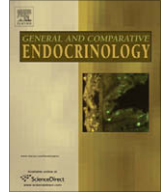




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The reproductive biology of male cottonmouths (*Agkistrodon piscivorus*): Do plasma steroid hormones predict the mating season?

Sean P. Graham^{*,1}, Ryan L. Earley³, Shannon K. Hoss², Gordon W. Schuett, Matthew S. Grober

Center for Behavioral Neuroscience, Georgia State University, 33 Gilmer Street, S.E., Unit 8, Atlanta, GA 30303-3088, USA

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ABSTRACT

To better understand the proximate causation of the two major types of mating seasons described for North American pitvipers, we conducted a field study of the cottonmouth (*Agkistrodon piscivorus*) in Georgia from September 2003 to May 2005 that included an extensive observational regime and collection of tissues for behavioral, anatomical, histological, and hormone analysis. Enzyme immunoassays (EIA) of plasma samples and standard histological procedures were conducted on reproductive tissues. Evidence from the annual testosterone (T) and sexual segment of the kidney (SSK) cycle and their relationship to the spermatogenic cycle provide correlative evidence of a unimodal mating pattern in this species of pitviper, as these variables consistently predict the mating season in all snake species previously examined under natural conditions. In most reptiles studied to date, high plasma levels of T and corticosterone (CORT) coincide during the mating period, making the cottonmouth an exception to this trend; we suggest two possible explanations for increased CORT during spring (regulation of a spring basking period), and decreased CORT during summer (avoiding reproductive behavioral inhibition), in this species.

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1. Introduction

Owing to their diverse reproductive strategies, snakes provide an ideal model system to explore the role of steroid hormones in the regulation of seasonal reproductive events (Shine and Bonnet, 2000; Shine, 2003; Schuett et al., 1997, 2002, 2005, 2006; Taylor et al., 2004; Taylor and DeNardo, 2005). In particular, vipers and pitvipers (Viperidae) show great potential in this regard, due to their abundance in certain regions, large body size, variation of reproductive modes and strategies, and phylogenetic diversity (Bonnet et al., 2002; Salomão and Almeida-Santos, 2002; Almeida-Santos et al., 2004; Schuett et al., 2006).

However, fundamental information on the physiological regulation of reproduction (e.g., studies on the hypothalamo-pituitary-gonadal and hypothalamo-pituitary-adrenal axes) is lacking for most of the approximately 3000 extant species (Moore and Lindzey, 1992; Schuett, 1992; Whittier and Tokarz, 1992; Aldridge and Duvall, 2002; Shine, 2003; Schuett et al., 2002, 2005, 2006).

Due in part to these gaps in our knowledge, there are discrepancies in the literature concerning seasonal timing of sexual behavior (mating seasons) in many well-known and abundant snake taxa. The cottonmouth (*Agkistrodon piscivorus*), a viperid of North America, is one such example (Gloyd and Conant, 1990). Most sources have reported the cottonmouth as mating in both late summer and spring (see Schuett et al., 2002; Aldridge and Duvall, 2002); however, field observations of spring mating are rare, lack specifics, and predate our current understanding of reproduction in snakes (e.g., long-term sperm storage by females; Beyer, 1898; Wharton, 1966; Table 1). Anatomical and physiological indicators of the mating season in male pitvipers have been identified, such as patterns of annual testosterone (T) and kidney sexual segment (SSK) hypertrophy (e.g., Aldridge and Duvall, 2002; Schuett et al., 2002, 2005), but here too evidence is conflicting regarding the cottonmouth (Johnson et al., 1982; Zaidan et al., 2003; Table 1).

Although the causal role of sex steroids (e.g., androgens) in eliciting and modulating sexual behavior in male snakes (including vipers) remains controversial due to lack of strict experimental work (overviewed in Moore and Lindzey, 1992; see Schuett et al., 2006), mounting empirical evidence shows that seasonal peaks and troughs of circulating levels of sex steroids in males are coincident with presence and absence of sexual behavior, respectively (Schuett et al., 2006). North American pitvipers have been characterized as exhibiting two major types of mating seasons, with courtship, copulation, and male–male combat restricted to either: (i) late

* Corresponding author.

E-mail address: grahasp@auburn.edu (S.P. Graham).¹ Present address: Department of Biological Sciences, 331 Funchess Hall, Auburn University, Auburn, AL 36849-5941, USA² Present address: Department of Biology, San Diego State University, 5500 Campanile Drive, San Diego, CA 92182-4614, USA³ Present address: Department of Biological Sciences, University of Alabama, Box 870344, Tuscaloosa, AL 35487, USA

Table 1
Available evidence regarding the mating season in *A. piscivorus*

Source	Mating season			Evidence	Study type	
	Spring	Summer	Fall		Captive	Field
Beyer (1898)	X			“Mated pair”		X
Carr and Carr (1942)	X			Male–male combat		X
Ramsey (1948)		X		Male–male combat		X
Allen and Swindell (1948)			X	Copulation	X	
Wharton (1966)	X	X		Bisexual pairing—individuals <3 m apart; actual copulation not observed; also evidence from sperm smears		X
Perry (1978)		X		Male–male combat		X
Gloyd and Conant (1990)	X	X	X	Various references	X	
Johnson et al. (1982)	X	X		Androgen/SSK activity		X
Martin (1984)			X	Male–male combat		X
Fogelman et al. (1986)			X	Male–male combat		X
Zaidan et al. (2003)		X		Androgen activity/anecdotal reports of copulation		X
Hill and Beaupre (2008)*		X		Copulation		X

From anecdotal information it is apparent that male–male combat can occur at any time during the active season, however, a detailed report (*) of copulation has only been reported once and occurred during the late summer.

summer/fall, or (ii) late summer/fall and spring (Schuett, 1992; Aldridge and Duvall, 2002; Schuett et al., 2002, 2006). A mating season restricted to spring appears to be rare and limited to a single taxon (e.g., *Crotalus ruber*; Aldridge and Duvall, 2002). Thus, the annual cycle of plasma testosterone of North American pitvipers exhibits either a unimodal or bimodal pattern, and is associated with and considered a robust predictor of the mating season (Aldridge and Duvall, 2002; Schuett et al., 2002, 2005).

