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AN INTEGRATIVE ANALYSIS OF REPRODUCTION AND STRESS IN FREE-LIVING MALE COTTONMOUTHS, *AGKISTRODON PISCIVORUS*

by

SEAN GRAHAM

Under the Direction of Matthew Grober

ABSTRACT

I conducted an integrative field study on male cottonmouths (*Agkistrodon piscivorus*), a common pitviper of the southeastern United States, to investigate the evolution of contrasting mating patterns in North American pitvipers (bimodal and unimodal annual patterns) and resolve conflicting information about the pattern exhibited by the cottonmouth. I determined a unimodal late summer peak of testosterone (T) and a muted unimodal seasonal cycle of the sexual segment of the kidney (a secondary sexual characteristic), both of which were correlated with the single peak of spermatogenesis in late summer. I also conducted a study to determine diel and seasonal variation of corticosterone (CORT), the effect of captive handling on CORT, and the relationship between CORT and T after captive handling, and detected a significant elevation of CORT and a significant decrease of T after capture in male cottonmouths, as well as a significant negative correlation between CORT and T.

INDEX WORDS: Reptilia, cottonmouth, *Agkistrodon piscivorus*, testosterone, corticosterone, spermatogenic cycle, sexual segment of the kidney, handling stress, seasonal

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of
Master of Science
in the College of Arts and Sciences
Georgia State University

2006

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LIVING MALE COTTONMOUTHS, *AGKISTRODON PISCIVORUS*

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SEAN GRAHAM

Major Professor: Matthew Grober

Committee: Andrew Clancy

Gordon Schuett

Electronic Version Approved:

Office of Graduate Studies

College of Arts and Sciences

Georgia State University

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LIST OF ABBREVIATIONS

ANCOVA = analysis of covariance	d. = distance
<i>A. piscivorus</i> = <i>Agkistrodon piscivorus</i> , the cottonmouth	df = degrees of freedom
<i>A. contortrix</i> = <i>Agkistrodon contortrix</i> , the copperhead	e.g. = <i>exempli gratia</i> , for example
°C = degrees Celsius	EIA = enzyme immunoassay
c. = <i>circa</i> , about	est. = estimated
cc = milliliter	F = ratio of variances
CORT = corticosterone	fig. = figure
<i>C. atrox</i> = <i>Crotalus atrox</i> , the western diamond-backed rattlesnake	g = gram
<i>C. horridus</i> = <i>Crotalus horridus</i> , the timber rattlesnake	GCs = glucocorticoids
<i>C. molossus</i> = <i>Crotalus molossus</i> , the black-tailed rattlesnake	HPA = hypothalamo-pituitary-adrenal
<i>C. oreganus</i> = <i>Crotalus oreganus</i> , the northern Pacific rattlesnake	HPG = hypothalamo-pituitary-gonadal
<i>C. scutulatus</i> = <i>Crotalus scutulatus</i> , the Mohave rattlesnake	i.e. = <i>id est</i> , that is to say
<i>C. viridis</i> = <i>Crotalus viridis</i> , the prairie rattlesnake	J = Joules
	ln = the natural log
	max. = maximum
	m = meter
	M = molar
	min. = minutes
	ml = milliliter
	mm = millimeter
	n = number of subjects
	p = probability value

pg = picogram

r = correlation coefficient

R^2 = correlation coefficient squared

RIA = radioimmunoassay

Sc = spermatocytes

SE = standard error

Sg = spermatogonia

St = spermatids

Sz = spermatozoa

SSK = sexual segment of the kidney

SSKD = sexual segment of the kidney

tubule diameter

SSKE = sexual segment of the kidney

epithelial cell height

SSKL = sexual segment of the kidney

lumen diameter

STD = seminiferous tubule diameter

STE = seminiferous tubule epithelial

cell height

STL = seminiferous tubule lumen

diameter

SVL = snout-vent length

t = sample statistic of t distribution

T = testosterone

TL = tail length

T. s. parietalis = *Thamnophis sirtalis*

parietalis, the red-sided garter

snake

T. s. sirtalis = *Thamnophis sirtalis*

sirtalis, the eastern garter snake

V. aspis = *Vipera aspis*, the Aspic viper

μm = micrometer

μl = microliter

GENERAL INTRODUCTION

The conserved physiological mechanisms throughout vertebrates involved in the modulation of behavioral and physiological suites such as male and female reproductive behavior (i.e., the hypothalamo-pituitary gonadal axis) and the acute response to stressors (the hypothalamo-pituitary adrenal axis) have been thoroughly examined in many taxa (Crews, 1987; Norris, 1997; Wingfield et al., 1998; Thornton, 2001; Rhen and Crews, 2002; Sapolski et al., 2000; Romero, 2002; Seasholtz et al., 2002; McEwen and Wingfield, 2003), and these generalizations can now be critically applied to less studied and more secretive groups, such as reptiles. These vertebrate lineages should be studied to not only verify the generalizations determined from studies of more typical taxa (e.g., mammals and birds), but also to determine if and how novel physiological mechanisms evolve and under what circumstances they originated (Crews and Moore, 1986; Drickamer and Gillie, 1998). Secretive taxa offer an interesting challenge for researchers, but their intractability to research need not discourage further study. Indeed, the disciplines of comparative endocrinology and morphology may allow appropriate methodological solutions to this problem. This approach may lead to the identification of previously undescribed physiological mechanisms, illuminate interesting patterns and problems, and broaden our comprehension of physiological evolution.

Snakes exemplify a group whose secretive nature and bad reputation (along with intrinsic taxonomic biases in research programs; e.g., Bonnet et al., 2002) may have dissuaded research (overviewed by Shine and Bonnet 2000; Shine, 2003), yet the few studies conducted on exceptional cases has lead to the formulation of interesting endocrinological paradigms (Crews, 1984; Crews and Moore, 1986, 2005). Snakes are

notoriously difficult to study in the field, as their cylindrical bodies allow them to avoid observation in such a way that at any given time at a study area only a small percentage of the population is apparent to the observer (Fitch and Shirer, 1972; Parker and Plummer, 1987; Shine, 1993). There are essentially two ways to overcome the intractability of snakes to behavioral study: (a) conduct laboratory studies or (b) study snakes in exceptionally large concentrations. Laboratory studies on snakes have paid off with unique insights (e.g., Crews, 1984; Crews, 1991; Schuett et al., 1996; 1997; 2004a, b; Schuett and Grober, 2000), and field studies offer their own unique rewards and the benefit of appropriate context (e.g., Fusani et al., 2005; Schuett et al., 2005; Schuett et al., 2006). Both of these approaches have been used to study the red-sided garter snake (*Thamnophis sirtalis parietalis*), which has become the representative snake model organism for neuroendocrinological, ecological, and behavioral research (Crews, 1984; Crews, 1991; Rossman et al., 1996; Krohmer, 2004a; Shine et al., 2004). Studies on the red-sided garter snake from Manitoba, Canada derive from populations that occupy communal hibernacula; these sites occur at higher latitudes than any North American snake (Krohmer, 2004a). The mating system consists of mating “balls” of dozens to hundreds of snakes with highly skewed sex ratios in favor of males (e.g., 100:1), and are also exceptional in that individuals will continue mating behavior despite an observer being present and manipulating them in the field (Shine et al., 2001; Krohmer, 2004a). Although this situation has allowed researchers to overcome the difficulty of studying snakes under natural conditions, given that there are nearly 3000 species of extant snakes and their mating systems can be disparate from those of garter snakes from Manitoba (e.g., Duvall et al., 1992), other models should also be considered for study.

Pitvipers can make excellent subjects for behavioral and physiological research, because they: (a) can be found in high densities, (b) are relatively easy to collect, (c) are sufficiently large enough for tissue sampling, and (d) in some cases (e.g., copperheads and cottonmouths) there is abundant ecological and natural history data on certain populations (Gloyd and Conant, 1990). Furthermore, pitvipers have undergone several recent adaptive radiations (e.g., North American rattlesnakes—Murphy et al., 2002; Douglas et al., 2006, tropical highland forms—Crother et al., 1992, insular tropical radiations— Tsai and Tu, 1992; Molhatra and Thorpe, 1999) that allow interesting interspecific comparisons and the formulation of robust phylogenetic hypotheses.

Pitvipers are so-named for the loreal pit organ—the most sensitive infrared receptor in the animal kingdom (Moleenar, 1992). Rattlesnakes have evolved a characteristic anti-predatory warning system (the rattle) that involves one of the most efficient vertebrate muscle systems known (i.e., the tailshaker muscle—Schaeffer et al., 1996). The pitvipers are of course venomous, and have evolved sophisticated weapons that are used in feeding and defense (Greene, 1997; Fry and Wüster, 2004). There is no doubt that along with these fascinating characteristics, pitvipers have also developed many interesting physiological and behavioral mechanisms that will enrich our general understanding of the ecology and evolution of vertebrates. The behavioral studies of copperheads and rattlesnakes by Schuett and colleagues (Schuett, 1992; 1997; Schuett and Grober, 2000; Schuett et al., 1996; 1997; Schuett and Grober, 2000; 2002; 2005; 2006), and Taylor and colleagues (Taylor et al., 2004a,b; Taylor and DeNardo, 2005)—which have included aspects of both the hypothalamo-pituitary-gonadal (HPG) and

hypothalamo-pituitary-adrenal (HPA) axes—are good examples, and future research will undoubtedly reveal similar rewards.

CHAPTER 1

Seasonal Concentrations of Plasma Testosterone in Male Cottonmouths (*Agkistrodon piscivorus*) and their Relationship to Gonadal State and Activity of the Kidney Sexual Segment.

SUMMARY

To better understand the proximate causation of two observed mating patterns in North American pitvipers (unimodal and bimodal annual patterns), I conducted an integrative field study of the cottonmouth (*Agkistrodon piscivorus*) in Georgia from September 2003 through May 2005 that included an extensive observational regime and collection of tissues for behavioral, anatomical, histological, and hormone analysis. An enzyme immunoassay (EIA) of plasma samples and standard histological procedures were conducted on collected 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.

INTRODUCTION

Uncoupling mechanisms have evolved repeatedly in vertebrates and allow flexibility with regard to timing of important reproductive events; examples include embryonic diapause in mammals (Renfree and Shaw, 2000), sperm storage in most major

vertebrate lineages (Birkhead and Møller, 1993), and an apparent complete dissociation between reproductive behavior and sex steroid secretion in a population of snakes (Crews, 1984). The diversity of these mechanisms was characterized by Crews (1984) as a continuum of strategies ranging from the mammalian norm of complete association of gonadal (including sex steroid secretion) function and mating behavior to complete dissociation of gonadal function (including sex steroid secretion) and mating behavior, best exemplified by the red-sided garter snake (*Thamnophis sirtalis parietalis*).

In this species, manipulation studies failed to support the activational role of sex steroids on male sexual behavior, as only an artificial hibernation period followed by warming appeared to rescue the courtship ability of gonadectomized or intact males (Gartska et al., 1982). Originally it was hypothesized that the extreme environmental conditions experienced by this ectotherm (it is found at a higher latitude than any other North American snake, and has only a three month activity period—Krohmer, 2004a) would not allow enough time for the secretion of sex steroids during the brief mating period, and led to the evolution of this interesting uncoupling mechanism (Crews and Moore, 1986). However, field studies have determined that sex steroids are elevated during the brief mating period (Krohmer et al., 1987; Moore et al 2000a; Moore et al., 2000b). Unfortunately, the laboratory studies on captive *T. s. parietalis* and *T. s. sirtalis* are the only removal and replacement manipulation experiments ever performed on a snake, and may have led to the misinterpretation that all snakes are capable of a completely dissociated reproductive mode. Although correlative studies conducted on pitvipers and other snake species support the role of sex steroids in the activation of sexual behavior, the dissociated-associated paradigm and widespread mechanisms of

sperm storage in both male and female snakes have led to a lack of consensus about the appropriate proximate correlates of mating in these secretive animals (Schuett et al., 2006).

Numerous studies support the role of testosterone (T) as the primary androgen in reptiles, including snakes (Moore and Lindzey, 1992; Schuett et al., 2002; Schuett et al., 2005, 2006). Contrasting unimodal and bimodal annual patterns (with maximum activity in late summer-fall or late summer-fall and again in spring) of T secretion and mating persist despite the retention of an identical spermatogenic pattern (i.e. aestival or “postnuptial” spermatogenesis, with peak spermiogenesis occurring in late summer—St. Girons, 1982) in North American pitvipers (Schuett, 1992; Aldridge and Duvall, 2002). Peak hypertrophy of the sexual segment of the kidney (SSK), an androgen-dependant secondary sexual characteristic involved in seminal fluid formation (Prasad and Reddy, 1972), also has been characterized as tracking the general mating pattern in North American pitvipers (with bimodal or unimodal patterns of SSK hypertrophy—Aldridge, 1993, Aldridge and Brown, 1995; Aldridge 2000, Schuett et al., 2002). Together, the seasonal T and SSK cycle—and their relationship to the spermatogenic cycle (i.e., if both T and the SSK are maximal during the single peak of spermatogenesis, correlations between all of these variable are to be expected)—are robust predictors of the seasonal mating pattern in pitvipers (Aldridge and Duvall, 2002; Schuett et al., 2002; also see Fig. 1 and 2). The bimodal and unimodal mating pattern are presumed to have evolved in response to the contrasting patterns of estrous exhibited by females (Aldridge and Duvall,

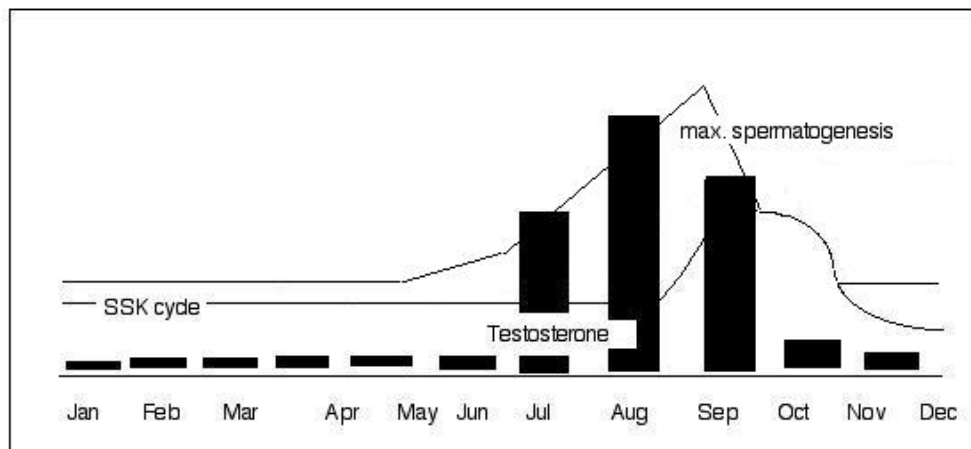


Figure 1 Unimodal mating pattern. Composite based on several descriptive studies of rattlesnake species (*Crotalus viridis*, Aldridge, 1993; *C. horridus*, Aldridge and Brown, 1995; *C. molossus*, Schuett et al., 2005). Maximum spermatogenesis in late summer (aestival pattern), muted SSK peak in late summer, and significant elevation of testosterone (and other sex steroids) in late summer. Following spermatogenesis, sperm is stored in the ductus deferens throughout the year, and presumably voided before or during testicular recrudescence in spring. Mating occurs in a single mating season during mid- to late summer and up to autumn (Schuett et al., 2005). Mated females store sperm in the oviducts throughout the winter until ovulation in spring.

