REPRODUCTIVE PHYSIOLOGY OF THE BROAD BANDED WATERSNAKE, *NERODIA FASCIATA CONFLUENS*, IN SOUTHEASTERN LOUISIANA

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Abstract.—Reproductive cycles in temperate zone colubrid snakes are generally characterized by summer spermatogenesis (postnuptial) in males and spring vitellogenesis (Type I) in females. Watersnakes and gartersnakes in southern North America have shorter hibernation periods than confamilial northern species and potentially different timing of hormonal cues regulating gametogenesis. We studied the reproductive cycles of male and female Broad Banded Watersnakes (*Nerodia fasciata confluens*) to uncover whether hormone cycles are more similar to closely related northern natricine species or to more distantly related southern colubrids. We collected *N. f. confluens* from southeastern Louisiana and maintained them in outdoor enclosures. The first experiment quantified circulating sex steroid hormones, follicle growth, and presence or absence of sperm each month from February 2000 to February 2001. The second experiment measured the same variables weekly during the spring breeding season, from early April 2001 to late June 2001. Male androgen concentrations were elevated in the fall and the spring. Female estradiol-17β was elevated in the spring only. Ovarian follicle growth began in early May, after mating. The male reproductive cycle was classified as postnuptial spermatogenesis and the female cycle as Type I vitellogenesis; rapid follicle growth in the spring. *Nerodia f. confluens* has a reproductive pattern more similar to other southern temperate colubrids than to its more closely related northern temperate natricines, suggesting that local climate has a stronger influence on reproductive timing than phylogeny.

Key Words.—colubrids; hormone; natricine; Nerodia; physiology; reproduction; reptile; snakes

INTRODUCTION

Characterization of spermatogenesis and vitellogenesis patterns in colubrid snakes is largely based on studies of species north of 37° N (Aldridge 1981; Weil and Aldridge 1981; Licht 1984; Aldridge et al. 1990; Greene et al. 1999). Natricines, including the Northern Watersnake (*Nerodia sipedon*), Red-sided Garter Snake (*Thamnophis sirtalis parietalis*), and Southern Watersnake (*Nerodia fasciata*), mate in the spring shortly after emergence from a hibernation of three to six months. After the spring mating period, males produce sperm in the fall (postnuptial spermatogenesis), then mate after emergence the following spring (Weil and Aldridge 1981). Circulating testosterone is high in the fall at the beginning of hibernation and in the spring upon emergence (Weil and Aldridge 1981; Garstka and Crews 1982). Female natricine snakes undergo Type I vitellogenesis, defined as rapid yolk deposition and follicle growth, in the spring following emergence from hibernation (Aldridge 1979). In Red-sided Gartersnakes,

yolk deposition may be induced by post-emergence mating, although yolk production is independent of mating stimuli (Whittier and Crews 1986; Whittier and O'Conner 1991).

The Rough Greensnake (*Opheodrys aestivus*), a southern colubrid, has postnuptial spermatogenesis, but does not maintain a constantly high level of androgens over the winter (Aldridge et al. 1990). Instead, these males have high testosterone levels in the fall and spring, with low testosterone concentrations in winter and summer (Aldridge et al. 1990). Postnuptial spermatogenesis has also been documented in another southern colubrid, the Black Swampsnake (*Seminatrix pygaea*), in South Carolina (Gribbins et al. 2005), but seasonal variation in hormones has not been studied in this species. The strategy of postnuptial spermatogenesis is employed by many male colubrids of warmer climates; however, there is no evidence of a sustained high level of androgens over winter months in any family of southern temperate or tropical snakes.

FIGURE 1. *Nerodia fasciata confluens* from our study site approximately 20 km north of New Orleans, Louisiana, USA. (Photographed by Tom Lorenz)

Patterns of vitellogenesis for colubrid snakes, both northern and southern, appear relatively conserved. Type I vitellogenesis is the norm, including southern colubrids such as *O. aestivus* in Arkansas (Plummer 1984). Hormonal studies on female southern colubrids are lacking, but laboratory studies have indicated a spring surge in estrogen (Kleis-San Francisco and Callard 1986), as is seen in northern colubrids. The tropical elapid *Naja naja* also has this spring surge (Bona-Gallo et al. 1980), suggesting that this may be the norm for many snake species.

Nerodia fasciata confluens (Fig. 1) ranges through much of the coastal plain of the Gulf of Mexico and is found in a variety of aquatic habitats (Mushinsky et al. 1980). Follicle growth occurs in spring and early summer, based on measurement of follicle size in preserved specimens captured throughout the year in southern Louisiana (Kofron 1979). Laboratory studies of the ovarian cycle showed that vitellogenesis is regulated by estradiol-17β (Riley and Callard 1988), as it is in other oviparous vertebrates (Norris 1997).