Furthermore, male snakes and other squamates possess a unique region of the kidney—the sexual segment (SSK)—which is thought to contribute to seminal fluid and nourish spermatozoa (Prasad and Reddy, 1972; Fox, 1977). Because SSK cells are androgen-dependent and become hypertrophied when circulating testosterone levels are high (Bishop, 1959; Krohmer, 2004b), their activity is also associated with the mating seasons (Aldridge, 2002; Aldridge and Duvall, 2002), although sometimes the association is subtle in snakes (i.e., Aldridge and Brown, 1995; Clesson et al., 2004). These unimodal and bimodal patterns of sex steroid secretion, secondary sex characteristics (e.g., the SSK), and behavior persist despite a conserved sequence of spermatogenesis, which peaks during the late summer/fall in all North American pitvipers studied to date (the aestival, or Type I pattern; Saint Girons, 1982; Schuett, 1992; Aldridge and Duvall, 2002).

Studies examining the role of corticosterone (the primary glucocorticoid of squamate reptiles) in reproductive events of male reptiles suggest that levels of plasma corticosterone also rise during the mating period, likely due to the energy requirements of reproduction (Romero, 2002; Taylor et al., 2004). More typical, basal levels of corticosterone have been linked to diel and seasonal energetic homeostasis, and there is evidence that corticosterone collaborates with insulin to regulate energy balance (Dallman et al., 1993). However, levels of corticosterone during reproduction in male snakes, including pitvipers, have been examined in only a few species (e.g., Schuett and Grober, 2000; Taylor et al., 2004); hence, its function in the reproductive cycle is largely unexplored and unknown.

Here, we examined endocrinological and morphological parameters pertaining to the reproductive biology of a population of *A. piscivorus* from Georgia, USA. Based on several recent studies of this species (Zaidan et al., 2003; Hill and Beaupre, 2008), we predicted that: (i) males would show hypertrophy of SSK cells coincident with elevated levels of testosterone in late summer and/or fall, (ii) plasma testosterone and corticosterone in males would be at peak levels in late summer and/or fall, coinciding with aestival spermatogenesis and the mating season, whereas levels in spring would be lowest, and (iii) courtship and copulation (and acts related to these activities, such as male–male combat) would

be restricted to late summer and/or fall. To achieve this goal we made direct observations of wild-living individuals, used anatomical evidence derived from the male urogenital tract, and determined concentrations of circulating testosterone and corticosterone in males throughout the active season (March–October). In most cases we were able to obtain histological and hormonal samples from the same individual, allowing us to evaluate parallel changes in reproductive morphology and physiology.

2. Materials and methods

2.1. Study animal and site

The cottonmouth is a common semi aquatic pitviper often found at high densities (>700 per hectare—Gloyd and Conant, 1990). This snake is readily detectable in its habitat because it is relatively large-bodied and less likely to flee compared to other snakes (Gloyd and Conant, 1990). The study site is a ~ 240 ha floodplain forest/beaver dam marsh complex located at the confluence of Morning Creek with the Flint River in the Piedmont region of Georgia (N33°28'01.83", W84°23'12.75"). The habitat is a mosaic of wetlands/uplands and bounded on all sides by suburban or rural development. Wharton (1978) provides a complete description of river swamp and beaver marsh habitat in Georgia.

2.2. Field observations and processing

Males of *A. piscivorus* were collected and/or observed at the study area from September 2003 to September 2005. Captured animals were processed immediately to collect blood samples for steroid analysis. Animals were captured with metal tongs and gently secured for processing in plastic tubes or plastic buckets with lids. At the time of capture, habitat, date, time of day, and behavioral information (e.g., associations, courtship behavior, copulation, agonistic encounters) were recorded. Behavioral notes followed the nomenclature of Carpenter and Ferguson (1977) and Carpenter and Gillingham (1990). Adult snakes were measured (snout-vent-length—SVL, and tail length—TL) to the nearest 0.5 cm by stretching flexible tape along their side in the tube, and weighed to the nearest 3 g using a Pescola spring scale. All snakes from which only a blood sample was taken were marked with a unique scale-clip (e.g., Fitch, 1960) before release at their point of capture.

Observations and sampling took place evenly throughout the active season (March–October 2004) to eliminate sampling bias. Eight person-hours per week were spent searching for cottonmouths during the spring (March 1–May 31, 2004) and late summer (August 1–October 31, 2004) periods. These search-hours

did not include time spent observing or processing cottonmouths. Two person-hours per night were spent observing from May 15 to August 31, 2004 (designated summer period when cottonmouths potentially switch to a nocturnal pattern; see Gloyd and Conant, 1990), as well as a six person-hour per week daylight schedule. Winter observations took place opportunistically on warm days from November to February 2004 when cottonmouths can sometimes be found basking outside of hibernacula (Gloyd and Conant, 1990; Ernst and Ernst, 2003). During the 2005 field season, searching, observations, and processing took place only during the potential breeding seasons (March–May; August–September, 2005) for an equal amount of time (32 total person-hours searching during each period).

2.3. Blood collection

From September 2003 to June 2004, subjects were sampled for blood while secured in a plastic tube. To achieve light anesthesia, a small cotton ball with 0.5 ml of isoflurane was placed in the restraint tube until the snake exhibited lack of a righting reflex. Evidence suggests that the stress response of reptiles can cause a marked negative effect on circulating sex steroids in as few as 2 h (Moore et al., 2000a; Lance et al., 2004). Therefore, a small sample (1 ml) of blood was collected as soon as possible (mean \pm SE, 22.15 min \pm 8.24; range, 3–30 min) in a labeled plastic vial by cardiocentesis using a disposable 1-cc heparinized tuberculin syringe. From June 2004 to May 2005 blood samples were collected from the caudal sinus without anesthesia (mean \pm SE, 13.33 min \pm 8.96; range, 3–30 min). Possible effects of these two techniques on hormone levels are discussed below. Blood samples were put on ice packs in a cooler for no more than 24 h (Taylor and Schuett, 2004), were then centrifuged and plasma was drawn off and placed in a new labeled micro-centrifuge tube, which was placed at -20°C until assays were performed. All subjects not collected for reproductive tissues (see below) were released at the point of capture with a unique scale-clip, and these snakes were not re-sampled during this study.