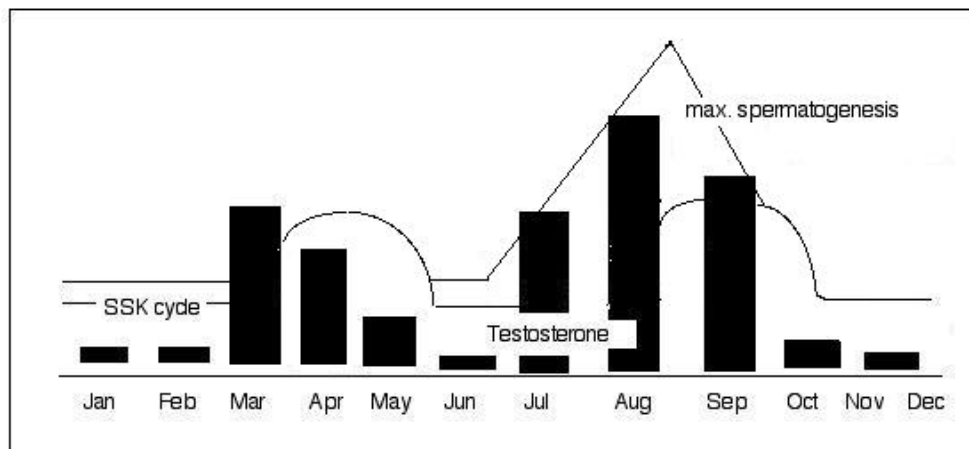


Figure 2 Bimodal mating pattern. Composite based on several descriptive studies of rattlesnakes and the copperhead (*Agkistrodon contortrix*, Schuett et al., 1997; *Crotalus oreganus*, Aldridge, 2001; *C. scutulatus*, Schuett et al., 2002; *C. atrox*; Taylor et al., 2005). Maximum spermatogenesis in late summer (aestival pattern), significant SSK peaks in late summer and spring, significant elevation of testosterone (and other sex steroids) late summer and spring. Sperm stored throughout the year in the ductus deferens, and presumably voided before testicular recrudescence in spring. Mating occurs twice—in late summer/ and or autumn and in spring following hibernation. Mated females store sperm either throughout the winter or temporarily (~ 1-2 months) in the oviducts during spring before ovulation. The degree to which individual females utilize only one or both sperm storage strategies has not been determined; the pattern observed is a population-level phenomenon.

2002, see Fig. 3), and because there are many more studies of the male pattern in snakes, for comparative purposes I selected the male pattern for study.

The discovery of contrasting mating patterns between closely related, sympatric pitvipers (e.g., *Crotalus atrox* and *C. molossus*—Schuett et al., 2005) suggests an interesting ultimate explanation and warrants examination of more cases. The copperhead (*Agkistrodon contortrix*), a sympatric congener of *A. piscivorus*, has been well characterized as exhibiting a bimodal mating pattern by both field observations and laboratory studies of the T cycle and sexual behavior (Fitch, 1960; Schuett et al., 1997). However, in the case of the cottonmouth, various forms of evidence are conflicting.

Endocrinological studies indicate a unimodal pattern of T secretion, with reported peak values of testosterone considerably less than those reported for other snakes (Johnson et al., 1982; Zaidan et al., 2003; Schuett et al., 2005). One paper reported a unimodal peak of T secretion and a bimodal pattern of SSK hypertrophy, the only reported case of this condition in any snake (Johnson et al., 1982). Others have commented on the low sample sizes used to support the spring peak of SSK hypertrophy in this study (Aldridge and Duvall, 2002). Most sources have reported the cottonmouth as mating in both late summer and spring; however, field observations of spring mating are rare, lack detail, and predate our current understanding of snake reproduction and sperm storage (e.g., Beyer, 1898; Wharton, 1966). Detailed descriptions of mating in either period have not been published (Ernst and Ernst, 2003). Therefore, the sum of correlational evidence in this species may help resolve the issue.

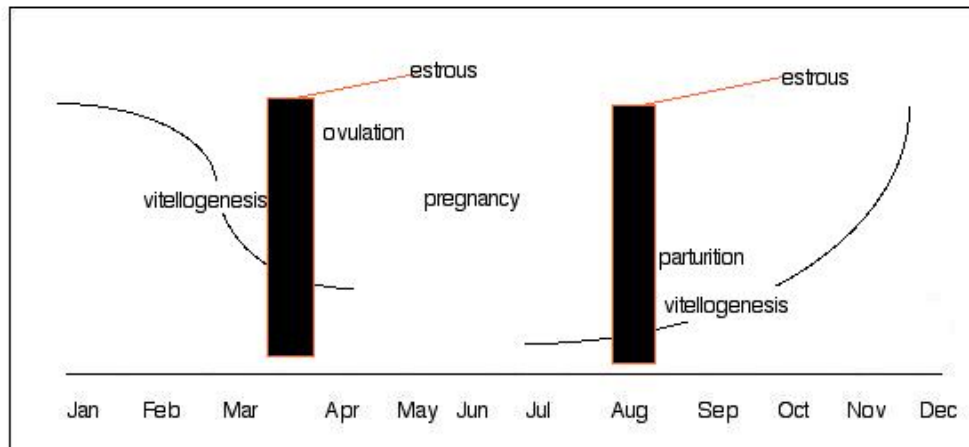


Figure 3 Female mating pattern of North American pitvipers. The type II vitellogenic cycle (Aldridge, 1979; but see Taylor and DeNardo, 2005) is initiated in late summer, presumably pauses during winter, and continues until ovulating in spring. Pregnancy occurs throughout the summer months, parturition in late summer-fall. Estrous and mating take place during some part of the vitellogenic cycle, usually associated with ecdysis, either once or twice during the year (unimodal or bimodal), and it is presumed the male pattern evolved in response to this (Schuett, 1992; Aldridge and Duvall, 2002). Most populations contain varying proportions of females undergoing either vitellogenesis (attractive-receptive) or pregnancy (reproductive) each year. Females without sufficient energy reserves to reproduce will delay reproduction until another time, and vitellogenesis is typically initiated at the same time period from year to year.

The purpose of this study was to examine the gonadal, endocrine, and behavioral factors associated with mating in male free-living cottonmouths. Since the contrasting patterns of mating in pitvipers may be influenced by environmental factors (i.e., zeitgebers—Aldridge, 1975; Duvall et al., 1982; Aldridge and Duvall, 2002; Zaidan et al., 2003; Schuett et al., 2005), for comparative purposes it was important to conduct this study in the field with wild snakes. The high density and detectability of this species at the study area allowed collection of large histological and hormone samples, and a vigorous attempt was made to capitalize on this situation to make detailed field observations of reproductive behavior. Consistent with previous reports of the androgen cycle in this species, I hypothesized that a unimodal mating pattern occurs in this species, with male sexual behavior (courtship and male-male combat) most prevalent during or immediately following a single seasonal peak of T, SSK hypertrophy, and spermatogenesis in late summer. I further predicted that since these variables are occurring concomitantly, there should be several significant correlations between them.

MATERIALS AND METHODS

Study Animal and Site The cottonmouth is a semi aquatic pitviper often found at high densities (>700 per hectare—Gloyd and Conant, 1990), meeting this requirement for tractability of study (see general introduction). This snake is readily detectable in its habitat, because it is relatively large-bodied and more likely to stand its ground than to flee compared to other snakes (Gloyd and Conant, 1990). The cottonmouth is found in the southeastern United States, a region of high reptile diversity with a regional climate typical of snake habitat (Gloyd and Conant, 1990; Gibbons and Dorcas, 2005). The study site is a c. 240-hectare floodplain forest/beaver dam marsh complex located at the

confluence of Morning Creek with the Flint River in the Piedmont region of Georgia. The study area is a mosaic of wetland/upland habitats, and is bound on all sides by suburban or rural development. For a complete description of river swamp and beaver marsh habitat in Georgia, see Wharton (1979).

Field Observations and Processing. Free-living male *A. piscivorus* were collected and/or observed at the study area from September 2003 through May 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, coitus, agonistic encounters.) was recorded. Behavioral notes followed the nomenclature of Carpenter and Ferguson (1977) and Carpenter and Gillingham (1990) for reproductive behavior, and Savitzky (1992) for foraging behavior. Adult snakes were measured (snouth-vent-length—SVL, and tail length—TL) to the nearest 1 cm by stretching flexible tape along their side in the tube, and weighed to the nearest 3g using a Pescola spring scale. All processed snakes received a unique scale-clip mark (e.g., Fitch, 1960) before release at their point of capture.

Observations and sampling took place evenly throughout the active season (March through October) to eliminate sampling bias. In brief, at least eight person-hours per week were spent searching for cottonmouths during the crucial spring and late summer periods, defined here as March 1 to May 31 (spring) and August 1 to October 31 (late summer). These search-hours did not include time spent observing or processing cottonmouths. At least two person-hours per night were spent observing from May 15-

August 31st (the designated summer period when the cottonmouth may switch to a nocturnal pattern; see Gloyd and Conant, 1990), as well as a six person-hour per week daylight schedule. Winter observations and collection took place opportunistically on warm days from November through February when cottonmouths can sometimes be found basking outside of their 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) for an equal amount of time (32 total person-hours each searching during each period).

Blood Collection. From September 2003 to June 2004, subjects sampled for blood for sex steroid analyses were anesthetized using isoflurane in the field for the researcher's safety. All blood samples were collected with the snakes secured in a plastic tube. To achieve light anesthesia, a small cotton ball with 0.5 ml of anesthetic was placed in the opposite side of 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 two hours (Moore et al., 2000; Lance et al., 2003). Therefore, a small sample (1 ml) of blood was collected as soon as possible in a labeled plastic vial (in less than 30 min.) by cardiocentesis using a disposable 1-cc heparinized tuberculin syringe (mean 22.15 min; $SE = 8.24$; range 3-30 min.). From June 2004 through May 2005 the sampling regime changed as the author gained more experience with a different approach; blood samples were collected (within 3-30 min, $SE = 8.96$; mean 13.33 min.) from the caudal sinus without anesthesia (possible differences between these techniques discussed below).

All subjects not collected for tissues (see below) were released at the point of capture with a unique scale clip (e.g., Fitch, 1960), and these snakes were not re-sampled for this study. A subset of the population was collected for reproductive tract analysis and to determine the effects of captive restraint on plasma hormone concentrations (see below). These animals were transported to a staging area in a tied bag secured in a bucket. They were kept in ambient (outdoor) temperature and humidity conditions, and all but 4 subjects were never transported more than 1km from the study site. After 2-24 hours (mean = 8.59 hr; *SE* = 7.5) of exposure under this experimental protocol, the animals were anesthetized as described above. A large sample of blood (>2 cc) was then collected by draining blood directly from the heart. This sample was subsequently processed as described and represents the second treatment taken under captive conditions (N = 33; see Chapter 2). Blood samples were put on ice packs in a cooler for no more than 24 h (Taylor and Schuett, 2004). Blood samples were centrifuged and plasma drawn off and placed in a new labeled plastic vial before being placed at – 20° C for storage.

Testosterone Assay. Enzyme immunoassays were conducted on plasma samples to determine concentrations of testosterone. Hormones were extracted from thawed plasma samples using an ether extraction method. In brief, 2ml of diethyl ether was added to each 225µl sample of plasma and mixed for 3 min. in borosilicate vials using a multi-tube vortexer. After an additional 3 min. and a qualitative determination that the sample had achieved phase separation, the sample was fast-frozen by placing it on a rack in a methanol/dry ice bath. The ether layer was decanted into a 16x30mm borosilicate vial and the aqueous phase layer was submitted to another round of diethyl ether and fast

freezing. This ether layer was then decanted into the same vial as the first sample to extract as much of the hormone as possible. The ether was gradually evaporated under a fume hood and nitrogen stream at 40°C. The hormone film left at the bottom of each vial was then re-suspended in 225µl EIA buffer (provided in the kits), covered with parafilm, and placed in a refrigerator overnight. Enzyme immunoassays were performed on these extracted hormone samples using the instructions of the kit (Cayman Chemical Company, Ann Arbor, Michigan).

The assays were validated for *A. piscivorus* testosterone by assessing parallelism and comparing samples of unknown T concentration with those spiked with T standards provided in the kit. A pooled sample was created by adding 10 ether-extracted samples together. A serial dilution of these samples was made to determine a dilution curve. I then transferred 225 µl of the pooled sample into a 1.5ml Eppendorf centrifuge tube and mixed this with 225 µl 0.1 M phosphate buffer on a single-sample vortexer. This created a 1:2 dilution (the pooled sample representing a 1:1 concentration). 225 µl of this dilution was then mixed with 225 µl 0.1M phosphate buffer to create a 1:4, 1:8, 1:16, 1:32, and finally 1:64 solution. These serial dilutions were run in quadruplicate per the instructions of the kit. The dilution curve was log-logit transferred, and compared to the standard curve. The slopes of the two curves were parallel (comparison of slopes: $t_{11} = 0.07$, $p = 0.95$; Zar, 1996), indicating that the kit detects *A. piscivorus* T.

Recovery was estimated using an 880 µl sample of pooled ether-extracted hormone. 110 µl of this sample was distributed into 8 1.5ml Eppendorf centrifuge tubes and mixed with a known amount of standard provided by 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

concentrations present in the sample. Minimum recovery ranged from 85.1-105% (85.1% minimum recovery), and the slope of the expected versus observed curve was 0.81, which indicates a tight, linear relationship between these variables ($R^2 = 0.96$, $p = 0.95$). 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%.

Tissue Collection, Processing, and Quantification. A subset of study subjects was selected for destructive sampling for gross and histological examination of reproductive tissues. Most procedures followed Schuett and colleagues (2002). Snakes to be used for histological analysis were taken to a staging area (see above for details) and administered isoflurane for anesthesia. Anesthesia was induced as described above. SVL and TL (nearest mm) was recorded by measuring the anesthetized snake positioned straight, and body mass (nearest 0.1g) was measured by a triple beam balance before removal of tissues. Blood samples for hormone assay were taken (see above), and the head was then removed by a modified guillotine procedure.

Following this, the right and left reproductive tract was measured *in situ* to the nearest mm and removed. These organs were then fixed in 10% water buffered formalin for two weeks to ensure penetration of the preservative. The tissues were then stored in 95% ethanol for later gross and histological analysis. Gross measurements of the right testis and kidneys (to the nearest 0.1 mm) were recorded using Spi 2000 calipers and a Mettler Toledo scale (to the nearest 0.01 g). These measures included testis length, width, height, and mass, and kidney length and mass. Measurements of the right reproductive tract were used to compare to other studies in snakes; the right reproductive tract is measured as a matter of convention (Schuett et al., 2002).

5-8mm sections from the right reproductive tract (mid-testis, mid-vas deferens, and anterior kidney) were taken. The tissues were imbedded in paraffin by serial dehydrations in an automated citadel, sectioned (at 10 μm) using a rotary microtome, and stained using Erlich's Hematoxylin and Eosin. Diameter, lumen diameter, and epithelial cell height of both Seminiferous (STD, STL, STE) and SSK (SSKD, SSKL, SSKE) tubules were measured to the nearest μm using Zeiss Axiovision 4.0 software for light microscopy. The median of twelve of these values for each sample 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).

Data Analysis. SVL and body mass were compared using linear regression and were positively correlated ($F_{1,53} = 168.4$, $p = 0.0001$, $R^2 = 0.76$). I therefore used SVL as the covariate in subsequent analyses. For most analyses, I used only the initial T samples collected in the field, unless specifically addressing the effects of captivity on T. Three T samples were eliminated from further analyses; two were more than two standard deviations above the mean (Zar, 1996) and one represented the only sample for a month (January). I compared spring (April to May) mass, SVL, and T values for 2004 and 2005 using an ANOVA with month, year, and month x year as the main effects. I found no effect of year on body mass, SVL, T, and captive T (mass overall model: $F_{5,15} = 0.66$, $p = 0.66$; SVL overall model: $F_{5,15} = 0.29$, $p = 0.91$; T overall model = $F_{3,16} = 0.33$, $p = 0.80$), so I combined these data for analysis. Thus, in effect, I tested for differences between two sampling protocols (anesthesia, Spring 2004; non-anesthesia, Spring 2005),

and found no differences in plasma T. Similarly, I tested the effect of the different histology sample years (fall 2003 and 2004) on representative histology variables (testis mass, median SSKD, and median STD). I found no effect of year, and therefore histology data were combined for analysis (testis mass overall model: $F_{3,5} = 0.84$, $p = 0.53$; SSKD overall model $F_{3,5} = 0.42$, $p = 0.75$; STD overall model $F_{3,5} = 0.64$, $p = 0.62$). In addition, there were no significant effects between T and time of day sampled, and between T and time after capture the sample was taken (linear regression, time day/T: $F_{1,49} = 0.53$, $p = 0.47$, $R^2 = 0.07$; time till bleed/T: $F_{1,48} = 0.399$, $p = 0.53$; $R^2 = 0.008$).

To achieve normality, T values were ln transformed. With the exception of median STD, gross and histological variables met the assumption of normality and were not transformed. However, unless specified, the original untransformed data is presented in the figures. There was homogeneity of variance for gross, histological, and hormone values across months. I analyzed the monthly differences in T, testis mass, and histological variables (median SSKD, SSKE, SSKD, STD, STE, and STL) using an ANCOVA with month as the main effect with SVL and SVL*month as covariates. For comparison, I similarly analyzed the month effects on log-transformed T values using only samples from animals under captive conditions. I also analyzed the variation of T, testis mass, and the histological variables using ANCOVA with stage of spermatogenesis as the main effect and SVL as the covariate.