Because *N. f. confluens* is closely related to *T. s. parietalis*, it could be expected that their reproductive cycles would be similar. However, *N. f. confluens* is a southern temperate species with an abbreviated brumation period of approximately three to five months, which can be interrupted by emergence to bask or feed during warm spells (pers. obs.). Therefore, we predict that *N. f. confluens* reproductive cycles are more similar to southern colubrid species, some of which are not natricine snakes, such as *O. aestivus*. Evidence for this would include a lack of maintained androgens over the winter, which has been observed in northern male natricines (Weil and Aldridge 1981; Krohmer et al. 1987; Clesson et al. 2002). Cloacal washes of females may also give further understanding of the timing of mating. Comparisons can then be made to colubrids with fall mating (Krohmer and Aldridge 1985b) and southern temperate vipers with dissociated hormone profiles (Graham et al. 2008). Understanding the reproductive cycle of a subtropical snake can be valuable for understanding the interaction between the sexes over time (i.e., seasons) as well as an examination of the effects of a subtropical climate on reproduction.

MATERIALS AND METHODS

Study animals.—We collected *N. f. confluens* by hand or by minnow traps in the Manchac and Ruddock swamps, approximately 20 km north of New Orleans, Louisiana, USA (30° 17' 30" N / 90° 24' 7" W). As required by Louisiana Department of Wildlife and Fisheries, snake collection was permitted under a state fishing license. We housed snakes in outdoor enclosures at Southeastern Louisiana University. Enclosures were cylindrical 3,000 L fiberglass tanks that we placed in partial shade. Each tank was 250 cm in diameter and 90 cm high, which prevented any snakes from escaping the enclosures. We covered the bottom of each tank with 4 cm gravel (5–10 mm diameter) for drainage and 6 cm cypress mulch for substrate. We provided a variety of cover objects including: PVC pipes (5–7 cm diameter, approximately 1 m long), wooden boards (60 cm x 120 cm), black foam insulation (90 cm x 90 cm), and plastic lids (30 cm x 60 cm). We placed flagging material across the top of each enclosure to discourage avian predators. We fed the snakes *ad libitum* from February through November by stocking two shallow (20 cm deep) plastic containers (60 L total volume) with small fish (approximately 4–10 cm total length). Fish that we fed to snakes were either Mosquitofish (*Gambusia affinis*), Mollies (*Poecilia latipinna*), or shiners (*Notropis* sp.) captured or purchased locally. We fed frogs (usually the Green Frog; *Lithobates clamitans*) and tadpoles (usually the American Bullfrog; *L. catesbeiana*) to snakes when available. Kofron (1978) found that fish and anurans are the principal dietary items of this species. No live prey were added from mid-November through early February because the snakes stopped eating in mid-November and only began feeding again in late February. During this time average daytime temperatures were 15° C ± 12° C and average nighttime temperatures were 5° C ± 13° C. To provide substrate for burrowing and insulation, we added an additional 30–40 cm of mulch to each enclosure in November.

Experiment 1: annual cycle.—The purpose of this first experiment was to describe the annual cycle of hormone fluxes in male and female *N. f. confluens,* and follicle growth in female *N. f. confluens.* We collected snakes for this experiment in February and March 2000. Each snake was randomly assigned to a group of four females and four males in each of three replicate enclosures, for a total of 24 snakes. We acclimated newly introduced snakes for at least one week before the first sampling to reduce the effects of capture stress on sex steroid hormone levels. Every month from February 2000 to February 2001 (except December and January), we captured snakes by hand from within the enclosures and bled them immediately. We drew approximately 0.1–0.5 mL of blood from the caudal vein with a heparinized (6 mg/mL ammonium heparin in 0.9% NaCl) 1 mL syringe fitted with a sterile 26 or 27-gauge, 1.6 cm needle. We transferred whole blood to microcentrifuge tubes and held tubes on ice until all samples were collected (over an average time of 60 min). Following collection, we brought blood samples to the lab and centrifuged them at 14,000 rpm at 4° C for 5 min. We transferred plasma to a clean vial and stored vials at -80° C for later hormone analysis. After blood sampling, we identified the individual by unique ventral markings, and measured snout-vent length (SVL), tail length (TL), and mass of each snake.

We performed ultrasonography on all females one day after bleeding using a portable Aloka SSD-900V ultrasound unit (Aloka America, Wallingford, Connecticut, USA). We transported females to a lab < 1 km from the enclosures. During examination, we placed snakes on their backs in warm water and a variable range abdominal probe was moved across the ventral side. We printed ultrasonic images and used the gall bladder as a point of reference to locate the ovaries/follicles. The gall bladder appears as a dark circle immediately anterior to the ovaries. We measured the length and width of follicles or embryos using the calibrated measurement function. Because not all follicles were visible, we did not count the number of follicles. We measured the largest follicles and typically, all visible follicles were approximately the same size. To determine if body size affected which females were gravid, we compared lengths, weights, and the ratio of weight/length between gravid and nongravid females.

In October 2000 and in February 2001, we surveyed for the presence of sperm using cloacal washes from males and females to determine the possibility of fall and spring mating. We injected approximately 1 mL of 0.9% NaCl in deionized, distilled water into the cloaca using a 3 mL syringe with a ball tipped needle. The belly was then massaged and the solution was drawn back into the syringe. Then, we examined the samples under a light microscope for the presence of sperm. This method has been shown to be an effective and nondestructive technique with watersnakes (Green et al. 1999).

Experiment 2: reproductive season.—We collected a second group of animals, seven males and eight females, in February and March 2001. These animals were housed similarly to the first experiment; however, one enclosure contained four females and only three males. We provided cover and food as in Experiment 1. However, to facilitate cleaning, no gravel or soil substrate was added.

