 taxa. Due to lack of data, 10 of 30 ingroup taxa were scored as unknown (?). Three taxa were scored as polymorphic (0&1). Two equivocal regions are present. This tree is modified from Murphy et al. (this volume; Fig 6).

of reptiles, but not in snakes. The same can be said for sex steroid receptors. Descriptive studies of seasonal variation in SHBGs and SSRs are necessary, and experimental work lies ahead. Because the anterior hypothalamus-preoptic area (AH-POA) has important functions in regulating male sexual behavior in reptiles, it is necessary to investigate potential seasonal changes in size of nuclei and their frequencies (e.g., Crews et al. 1993; O'Bryant and Wade, 2002), as well as seasonal changes in steroid-receptor populations.

Finally, as discussed by Schuett (1992), Saint Girons et al. (1993), and Schuett et al. (1997), the dichotomous paradigm of "associated" vs "dissociated" reproduction as developed and promulgated by Crews (1984, 1991) and Crews et al. (1984) is confusing and masks the continuous nature of the phenomena under inspection (R. Aldridge, unpublished; pers. comm.). The results of this study further reinforce this view. For example, meiotic components of the testis can be regressed, yet the gonad itself is active with respect to sex steroid production and secretion. Similar problems have arisen in the organization-activation hypothesis of hormone action (Moore and Lindzey, 1992), and abandonment of strict adherence to this paradigm required years.

Phylogeny and Seasonal Timing of Mating

A body of evidence from studies of free-ranging populations of rattlesnakes (*Crotalus* and *Sistrurus*) indicates that seasonal timing of mating in species from temperate regions is not a uniform phenomenon. Instead, it appears that the pattern of mating falls into one of two basic categories: unimodal [summer (fall) only] or bimodal [summer (fall) and spring] (Duvall et al., 1992, 1993; Schuett, 1992; Aldridge and Duvall, 2002). In species that show a unimodal pattern, females show obligatory long-term sperm storage, LTSS (Schuett, 1992; Aldridge and Duvall, 2002; Sever and Hamlett, 2002). As briefly discussed by Schuett (1992:180), gaining an understanding of the origin, evolution, and maintenance of LTSS (and other reproductive characters) will require phylogenetic analysis to provide insights to character evolution. Over the past decade, several robust phylogenies of vipers have been published, including one for rattlesnakes (Murphy et al., this volume), which can be used to analyze character evolution (for examples in reptiles, see Greene and Burghardt, 1978; Rodríguez-Robles and Greene, 1996; Schuett et al., 2001a; Meylan et al., 2002; Greene et al., this volume; Martins et al., this volume).

With the exceptions of Schuett (1992) and Aldridge and Duvall (2002), who present verbal models on the evolution of mating patterns in North American pitvipers (*Agkistrodon*, *Crotalus*, *Sistrurus*), no other studies have examined seasonal timing of mating in vipers. Using several phylogenetic hypotheses of New World pivipers (e.g., Kraus et al., 1996; Parkinson, 1999; Douglas et al., this volume; Murphy et al., this volume; Parkinson et al., this volume) as a foundation, we reconstructed a preliminary hypothesis of the mating patterns of rattlesnakes (*Crotalus* and *Sistrurus*). Based on several phylogenetic studies (Kraus et al., 1996; Parkinson, 1999; Parkinson et al., this volume), we used *Agkistrodon* as the outgroup (Fig. 7).