I used correlational analysis to determine significant interactions between T, SVL, testis mass, and the histological variables. A paired t test was conducted to compare the initial T samples collected in under 30 min. in the field and those sampled under captive

conditions from the same animal. All data were analyzed using the statistical program JMP (SAS Institute, Inc. Cary, NC.) with the α value set at $p = 0.05$.

RESULTS

Behavioral Observations. Sampling effort is illustrated in table 1. In the spring observation period I observed two bisexual pairs in close association (<1m of each other). In the first case (14 May 2004, 1132 h) the snakes were both in the typical foraging posture of pitvipers. In the second case (13 March 2005, 1415 h) a male snake (in shed cycle) was discovered in leaf litter near a boulder hibernaculum, and during its capture a female was discovered underneath him in physical contact. In addition, on 11 April 2005 (1845 h), two snakes were discovered in close association (est. d. = 25-50 cm) near a windthrow (often used as hibernaculum). Only one snake was captured, and was sexed as female. On 21 April 2005 (1910 h) a male snake (possibly the uncaptured snake from 11 April) was captured coiled in the exact place where the female was captured on 11 April 2005. None of the female snakes from these spring observations appeared to be approaching their shed cycle. During the late summer I found three bisexual pairs in close association. In one case (8 August 2004, 2116 h) the male snake passed within 25 cm of a non-pregnant female during what appeared to be a mobile foraging bout. The female seemed to notice the male and retreated in the opposite direction. Another case (19 August 2004, 1948 h) consisted of a male and non-pregnant (eyes opaque in shed cycle) female tightly coiled together in contact with one another on a marsh hummock. On 28 August 2004 (1016 h), a similar pair was seen in a similar microhabitat. In this case, however, the female was sexed visually (by observing the tail morphology) during

Table 1 Sampling effort for this study. Number of person hours spent searching per month, snakes observed and captured per month, and total blood and histological samples collected per month.

Month	Effort (est. person-hrs.)	Observations (number of individuals)	Captures	Blood samples	Sacrifices
Feb. 2004	34	5	4		
Mar. 2004	36	12	9	3	3
Apr. 2004	45	46	36	10	4
May 2004	43	50	33	7	4
Jun. 2004	47	58	26	8	4
Jul. 2004	42	54	39	11	4
Aug. 2004	35	64	39	6	4
Sep. 2003/4	32.5	24	17	3	5
Oct. 2003/4	32.5	15	11	3	4
Jan. 2005	1	1	1	1	1
Mar. 2005	8	9	9	2	
Apr. 2005	8	11	10	3	
May. 2005	8	6	6	1	
Aug. 2005	20	26	20		
Sep. 2005	8	12	8		
Total	400	393	268	58	33

escape. Another instance suggestive of mating activity was the observation of three snakes in close association atop a windthrow on 14 August 2004 (1255 h). Only two were captured, a pregnant female, and an opaque, non-pregnant female. The other escaped without verification of sex. However, on 28 August 2004 (1148 h), a male (possibly the third, uncaptured snake) was found in this exact location. Finally, an observation of courtship (consisting of a cottonmouth approaching a shedding individual and vigorously tongue flicking and head jerking) was made at this study site on 28 August, 1996. Despite > 400 person hours searching, no instances of copulation were observed during the study.

Spermatogenic Cycle. The spermatogenic stage for each cottonmouth is plotted by month in Fig. 4 (see also fig. 5). The peak of spermatogenesis was in late summer (July) when STE values were significantly largest ($F_{15,16} = 3.87$, $p = 0.0054$, Fig. 6, see also Fig. 16), and testis mass was greatest ($F_{15,16} = 2.87$, $p = 0.0221$). Testis mass and STE varied significantly with spermatogenic stage, with the greatest testis mass during stage 4 and 5, and maximum STE during stage 4 (testis mass: $F_{6,22} = 5.29$, $p = 0.0016$; STE: $F_{6,22} = 5.73$, $p = 0.0010$). A significant positive correlation across months between testis mass and STD ($r_{24} = 0.597$, $p = 0.0021$, Fig. 7) and STE ($r_{24} = 0.648$, $p = 0.0006$) was detected. STE was also positively correlated with STD ($r_{24} = 0.568$, $p = 0.0038$).

Ductus deferens . The ductus deferens had dense populations of sperm in 31 of 33 specimens examined (Fig. 8). The two specimens that could not be scored as having a sperm mass may have actually contained sperm and its lack of detection could be an artifact of poor histological preparation.

Sexual Segment of the Kidney. Monthly median values of SSK diameter did not reveal a statistically significant trend (Fig. 9, 10; see also Fig. 15). Testis mass was positively

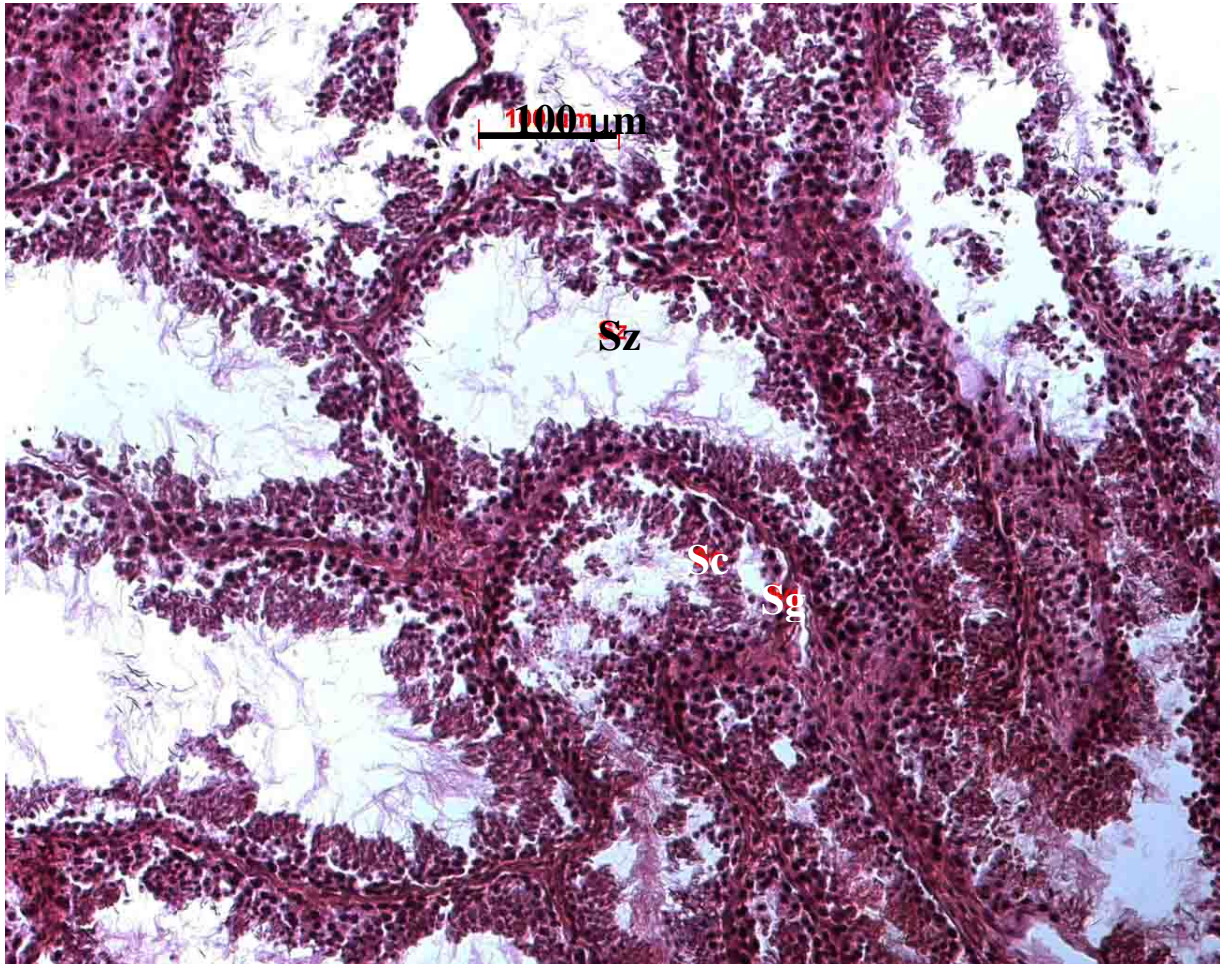


Figure 5 Stage 5 Spermatogenesis. September specimen showing seminiferous tubules during late spermiogenesis. 400x magnification
Sc = Spermatocytes; Sg = Spermatogonia; Sz = mature spermatozoa

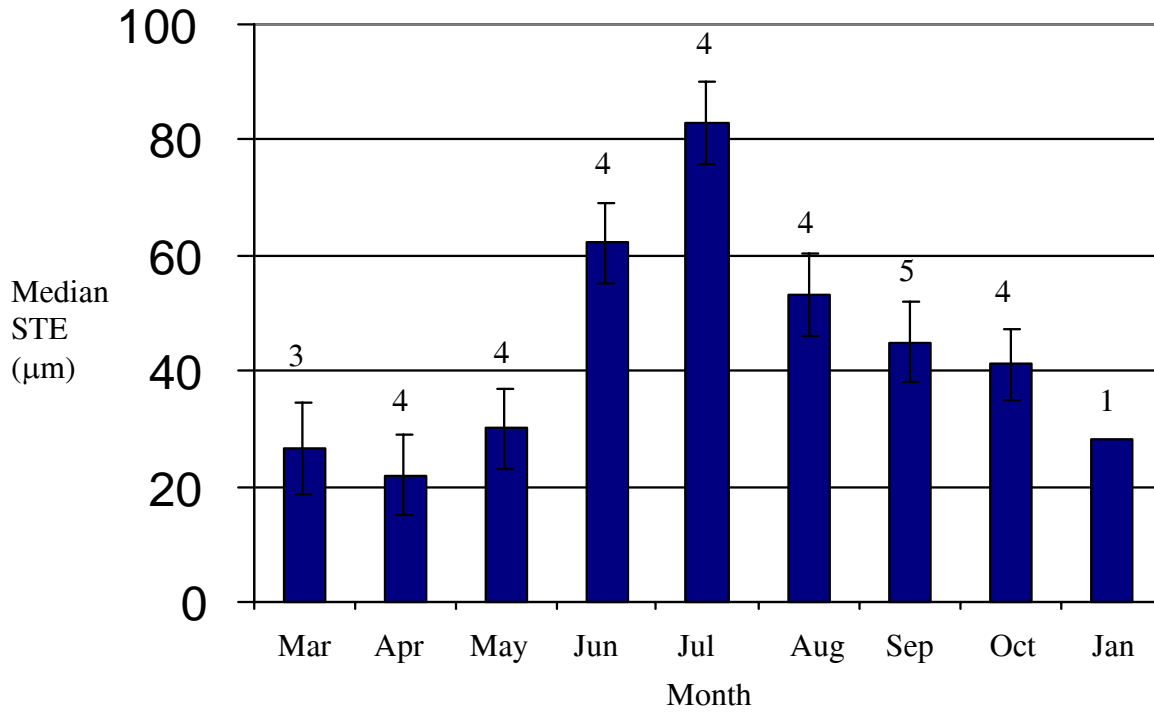


Figure 6 Monthly variation of median STE. There is a significant peak in July. Sample sizes labeled above standard error bars.

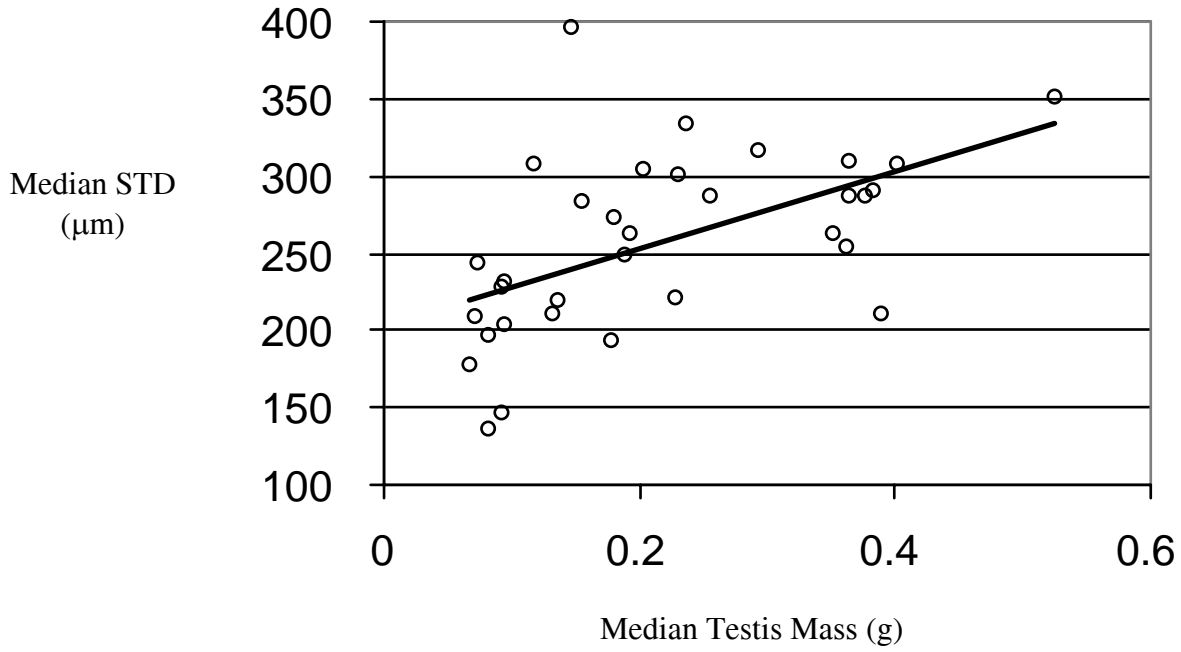
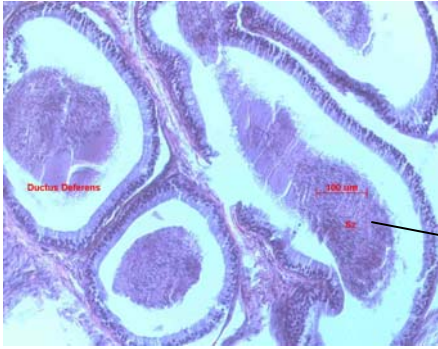
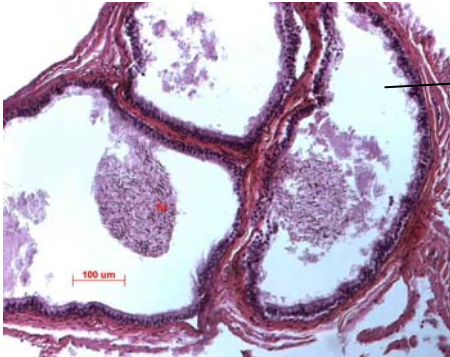


Figure 7 Relationship between median STD and median testis mass ($r_{24} = 0.597$, $p=0.0021$).



March

spermatozoa



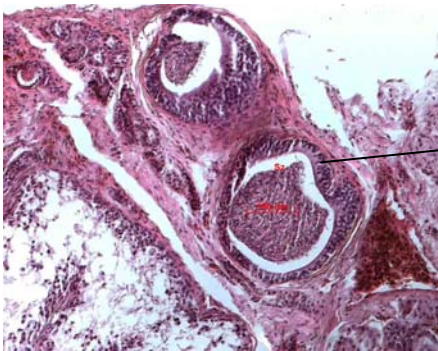
lumen of
ductus
deferens

July



October

100 μm



January

ductus
deferens

Figure 8

Ductus deferens sectioned between the testis and anterior kidney.

Large populations of spermatozoa were present in all months sampled. Differences in structure of ductus due to position of section, not seasonal changes.

400x magnification.