To develop a more detailed understanding of hormonal changes during the reproductive period, we collected approximately 0.1–0.3 mL of blood from each snake once a week beginning 4 April and ending 30 June 2001 and processed samples as described above. The day after bleeding, we performed ultrasounds on each female. For the first five weeks, we collected cloacal washes weekly from all males and females until all females were thought to have ovulated, based on the presence of large follicles in all gravid females. To determine if body size influenced which females were gravid, we compared SVL, weight, and the ratio of weight/length between gravid and nongravid females.

Hormone analysis.—We measured blood plasma levels of estradiol-17β (E2) and testosterone (T). Pure steroids (HPLC grade estradiol-17β, catalog number E1132 and testosterone, catalog no. 86500) were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Immediately prior to each assay, we prepared serial dilutions (3.95–1,000 pg/mL) of authentic steroid and analyzed these at the same time as the plasma samples. For each hormone, we extracted duplicate aliquots (E2, 50 µL; T, 6.25 µL for females, 0.33 µL for males) of each sample with two, 1.5 mL aliquots of anhydrous diethyl ether. Extracts were evaporated to dryness under nitrogen at 37° C, then reconstituted in 200 µL RIA buffer (9 mM sodium phosphate, pH 7.0, 140 mM NaCl, 0.1% gelatin, 250 µM thimerosal) for E2 analysis or 60 µl EIA buffer (1 M sodium phosphate, pH 7.4, 1% BSA, 4 M NaCl, 10 mM EDTA, 0.1% sodium azide) for T analysis.

We analyzed E2 by radioimmunoassay (RIA) following the methods of Cheek et al. (2000). Briefly, we incubated standards and reconstituted samples with antiserum (Endocrine Sciences, Calabasas Hills, California, USA; diluted 1:4000) for 30 min at room temperature, then we added 5000 cpm tracer ([2,4,6,7-^{3}H] estradiol (New England Nuclear, Boston, Massachusetts, USA), which we incubated overnight (12–16 h) at 4° C. The assay was ended by placing the tubes on ice and adding 500 µl charcoal-dextran to absorb free tracer. We then centrifuged the mixture at 3,000 rpm for 20 min at 4° C. We then poured the supernatant into scintillation vials containing 4 ml of
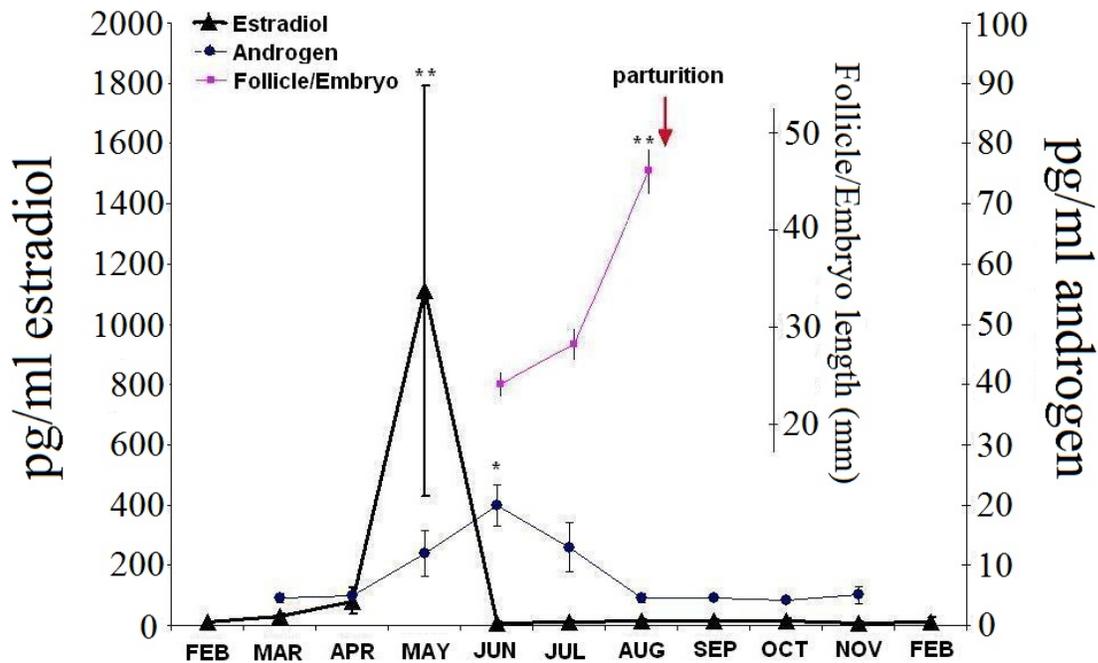
FIGURE 2. Annual cycle of androgen, estradiol-17β (E2), and follicle/embryo size in female *Nerodia fasciata confluens* (significant differences: * indicates $P < 0.05$, ** indicates $P < 0.01$) from Louisiana, USA. Follicle/embryo growth was not measured before June. All females had given birth by September. Points represent mean of 4–10 females per month and error bars are ± 1 SE

ScintiSafe Plus LSC Scintillation Cocktail (Fisher Scientific, Atlanta, Georgia, USA). We counted samples using a Tricarb 1900TR Liquid Scintillation Counter Analyzer (HMI, Ramsey, Minnesota, USA). Duplicate determinations were made for controls, standards, and samples.