The analysis was performed with MacClade (Maddison and Maddison, 2001), and we followed the procedures of Schuett et al. (2001a). The two-state behavioral character under investigation (mating pattern: 0 = bimodal, 1 = unimodal) was reconstructed parsimoniously onto a tree slightly modified from Murphy et al. (this volume; see Fig. 6). Knowledge of the outgroup was complete, but in 10 of 30 (33.3%) ingroup taxa we lacked information. Accordingly, their states were treated as unknown (?) as per the recommendation of Maddison and Maddison (2001). In a recent analysis (Aldridge and Duvall, 2002), *Sistrurus catenatus* and *Crotalus horridus* were scored as polymorphic (0&1) with respect to mating season, and that information was incorporated herein. Finally, recent mtDNA analyses by Douglas et al. (this volume) show that the species *C. viridis* is not monotypic, and is best viewed as a species group. One species (*C. viridis*) largely occurs east of the Rocky Mountains, and six species (*C. abyssus*, *C. cerberus*, *C. concolor*, *C. helleri*, *C. lutosus*, and *C. oreganus*) are found west of that range. To avoid a cumbersome topology, the western group according to Douglas et al. (this volume) is presented as *C. "viridis"* and is polymorphic (0&1) with respect to mating pattern (Aldridge and Duvall, 2002). The ingroup (*Crotalus* and *Sistrurus*) was composed of 30 species.

The present reconstruction required eight steps (i.e., five gains and/or losses of character states 0 and 1; plus three polymorphic sites), and two equivocal regions are present (Fig. 7). The first equivocal region occurs in the common ancestor of the outgroup and ingroup, and the second occurs in the *C. polystictus* species group. The ancestral character state as represented in the outgroup is the bimodal mating pattern (0).

To resolve the equivocal regions, the Equivocal Cycling routine was used and showed that there are four equally most-parsimonious reconstructions. These reconstructions are not shown, but in this case MacClade simultaneously reconstructed both equivocal regions in each of the four reconstructions. It is important to note that use of additional outgroups and more data are required to empirically resolve the equivocal regions. In the first equivocal region, for example, it is unclear whether the unimodal pattern of mating was gained at the basal-most region of the ingroup, or present in the common ancestor of *Agkistrodon* + rat-

tlesnakes but lost in *Agkistrodon* (outgroup) and several clades of the ingroup. If we accept the first scenario, then the unimodal mating pattern was gained once in the ingroup, and reversals to the ancestral state (0) occurred at least four times.

We want to emphasize, however, that our reconstruction of the seasonal mating pattern in rattlesnakes can only be viewed as a heuristic exercise, due to the considerable lack of natural history data. Moreover, the tree itself is incomplete in that several species are absent (e.g., *C. angelensis*, *C. lannomi*, and *C. stejnegeri*). Despite the deficiencies of this analysis, it serves the important function of providing a foundation from which future research can proceed. Although it is clear that data on species from Mexico and the Neotropics are sorely needed, there is a paucity of data on temperate zone species, especially those from the Colorado Plateau and the Pacific Northwest. Verbal models on the evolution of mating seasons, such as those recently produced by Aldridge and Duvall (2002), are important and provide direction, but comparative evolutionary analyses of the components of mating systems, such as the mating seasons, cannot advance with much rigor outside of a phylogenetic framework. The explicit use of phylogenies to address comparative problems has been demonstrated to provide invaluable insights in numerous systems (e.g., Schwenk, 1993; Martins, 1996; Schuett et al., 2001a; Greene et al., this volume; Martins et al., this volume). As more robust trees on rattlesnakes are produced, and a more complete understanding of mating seasons is obtained, the hypothesis presented herein will inevitably be improved. Ultimately, this approach will provide a powerful tool to explore proximate and ultimate questions of mating seasons.

Concluding Remarks

One important question regarding the mating season is the degree to which seasonal timing of mating for a particular population (species) is stable (e.g., bimodal vs unimodal seasonal pattern). Although it is desirable to have sound knowledge of seasonal timing and stability of mating seasons prior to performing phylogenetic analyses, it is our working hypothesis that this character (suite of characters) is stable in populations. For example, the mating system of a species is an emergent property of the interplay of suites of morphological and physiological traits (characters) of males and females that function as coordinated units. This system can be so sensitive that disruption to or malfunction of any one component can result in reduced or zero fitness. Thus, functional integration of numerous characters is necessary for successful reproduction; as per Schwenk and Wagner (2001:554), we suggest that the mating system of a population is under some degree of "internal" stabilizing selection. Recent conceptual developments on character evolution by Wagner and Schwenk (2000), and Schwenk and Wagner (2001) have emphasized functional integration and functional trade-offs, and they have developed the model of evolutionarily stable configurations (ESCs). ESCs are defined as (Schwenk and Wagner, 2001:553) "...suites of characters (from two to many) that functionally interact to produce a particular output." Using a reptilian example, these authors provide an intriguing discussion of ESCs and feeding systems in lizards. If we borrow from their perspective on ESC, characters of mating systems related to seasonal timing of mating are predicted to be stable. The HPGK-axis (see above) in male snakes, for example, operates in a coordinated manner (and with other systems) in temperate zone species to produce various outputs (e.g., sex steroid synthesis and secretion, spermatogenesis, and sexual behavior) at a particular time. These outputs, we predict, will show temporal stability in populations.