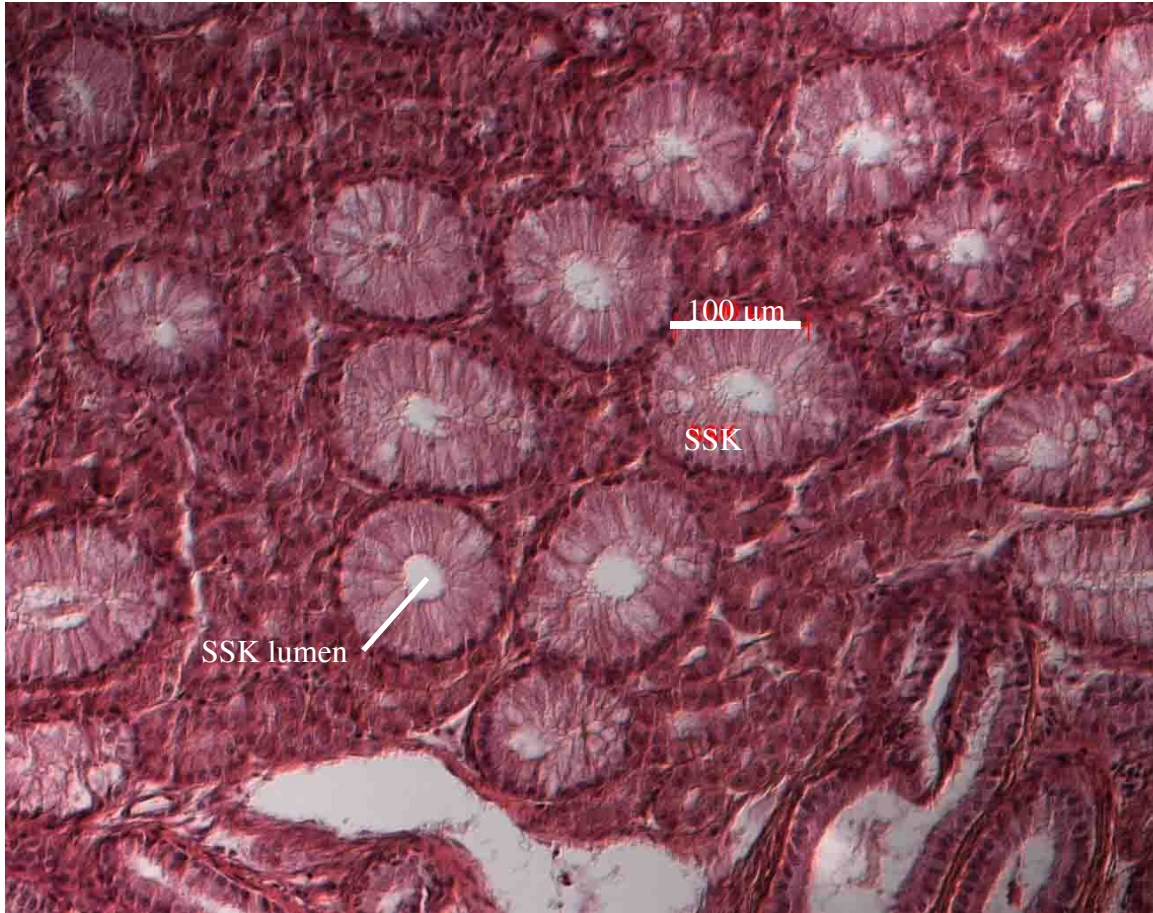


Figure 9 Sexual segment of the kidney from a July specimen. 400 X magnification.

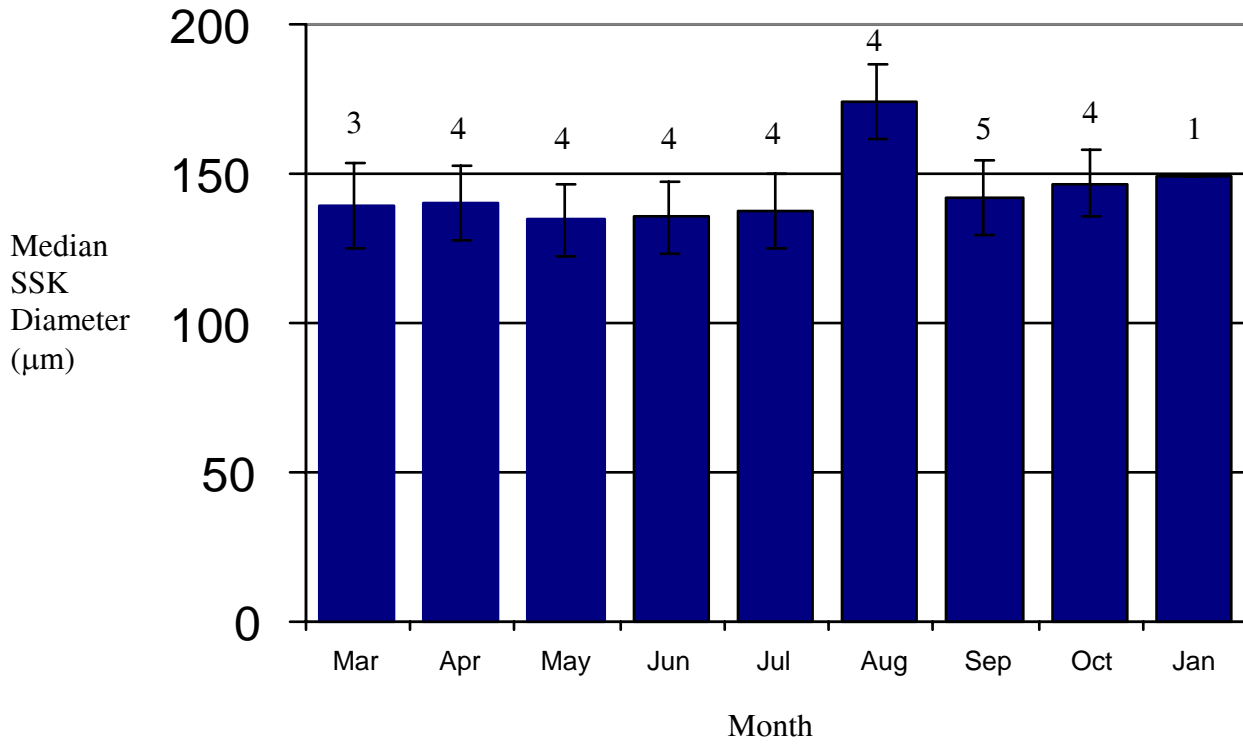


Figure 10 Monthly variation of median SSKD. There is a non-significant peak in august. Sample sizes labeled above standard error bars.

correlated with SSKE and SSKD (SSKE: $r_{24} = 0.579$, $p = 0.0031$; SSKD: $r_{24} = 0.5913$, $p = 0.0023$; Fig. 11); SSKE and SSKD were positively correlated with each other ($r_{24} = 0.934$, $p < 0.0001$).

Testosterone. The mean value of plasma T detected by the EIA was 5.6 ng/ml (range = 0.62 – 35.72). Testosterone was significantly elevated in summer (Jun. Jul. Aug.= Sep. Oct.; Mar., Apr., May = Sep., Oct.; Jun. Jul. and Aug. > Mar., Apr., and May; $F_{15,33} = 6.128$; $p = < 0.0001$, Fig. 12; Fig. 16), and levels in spring were basal in both 2004 and 2005. T was significantly elevated during spermatogenic stage 4 relative to other stages ($F_{5,17} = 3.19$, $p = 0.032$). Data from snakes held in captivity for 2-24 hours revealed no seasonal peak of T ($F_{15,13} = 2.197$, $p = 0.08$). The T concentrations were significantly higher in field samples compared to samples taken from animals under captive restraint. (paired t test: $t_{20} = 2.46$, $p = 0.012$, Fig. 13). Plasma T levels were positively correlated with median testis mass and median STE (testis mass: $r_{24} = 0.424$, $p = 0.038$, Fig. 14; STE: $r_{24} = 0.558$, $p = 0.0046$).

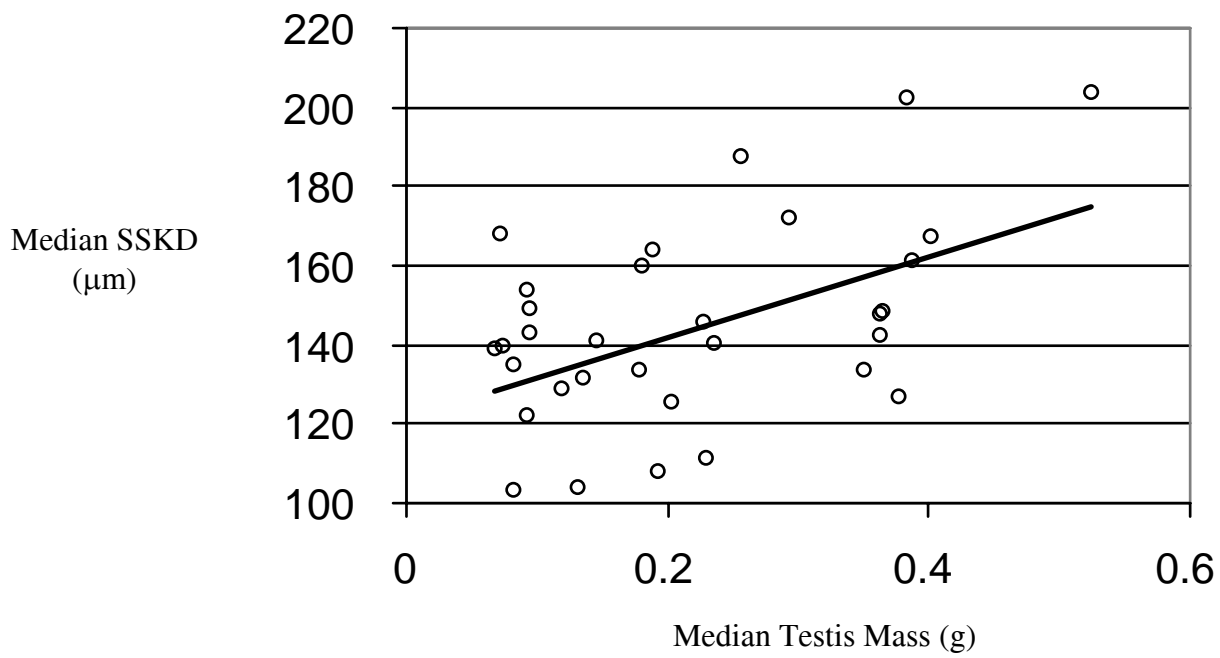


Figure 11 Relationship between median SSKD and median testis mass ($r_{24} = 0.5913$, $p = 0.0023$).

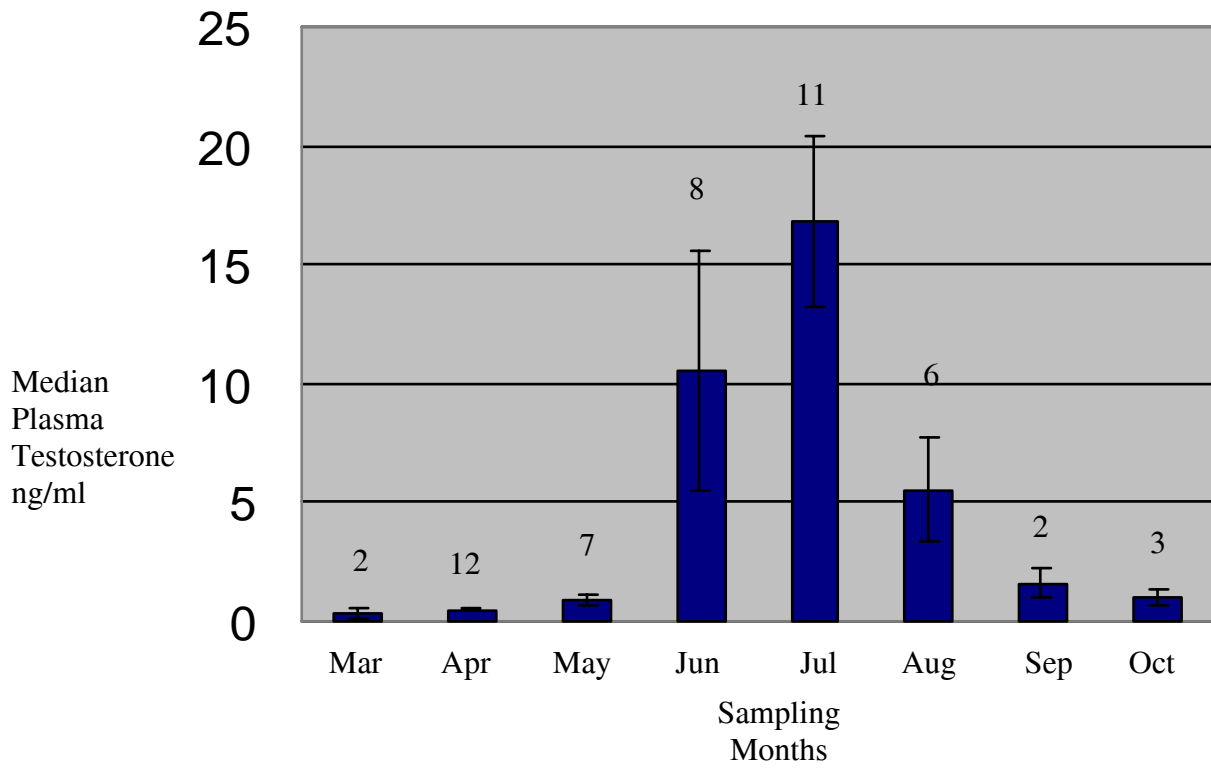


Figure 12 Median T plotted by month. June, July, and August values significantly elevated ($F_{15,33} = 6.128$; $p = 0.0001$).

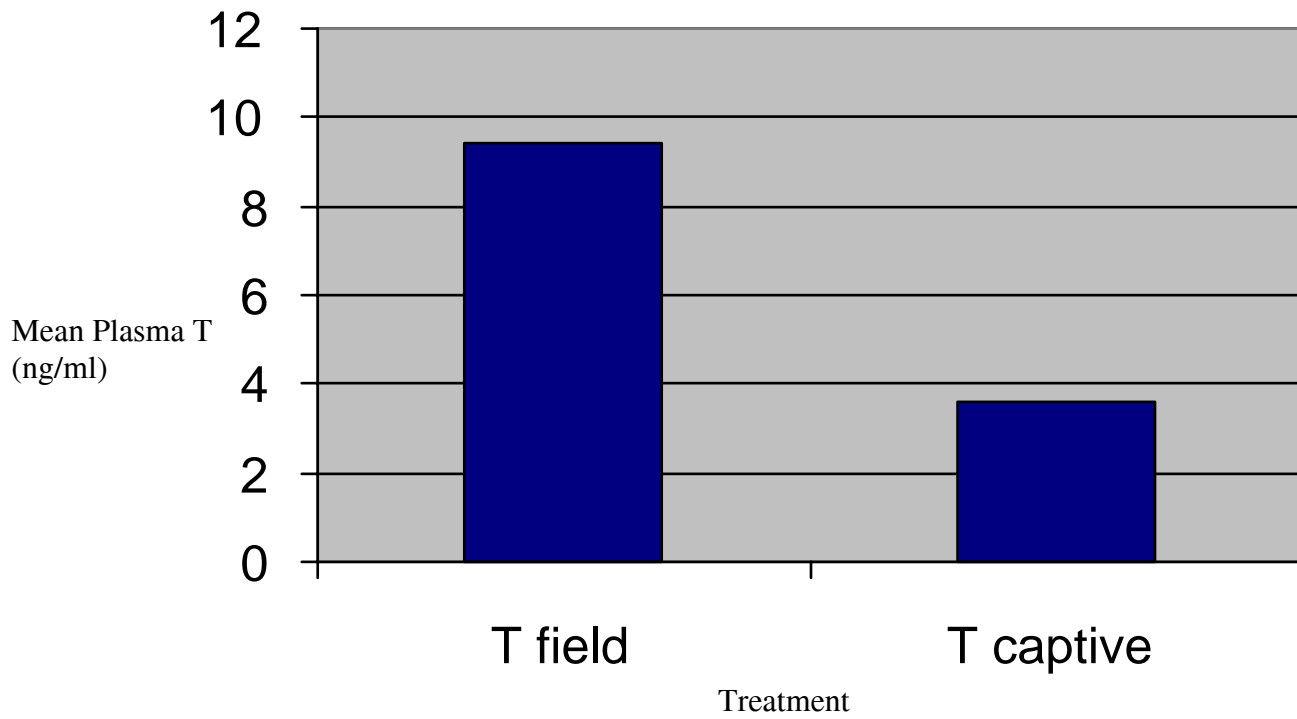


Figure 13 Comparison of mean plasma T levels: field samples vs. captive handling treatment (paired t test: $t_{20} = 2.46$, $p = 0.012$).