We analyzed T by acetylcholinesterase (AChE)-based competitive enzyme-linked immunoassay (ELISA) following the methods of Fentress et al. (2006). The cross reactivity of the T antibody for other androgens is 21% for 5α-dihydrotestosterone, 12.4% for 11-ketotestosterone, 10% for 5β-dihydrotestosterone, 3.6% for androstenedione, 1.2% for 11-hydroxytestosterone and less than 0.02% for estradiol, corticosterone, and progesterone. Plasma samples were not fractionated prior to analysis, so we present results as total androgens.

Parallel dilution of circulating steroid hormone compared to pure steroid was demonstrated for E2 and T. Recovery of known steroid concentrations was 88.0% for E2 (n = 4 replicates) and 82.9% for T (n = 4 replicates). Intra-assay variability (coefficient of variation) was 11.7% for E2 (n = 20 replicates in one assay) and 14.8% for T (n = 20 wells in one plate). Inter-assay variability was 9.8% for E2 and 13.7% for T (n = 3 replicates measured in 11 different assays). The detection limits of the assays (the dose of authentic

steroid that displaced 5% of labeled steroid) were 13.0 pg/mL for E2 and 15.0 pg/mL for T.

Statistical analyses.—We log transformed the hormone data prior to repeated measures analysis of variance. To test for the effect of time, we used each month as the fixed effects for the first experiment and weeks were fixed effects in the second experiment. We used orthogonal contrasts to test the differences between specific months, seasons, or weeks. We used analysis of variance (ANOVA) to compare length (SVL), mass, and a weight index (body mass/SVL) between gravid and non gravid *N. f. confluens*. Results were accepted as significant at α ≤ 0.05.

RESULTS

Annual cycle; females.—E2 concentrations remained near zero through most of the year with a single peak in May representing a 10-fold increase over April levels ($F_{10,94} = 2.619$, $P < 0.001$; Fig. 2). Concentrations of E2 were elevated in eight of the 12 females. In these eight females, growing embryos were visible by ultrasonography from mid-June through parturition in August and September. Embryo size increased throughout the summer (Fig. 2 inset). Female snakes that were gravid were, on average, longer and heavier, but not significantly greater in length ($F_{1,10} = 1.764$, $P =$
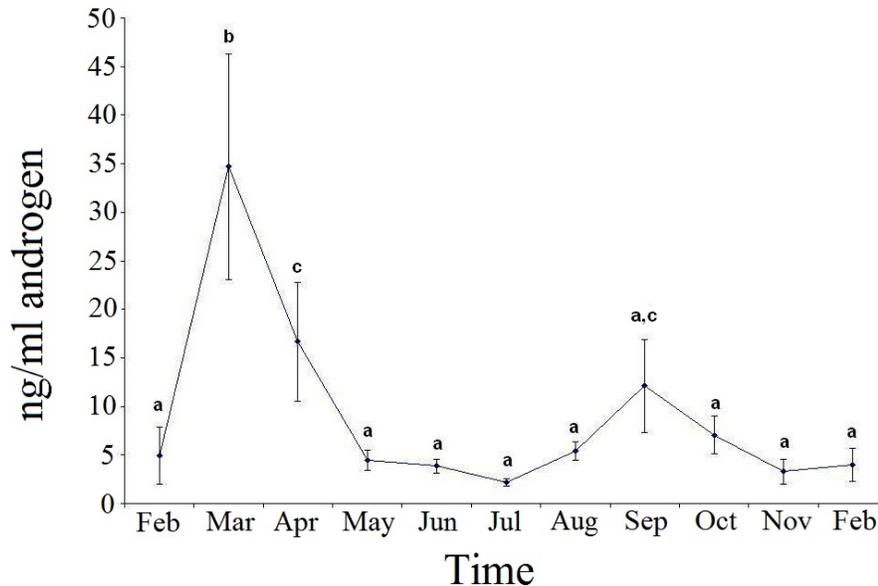
FIGURE 3. Annual cycle of androgen levels in male *Nerodia fasciata confluens* from Louisiana, USA. Points represent the mean of 4–12 males per month and error bars are ± 1 SE. Letters indicate similar mean values.

0.21), weight ($F_{1,10}$ = 2.817, P = 0.12), or condition (weight index; $F_{1,10}$ = 1.764, P = 0.11) than non-gravid females. There were gravid snakes in all three tanks.

Androgen concentrations in gravid females were not significantly different between months ($F_{6,94}$ = 8.642, P > 0.84). Concentrations were highest in June when embryos were first visible. Androgens increased from May to June and then decreased gradually throughout embryo incubation. The timing of mating could not be precisely determined because we collected cloacal washes only at the end of the experiment in October 2000 and February 2001. No sperm were present at either time, indicating that fall mating and subsequent sperm storage are unlikely.

Annual cycle; males.—Male androgens varied significantly throughout the year ($F_{9,80}$ = 6.585, P < 0.001; Fig. 3). March was significantly different from all other months, with a seven-fold increase followed by a decline to winter levels by May (P < 0.001). Androgen concentrations increased slightly in September then returned to low levels by November (P < 0.001). Most males (nine of 12) followed this two-peak pattern but three had low and constant androgen concentrations throughout the year. Sperm were present in most males in October 2000 (six of nine) and February 2001 (six of eight), even though androgen concentrations were low compared to peaks in March and September.

