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Fine-scale hormonal patterns associated with birth and maternal care in the cottonmouth (*Agkistrodon piscivorus*), a North American pitviper snake

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ABSTRACT

Steroid hormones regulate many aspects of reproductive physiology and behavior, including parental care. Reptiles display a variety of egg- and neonate-directed parental behaviors, yet few studies have addressed their endocrine correlates. Viviparous female pitvipers remain at the birth site with their young for one to two weeks until neonates complete their first shed cycle ('ecdysis'). To study possible relationships between steroid hormones and these behaviors, we conducted a captive study on wild-caught pregnant cottonmouths. Females were divided into two treatment groups: Maternal Attendance (MA) – females were allowed a maternal attendance period, where neonates were left with the mother until they completed ecdysis and then were removed; Separated (SE) – females had their neonates removed within 24 h of birth. Serial blood samples were collected from MA females at various points during and after attendance; SE females had samples collected on a similar temporal schedule. Plasma levels of progesterone (P), estradiol (E2), testosterone (T), and corticosterone (CORT) were measured in all samples. We did not find a difference in the overall pattern of P, E2, or T between MA and SE females; however, MA females exhibited a significant peak in CORT on the day that neonates shed that was not observed in SE females. It is possible that the elevated CORT observed in MA females was stimulated by increased activity and/or changing chemical cues of shedding neonates. Based on evidence that free-ranging pitvipers cease MA when all offspring complete ecdysis, we hypothesize that CORT has a role in signaling mothers to terminate care and disperse.

1. Introduction

Many aspects of reproductive physiology and behavior are regulated by steroid hormones, the molecular structures of which are highly conserved across vertebrates (Norris, 1997). This allows for a comparison of the reproductive functions of these hormones among taxa with contrasting life histories that have been shaped by their unique physiology and ecology (e.g., Hirschenhauser and Oliveira, 2006; Romero, 2002; Staub and De Beer, 1997; Wingfield et al., 1997). One life history characteristic that varies markedly among vertebrate groups with substantially different physiology is parental care, defined in the narrow sense as "parental behavior that occurs post-fertilization, is directed at offspring, and appears likely to increase offspring lifetime reproductive success" (Klug et al., 2012; p. 21). Parental care is present in almost all endothermic vertebrates (i.e., birds and mammals), but relatively rare among ectothermic species (i.e., fishes, reptiles, and amphibians) (Clutton-Brock, 1991). Furthermore, in contrast to the precocial young of ectotherms, the young of most endotherms are nutritionally and physiologically dependent upon parents, making the costs of not providing care particularly large for this group. These costs presumably resulted in the strong hormone-behavior relationships that have been documented during obligatory parental care in birds (reviewed in Buntin, 1996) and mammals (reviewed in Gonźalez-Mariscal and Poindron, 2002), but very little is known about the endocrine control of the more rudimentary parental behaviors characteristic of terrestrial ectotherms (fishes have been investigated more thoroughly; e.g., Oliveira et al., 2002; Whittington and Wilson, 2013).

For example, despite the widespread occurrence of egg-directed parental care (e.g., nest guarding and egg brooding) in reptiles

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(reviewed in Shine, 1988; Somma, 2003) we know of only four studies that reported steroid hormone levels of females engaged in these behaviors (alligators: Elsey et al., 1990; tuataras: Guillette et al., 1990; marine iguanas: Rubenstein and Wikelski, 2005; pythons: Stahlschmidt et al., 2013). Also, none of these studies measured hormones in non-guarding/brooding females in the same reproductive state (i.e., same number of days post-oviposition), so it cannot be concluded that the hormonal changes documented are regulating (or are at least associated with) parental behaviors, per se, rather than a simple consequence of reproduction. Neonate-directed parental care (e.g., attendance with documented or putative guarding) is rare in reptiles, but does occur in crocodilians, viviparous lizards in the genus *Egernia* (While et al., 2009), and viviparous snakes in the subfamily Crotalinae (pitvipers) (reviewed in Greene et al., 2002; Shine, 1988; Somma, 2003); to date, there have been no studies measuring steroid hormones during neonate care in any reptile. To address this paucity of information, we chose to examine the hormonal correlates of neonate-directed parental behavior in a pitviper.

Although empirical data are limited, a large number of anecdotal observations have documented extended maternal attendance of young in the majority of New World pitviper species that inhabit temperate zones (i.e., *Agkistrodon, Crotalus, Sistrurus*; commonly known as rattlesnakes and moccasins; reviewed in Greene et al., 2002). Mothers generally remain with young at the birth site until neonates complete the first shed cycle, which occurs approximately 10–14 days after parturition; once neonates shed, they and the mother disperse. During attendance, the defensive behavior of mothers varies from complete quiescence to elevated aggression, and sometimes includes 'chasing' behavior that involves the mother making a quick advance toward the human observer. These observations led to the hypothesis that maternal attendance of young in pitvipers might function to increase the survival of offspring via predator deterrence (Butler et al., 1995; Greene et al., 2002), and a few empirical studies have found evidence supporting this hypothesis (Graves, 1989; Greene et al., 2002; Hoss and Clark, 2014).