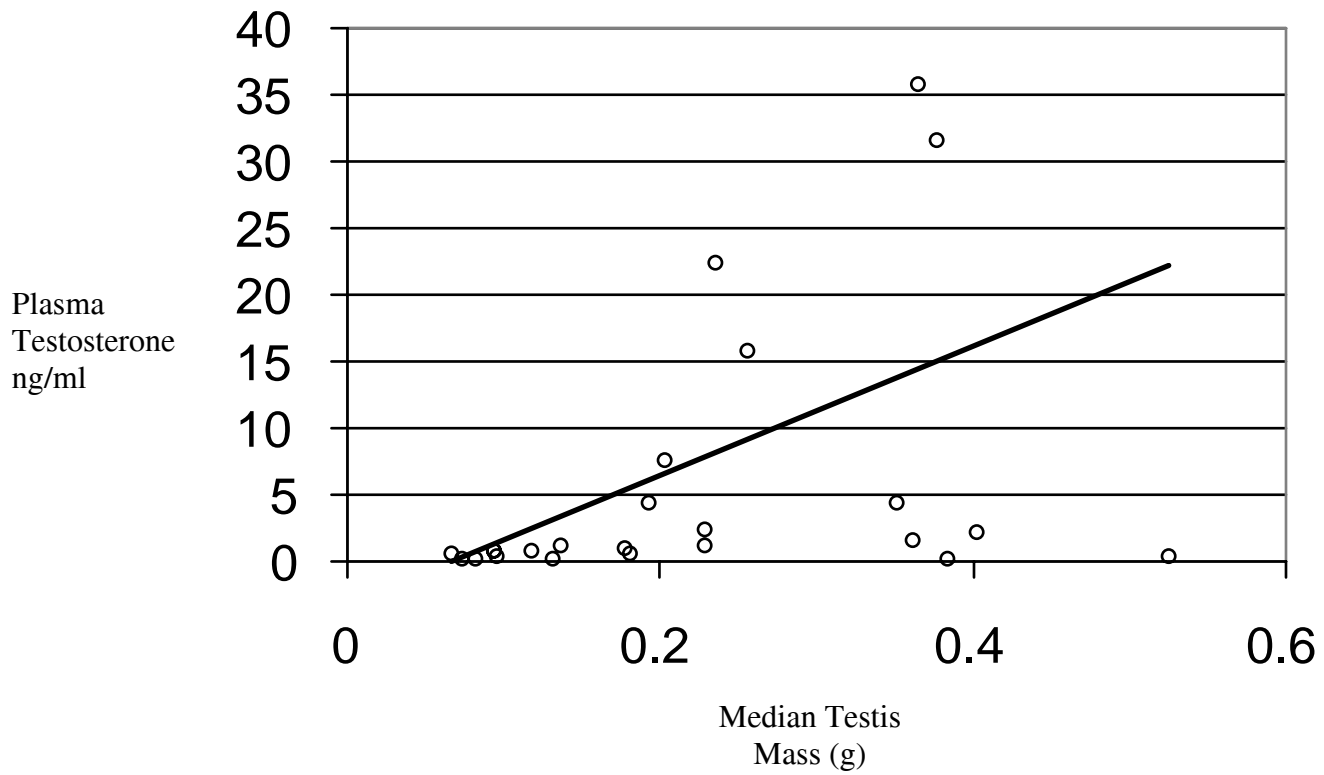


Figure 14 Relationship between testosterone and testis mass ($r_{24} = 0.424$, $p = 0.038$).

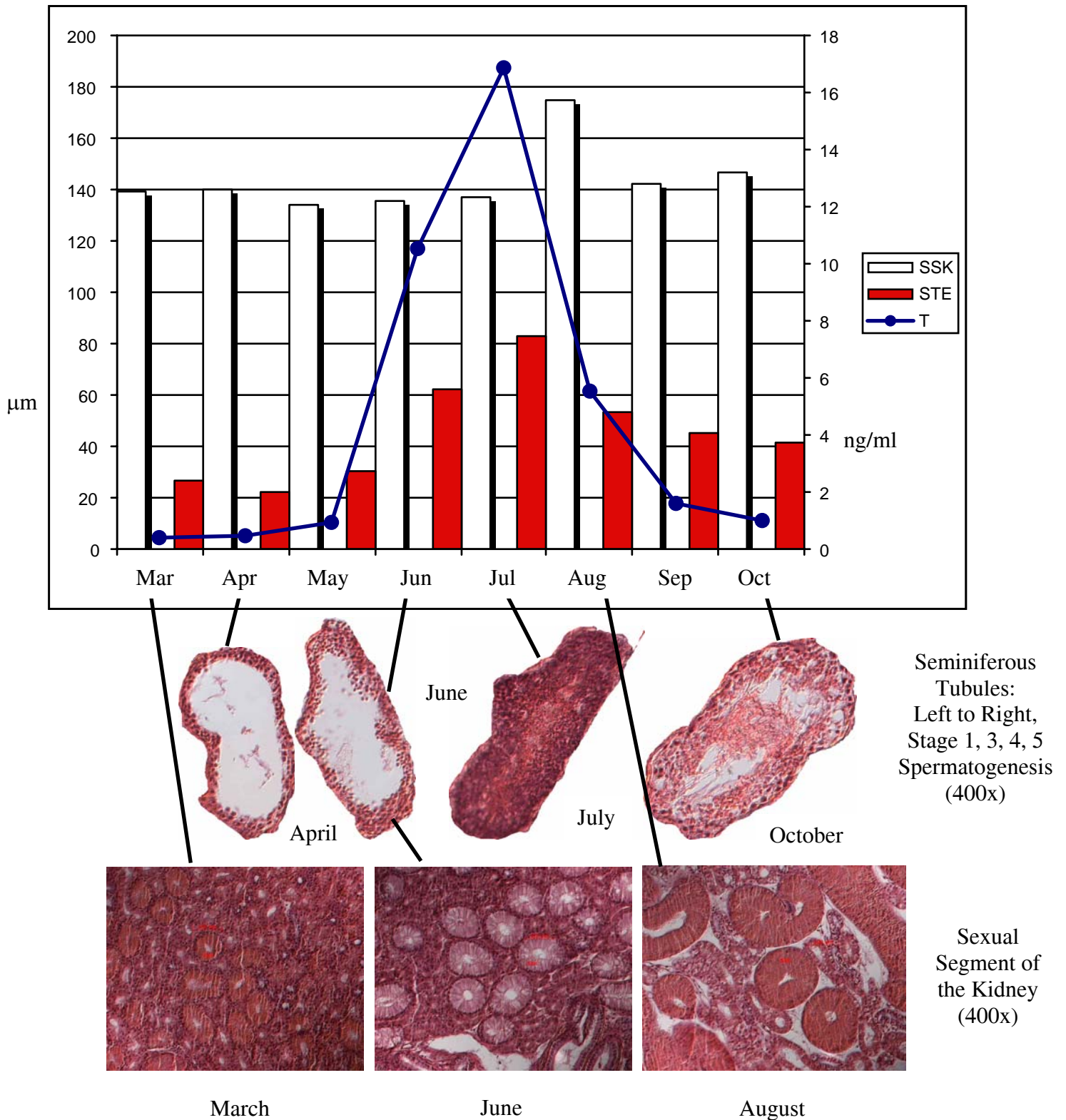


Figure 15 Three predictors of the unimodal mating pattern: a synthetic view. Significant peak of T and STE in July, with a delayed, nonsignificant peak of SSKD in August.

DISCUSSION

Due to the plasticity of snake reproductive adaptations (Moore and Lindzey, 1992; Whittier and Tokarz, 1992; and Schuett, 1992), it can be difficult to identify the appropriate correlative predictors of the mating pattern in these secretive animals. Obviously, although documentation of mating behavior would be optimal to determine the mating pattern, other factors may also prove useful. For example, despite a large amount of time spent searching and observing cottonmouths (> 400 person hours searching, see Table 1), only one trend emerged—cottonmouths apparently are very secretive during when they are mating. There was some evidence of male-female associations in spring and late summer, but it is impossible to compare the two periods with such low sample sizes. It is generally acknowledged that pitvipers do not typically mate at or near winter hibernacula (e.g., Aldridge and Duvall, 2002), though exceptions do exist (e.g., *Crotalus atrox*—Schuett et al., 2005, 2006). From my limited observations in spring, it is apparent how a bimodal mating pattern has been assumed for cottonmouths (e.g., Schuett, 1992; Aldridge and Duvall, 2002; Schuett et al., 2002). However, the spring associations I observed might simply be the case of males and females emerging from a common hibernaculum but not actually mating. The fact that the late summer associations included females about to undergo ecdysis and the spring associations did not is a good indication that mating takes place during the late summer, as it is well known that shedding is an indicator of attractivity and receptivity in female snakes (Mason, 1992; Schuett, 1992).

The fact that reproductive behavior was not observed in my study was not altogether unexpected and can possibly explain why a detailed description of the courtship and mating of this animal has not been published (Ernst and Ernst, 2003). A recent unpublished four-year radiotelemetry effort that tracked several adult cottonmouths of both sexes described a single observed copulation on 18 August, 1998 (Hill, 2004). Other researchers have commented on cryptic mating in snakes, with field documentation of mating success differing drastically from mating and reproductive success informed from paternity analyses (Prosser et al., 2002).

The anatomical and histological analyses I conducted yielded similar results to the previous study by Johnson and colleagues (1982) for the cottonmouth, and studies of pitvipers exhibiting a unimodal mating pattern (Aldridge, 1993; Aldrige and Brown, 1995). The spermatogenic cycle of *A. piscivorus* is essentially identical to that of most temperate zone snakes, with recrudescence of the seminiferous tubules beginning in the spring, and peak spermatogenesis occurring during the summer (Saint Girons, 1982; Schuett, 1992; Aldridge and Duvall, 2002). Sperm is subsequently stored throughout the year, indicated by the observation of sperm in the ductus deferens in all months sampled.

One major departure in the histological results compared to a previous study in this species (Johnson et al., 1982) was the lack of support for definite peaks in SSK activity. Only small sample sizes ($n = 9$) were reported in the Alabama study by Johnson and colleagues (1982; see Schuett, 1992; Aldridge and Duvall, 2002) to support a spring peak of SSK, though, admittedly, the sample sizes I present are also limited ($n = 11$, this study). The fact that a T peak was not even qualitatively linked with the spring SSK peak the study by Johnson and colleagues (1982) raises other questions; it is well established

that the SSK is responsive to T (Bishop, 1959; Prasad and Reddy, 1972). Unlike the pronounced SSK hypertrophy reported in lizards, snakes appear to maintain hypertrophied SSK tubules throughout the active season with only moderate increases during the mating period (Krohmer, 2004), although these increases consistently track the mating season in nearly all cases examined to date (Saint Girons, 1982; Krohmer et al., 1987; Aldridge, 1993; Aldridge and Brown, 1995; Aldridge, 2001; Aldridge, 2002; Schuett et al., 2002; Krohmer, 2002—for a possible exception see Weil and Aldridge, 1981, and discussions in Aldridge, 2001; Aldridge, 2002). Consistent with other reports of temperate zone pitvipers exhibiting a unimodal mating pattern (Aldridge, 1993; Aldridge and Brown, 1995, but see Tsai and Tu, 2000), I detected only a qualitative increase in SSK tubule diameter during the late summer.

There is a significant positive correlation between testis mass, STE, and SSK tubule diameter; I interpret these results as evidence that testicular and SSK variables are linked in *A. piscivorus*, with maximum activity of both corresponding to the late summer peak of T (see below). Studies of pitvipers with a bimodal mating pattern have not reported these types of significant correlations; this is the case because during the spring mating period T and SSK activity occur during the regressive stages of spermatogenesis (Schuett et al., 2002; Schuett et al., 2006). It is thus possible that this muted SSK cycle (with SSK characters linked to testicular factors) is characteristic of pitvipers with the unimodal mating pattern, possibly due in part to low plasma concentrations of T compared to those exhibiting a bimodal pattern. In cottonmouths and other cases, peak SSK hypertrophy appears to be in August, one month after the largest peak of T (see below), STE width, and testis mass. Possibly, a threshold amount of T must be secreted

and in circulation to initiate the mechanisms involved in SSK hypertrophy (i.e., androgen receptor recruitment). Other researchers have reported delayed responses of SSK hypertrophy in relation to testosterone treatment (Krohmer et al., 2004b), testis mass (Tsai and Tu, 2000), and interstitial cell development (Bishop, 1959).

The seasonal cycle of T in *A. piscivorus* is similar to that reported for *Crotalus molossus* by Schuett and colleagues (2005), with a single peak in summer that corresponds to a single mating season. Although other species of pitvipers are predicted to show a unimodal seasonal pattern of plasma T (and other sex steroids) based on recent behavioral field studies (e.g., *Crotalus horridus*, Aldridge and Brown, 1995; and *C. viridis*—Aldridge 1993; see Schuett et al., 2002; Aldridge and Duvall, 2002), *A. piscivorus* and *C. molossus* are the only two snakes of which I am aware that exhibit a unimodal pattern of T secretion (see Schuett et al., 2005). This pattern differs markedly from other snakes that exhibit a bimodal pattern of T secretion and mating (*T. s. parietalis*, Krohmer et al., 1987; *T. s. sirtalis*, Clesson et al., 2002; *Agkistrodon contortrix*, Schuett et al., 1997; *Crotalus scutulatus*, Schuett et al., 2002; and *C. atrox*, Taylor et al., 2004; Schuett et al., 2005, 2006). Anecdotal evidence for a spring mating period in *A. piscivorus* (e.g., Beyer, 1898; Wharton, 1966) is probably erroneous, but further work will need to be done to verify this conclusion. Therefore, it appears that both SSK hypertrophy and plasma T concentrations are indicative of either mating or spermatogenesis (or both) in all snakes examined, and no snake examined under natural conditions to date is known to exhibit a mating season without a correlated peak of T (see Krohmer et al., 1987). If the cottonmouth does mate in the spring without a concomitant peak of T, it would be the only exception to this generalization.

Previous studies also described a single androgen peak in the cottonmouth corresponding to the late summer breeding season (Johnson et al., 1982; Zaidan et al., 2003), with peak hormone values reported similar to those reported here. Though my field values were significantly higher than those derived from captive animals, they were comparable to previously published results (Johnson et al., 1982; Zaidan et al., 2003). Moreover, this was the case despite differences in methodological approaches (sampling captive animals transported from the field vs. field sampling; RIA vs. EIA methods to quantify sex steroids). Thus, these studies appear to corroborate each other and support the view that cottonmouths have lower plasma T concentrations compared to copperheads, rattlesnakes, and other species of snakes (Schuett et al., 2005).

I have described a single peak of testosterone, SSK hypertrophy, and testicular activity in cottonmouths from Georgia, and conclude that the mating pattern is unimodal with peak mating likely occurring only in the late summer. Because I have shown that data from two consecutive spring periods indicate basal T levels, it does not appear that this is a temporally variable phenomenon. Others have reported similar between-year consistency in seasonal hormone patterns in snakes (Weil and Aldridge, 1981; Naulleau et al., 1987; Schuett et al., 2005). Comparison of this study to those conducted on cottonmouths in other populations (Alabama: Johnson et al., 1982; Arkansas: Zaidan et al., 2003) suggests that this pattern is geographically stable and is possibly found throughout the range of *A. piscivorus*. Experimental studies are still required to confirm the androgenic activation of male sexual behavior in *A. piscivorus*, but descriptive and correlative analyses are valuable for generating hypotheses that facilitate meaningful comparisons.

The findings of this study are surprising considering that a sympatric congener of *A. piscivorus*, *A. contortrix*, has been described as having a bimodal pattern of mating with evidence from steroid hormone profiles and behavior from extensive laboratory studies, with peak hormone values much larger than those reported here (Schuett et al., 1997; Schuett, et al., 2005). However, there are many other examples of closely related viperids with contrasting mating patterns. The well-studied *Vipera aspis* and *V. berus* have distinct mating (spermatogenic and behavioral) cycles even though they are sympatric in certain areas (Saint Girons, 1982). *Crotalus oreganus* and *C. viridis* are closely related rattlesnake taxa, yet the former has a bimodal breeding pattern, while the latter has a unimodal pattern based on behavioral evidence and the SSK cycle (Aldridge, 1993; Aldridge, 2002). Finally, a recent study that relied heavily on endocrine evidence showed that two species of sympatric congeners (*C. atrox* and *C. molossus*) exhibit contrasting mating patterns (Schuett et al., 2005). The ultimate factors that led to the development of these contrasting patterns are now under debate (Aldridge and Duvall, 2002; Schuett et al., 2002; Schuett et al., 2005, 2006), and more studies that identify the proximate mechanisms involved will aid in the development of robust hypotheses.