Reproductive season; females.—Four of the eight females we collected in spring 2001 had sperm in the cloaca at least once during the first five weeks after capture (Table 1), indicating that mating occurred before or during the time of this experiment. Increases in E2 and follicle growth occurred only in females with sperm present in the cloaca. Overall, there was a significant increase in E2 for all female snakes ($F_{11,63}$ = 9.966, P < 0.001; Fig. 4). E2 was high for vitellogenic snakes from mid-April through mid-May. Females that did not have sperm present in the cloaca (n = 4) during the first five weeks after capture did not become gravid and did not have significantly higher E2 concentrations during any

TABLE 1. Sperm presence/absence in cloacal washes from gravid *N. f. confluens* from Louisiana, USA, in Experiment 2. No nongravid snake had sperm at any sampling point. Negative sign indicates no sperm, positive sign for very few sperm, double positive for abundant sperm. No sperm was found before 19 April 2001 or after 3 May 2001. Follicles appeared in all females on 3 May 2001. An asterisk (*) indicates levels of estrogen were > 0.3 ng/mL.

	19 April 2001	26 April 2001	3 May 2001	11 May 2001
Gravid Female 1	-	++*	+*	-*
Gravid Female 2	++*	-	-	-
Gravid Female 3	-*	-	+	-*
Gravid Female 4	++*	-	-	-*

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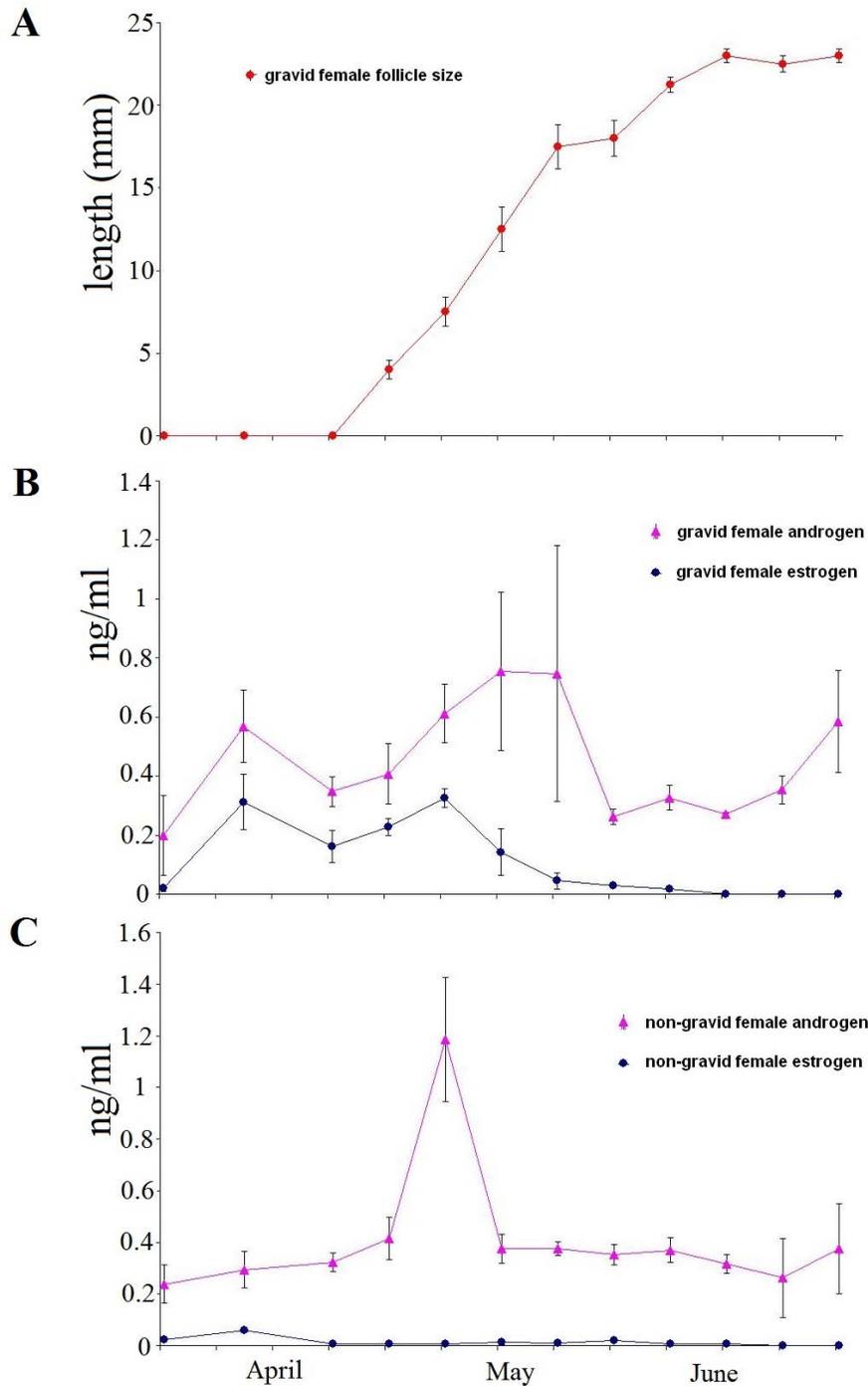
FIGURE 4. Reproductive status of female *Nerodia fasciata confluens* during spring 2001 from Louisiana, USA. A: Follicle growth in gravid females (n = 4). B: Androgen and estradiol-17β (E2) concentrations in mated females. C: Androgen and E2 in unmated females (n = 4). Error bars are ± 1 SE.