2.4. Hormone assays

Enzyme immunoassays (EIA) were conducted on plasma samples to determine concentrations of testosterone (T) and corticosterone (CORT). Hormones were extracted from thawed plasma samples using an ether extraction method. Briefly, 2 ml of diethyl ether was added to each 225 μl sample of plasma and mixed for 3 min in 16×125 mm borosilicate vials using a multi-tube vortexer. Samples were left undisturbed for 3 min to allow phase separation, after which time the aqueous layer was fast-frozen in a methanol/dry ice bath. The ether layer was decanted into a 16×125 mm borosilicate vial and the remaining aqueous phase layer was thawed and submitted to another round of diethyl ether and fast freezing. The second ether layer was decanted into the same vial as the first. Ether was gradually evaporated under a gentle stream of nitrogen at 40°C , leaving a hormone residue that was resuspended in 225 μl EIA buffer, covered with parafilm, and placed at 4°C overnight. EIAs were performed on extracted hormone samples, and manufacturer's instructions were followed (Cayman Chemical Company, Ann Arbor, Michigan); standards also were extracted prior to performing assays.

The assays were validated for *A. piscivorus* T and CORT by assessing parallelism and by assessing recovery, which entailed spiking T/CORT samples of known concentration with standards provided in the kit. Ten ether extracted samples obtained from animals not used in this study were combined to form a pooled control, serially diluted from 1:1 to 1:64 in 0.1 M phosphate buffer, and run in quadruplicate. The dilution curve was log-logit trans-

formed, and compared to the standard curve. The slopes of the two curves were parallel (T: $t_n = 0.07$, $p = 0.95$; CORT: $t_{14} = 0.19$, $p = 0.85$; Zar, 1996), indicating that the kit effectively detects *A. piscivorus* T and CORT.

Recovery was estimated using an 880 μl sample of the pool. This sample (110 μl) was distributed into eight micro-centrifuge tubes and mixed with an equal volume of standard provided with the kit (3.8, 7.8, 15.6, 31.3, 62.5, 125, 250, and 500 pg/ml). Recovery amounts were based on the known T/CORT concentrations present in the pooled sample. Minimum recovery ranged from 85.1% to 105%, but a linear relationship was established between expected and observed concentrations (T: $R^2 = 0.96$, $p = 0.95$, slope = 0.81; CORT: $R^2 = 0.90$, $p = 0.85$, slope = 0.86). The intra-assay coefficients of variation for the T assays were 5.96%, 8.30%, and 10.95%. The inter-assay coefficient of variation for the T assays was 12.06%. The intra-assay coefficients of variation for the CORT assays were 3.53%, 3.68%, and 15.88%. The inter-assay coefficient of variation for the CORT assays was 9.97%. Late summer 2005 T samples ($n = 5$) were analyzed on a separate EIA; the intra-assay coefficient of variation was 6.14% and the inter-assay coefficient of variation could not be calculated because the control samples used in the initial assays were unavailable.

2.5. Tissue collection, processing, and quantification

A subset of study subjects were selected for gross and histological examination of reproductive tissues. Most procedures followed Schuett et al. (2002). Snakes used for histological analysis were taken to a staging area in a bag secured in a bucket. They were kept in ambient (outdoor) temperature and humidity conditions, and all but four subjects were never transported more than 1 km from the study site. They were then anesthetized with isoflurane (see Section 2). SVL and TL (nearest mm) was recorded by measuring the anesthetized snake positioned straight, and body mass (nearest 0.1 g) was determined by a triple beam balance. The animal was then killed by decapitation.

The right reproductive tract was removed, fixed in 10% water buffered formalin for 2 weeks, and stored in 95% ethanol. Gross measurements of the right testis and kidneys were recorded using Spi 2000 calipers (nearest 0.01 mm) and a Mettler Toledo balance (nearest 0.01 g). Testis length, width, height, and mass, and kidney length and mass were recorded. Measurements of the right reproductive tract were taken so that they could be compared to most previous studies in North American snakes (Schuett et al., 2002).

Sections (5–8 mm) from the right reproductive tract (mid-testis, mid-vas deferens, and anterior kidney) were excised, embedded in paraffin after automated serial dehydration, sectioned (at 10 μm) using a rotary microtome, and stained with Erlich's Hematoxylin and Eosin. For each snake, 12 measurements of kidney diameter, lumen diameter, and epithelial cell height of both seminiferous (STD, STL, STE) and SSK (SSKD, SSKL, SSKE) tubules were taken to the nearest μm using Zeiss Axiovision 4.0 software for light microscopy. The mean of these 12 values was determined and analyzed statistically. Only tubules that appeared nearly circular were measured. Presence of sperm was diagnosed from the ductus deferens at a point between the testis and the kidney, or at the posterior ductus. Spermatogenic stage was determined using the terminology of Goldberg and Parker (1975).

2.6. Data analysis

Although for many individuals ($N = 24$ out of 65) we obtained both histological and field hormone samples, we were not able to collect both types of samples for all individuals, resulting in differing samples sizes among analyses. Some variables (SVL, body mass, T, testis mass) were log transformed to meet the assumption of

normality for parametric statistics; however, figures illustrate untransformed adjusted means (least squares means). SVL and body mass were significantly positively correlated ($F_{2,10} = 2.95$, $R^2 = 0.64$, $p < 0.0001$). We therefore used SVL as our body size covariate in subsequent analyses. All analyses involving CORT included bleed time (time from capture to blood draw) as an additional covariate, due to a significant positive relationship between the two variables ($F = 8.20$, $R^2 = 0.14$, $p = 0.006$). Two samples were eliminated from the analysis because they represented the only sample for a given month (November and January). We compared spring (April–May) and late summer (August/September) T levels between 2004 and 2005 and found no significant difference in T between years or seasons (two-way ANCOVA; season \times year effect = $F_{1,30} = 0.94$, $p = 0.34$). CORT levels between 2004 and 2005 were also compared with an ANCOVA (season \times year effect: $F_{1,28} = 3.52$, $p = 0.07$). These results suggest no differences in T or CORT stemming from different sampling protocols (anesthesia, spring 2004; non-anesthesia, spring 2005). We found no effect of year (2003 and 2004) on histology variables (ANCOVA; testis mass: $F_{1,29} = 0.01$, $p = 0.94$; SSKD: $F_{1,29} = 0.03$, $p = 0.86$; STD: $F_{1,29} = 0.77$, $p = 0.39$). In all of these cases (field T and histology), years were combined for further analysis. In addition, neither time of day sampled ($F_{1,52} = 1.35$, $R^2 = 0.03$, $p = 0.25$) nor bleed time ($F_{1,51} = 0.13$, $R^2 = 0.00$, $p = 0.72$) significantly affected T.