This study has implications for the study of the evolution of pitviper mating patterns, since it appears to contradict some of the predictions of current hypotheses. These can be categorized broadly into “phylogenetic” and “ecological” categories. Attempts to infer phylogenetic constraint in pitviper mating patterns (for example, Almeida-Santos and Solomao, 2002, who report similar mating patterns for tropical and temperate taxa, and Schuett et al., 2002, who used *Agkistrodon* as the outgroup for a phylogeny of mating patterns of North American rattlesnakes) should consider the

congeneric *A. contortrix* and *A. piscivorus*—another example of many closely-related pairs of vipers with contrasting mating patterns (Aldridge and Duvall, 2002). A simplistic explanation of pitviper mating patterns as a response to local ecological conditions (e.g., Aldridge and Duvall, 2002) should consider *C. atrox* and *C. molossus* (Schuett, et al., 2005), and *A. contortrix* and *A. piscivorus*, which are found in sympatry throughout most of the range of both species and yet retain contrasting mating patterns. The study of pitviper mating systems, and especially the evolution of seasonal patterns of mating, offers an excellent opportunity to examine physiological evolution and to weigh the relative importance of the above and other contributing factors.

The use of a suite of correlative factors to characterize and predict mating patterns of snakes has tremendous benefits considering the technical problems in conducting field research on the behavior of secretive animals. For some vertebrate groups (i.e., abyssal fishes, fossorial mammals, canopy-dwelling amphibians), field observation of behavioral patterns may never be possible in nature; yet, I hold that interesting insights can still be gleaned by examining proximate correlates to these behaviors from field collections.

CHAPTER 2

Plasma corticosterone variation in free-ranging male cottonmouths (*Agkistrodon piscivorus*): diel, seasonal, and captive handling effects, and interactions with plasma testosterone.

SUMMARY

Reptiles have been neglected with respect to hypothalamo-pituitary-adrenal (HPA) axis research, despite their possible value as comparative models useful for testing hypotheses generated from studies of mammals and birds. In reptilian taxa that have been studied, diel and seasonal variability in glucocorticoids (GCs) are demonstrated, with peak levels of corticosterone (CORT) coincident with the mating season. These results are in contrast to research demonstrating that social or captive stress activation of the HPA-axis can down-regulate the reproductive (hypothalamo-pituitary-gonadal—HPG) axis. I investigated diel and seasonal variation in CORT in wild male cottonmouths (*Agkistrodon piscivorus*), and compared field samples to those collected under a captive handling protocol where sampling of blood occurred from 2 to 24 hours later. Statistical associations between CORT and testosterone (T) were also examined. My study revealed no significant diel variation of plasma CORT, but did observe a non-significant trend with increased levels during the day and toward the evening. Such increases during late photophase and early scotophase were expected based on metabolic patterns described in earlier studies by other researchers on this species. I demonstrated significant seasonal

variation of plasma CORT, with the highest values in April to May, and the lowest values in July. Thus, this pitviper appears to be an exception to the general trend observed in male reptiles that plasma CORT levels increase during the mating period. The pattern of CORT in male cottonmouths may possibly be related to the negative impact of the HPA-axis on the HPG-axis in pitvipers; there was a significant elevation of CORT within 30 minutes of capture and a significant decrease of T in snakes held in captivity for 2-24 hours. Both elevated CORT and depressed T inhibit reproductive behaviors in other species of *Agkistrodon*. I suggest that increased CORT during the mating season, as reported for most reptiles, may not be advantageous for species with CORT-modulated reproductive suppression.

INTRODUCTION

In vertebrates the hypothalamo-pituitary-adrenal (HPA) axis mediates a response to stressors that is possibly the single most important physiological reflex in terms of evolutionary fitness. This stress response begins with the release of catecholamines by the sympatho-adrenal (SA) axis within seconds, orchestrating a rapid enhancement of cardiovascular and energetic physiology—the so-called “fight or flight” response (Cannon, 1929; Sapolski et al., 2000). This is followed and modulated by the HPA-axis and increases of glucocorticoids (GCs) within minutes (Seyle, 1978; Wingfield et al., 1998; Sapolski et al., 2000). Since many acute stressors (e.g., predation events) are over within seconds, the adaptive significance of slower (e.g., minutes to hours, with a longer delay between their elevation and action at target tissues) GC responses after stressors has been less clear. Diverse effects have been identified; these include continued

mobilization of energy reserves and suppressive effects on reproduction and aspects of the immune system (Wingfield et al., 1998; Sapolski et al., 2000; McEwen and Wingfield, 2003).

Several recent attempts have been made to interpret the diverse actions of GCs and characterize them in terms of predictive hypotheses. Three prominent examples are (a) the emergency life history stage (Wingfield et al., 1998), (b) permissive, stimulatory, suppressive, and preparative effects (Sapolski et al., 2000), and (c) the concept of allostasis (McEwen and Wingfield, 2003). These hypotheses have as a common theme determined diverse effects of GCs that suggest their action is a generalized response to perturbations or environmental variations that facilitate the return to a “normal” state, or equilibrium, of physiological activity.

In addition to the “classic” HPA-axis response to stressors, there are seasonal patterns of glucocorticoid (GC) release, with many species exhibiting increased GCs during the mating season (Licht et al., 1983; Krohmer et al., 1987; Romero and Wingfield, 1999; Romero, 2002; Moore and Jessop, 2003). Diel GC variation in plasma CORT levels is known, but this pattern has received little attention in most taxa (Tyrell and Cree, 1998). In general, GCs track diel patterns of metabolism and activity, e.g., nocturnal or diurnal trends (Dauphin-Villement and Xavier, 1987; Summers and Norman, 1988; Cree et al., 1990a; Tyrell and Cree, 1998; Breuner et al., 1999; Romero and Wikelski, 2006). In mammals and birds, corticosterone (CORT) appears to have a circadian pre-active peak prior to the normal activity period (Breuner et al., 1999). These studies, which have targeted diel and seasonal variations of GCs, show that baseline (i.e.,

non-stressor induced) levels of GCs may play have a general role in regulating metabolism (Dallman et al., 1993; McEwen and Wingfield, 2003).

As ectotherms, reptiles are potentially good models for elucidating the actions and adaptive significance of GC variation in vertebrates. Since many of the current concepts of GC action (e.g., allostasis) were developed from endothermic models in which energy flux is known to have profound effects on evolutionary fitness (McEwen and Wingfield, 2003), it is important to consider model organisms that are less heavily dependant on these factors and are comparatively more flexible with regard to energetic and metabolic homeostasis (Pough, 1980; Pough et al., 2002). Like mammals and birds, reptiles exhibit diel, seasonal, and handling-induced changes in GCs, and therefore make excellent comparative models to test hypotheses developed from studies of endotherms about the adaptive significance of GC action. CORT is the primary GC in reptiles (Norris, 1997).

Very few studies have examined diel CORT variation in reptiles (see Tyrell and Cree, 1998), although several have described seasonal CORT profiles with most species showing peaks coincident with the mating season (Romero, 2002). These results are paradoxical given the general view that stressors have negative impacts on many facets of the hypothalamo-pituitary-gonadal (HPG) axis in reptiles (Greenberg and Wingfield, 1987; Tyrell and Cree, 1998; Lance et al., 2004). Sapolski and colleagues (2000) suggest the possibility that even baseline levels of GCs can inhibit aspects of the reproductive axis.

In addition to diel and seasonal CORT variation in reptiles, there are suppressive effects of handling stress on the HPG-axis (earlier literature reviewed by Greenberg and Wingfield, 1987; Cree et al., 1990b; Moore et al., 1991; Valverde et al., 1999; Malthies et

al., 2001; Lance et al., 2003; Moore et al., 2000b, Moore et al., 2001; Moore and Mason, 2001; Moore et al., 2005). Furthermore, some researchers have examined the physiological-behavioral effects of social stress and male agonistic behavior on reproduction in lizards (Greenberg and Crews, 1990; Denardo and Licht, 1993; Knapp and Moore, 1995; Rubenstein and Wikelski, 2005) and snakes (Schuett et al., 1996; Schuett and Grober, 2000). A trend emerging from these studies is that there is often a negative interaction between hormones of the HPA-axis (e.g., corticosterone) and hormones of the HPG-axis (e.g., testosterone), which might indicate that CORT directly suppresses T. Lance and colleagues (2003) reported that this interaction occurs in captive-handled American alligators in as little as two hours.

In this study I attempt to advance our understanding of the HPA-axis in vertebrates by describing the diel and seasonal variation in CORT secretion in a wild snake population, as well as to characterize the effects of handling on CORT levels and potential interaction between CORT and plasma T. To determine seasonal patterns of CORT variation and the effects of handling stress on naïve snakes it was important to conduct this study in the field. I chose the cottonmouth (*Agkistrodon piscivorus*) as a study organism because they are large, easy to detect and collect, occur at high densities in certain habitats (Gloyd and Conant, 1990), and have previously been characterized as having low plasma T levels compared to other snakes (Johnson et al., 1982; Zaidan et al., 2003; Schuett et al., 1996, 2005), which suggests the possibility that this outcome was an artifact of the collection protocol or hormone assay method of previous studies (Johnson et al., 1982; Zaidan et al., 2003). In these studies, snakes were brought from the field into

the laboratory before blood sample collection, raising the possibility that the low T levels resulted from the activation of the HPA-axis by captive handling.

Several studies have determined the hormonal and histological correlates to mating in this snake (Johnson et al., 1982; Zaidan et al., 2003; Graham et al., in prep; see Chapter 1 of this study). A detailed description of courtship and male agonistic behavior in this species is still lacking (Ernst and Ernst, 2003), although the mating system of *A. piscivorus* is probably similar to other pitvipers with an operational sex ratio skewed towards males during the breeding season (e.g., prolonged mate-searching polygyny of Duvall et al., 1992). This mating system is presumed to be costly to males, both in terms of predation risk (Aldridge and Brown, 1995; Aldridge, 2001), and energy, as male snakes often forgo feeding during the mating season (Olsson et al., 1997; O'Donnell et al., 2004). In studies of the red spotted garter snake (*Thamnophis sirtalis concinnus*), CORT was significantly correlated with negative relative residual body condition (Moore et al., 2000), presumably due to the need to mobilize energy reserves. Snakes therefore make an excellent candidate system to test whether energy mobilization during the mating season is responsible for increased CORT (see the “Energy Mobilization Hypothesis” in Romero, 2002).

The copperhead (*Agkistrodon contortrix*), exhibits pronounced courtship inhibition after losing fights for potential mates, and this response is associated with elevated levels of plasma CORT relative to winners and controls (Schuett et al., 1996; Schuett and Grober, 2000). Therefore, it possible that the typical reptile mode of increased CORT during the mating season would be maladaptive for this species. Describing the seasonal variation of CORT in its close relative the cottonmouth—which

also exhibits similar courtship and male agonistic behaviors—could possibly lend credence to behavioral hypotheses for seasonal CORT variation (e.g., the “Behavioral Hypothesis” in Romero, 2002, and the “Energetics-Hormone Vocalization model” of Moore and Jessop, 2003). In the only paper of the seasonal profile of CORT in a pitviper species (*C. atrox*—Taylor et al., 2004), there was no significant seasonal variation of GCs in males. Those results are in contrast to the predictions of Romero (2002).

The circadian metabolic pattern of *A. piscivorus* was previously described in laboratory studies (Blem and Kileen, 1993; Zaidan, 2003), and both determined likely endogenous rises of metabolic rate most likely occurred during the day and peaked after dark. Most authors have referred to cottonmouths as nocturnal during most of the active season (Gloyd and Conant, 1990; Ernst and Ernst, 2003), although diurnal activity has been noted (Gloyd and Conant, 1990; Ernst and Ernst, 2003). The extent to which the diel CORT pattern is associated with the activity and metabolic pattern has not been described, and to my knowledge diel variation of CORT has not been examined in any snake.

My study was designed to test the following hypotheses: (1) The low plasma T levels reported by previous authors (Johnson et al., 1982; Zaidan et al., 2003) were an artifact of their sampling protocol, and cottonmouths have plasma T levels comparable to other pitvipers. (2) The stress of capture and confinement causes significant increases of CORT, and a subsequent decrease in plasma T. (3) The trends described for seasonal CORT variation in reptiles apply to *A. piscivorus*, predicting that in an animal with increased energetic demands during the mating season, CORT levels should peak and be negatively correlated with body condition. (4) CORT should be correlated with diel

metabolic activity based on comparisons to previous studies of cottonmouth metabolism (Blem and Kileen, 1993; Zaidan, 2003).

MATERIALS AND METHODS

Field Processing. Adult male *A. piscivorus* were collected and/or observed at the study site from September 2003 through May 2005. When captured, all animals were immediately processed to collect blood samples for steroid analysis. Animals were captured with metal tongs and gently secured in plastic tubes or plastic buckets with lids. At the time of capture, date and time of day, habitat, and behavioral information (male-female associations, basking, foraging, etc.) were recorded.

Adult snakes were measured (snout-vent-length, SVL; tail length, TL) to the nearest 1 cm by stretching a flexible tape along their side. Mass was determined to the nearest 3g using a Pescola spring scale. All individuals that were returned to their capture site received a unique scale-clip (e.g., Fitch, 1960), and no repeated samples were obtained for initial hormone values. Behavioral observations and sampling occurred regularly throughout the active season (March through October). Minimally, 8 person hours per week were spent searching for, observing, and/or processing cottonmouths in spring (1 March to 31 May), and in late summer to fall (1 August to 31 October). During other times of the active season (1 June to 31 July), observations were conducted for a minimum of 2 person-hours per day in the evening, and a minimum of 6 person-hours per week during daylight hours. During the 2005 field season, searching, observations, and processing took place only during the potential breeding seasons (March to May; August to September) for an equal amount of time (32 total person-hours each searching). Winter

observations and collecting took place opportunistically on warm sunny days from November through February when cottonmouths can sometimes be found basking outside of hibernacula (Ernst and Ernst, 2003).

Blood collection and handling. From September 2003 to June 2004, subjects sampled for hormone analyses were anesthetized using isoflurane in the field for the researcher's safety. To achieve light anesthesia, a small cottonball with 0.5 ml of anesthetic was placed in the opposite side of the restraint tube until the snake showed lack of a righting reflex (usually <15 min). A small sample (1.0 ml) of blood was obtained within 30 minutes (range = 3-30; mean = 22.15 min.; *SE* = 8.23) by cardiocentesis per methods described by Schuett and colleagues (1997) using a disposable 1-cc heparinized tuberculin syringe. All blood samples were placed in 1.5 ml centrifuge tubes, labeled, and put on ice packs in a cooler for various periods under 24-hr (Taylor and Schuett, 2004). Blood samples were centrifuged and plasma was placed in new sterile 1.5 mL centrifuge tubes, and stored at -20° until steroid assays could be performed. From June 2004 through May 2005, protocol for the collection of blood samples was altered as the author became experienced with a different sampling method. Blood (1.0 ml) was obtained within 3-30 min (mean = 13.33; *SE* = 8.96) minutes from the caudal vessels without the use of isoflurane anesthetic. Possible differences between collection protocols are discussed below. A subset of the population (*N* = 33, see Chapter 1) was collected for destructive sampling for reproductive tissues used in a separate study (see Chapter 1; Graham et al., in prep). After blood sampling in the field, these animals (representing my "handling stress" treatment) were transported in a bag and secure bucket to a staging area near the study site, kept in ambient (outdoor) temperatures and humidity conditions until

processed, and all but 4 were never transported more than 1km from the study area. At the time of sacrifice (2-24 hr. after capture; mean = 8.59 hr.; $SE = 7.5$), these animals were anesthetized as described above, and an additional sample was obtained by cardiocentesis and processed as described above.