week ($F_{1,6}$ = 65.09, P < 0.001). There was a significant effect of week on the level of androgens in female snakes ($F_{11,77}$ = 4.302, P < 0.001). Estidiol-17β concentrations during weeks four through seven were all significantly higher than the first week (P < 0.001). There was no difference in androgen levels between gravid and nongravid females ($F_{1,6}$ = 0.177, P = 0.688).
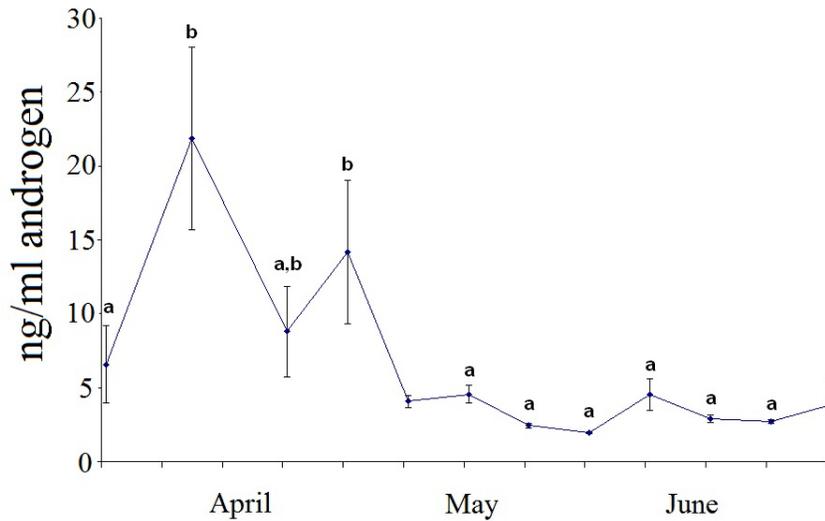
FIGURE 5. Androgen concentrations in male *Nerodia fasciata confluens* during spring 2001 in Louisiana, USA. Points represent the mean of seven males and error bars are ± 1 SE . Letters indicate similar mean values.

In vitellogenic females, follicle size increased during the eight-week measurement period, with the most rapid growth during May (Fig. 4). Developing embryos were clearly visible by 21 June 2001. Female androgen concentrations varied over the mating season in all females and were elevated from mid to late May (10 May to 24 May 2001) for gravid females. Year two gravid females were initially significantly greater in weight ($F_{1,5}$ = 7.334, P = 0.04) and condition ($F_{1,5}$ = 6.624, P = 0.05), and were significantly longer ($F_{1,5}$ = 6.805, P = 0.04) than non-gravid females (Table 2). Similarly, pooled data on females from 2000 and 2001 showed significantly greater weight ($F_{1,17}$ = 8.062, P = 0.01) and weight index ($F_{1,17}$ = 8.734, P = 0.009) in gravid females versus non-gravid females but not significantly different in length ($F_{1,17}$ = 3.965, P = 0.06; Table 2).

Reproductive season; males.—All males had sperm during some of the spring sampling periods, but presence or absence of sperm on a given sampling date appeared independent of androgen concentration. Male androgen concentration varied significantly over the reproductive season ($F_{11,59}$ = 6.495, P < 0.001; Fig. 5). Androgen levels were significantly higher in week two than all other weeks (P < 0.001). Gravid female E2 and androgen were also high during this period.

DISCUSSION

To our knowledge, this is the first longitudinal and nondestructive study of hormonal cycles in cohabiting male and female natricine snakes from a southern temperate climate. Hormone and mating profiles demonstrate a reproductive strategy that appears more

TABLE 2. Mean snout-vent length (± SE) , mass, and weight index for gravid and nongravid female *N. f. confluens* from monthly (2000) and weekly (2001) samples from Louisiana, USA. Significant differences between gravid and non-gravid females indicated as * P < 0.05 and ** P < 0.01.

	SVL (cm)	Mass (g)	weight index (mass/SVL)
2000			
Gravid (n = 8)	60.56 ± 3.03	194.9 ± 30.22	3.13 g/cm (± 0.34)
Nongravid (n = 4)	54.25 ± 4.12	125.8 ± 7.79	2.32 g/cm (± 0.07)
2001			
Gravid (n = 4)	67.00 ± 2.39*	255.5 ± 44.78*	3.79 g/cm (± 0.57)*
Nongravid (n = 3)	58.50 ± 3.14	125.0 ± 21.50	2.14 g/cm (± 0.38)
Combined			
Gravid (n = 12)	62.71 ± 2.25	215.1 ± 24.50*	3.35 g/cm (± 0.28)**
Nongravid (n = 7)	56.07 ± 2.49	125.4 ± 8.16	2.24 g/cm (± 0.13)

TABLE 3. Timing of mating, season of elevated levels of androgens (High T), and season of vitellogenesis of nine species of temperate snakes. Seasons of High T are fall through winter (F-W) and peaks in fall and spring (F/Spr).