We analyzed monthly patterns of T, testis mass, and histological variables (mean SSKD, SSKL, SSKD, STD, STE, and STL) using ANCOVA. We also analyzed the variation of T, testis mass, and the histological variables using ANCOVA with stage of spermatogenesis as the main effect. Monthly variation of CORT was similarly analyzed. The assumption of homogeneity of slopes was met for all ANCOVAs. Tukey's post hoc tests (using least squares means) were used to determine significant variation among months and spermatogenic stage for the above comparisons. We used correlation analysis to determine significant relationships between T, CORT, SVL, body condition index (BCI–SVL/body mass, or residuals of body mass vs. SVL regression), testis mass, and histological variables. All data were analyzed using the statistical program SAS 9.1 (SAS Institute, Inc. Cary, NC) with the α value set at $p = 0.05$. The p -values derived from multiple correlations were compared against an adjusted

p -value derived from the sequential Dunn-Sidak method, which alleviates compounding of Type I error, to determine significance.

3. Results

3.1. Spermatogenic cycle

Seasonal differences were detected in testis parameters (STE: $F_{7,23} = 8.12$, $p < 0.0001$; testis mass: $F_{7,23} = 5.07$, $p = 0.0014$; Fig. 1; Table 2) with highest values in July–August, which corresponded with the progression of spermatogenesis (Fig. 2; Fig. 3; Table 2). Testis mass and STE varied significantly with spermatogenic stage, with maximum STE and testis mass corresponding to late spermatogenic stages (testis mass: $F_{5,25} = 12.4$, $p < 0.0001$; STE: $F_{5,25} = 12.63$, $p < 0.0001$; Fig. 3; Table 2), and significant positive correlations between testis mass and STD ($r_{30} = 0.59$, $p = 0.0003$) and STE ($r_{30} = 0.74$, $p < 0.0001$) were detected across months. STD was also positively correlated with STE ($r_{30} = 0.48$, $p = 0.005$), and negatively correlated with STL ($r_{30} = -0.45$, $p = 0.01$); however,

Table 2

Summary of statistical analyses performed on anatomical, histological, and hormonal data from *A. piscivorus* collected during the present study

Reproductive parameter	Month			Spermatogenic stage		
	F	df	p	F	df	p
SSKD	0.91	7.23	0.52	1.81	5.25	0.15
SSKL	0.94	7.23	0.50	0.32	5.25	0.90
SSKE	0.73	7.23	0.65	2.52	5.25	0.06
STD	1.27	7.23	0.31	2.35	5.25	0.07
STL	2.99	7.23	0.02	1.33	5.25	0.28
STE	8.12	7.23	<0.0001	12.63	5.25	<0.0001
Testis mass	5.07	7.23	0.001	12.40	5.25	<0.0001
Plasma testosterone	13.19	7.47	<0.0001	3.23	4.18	0.04
Plasma corticosterone	3.56	7.43	0.004	3.86	4.16	0.02

F-values (F), degrees of freedom (df), and p-values (p) are from ANCOVA, in which the reproductive parameter was the dependent variable and either month or spermatogenic stage was the class variable. Snout-vent length (SVL) was used as a covariate in all analyses and blood collection time was used as an additional covariate in the corticosterone analysis. Significant p-values ($\alpha \leq 0.05$) are in bold.

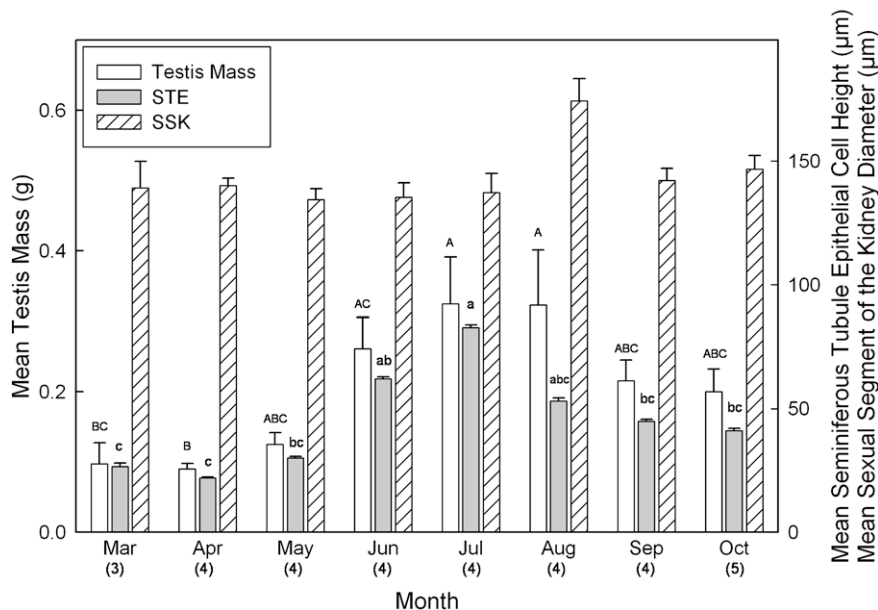


Fig. 1. Anatomical and histological parameter of the reproductive cycle in *A. piscivorus*. Months with significantly different anatomical and histological values are designated with different letters. Upper and lower case letters indicate different anatomical and histological parameters. Sample sizes are in parentheses below each month.