Corticosterone assay. Enzyme immunoassays (EIAs) were performed to determine the plasma concentrations of corticosterone (CORT). Hormones were extracted from thawed plasma samples using an ether extraction method. In brief, 2 ml diethyl ether was added to each 225 μ l sample of plasma and mixed for 3 min on a multi-tube vortexer, followed by 3 min of phase separation prior to fast-freezing in a methanol/dry ice bath. After fast-freezing, the ether layer was decanted into a 16 x 30 mm borosilicate vial and the aqueous layer was subject again to the same treatment as above. The second ether layer was decanted into the same 16 x 30 mm borosilicate vial as the first. Diethyl ether was evaporated under a gentle stream of nitrogen at 40°C under a fume hood and the resulting hormone pellet was re-suspended in 225 μ l of EIA buffer (provided in the kits). Enzyme immunoassays were performed on the ether-extracted hormones run in duplicate following procedures of the kit (Cayman Chemical Company, Ann Arbor, Michigan).

The assays were validated for *A. piscivorus* by assessing parallelism and by calculating expected versus observed corticosterone concentrations from known samples spiked with standards. A pooled sample (1:1) was created from ten ether-extracted samples. For serial dilutions, 225 μ l of this sample was transferred to a 1.5 ml Eppendorf tube and mixed by vortexing with 225 μ l of 0.1 M phosphate buffer to create a 1:2 dilution; 225 μ l of 1:2 dilution was mixed with an equal volume of 0.1 M phosphate buffer to create a 1:4 dilution, and so on until 1:64. The serial dilutions were run in

quadruplicate using the EIA protocol described above. The log-logit transformed dilution curve was parallel to the standard curve (comparison of slopes: $t_{14} = 0.19$, $p = 0.85$; Zar 1996). An 880 μl sample of pooled hormone extract was used to assess recovery. 110 μl of the large sample was then transferred into 8 additional tubes and mixed with an equal volume of each standard provided with the EIA kit (3.8, 7.8, 15.6, 31.3, 62.5, 125, 250, and 500 pg/ml). Expected recovery concentrations were based on the known amount of corticosterone in the *A. piscivorus* control sample. Minimum recovery was 85.2% (range 85.2 – 116%) and the slope of the observed vs. expected curve was 0.83, demonstrating a highly linear relationship between observed and expected recovery ($R^2 = 0.90$, $p = 0.85$). 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%.

Data Analysis . SVL and body mass were compared using linear regression and were positively correlated ($F_{1,53} = 168.4$, $p = 0.0001$, $R^2 = 0.76$). I therefore used SVL as the covariate in subsequent analyses. CORT and T values were log transformed, and I determined homogeneity of variance in both hormone datasets across months. However, unless specified, the original untransformed data are presented in the figures. I compared mass, SVL, and CORT for spring 2004 and 2005, and fall 2003 and 2004, using ANOVA with month, year, and month x year as the main effects. I found no response in these variables (2004/2005 overall model: $F_{3,16} = 2.30$, $p = 0.12$) and combined them for subsequent analysis. Therefore, I tested the differences between the anesthetized (spring 2004) and non-anesthetized (spring 2005) snakes and found no significant differences. I compared fall 2003 and 2004 captive samples as in above and found no differences. I used linear regression to determine the effect of time of day and the time between capture

and blood draw on CORT. Linear regression was also used to determine the effect of body condition index (SVL/mass) and residual body mass on CORT.

I analyzed the effect of time of year (month) on CORT using an ANCOVA using SVL, time from capture to blood collection, and time of day as the covariate in three separate analyses. In addition, I tested for monthly CORT variation using an ANCOVA with SVL and SVL*month as covariates. A paired t test was used to determine significant differences between field and captive T and CORT values.

RESULTS

The mean value of CORT detected by the EIA was 34.25 ng/ml (range = 0.62 – 143.72). I found no effect of any indicator of body condition (body condition index or residual body mass) on CORT. There were no differences between Spring 2004 and 2005 plasma CORT, when two different sampling methods were used (anesthesia vs. non-anesthesia). I combined all samples to examine the effects of time of day and time after capture. I found no significant correlation between CORT and time of day sampled ($R^2 = 0.03$, $F_{1,48} = 1.79$, $p = 0.18$, Fig. 16), although there was a positive trend. I ruled out possible sampling bias by comparing the time of sample collection by month and amount of time it took to obtain the sample (e.g., it was possible that it was more difficult for the researcher to obtain samples during the hottest part of the day or year, and this could result in increased latencies to blood acquisition). These comparisons yielded no significant effects.

There was significant seasonal variation of CORT, with three separate ANCOVA analyses (using SVL, time until blood collection, and time of day as covariates) revealing the same general trend; a significant peak of CORT in April and May, and a significant

decline of CORT in July (overall model, SVL: $F_{8,39} = 3.95$, $p = 0.0017$, Fig. 17; time until blood collection; $F_{8,39} = 6.16$, $p = < 0.0001$; time of day: $F_{8,39} = 4.57$, $p = 0.0005$). However, using a more conservative ANCOVA with SVL and the SVL*month interaction as covariate pushed the p value below significance ($F_{15,35} = 3.06$, $p = 0.0781$), indicating a slight body size/CORT covariance across months.

The initial samples taken in the field contained significantly lower CORT values than those taken 2-24 hours later ($t_{20} = 3.68$, $p = 0.0015$, Fig. 18), with an abrupt and significantly positive rise in CORT after capture, starting within 30 minutes ($F_{1,47} = 5.00$, $p = 0.03$, Fig. 19). There was a significant negative relationship between CORT and T ($R^2 = 0.11$, $F_{1,46} = 6.44$, $p = 0.019$; Fig. 20), and T was significantly lower in samples taken later compared to field samples (Fig. 17; see Chapter 1).

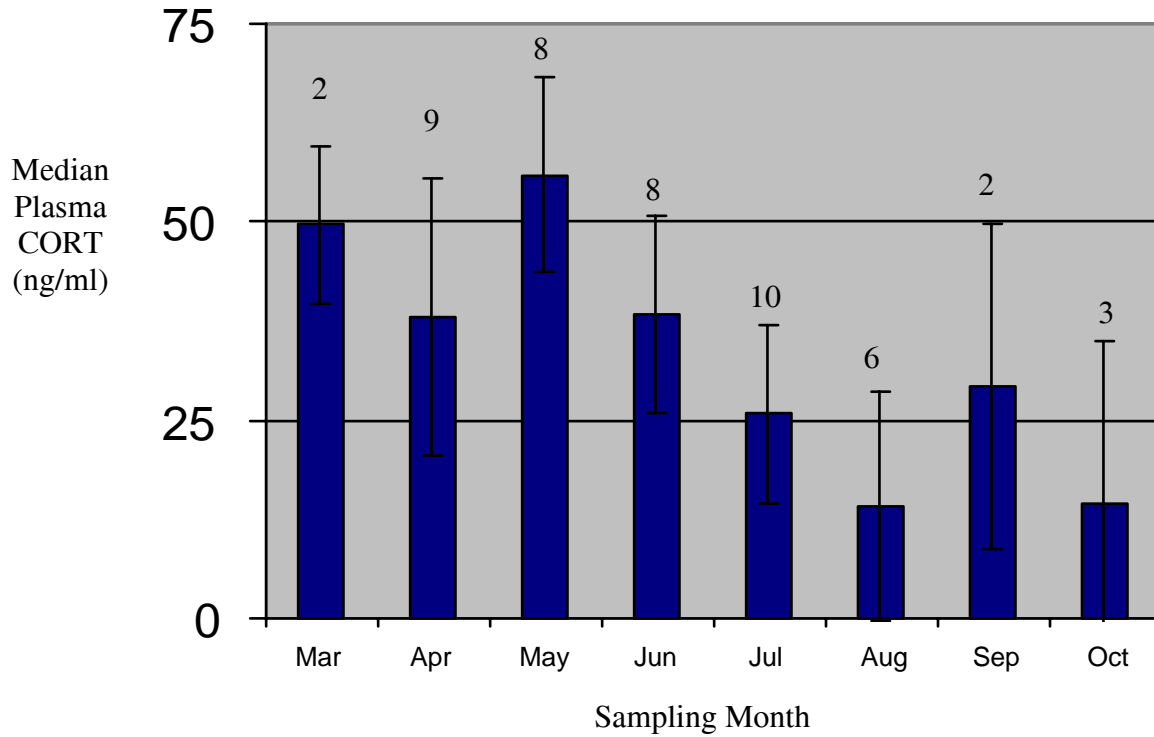


Figure 17 Median monthly CORT concentrations. In CORT was significantly highest in April-May, and significantly lowest in July. Sample sizes for each month labeled above standard error bars ($F_{8,39} = 3.95$, $p = 0.0017$).

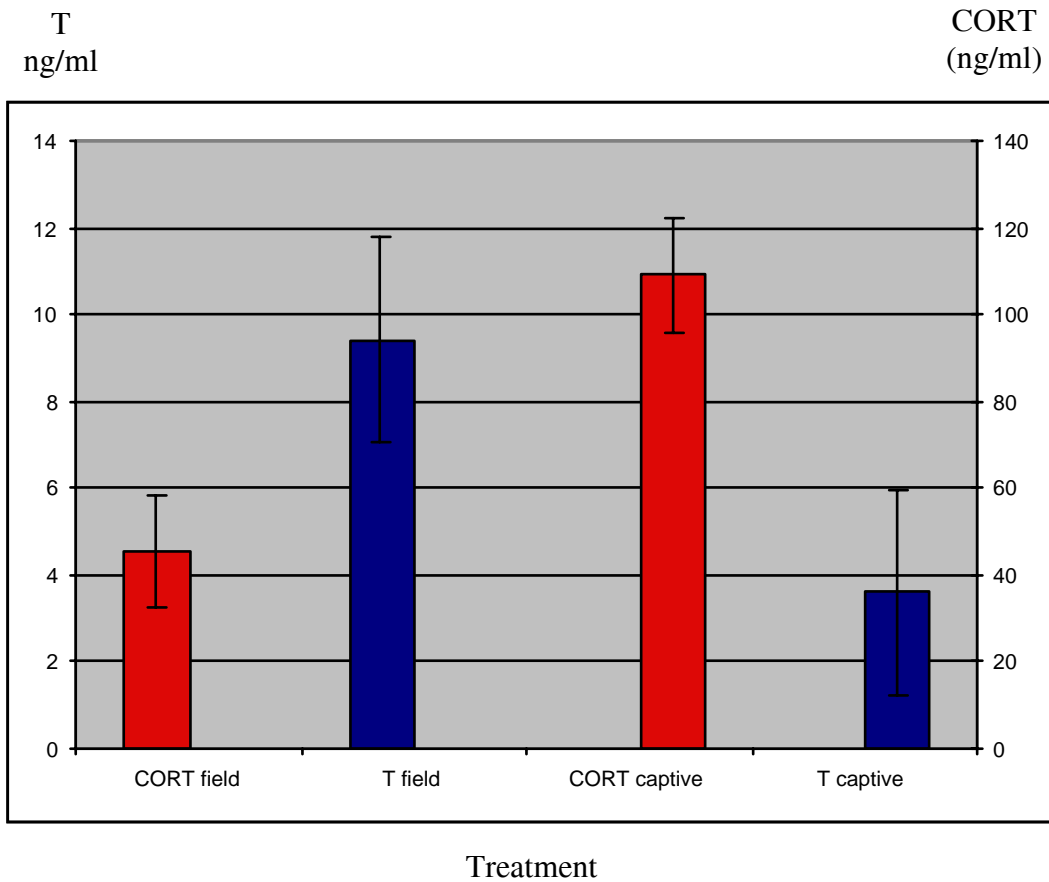


Figure 18 Comparison of mean CORT and T from field samples and samples taken after captive handling treatment. CORT is significantly elevated after captive handling ($t_{20} = 3.68$, $p = 0.0015$, Fig. 9). T levels are significantly lowered ($t_{20} = 2.46$, $p = 0.012$).

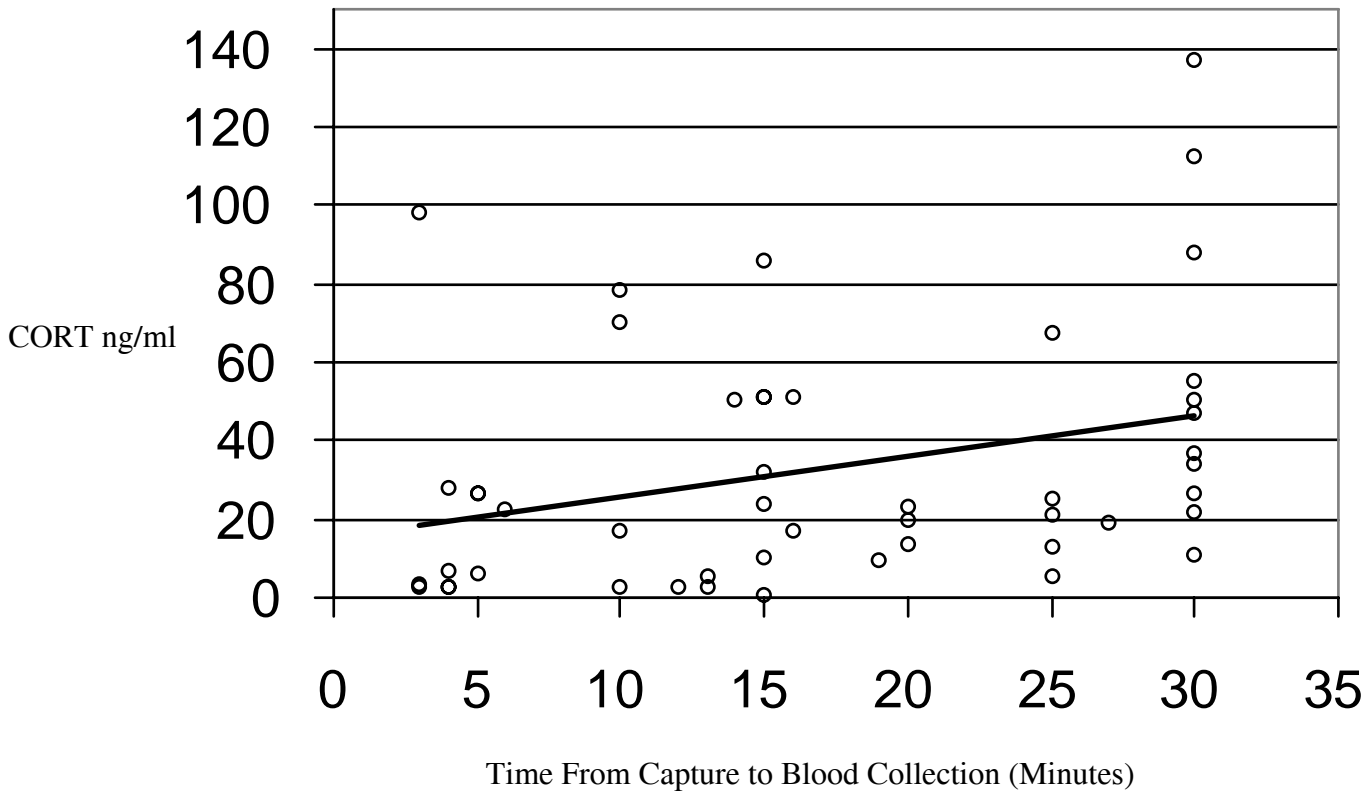


Figure 19 Relationship between CORT and time until blood collection. Levels of CORT rise significantly after capture within 30 minutes ($R^2 = 0.16$, $F_{1,47} = 8.99$, $p = 0.0043$).