Given this brief scenario of ESCs, perhaps the most pertinent questions are those related to *why* and *how* different mating systems evolve in different conspecific populations and in closely related species. Although we provide evidence of the seasonal timing of mating (mating season) in *C. scutulatus* from the northern part of its range, a comprehensive knowledge of the mating season and sex steroid profiles throughout its geographic range would be ideal (Schuett et al., 2001b). A complementary goal would be to follow the fate of same-population individuals through the use of radiotelemetry to detect possible differences in timing of sexual activity.

Obviously, several research directions have emerged in the study of reproduction in vipers and other snakes. Our study represents one perspective in understanding the evolution of mating systems in rattlesnakes. Moore and Lindzey (1992:77) state "...the type of mating system may be an important evolutionary determinant of hormonal control mechanisms... [b]ecause reptiles show little diversity in type of mating system, with most species being promiscuous or polygynous, this idea will be difficult to investigate in reptiles." Contrary to that view, we argue that the diversity of reptilian mating systems is impressive,

and are optimistic that neuroendocrine control of those systems will be equally diverse.

Acknowledgments.—We have greatly benefited from the assistance of numerous colleagues. Matthew Grober, Chuck Rau, Barney Tomberlin, the Schuett family (Laura, Laudon, and Emory), and Ryan Sawby assisted in collecting animals for hormonal analyses. Histological analysis was greatly accelerated through the help of Phil Fernandez. Sharma Hammond helped with histological preparation and measurements. Roger Repp, Dale DeNardo, Randy Reiserer, and Mike Cardwell generously provided information on the breeding biology of *C. scutulatus*. Matthew Grober, Mats Höggren, and Brian Sullivan offered helpful suggestions in the early development of this manuscript; also, they were good listeners. GS thanks Kurt Schwenk for explaining his research on evolutionarily stable configurations and character evolution, and Bob Aldridge for his opinions on associated and dissociated reproduction in reptiles. Two anonymous referees also provided guidance

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APPENDIX I

Localities (counties) in Arizona for *Crotalus scutulatus* used in the morphological analyses. Specimens are from the collection at Arizona State University at Tempe (ASU) or the private collection of Andrew T. Holycross (ATH) (specimens now deposited in the ASU museum).

Cochise Co. (ASU: 30088, 30094, 30105, 30143, 30181, 30184, 30192, 30238, 30240. ATH: 162, 1995-09, 1995-44, 1995-47, 1995-56, 1995-66, 1995-70, 1995-76, 1995-81, 1995-83, 1995-85, 1996-3, 1996-4, 1996-4, 1996-18, 1996-38, 1996-44, 1996-45, 1996-46, 1996-47, 1996-51, 1996-54, 1996-58, 1996-63, 1996-68, 1996-69, 1996-70, 1996-73, 1996-75, 1996-86). Maricopa Co. (ASU: 257, 541, 988, 1444, 1576, 1977, 2341, 2917, 2918, 14091, 14307, 14313, 14085, 22478, 24458, 24463, 27459, 29895). Pima Co. (ASU: 1048, 13871, 24019, 26491, 27844, 28021). Pinal Co. (ASU: 2352, 22518, 29481, 29636, 29637). Santa Cruz Co. (ASU: 3677). Yavapai Co. (ASU: 4114).