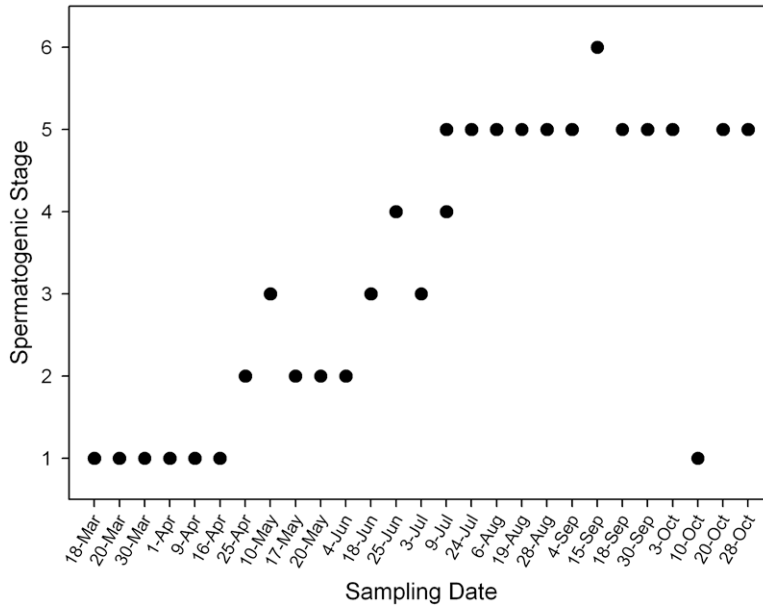


Fig. 2. Annual spermatogenic cycle of *A. piscivorus*. All samples were from 2004, with the exception of 15 and 30 September and 3 and 20 October, which were collected in 2003. Spermatogenic stages from Goldberg and Parker (1975): Stage 1 = completely regressed testes; only 1–2 spermatogonial cell rows present in seminiferous tubule. Stage 2 = early recrudescence; seminiferous tubule epithelial cells 3–4 rows deep. Stage 3 = late recrudescence; spermatogonia and secondary spermatocytes present. Stage 4 = early spermiogenesis; spermatids present. Stage 5 = spermiogenesis; mature spermatozoa present in seminiferous tubule lumen. Stage 6 = early regression; spermatogenic epithelium collapsing from lining of seminiferous tubule.

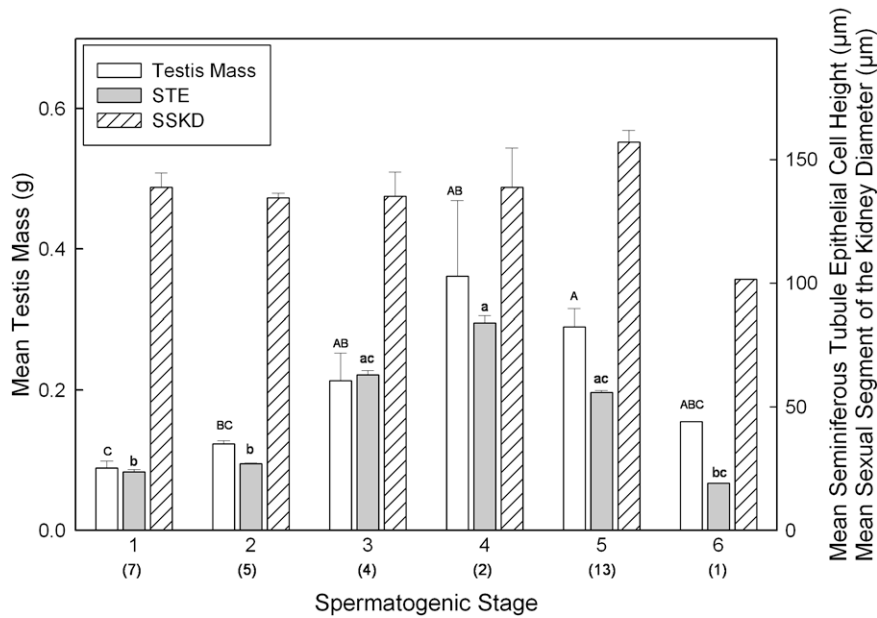


Fig. 3. Relationship of anatomical and histological parameters to spermatogenic stages. Stages with significantly different histological values are designated with different letters. Upper and lower case letters indicate different anatomical and histological parameters. Sample sizes are in parentheses below each spermatogenic stage.

these correlations did not retain significance after correcting for multiple comparisons (see Table 3).

3.2. Ductus deferens

The ductus deferens had dense populations of sperm in 31 of 33 specimens examined, and sperm was present in at least one sample from all seasons. The two specimens that could not be scored may have actually contained sperm that could not be detected due to the histological preparation.

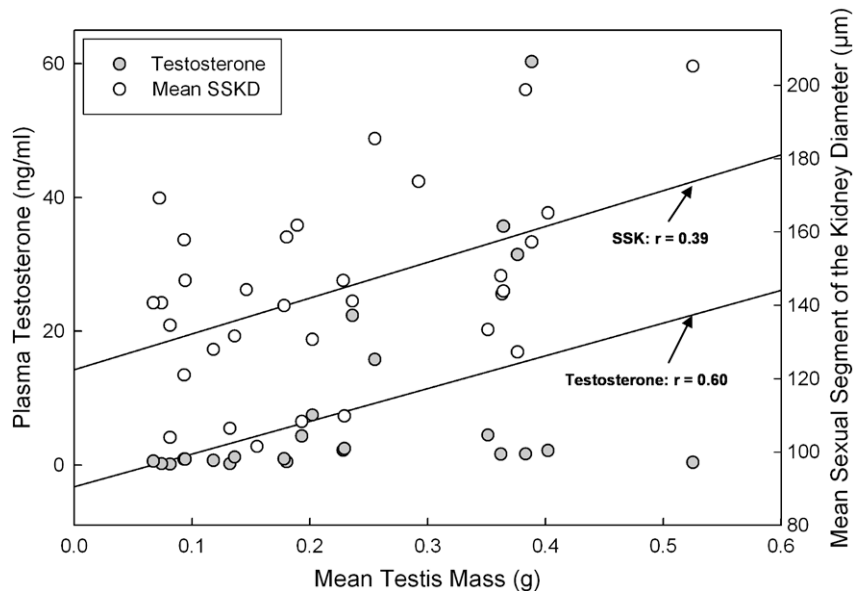
3.3. Sexual segment of the kidney

There was no significant difference across months in mean SSKD ($F_{7,23} = 0.91, p = 0.52$; Fig. 1; Table 2). SSKE and SSKD were positively correlated ($r_{32} = 0.93, p < 0.0001$; Table 3). Testis mass was positively correlated with SSKD and SSKE (SSKD: $r_{32} = 0.39, p = 0.03$; Fig. 4; SSKE: $r_{32} = 0.46, p = 0.008$), and STD was positively correlated with SSKD ($r_{32} = 0.35, p = 0.049$). However, these correlations did not remain significant after correcting for multiple comparisons (Table 3).