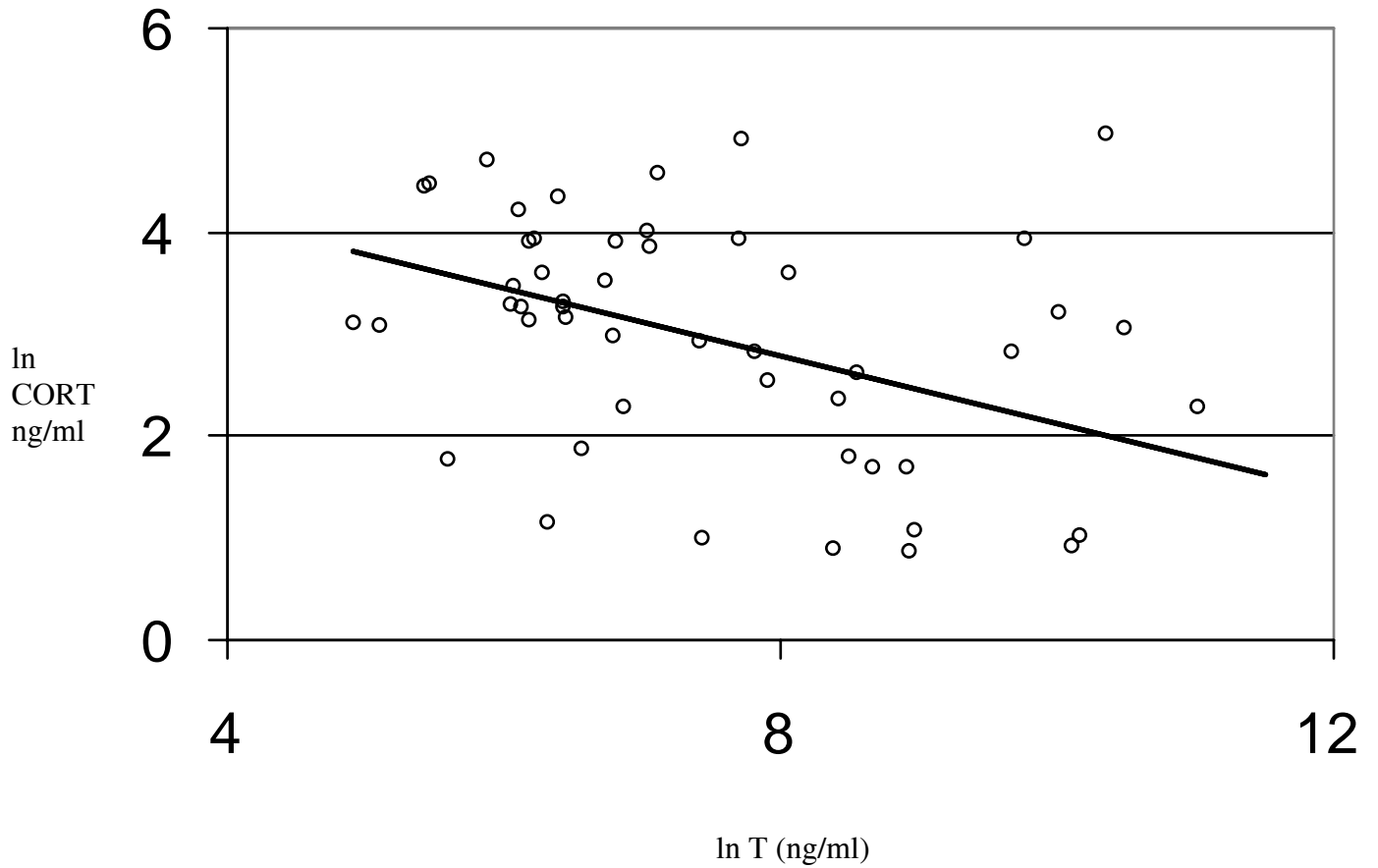


Figure 20 Relationship between ln plasma CORT and ln plasma T. There is a significant negative trend between CORT and T ($R^2 = 0.11$, $F_{1,46} = 6.44$, $p = 0.019$; Fig.19).

DISCUSSION

My results did not support the hypothesis that endogenous metabolic rhythms are mirrored by diel CORT variation, but I did detect a non-significant trend of CORT increasing during the day and into the evening. One potential problem in this study was that I did not sample consistently during the nocturnal period (e.g., only 9 of the samples were collected during evening hours); therefore, it is possible that increased sampling would have resulted in a significant correlation. Additionally, although there was no bias in time of day sampled vs. month, there were seasonal differences in CORT levels, and a time of day vs. seasonal effect cannot be ruled out. Nonetheless, based on my limited sample, it is possible that the CORT values peak just after dark; accordingly, this would be evidence for a pre-active peak as discussed by Breuner and colleagues (1999). In support of my findings, Blem and Kileen (1993) and Zaidan (2003) conducted laboratory studies on *A. piscivorus* and found evidence for circadian metabolic variation, with metabolic rate (J/ml) increasing during nighttime hours under total darkness. If a circadian CORT rhythm is endogenous and substantiated by future studies, it could lend support to studies in mammals (Kafka et al., 1986) birds (Bruener et al., 1999) and other reptiles (Dauphin-Villemant and Xavier, 1987; Summers and Norman, 1988; Tyrell and Cree, 1998; Romero and Wikelski, 2005) that demonstrated diel patterns of CORT secretion associated with circadian activity patterns. This CORT variation has been hypothesized by Dallman and colleagues (1993) to modulate circadian patterns of activity and metabolism in conjunction with insulin, with peaks occurring prior to and during the time of first activity—presumably a period when increased vigor is adaptive.

Romero (2002) presented several hypotheses to explain the adaptive significance of seasonal CORT variation in free-living vertebrates. The “Energy Mobilization Hypothesis” is similar to the above hypothesis concerning diel CORT variation, and it predicts peak plasma CORT levels should occur during the most energy-limited time of the year, with reproduction for both sexes presumably the most costly annual event. The “Behavior Hypothesis” predicts that variations of CORT may be important for the expression (or lack of expression) of CORT-modulated behaviors during a particular season. The “Preparative Hypothesis” is derived from views posited by Sapolski and colleagues (2000), and it predicts that increased CORT should be present during times of year where it is important to “prime” the stress axis to be ready for potential stressors. In addition, Moore and Jessop (2003) viewed elevated CORT levels during the mating period as an extension of the “Energetics-Hormone Vocalization” (EHV) model developed from studies on anurans by Emerson and colleagues (2001). This model, itself an extension of the “Challenge Hypothesis” (Wingfield et al., 1990), predicts increasing plasma testosterone levels during the mating season due to reproductive activities, and a concomitant rise of CORT to mobilize energy for behaviors and physiological activities. “At some point, plasma corticosterone levels surpass a threshold and trigger a short-term stress response. Plasma testosterone levels then decline, resulting in a negative association between the two hormones (Moore and Jessop, 2003).” Presumably, studies that show increases of CORT during the mating season are due to sampling at a population level, when in fact different individuals have increases or decreases of CORT and T occurring at different stages of this process.

I demonstrated significant seasonal variation in plasma CORT, with levels significantly highest in April and May and significantly lower during July. Consequently, I found no support for the hypothesis that CORT increases during the mating period. This peak and nadir corresponded to the beginning of the feeding period and the peak of spermatogenesis in male cottonmouths, respectively (Johnson et al., 1982; Hill, 2004; unpubl. data, this study). My results complement a study conducted on the pitviper *Crotalus atrox* (Taylor et al., 2004) as exceptions to the general trend of seasonal CORT variation observed in reptiles, e.g., CORT peaking with mating season (Romero, 2002; Moore and Jessop, 2003). In my study and in Taylor and colleagues (2004), lower relative concentrations of plasma CORT occurred during the late summer mating season in males. However, data from the present study must be viewed with caution, because the levels I present—though collected within 30 min. of capture—may not represent true basal levels (discussed below). According to Romero (2002), these data would have been characterized as representing CORT under a captive handling treatment, since they were collected after five minutes of capture. Regardless, in most reptiles basal and captive stress levels have similar seasonal trends (Romero, 2002). Moreover, by using the three separate analyses above, it is apparent that a monthly CORT trend probably exists independent of body size, and is in fact real. It should be noted that most studies of this type (e.g., Moore et al., 2000b; Taylor et al., 2004; Moore et al., 2005) do not include mass*month or SVL*month interactions in their comparative analyses of seasonal CORT variation.

It is also important to note that the importance of seasonal variation of binding proteins was not determined, and therefore I did not distinguish between free and total

steroid concentrations. Plasma binding proteins (and other factors, such as tissue sensitivity and metabolic clearance) can profoundly modulate the biological action of GCs (Romero, 2002; Breuner and Orchinik, 2002), and the action of these and other mechanisms in this and other reptile systems must await further study.

The cottonmouth has been described as having mating seasons in both late summer/fall and in spring (e.g., Schuett, 1992; Schuett et al., 2003) though recent evidence supports the view first suggested by Zaidan and colleagues (2003) that *A. piscivorus* mates only in the late summer and fall (see Chapter 1; Graham et al., in prep). Interestingly, in this study and in a study of *C. atrox* (Taylor et al., 2004) the Energy Mobilization Hypothesis seems to be refuted, and this could indicate that despite the costs of reproduction in terms of predation or energy mobilization for male pitvipers during the mating season (Aldridge and Brown, 1995; Taylor et al., 2004), CORT levels are equivalent or lower, not elevated, during mating. Moreover, unlike the study of Moore and colleagues (2000a), I found no significant interaction between CORT and indicators of body condition, suggesting cottonmouths are not energy-limited, or do not initiate CORT increases when moderately energy-limited. Perhaps the large body mass (including massive fat reserves), and low metabolic rate (McCue and Lillywhite, 2002; Zaidan, 2003) of pitvipers thus far studied present a buffer from the need to initiate HPA-axis responses during energy limitation or the mating season (Taylor et al., 2004).

On the other hand, there are other activities in cottonmouths that may require energy mobilization during certain times of the year. The peak CORT values (April-May) corresponded to a time of year when energy may be limited. At this time cottonmouths have emerged from hibernation, enter a period of basking (Hill, 2004; S.Graham, unpubl.

data) during early spring, and begin establishing ambush sites along watercourses (Savitsky, 1992); perhaps while they wait for their first meal they are running a negative energy balance. Glaudas and colleagues (2006) reported overland migrations by cottonmouths to a wetland in South Carolina during March and in October to November. The population they studied becomes active earlier and enters hibernation later than the population I examined (it located further south), and therefore the elevated CORT during April and May, significant decreases during July, and moderate increases again in autumn (September/October) noted in this study may be associated with migration to and from hibernacula to feeding areas. Although spatial ecology was not the primary goal of this study, and therefore recaptures were limited, migrations from hibernacula to feeding areas of at least 200m were observed. However, many hibernacula at this study area were located very close to or within feeding areas, so long-distance migrations are certainly not obligatory in this population. More detailed studies that incorporate CORT variation, metabolic measures and energy budgets, and spatial/behavioral ecology of this species and other vertebrates are necessary to further test the Energy Mobilization Hypothesis of seasonal CORT variation (Romero, 2002), and could contribute greatly to general energetic hypotheses of GC action (e.g., Dallman et al., 1993; Wingfield et al., 1998; McCue and Wingfield, 2003).

I found ample support for my hypothesis that under certain conditions (e.g., handling stress), CORT and T showed a marked reciprocal relationship. CORT levels were significantly elevated within 30 minutes, and T levels were significantly reduced within 2 to 24 hours after capture. There was an overall negative relationship between CORT and T throughout the active season. My sampling protocol allowed me to

determine the fine-scale time variation in plasma CORT concentrations. There was a positive correlation between time until blood collection from capture (mean 16.86 min.; range 3-30 min) and plasma CORT during the 30-minute field blood-sampling regime. This is important information, as there is still lack of consensus regarding the period after restraint CORT rises above baseline in certain species (Romero and Reed, 2005). This study is similar to that of Schuett and colleagues (2004), who reported significantly elevated corticosterone within 30 min. of handling in *Crotalus atrox*.

My results suggest that CORT may inhibit T, but at a slower rate than that reported in other reptiles, such as the American alligator (Lance et al., 2003), and similar to those reported for most vertebrates studied (Sapolski et al., 2000). Although T was significantly lower within 2-24 hours under captive conditions in *A. piscivorus*, these levels were nonetheless within the normal ranges for this species found in a previous study on seasonal patterns (Chapter 1, S. Graham et al., in prep.). The seasonal peak of plasma T presented in Chapter 1 (S. Graham et al., in prep.) and in Zaidan and colleagues (2003) was ~15 ng/ml. Thus, I found no support for the hypotheses that (i) cottonmouths have a similar T secretion pattern compared to other pitivipers, and (ii) previous studies failed to detect this due to their sampling protocol or method of hormone assay. This is remarkable given the differences in sampling methods (e.g., sampling captive animals transported from the field vs. field sampling), and different types of hormone assays employed (e.g., radio immunoassay vs. enzyme immunoassay). These studies thus corroborate each other and support the view first posited by Schuett and colleagues (1997; see Schuett et al., 2005) that the cottonmouth exhibits a very different pattern of

sex steroid secretion than other pitvipers thus far studied, including its sympatric congener the copperhead, *A. contortrix*.

Behavioral studies of the copperhead have described socially mediated suppression of reproductive behavior (e.g., courtship, mating) that is associated with elevated levels of plasma CORT (Schuett et al., 1996; Schuett and Grober, 2000). Males that lost staged fights had significantly elevated levels of CORT relative to winners, showed no or depressed courtship activities, and even retreated from challenges posed by females (Schuett and Duvall, 1996; Schuett and Grober, 2000). Thus, if CORT negatively affects both physiological reproductive aspects (e.g., plasma T) and downstream behavioral correlates in pitvipers, then the seasonal CORT profiles I describe are not unexpected. Lower basal CORT levels may make it more difficult for males to reach an upper limit that inhibits reproductive behavior. If this contention is correct, it appears to support the Behavioral Hypothesis of Romero (2002), suggesting that seasonal patterns of CORT variation in male cottonmouths are best explained as a strategy to avoid CORT-induced (or inhibited) behaviors during the mating season. Because there is ample evidence that male cottonmouths participate in similar agonistic encounters during mating (Gloyd and Conant, 1990; Ernst and Ernst, 2003) as do copperheads, these findings are at odds with the Energetics-Hormones Vocalization model as extended by Moore and Jessop (2003), which predicts increases in both T and CORT during courtship periods and contest-intensive activities.

The significantly reduced CORT levels during July correspond very closely with the significant elevation of plasma T (see Chapter 1) occurring at the same time. This significant elevation of T is intimately associated with maximum testis mass,

seminiferous tubule epithelial cell height, spermatogenesis, and possibly a secondary sexual characteristic (e.g., the kidney sexual segment; see Chapter 1 of this thesis), suggesting that lower CORT during this period may be important for avoiding down regulation of the HPG-axis during the production of gametes. It is possible that in the cottonmouth the HPA-axis is down regulated during this important period to allow for the production of gametes and mating behavior. None of the proposed hypotheses discussed by Romero (2002), or Moore and Jessop (2003) appear to consider the role of seasonal variation of CORT in maintaining reproductive condition in this way, a possibility suggested here and implied in studies of the brown tree snake (*Boiga irregularis*; Moore et al., 2005). Sapolski and colleagues (2000) acknowledged the possibility that even baseline GCs may inhibit reproduction.

Additional studies in male cottonmouths and other pitviper species might yield important insights into the effects of CORT on reproductive physiology and behavior. For example, does handling stress cause reproductive suppression similar to that described after male-male combat in pitvipers? Studies of the brown tree snake (*Boiga irregularis*), a colubrid, have suggested this possibility (Moore et al., 2005). Is there a difference between the magnitude, duration, or timing of the CORT response initiated after male-male combat and handling stress? My study and the results presented by Schuett and colleagues (1996) suggest a much larger CORT response to handling stress in the cottonmouth than that resulting from conspecific agonism in the copperhead; however, a direct comparison in the same species is needed to confirm this, especially when considering the diversity of hormonal adaptations possible even among congeners or conspecifics (Schuett et al., 1997; Moore et al., 2001; Schuett et al., 2005; see Chapter

1 of this thesis). More studies that specifically examine handling stress are needed, and would allow direct phylogenetic comparisons. For example, is there a difference in the magnitude, duration, or timing of the CORT response to handling between closely related pitvipers (e.g., sister taxa) that differ in anti-predatory tactics (e.g., crypsis in *A. contortrix* and aposematic “gaping” behavior in *A. piscivorus*)? To my knowledge, correlations between anti-predatory tactics and GC secretion patterns have not been examined in any reptile, but could provide interesting insights into the adaptive significance of GCs in modulating these behaviors or their outcomes (i.e., cottonmouths and rattlesnakes coil and “warn” large mammalian predators; are massive CORT responses necessary to help them survive encounters with vertebrates much larger than themselves?).

My study and others have underscored the diversity of physiological responses to stressors in reptiles, and the importance in considering the day-to-day and season-to-season “permissive” variations in GCs. Additional studies in snakes and other reptiles are needed to identify behavioral and physiological trends related to the overall biology of the HPA-axis, and may lend important insights into the ancestral state of these mechanisms in vertebrates (Crews and Moore, 1986). Ectotherms in general hold great promise when considering energy-oriented concepts of GC action (e.g., allostasis, McEwen and Wingfield, 2003), because these vertebrates are not nearly as energy-dependant as mammals and birds (Pough, 1980; Pough et al., 2002) and yet show similar diel, seasonal, life history, and stressor-induced variations of GCs.

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