Species	Mating season	High T	Vitellogenesis	Reference
Thamnophis sirtalis	Spring	F-W	Spring	Whittier et al. 1987
T. ordinoides	Summer	--	Spring	Hebard 1951
Nerodia sipedon	Spring	F-W	Spring	Bauman and Metter 1977; Weil and Aldridge 1981
N. f. confluens	Spring	F/Spr	Spring	Current paper
Opheodrys aestivus	Summer	F/Spr	Spring	Plummer 1984; Aldridge et al. 1990
Tropidoclonion lineatus	Summer	--	Spring	Krohmer and Aldridge 1985a, 1985b
Heterodon platirhinos	Summer	--	Summer	Trauth et al. 1994
Tantilla coronata	Spring	--	Summer	Aldridge and Semlitsch 1992a, 1992b
Agkistrodon piscivorus	Sum/Fall	Summer	Biennial	Aldridge and Duvall 2002; Graham et al. 2008

tied to climate than to the northern relatives of *N. f. confluens*. This study provides clear evidence that *N. f. confluens* undergoes vitellogenesis in the spring (Type I). Female E2 is non-detectable in the fall, increases dramatically in the spring, and is four-fold higher in mated females than in unmated females. Follicles begin growing after two weeks of sustained high E2 concentrations in mated females, possibly indicating that vitellogenesis is mating-induced.

Spring follicle growth is common among female natricines and colubrids (Table 3). *Thamnophis sirtalis parietalis*, a northern temperate natricine, also has an estrogen surge followed by follicle growth in the spring (Whittier et al. 1987). Some follicle growth occurs from parturition to hibernation in Missouri populations of *N. sipedon* (Aldridge 1981). This follicle growth was from approximately 5–9 mm but there was no rapid growth. In this study, it is possible that *N. f. confluens* had winter follicle growth that was smaller than could be detected by ultrasonography. However, our ultrasound did detect 5 mm follicles in the spring. Most female Diamondback Watersnakes (*Nerodia rhombifer*) in a tropical population (Veracruz, Mexico) had growing follicles in May (Aldridge et al. 1995). Rapid follicle growth is also confined to April and May in Arkansas of the confamilial *O. aestivus* (Plummer 1984). Rapid follicle growth from March to May appears to be consistent in temperate and tropical colubrid snakes, suggesting conservation of reproductive timing across the family range.

There were gravid and nongravid females in both 2000 and 2001. There was at least one gravid female in every enclosure for both studies and the size of the females may affect their likelihood to mate and/or develop follicles. Female *N. f. confluens* in the reproductive season showed a correlation of body mass to follicle production, the smaller females of this study never became gravid even with constant access to males. Energy availability ("body reserves") of female vipers has been shown to affect their reproduction effort (Lourdais et al. 2002). This has been observed in natricines as well, where vitellogenic female *T. sirtalis* had a higher body mass (Whittier and Crews 1990).

Female testosterone levels have rarely been measured in snakes; however, the simultaneous increase of androgens and E2 in mated females reflects the role of T as the precursor to E2 and this also occurred in female *T. sirtalis* (Whittier et al. 1987). Even though T is a precursor to E2, androgen levels were consistently higher in reproductively active females and low in unmated females. It is possible that androgen serves a function for female reproduction and is not just a precursor or metabolite. This is more often observed in non-reptilian vertebrates (Norris 1997).

We did not measure female progesterone concentrations because the primary focus of this project was to define the pattern of vitellogenesis, an estrogen regulated process. Circulating progesterone, progesterone receptor concentrations (*sensu* Kleis-San Francisco and Callard 1986), and progesterone measurements could have provided a more complete picture of the female *N. f. confluens* reproductive cycle. Progesterone may have a different profile in viviparous snakes and would have provided an additional point of comparison. However, estradiol concentrations and follicle measurements provide a clear indication that *N. f. confluens* undergoes vitellogenesis in the spring (Type I) and suggest that vitellogenesis is mating-induced. It is also possible that estrogen levels rise only in females that have enough energy availability to become reproductive and this rise affects mating behavior.

Monthly and weekly serial sampling clearly delineated the annual and seasonal male androgen profile. Androgen concentrations are low in February and increase seven-fold in March, remain high through April. They again decrease in the summer and increase somewhat in September (approximately two-fold higher than summer levels). Following this, they decline to low levels throughout the winter. This seasonal profile is almost identical to that of another southern temperate colubrid, *O. aestivus* in Arkansas (Aldridge et al. 1990), but is in apparent contrast to testosterone profiles in *N. sipedon* and *T. sirtalis*. These species have a sharp increase in testosterone in the fall that is apparently sustained over winter because T concentrations are still high at the time of spring emergence (Weil and Aldridge 1981; Krohmer et al. 1987). However, blood samples

could only be collected during the active season in these studies, so actual hormone concentrations during winter hibernation are unknown. In both species, T concentrations drop two to four weeks post-emergence.