Table 3List of significant correlations between gross anatomical, histological, and hormonal parameters in *A. piscivorus*

Variable 1	Variable 2	<i>r</i>	df	<i>p</i> -value	<i>k</i>	Adjusted α	Significant after adjustment?
Field T*	Handling T*	0.909	21	<0.0001	78	0.00065	Y
Mean SSKE	Mean SSKD	0.929	32	<0.0001	77	0.00066	Y
SVL*	Mass*	0.800	58	<0.0001	76	0.00067	Y
Field T*	Mean STE	0.762	24	<0.0001	75	0.00068	Y
Testis mass*	Mean STE	0.743	32	<0.0001	74	0.00069	Y
Testis mass*	Mean STD	0.594	32	0.0003	73	0.00070	Y
Handling T*	Mean STE	0.583	29	0.0009	72	0.00071	N
Field T*	Testis mass*	0.602	24	0.0018	71	0.00072	N
Mean STL	Mean STD	0.503	32	0.0033	70	0.00073	N
Testis mass*	Body mass*	0.495	32	0.0040	69	0.00074	N
Handling T*	Testis mass*	0.516	29	0.0041	68	0.00075	N
Field T*	Field CORT*	-0.378	55	0.0044	67	0.00076	N
Mean STE	Mean STD	0.481	32	0.0053	66	0.00077	N
Mean STL	Mean SSKL	0.464	32	0.0075	65	0.00078	N
Mean SSKE	Testis mass*	0.459	32	0.0082	64	0.00080	N
Mean STE	Mean STL	-0.449	32	0.0099	63	0.00081	N
Testis mass*	SVL*	0.397	32	0.0243	62	0.00082	N
Testis mass*	Mean SSKD	0.391	32	0.0267	61	0.00084	N
Mean SSKD	Mean STD	0.351	32	0.0490	60	0.00085	N
Field T*	Mean STL	-0.407	24	0.0499	59	0.00086	N

Correlations are listed in decreasing order of significance; *p*-values shown are uncorrected. The adjusted α value is based on the 78 possible pairwise correlations subjected to sequential Dunn-Sidak adjustments [$1 - (1 - \alpha)^{1/k}$] where '*k*' is the comparison number for the particular correlation being adjusted. To remain significant after adjustment, the uncorrected *p*-value must be less than the adjusted α value. Variables noted with an asterisk were natural-log transformed for analysis. Significant *p*-values ($\alpha \leq 0.05$) are in bold.

**Fig. 4.** Relationship between testis mass, SSKD, and plasma T.

3.4. Testosterone

Testosterone varied across months ($F_{7,47} = 13.19$; $p < 0.0001$; Fig. 5; Table 2), was significantly elevated in summer (Tukey's post hoc tests: Jul > all months, except Jun; Jun > Mar, Apr, and May; Fig. 5; Table 2), and was lower in spring relative to late summer in both 2004 and 2005. T varied significantly with spermatogenic stage and trended toward highest values during late spermatogenic stages ($F_{4,18} = 3.23$, $p = 0.037$; Fig. 2; Table 2; group $N = 3$ (stage 1), 5 (stage 2), 4 (stage 3), 2 (stage 4), 10 (stage 5), 0 (stage 6)). T was positively correlated with mean STE ($r_{22} = 0.76$, $p < 0.0001$) and mean testis mass ($r_{22} = 0.60$, $p = 0.002$; Fig. 4) and negatively correlated with STL ($r_{22} = -0.41$, $p = 0.049$). However, after correcting for multiple comparisons, only the T-STE correlation remained significant (Table 3).

3.5. Corticosterone

We detected significant seasonal variation in CORT ($F_{7,43} = 3.56$, $p = 0.0042$; Tukey's post hoc tests: Jul < Mar, Apr, May; Fig. 5; Table 2). CORT also varied by spermatogenic stage ($F_{4,16} = 3.86$, $p = 0.0222$; Fig. 2; Table 2; group $N = 3$ (stage 1), 5 (stage 2), 4 (stage 3), 2 (stage 4), 10 (stage 5), 0 (stage 6)), with a Tukey's post hoc test indicating higher CORT during stage 2 than spermatogenic stage 5 ($t = 3.49$; $p = 0.023$; Table 2). There were no other statistical associations between CORT and testicular parameters. There was an overall negative relationship between CORT and T across months ($r_{55} = 0.3785$, $p = 0.004$). However, this correlation disappeared after correcting for multiple comparisons (Table 3). There were no significant correlations between indicators of body condition and CORT (BCI: $r_{49} = 0.077$, $p = 0.60$; residual body mass:

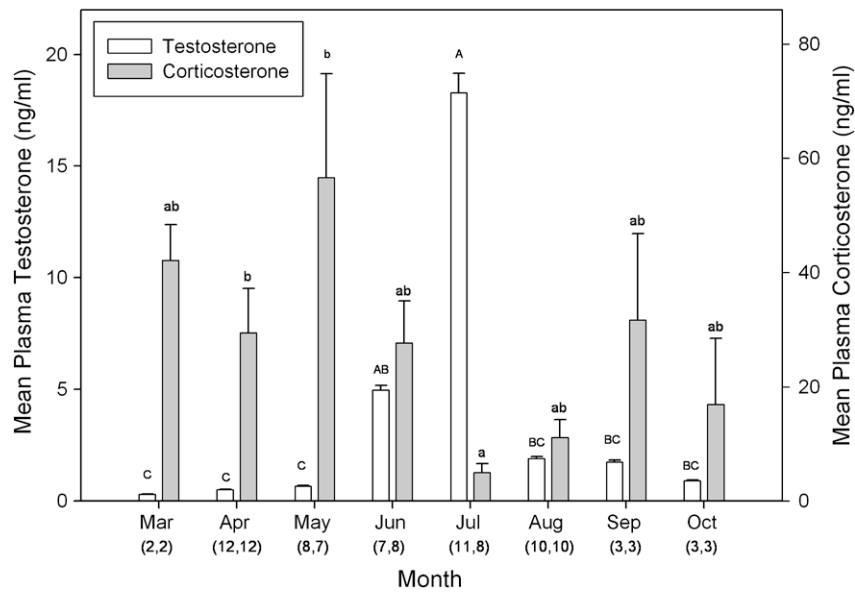


Fig. 5. Monthly profiles of plasma T and CORT in male *A. piscivorus*. Months with significantly different hormone values designated with different levels, with upper case letters for T and lower case letters for CORT. Sample sizes for testosterone and corticosterone, respectively, are in parentheses below each month.

$r_{49} = -0.245$, $p = 0.09$) before or after corrections. As stated earlier, there was a significant and positive relationship between bleed time and CORT ($F = 8.20$, $R^2 = 0.14$, $p = 0.006$).

3.6. Behavior

Over 400 person-hours were spent searching for and observing cottonmouths; during this time no instances of male–male combat, courtship, or copulation were observed.

4. Discussion

The anatomical and histological analyses of the urogenital tract of adult male *A. piscivorus* in this study yielded results similar to those reported for *A. piscivorus* from Alabama (Johnson et al., 1982). Furthermore, our data are consistent with the findings of other studies on temperate pitvipers exhibiting a single (=unimodal) mating season in late summer and fall (Table 4).

Table 4
Mating patterns of representative temperate zone snakes

Species	Mating season		Evidence			Source
	Bimodal	Unimodal	Behavior	SSK	T	
<i>Viperidae</i>						
Cottonmouth (<i>Agkistrodon piscivorus</i>) GA, AL, AR		X	X	X	X	This study; Zaidan et al. (2003), Johnson et al. (1982), Hill and Beaupre (2008)
Cottonmouth (<i>Agkistrodon piscivorus</i>) FL, LA, AL	X		?	X		Beyer (1898), Wharton (1966), Johnson et al. (1982)
Copperhead (<i>Agkistrodon contortrix</i>)	X	X	X	X	X	Fitch (1960), Schuett et al. (1997), Smith (2007)
Mohave rattlesnake (<i>Crotalus scutulatus</i>)	X		X	X	X	Schuett et al. (2002)
Southern/northern Pacific rattlesnake (<i>Crotalus oreganus</i>)	X		X	X		Aldridge (2002)
Western diamond-backed rattlesnake (<i>Crotalus atrox</i>)	X		X		X	Taylor et al. (2004) and Schuett et al. (2005)
Prairie rattlesnake (<i>Crotalus viridis</i>)		X	X	X		Aldridge (1993)
Timber rattlesnake (<i>Crotalus horridus</i>)		X	X	X		Aldridge and Brown (1995)
Black-tailed rattlesnake (<i>Crotalus molossus</i>)		X	X		X	Schuett et al. (2005)
European adder (<i>Vipera berus</i>)		X	X	X		Saint Girons (1982)
Aspic viper (<i>Vipera aspis</i>)	X		X	X	X	Saint Girons (1982) and Saint Girons et al. (1993)
<i>Colubridae</i>						
Red-sided gartersnake (<i>Thamnophis sirtalis parietalis</i>)*	X		X	X	X	Krohmer et al. (1987), Menconça and Crews (1989), Moore et al. (2001)
Eastern gartersnake (<i>Thamnophis sirtalis sirtalis</i>)	X		X	X		Clesson et al. (2002)
Lined snake (<i>Tropidoclonion lineatus</i>)		X	X	X		Krohmer and Aldridge (1985)
Northern watersnake (<i>Nerodia sipedon</i>)	X		X	X	X	Weil and Aldridge (1981), Ernst and Ernst (2003)
Southeastern crowned snake (<i>Tantilla coronata</i>)	X		X	X		Aldridge and Semlitsch (1992)
Rough greensnake (<i>Ophedryx aestivus</i>)	X		X	X	X	Aldridge et al. (1990), Ernst and Ernst (2003)

Behavior = X indicates that a source concluded the majority of mating reported or observed for each species was restricted to the period indicated; only three North American snakes for which there are data on proximate correlates of mating have been subjected to extensive reproductive behavioral studies: *Agkistrodon contortrix* (Schuett et al., 1996, 1997; Schuett and Grober, 2000), *Thamnophis sirtalis parietalis* (Crews, 1984, 1991; Krohmer et al., 1987; Shine et al., 2001) and *Nerodia sipedon* (Prosser et al., 2002; Weatherhead et al., 2002). SSK = X indicates either significant month/seasonal means (usually in the bimodal pattern), or a late summer increase trend (mostly in the unimodal pattern; e.g., this paper; Aldridge, 1993; Aldridge and Brown, 1995). T = X indicates significant monthly variation in plasma testosterone. *Indicates the snake which has been suggested to have a dissociated mating pattern, in which mating behavior is independent of androgens—even in this taxon, field studies have shown all three factors are coincident with mating. Additional information on these patterns from additional species (for which only behavioral information is known) is found in Aldridge and Duvall (2002).

The annual spermatogenic cycle of *A. piscivorus* is essentially identical to that of most other temperate zone snake species, with recrudescence initiated in spring, spermatogenesis progressing throughout summer, peak spermatogenesis in late summer, and regressive stages initiated in late summer through fall and continuing until the following spring (Saint Girons, 1982; Schuett, 1992; Aldridge and Duvall, 2002; Schuett et al., 2002; see Table 4). Following spermiogenesis, spermatozoa are subsequently stored in the sex ducts—primarily the ductus deferens—from fall through at least spring (Saint Girons, 1982; Schuett, 1992). Here, we found spermatozoa in the ductus deferens in all months sampled, a result which is similar to that described by Fitch (1960) in a Kansas population of copperheads (*Agkistrodon contortrix*). If mating occurs in late summer or fall, spermatozoa also can be stored by females throughout winter and up to the point of ovulation in mid- to late spring (Schuett, 1992; Sever and Hamlett, 2002).

Contrary to previous results for *A. piscivorus* (Johnson et al., 1982), the histological analysis of the SSK in this study did not reveal significant seasonal hypertrophy. Unlike the pronounced SSK hypertrophy reported in many species of lizards, snakes appear to maintain varying levels of hypertrophied SSK tubules throughout the active season, with comparatively modest increases during the mating season (reviewed by Krohmer, 2004a). Nonetheless, these increases in SSK parameters consistently parallel the mating season in snakes (Table 4; Saint Girons, 1982; Aldridge, 2002; Schuett et al., 2002; see discussions in Aldridge, 2001, 2002; for a possible exception, see Weil and Aldridge, 1981).

We did not detect a significant increase in SSKD during the late summer in male *A. piscivorus*, which is consistent with other reports of temperate zone pitvipers exhibiting a mating season restricted to late summer and fall in which qualitative increases were described (Table 4). However, we did detect correlations between testis mass, STE, and SSKD, suggesting testicular and SSK parameters are possibly linked in male *A. piscivorus*, with maximum activity of both corresponding to the late summer peak of plasma testosterone. Although many of these correlations disappeared after we employed a conservative statistical correction (Table 3), we suspect that these trends have biological significance. We suggest that SSK-testicular associations may be characteristic of pitvipers that exhibit a single mating season in late summer and fall, possibly due to low overall plasma concentrations of androgens compared to taxa exhibiting two mating seasons per annum. In general, the role of the SSK in reproductive biology of snakes and other squamates is not well understood, and efforts to investigate its function(s) with a range of molecular techniques (e.g., immunocytochemistry, in situ hybridization) would likely be fruitful (see Schuett et al., 2002; Sever and Hamlett, 2002).