Male *N. f. confluens* had sperm in their cloacas in October, February, April, and May (all times measured). Other temperate colubrids have mature sperm throughout the year (Weil and Aldridge 1981; Aldridge et al. 1990; Clesson et al. 2002), although sperm is more typically examined in the vas deferens in these studies (Aldridge et al. 1995). Androgen concentrations are not known, but the tropical natricine, *N. rhombifera*, begins spermatogenesis in September, with peak spermatogenesis occurring from November-January (Aldridge et al. 1995). The timing of spermatogenesis appears to be relatively conserved in colubrid snakes, regardless of climate.

Based on the presence of sperm in female cloacae, some natricines and colubrines have a fall mating period, including the Western Terrestrial Garter Snake (*Thamnophis elegans*; Storm and Leonard 1995), the Concho Watersnake (*Nerodia harteri*; Greene et al. 1999), and *O. aestivus* (Plummer 1984). In contrast, none of the > 500 female *N. sipedon* sampled in Missouri had sperm present in the cloaca in the fall (Bauman and Metter 1977). The absence of sperm in female cloacal washes that we collected in the fall indicate that *N. f. confluens* might not mate in fall at our site. We gave cloacal washes to roughly a dozen wild, mature female *N. f. confluens* in the field from August to December 2000 and no samples provided sperm.

We did not study sperm presence/absence in the detail necessary to completely understand spermatogenesis in *N. f. confluens*. Cloacal washes should have been performed every week and month of this study, but were not. Nevertheless, sperm presence in male snakes was observed in fall and spring, while female snakes only had sperm present in the spring. This demonstrates that even when housed together, this species showed no evidence of fall mating. This also indicated that sperm production in males was still postnuptial, even though no mating occurred in the fall. This contrasts with a predicted bimodal pattern of mating in temperate colubrids at more southern latitudes (Aldridge et al. 2009). Sperm storage can confound some of this information if the sperm is not stored in the cloaca.

While summer spermatogenesis in natricines may represent preparation for spring mating, the highest male androgen concentrations in this study are present in the spring, at the time of mating and when natricines have an increase in the sexual segment of the kidney (Weil and Aldridge 1981). Male and female hormonal cycles are closely coupled in the spring with the male androgen increase occurring just before the female E2 surge and subsequent follicle growth. Similar coordination occurs in other colubrids, including *N. sipedon* (Bauman and

Metter 1977; Weil and Aldridge 1981) and *T. s. parietalis* (Garstka et al. 1982). However, the high spring testosterone levels present in *N. sipedon* and garter snakes at the time of mating may not represent current T synthesis, but may represent unmetabolized circulating T present at the start of hibernation (Garstka et al. 1982). This study indicates T synthesis at the time of mating for *N. f. confluens*.

This study shows a dissociated hormone increase in the fall for male snakes, but no evidence of fall mating. Fall mating of temperate colubrids has been observed in *Tropidoclonion lineatum* (Krohmer and Aldridge 1985a), and is common for many pitvipers (Graham et al. 2008). Most temperate colubrids, however, mate in the spring and summer (Aldridge et al. 2009). This includes non-natricines such as *Tantilla coronata* (Aldridge and Semlitsch 1992a). Future study could be done on the nature of the climate versus phylogeny in the reproductive biology in temperate colubrid snakes. Aldridge and Duvall (2002) examined reproductive patterns of pitvipers. They determined that temperate vitellogenesis in temperate zone species is a modification of a tropical pattern. Similarly, colubrids may be modifying their patterns for northern and southern climates. A comparative study of southern populations of *N. sipedon* could provide useful data, as this species ranges into the same latitudes as *N. f. confluens* and *O. aestivus*. If *N. sipedon* has a hormone cycle more similar to southern colubrids than previous studies on *N. sipedon*, this would indicate an effect of climate. Museum samples of *N. f. confluens* could also be examined to determine if any follicle growth occurred in the fall and to see what the annual cycle is of the sexual segment of the kidney. It would also be interesting to describe hormone cycles for the tropical populations of *N. rhombifer*. Some of these species that range into northern and southern latitudes could be experimentally exposed to differing environmental conditions, provided the hibernacula temperatures experienced in each environment are known.

Male *N. f. confluens* and most male natricines have to contend with a dissociated mating pattern that exposes them to testosterone during times when there is no mating observed. Trade-offs between testosterone and physiological issues such as decreased survival and a suppressed immune system have been observed in lizards (Zera and Harshman 2001) and could be relevant to a dissociated testosterone strategy. Differences in climate may affect this as well; however, both spermatogenesis and timing of follicular development appear similar between tropical *N. rhombifer* and this subtropical *N. f. confluens*. There is also the possibility that some species have less of a negative tradeoff from elevated testosterone than others (Hau 2007).

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Acknowledgments.—We thank Steve Miller for his help with the snake ultrasound unit and Gary Schaffer for use of the mesocosms. We also thank Johel Chaves, Jonathan Willis, Arie Roth, and Stacy Steele for their help with data analysis and fieldwork. In the absence of a formal Animal Care and Use Committee at Southeastern Louisiana University, snake care and use were consistent with guidelines recommended by "Guidelines for Use of Live Amphibians and Reptiles in Field Research" (Herpetological Animal Care and Use Committee [HACC] of the American Society of Ichthyologists and Herpetologists, 2004).

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