Consistent with our predictions, the pattern of plasma T in male *A. piscivorus* during the active season was similar to previous work on this species (Johnson et al., 1982; Zaidan et al., 2003) and in other North American species of pitvipers (Table 4). In this pattern, there is a single peak of T in late summer that is coincident with a single mating season in late summer and fall (Table 4). Moreover, results of a recent study on a northeastern (Connecticut) population of *A. contortrix* revealed behavioral, histological, and hormonal results that were essentially identical to those we report here (Smith, 2007). Earlier studies of *A. contortrix* in the southern part of its range demonstrated that its mating season is the bimodal-type (Fitch, 1960; Schuett et al., 1997). Future studies of *A. piscivorus*, and other pitvipers, will likely show similar geographic variation in the timing and frequency of the mating season (Aldridge and Duvall, 2002; Schuett et al., 2002).

In most reptiles studied, corticosterone (CORT) and plasma sex steroids are elevated during the mating period (reviewed in Romero, 2002; Moore and Jessop, 2003). Several hypotheses have been proposed to explain this somewhat counterintuitive relation-

ship (e.g., even low levels of glucocorticoids can inhibit reproductive parameters; Sapolsky et al., 2000). For example, the “Energy Mobilization Hypothesis” (EMH) predicts that peak plasma CORT levels will occur during the most energy-limited time of the year (Romero, 2002), and reproduction is presumed to be the most costly annual event for both sexes. Our results complement a study conducted on males of the North American rattlesnake *Crotalus atrox* (Taylor et al., 2004) as exceptions to the usual pattern of seasonal CORT in reptiles (Romero, 2002; Moore and Jessop, 2003). It is possible that the snakes experience greatest energy limitation in the months immediately preceding reproduction, perhaps due to increased spermatogenic activity or other behavioral factors (e.g., increased foraging/searching) that we did not measure; if this were the case, then the EMH remains a viable option for explaining seasonal patterns in CORT. However, and in contrast to the study of Moore et al. (2000b), we found no significant interaction between CORT and indicators of body condition, a correlation that is predicted as support for the EMH. Perhaps the massive fat reserves and low metabolic rate (McCue and Lillywhite, 2002; Zaidan, 2003) of *A. piscivorus* and other pitvipers thus far studied buffer the need to initiate neuroendocrine stress responses during energy limitation or the mating season (see Taylor et al., 2004).

Alternatively, the peak CORT values (March–May) reported here might point to other activities modulated by CORT. The phenomenon of an early spring basking period (often coincident with ecdysis) has been widely reported in temperate zone snake species (e.g., *Elaphe obsoleta*—Prior and Weatherhead, 1996; *Vipera berus*—Olsson et al., 1997; *Lampropeltis triangulum*—Row and Blouin-Demers, 2006), but its adaptive significance remains largely unexplored. Because of its role in metabolism (e.g., Dallman et al., 1993), we propose that CORT may play a role in the regulation of this basking period.

In light of the behavioral and physiological studies on *A. contortrix* (Schuett et al., 1996; Schuett and Grober, 2000), lower plasma CORT levels during the period we propose as the mating season for *A. piscivorus* (i.e., late summer and early fall) is not entirely unexpected. For example, in male *A. contortrix*, rapid increases in CORT results from losing agonistic episodes, and high levels are associated with short-term inhibition of reproductive behavior (Schuett et al., 1996; Schuett and Grober, 2000). If CORT negatively affects reproductive parameters (e.g., plasma T) and downstream behavioral correlates in pitvipers and other snakes, then lower CORT levels may be a prerequisite for reproductive behavior. If this contention is correct, it appears to support the “Behavioral Hypothesis” (e.g., CORT modulates reproductive or other behaviors; Romero, 2002), suggesting that the seasonal pattern of CORT in male cottonmouths allows them to avoid CORT-induced (or inhibited) behaviors during the mating season.

From our extensive behavioral observations, we predicted a single late summer/fall peak of male–male combat, courtship, and copulation in this population. Although *A. piscivorus* is readily observable exhibiting certain behaviors (e.g., basking and foraging, Graham, unpublished data), we did not observe any reproductive behaviors during the period of this study. However, an observation of courtship was made at this site prior to this study on 28 August, 1996 (Graham, unpublished observation). The fact that courtship and copulation were not observed during this study was not altogether unexpected based on results of previous studies, and may explain (in part) why a detailed description of the reproductive behavior of cottonmouths in nature has not been published (Carpenter and Gillingham, 1990; Ernst and Ernst, 2003). For example, a recent 4-year radio-telemetric study of adult *A. piscivorus* in Arkansas yielded only a single observation of copulation, which occurred on 18 August 1998 (Hill and Beaupre, 2008).

Our description of the annual spermatogenic, SSK, and T cycles of *A. piscivorus* is consistent with studies of other North American

snake species that exhibit a unimodal mating pattern in which proximate indicators of mating and mating behavior covary, and contrasts with those exhibiting the bimodal pattern (see Table 4). We argue that both SSK hypertrophy and plasma sex steroid concentrations in male snakes are indicative of the mating season and/or spermatogenesis (occurring in mid- and late-summer) in all species examined to date (see Schuett et al., 2002). However, due to the apparent hormonal independence of reproductive behavior reported for an intensively studied snake population (the dissociated mating pattern of the red-sided garter snake, *Thamnophis sirtalis parietalis*, in Manitoba—Crews, 1984, 1991), the possibility that cottonmouths mate in spring without a concomitant peak of T and/or SSK hypertrophy in this population remains. Interestingly, however, in all studies in which male snakes were studied in nature, including the red-sided garter snake, the mating season and peak levels of sex steroids overlap (Table 4; Krohmer et al., 1987). If the present population of *A. piscivorus* does mate in spring without a concomitant peak of sex steroids, it represents the only exception.

In conclusion, we have described a suite of correlative factors that have been utilized by researchers to characterize and predict mating seasons of snakes. We argue that descriptive studies of sex steroid cycles and other factors provide reliable indirect evidence of mating seasons in snakes for the following reasons: (1) the mechanisms involved in modulation of reproduction in vertebrates are highly conserved (Wallen and Schneider, 2000); (2) the weakness or absence of other types of evidence that is to the contrary (e.g., unsubstantiated anecdotes); and (3) the few reliable exceptions to the generalizations we report herein, e.g., the red-sided garter snake and other examples of the dissociated mating pattern (Crews, 1984, 1991; see discussions in Schuett et al., 1997, 2006; Benner and Woodley, 2007).

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