Another conspicuous behavior that has been observed during the maternal attendance period is the formation of tight mother-young aggregations (reviewed in Greene et al., 2002). Aggregating behavior can greatly enhance thermoregulation and hydroregulation in snakes (Graves and Duvall, 1987; Lillywhite, 1987; Reiserer et al., 2008), both of which are particularly important during the shed cycle. The physiological benefits of aggregation might be substantial for pre-shed neonates, as their natal skin is highly permeable and they are more vulnerable to dessication while it is being replaced with thicker skin (Tu et al., 2002). Further evidence of the importance of the shed cycle in regulating maternal attendance stems from observations that C*rotalus unicolor* (formerly *C. durissus unicolor*) neonates shed and disperse within hours of birth, and *C. unicolor* is the only *Crotalus* spp with immediate post-birth dispersal of mothers (see Greene et al., 2002). The general pattern of mothers remaining at the birth site until neonates have completed their shed cycle, regardless of how long it takes, suggests that the delayed dispersal of mothers is not merely a result of the physiological stress of parturition, as suggested by Klauber (1972), and might be under endocrine control.

Recent research on the endocrinology of female reproduction in snakes (reviewed in DeNardo and Taylor, 2011) provides a starting point from which to examine the hormonal mechanisms of maternal attendance in pitvipers. A general pattern of steroid hormone levels during key reproductive events has been documented in females of several New World pitvipers, mostly by opportunistic sampling of free-ranging snakes. In general, estradiol is elevated during vitellogenesis, but low throughout pregnancy and the post-parturient period, while progesterone levels increase during pregnancy, but quickly return to baseline after parturition (reviewed in DeNardo and Taylor, 2011; Smith et al., 2012). Few studies have measured testosterone in females, but slight elevations were seen in *C. atrox* (Taylor et al., 2004) and *C. oreganus* (Lind et al., 2010) during the spring mating season, and testosterone was low or undetectable during gestation and post-parturition in *C. atrox* (Schuett et al., 2004) and *A. contortrix* (Smith et al., 2012). Corticosterone levels appear to be more variable than other steroid hormones, but pregnant *C. atrox* (Taylor et al., 2004) and *C. horridus* (Lutterschmidt et al., 2009) showed significantly higher concentrations than non-reproductive females. The only studies that repeatedly sampled females on a fine temporal scale demonstrated that corticosterone levels remained high throughout gestation, dramatically peaked at parturition, and then quickly returned to pre-parturition levels (Schuett et al., 2004; Smith et al., 2012). However, none of the above studies measured hormones in females while they were attending neonates.

Here, we examine hormonal correlates of maternal attendance behavior in the cottonmouth (*Agkistrodon piscivorus*), a semi-aquatic New World pitviper that is native to the southeastern United States and that is known to exhibit maternal attendance behavior (Walters and Card, 1996; Wharton, 1966). Reproductive female cottonmouths initiate vitellogenesis in late summer to early fall, suspend it over winter, and resume and complete it upon emergence from winter hibernacula in early spring (reviewed in Siegel et al., 2009). In vitellogeneic females, ovulation occurs in late spring, followed by an approximately 90 d gestation with parturition taking place in fall; thus, the reproductive cycle spans an entire year. Due to the large amount of stored energy required for reproduction in viviparous snakes (i.e., capital mode of reproduction; Bonnet et al., 1998) and pregnancy-associated anorexia (Bonnet et al., 1998; Crane and Greene, 2008; Lourdais et al., 2002; Macartney and Gregory, 1988; Webber et al., 2012; but see Schuett et al., 2013), annual reproduction is uncommon in New World pitvipers (reviewed in Klauber, 1972; Siegel and Ford, 1987; Taylor and DeNardo, 2010; but see Schuett et al., 2011). Though no long-term telemetry studies have been conducted on cottonmouths, available evidence suggests that most females reproduce on a less-than-annual basis (Scott et al., 1995; Siegel et al., 2009; Wharton, 1966; Zaidan III et al., 2003).

To determine whether steroid hormones regulate maternal attendance behavior in pitvipers, we conducted a captive study on wild-caught pregnant cottonmouths. Circulating levels of progesterone, estradiol, testosterone, and corticosterone were measured from blood samples that were collected serially from attending females before, during, and after the attendance period. The hormone concentrations of these females were compared to those of females that were not in attendance of neonates, but that had blood samples collected on a similar temporal schedule. We used this sampling scheme in order to disentangle general reproductive hormone profiles from hormonal changes stemming directly from the presence of neonates. If progesterone, estradiol, testosterone, and/or corticosterone regulate attendance behavior in cottonmouths, we predict that the pattern of one or more of these hormones will significantly differ between attending and non-attending females.

2. Methods

2.1. Animal collection and husbandry

A total of 29 pregnant cottonmouths were collected from Alabama (Monroe county) and Georgia (Clayton and Fayette counties), measured (snout-vent length), and transported to San Diego, California, where they were maintained in captivity. Females were col-

lected during two years (2010 and 2011) and captive maintenance differed between years due to animal holding facility limitations. Females collected in 2010 (Y1; $n = 13$) were housed in a temperature- and lighting-controlled (i.e., ~22.7–26.7 °C and 12L:12D cycle) room on the San Diego State University campus. Snakes were kept in individual cages (0.5 m × 0.4 m × 0.2 m) containing a small water dish. Females collected in 2011 (Y2; $n = 16$) were housed at the Sky Oaks Field Station in Warner Springs, California. Snakes were kept, individually, in large enclosures (1.8 m × 1.8 m × 0.9 m) containing a hide box (0.6 m × 0.6 m × 0.6 m) and water dish. These enclosures were inside an insulated warehouse with a concrete floor and skylights, which provided a natural light:dark cycle. Daytime substrate temperatures varied between 20.6 and 26.4 °C.

2.2. Experimental design and blood sample collection

Pregnant females (different individuals each year) were randomly assigned to one of two treatment groups: Maternal Attendance (MA; Y1: $n = 7$; Y2: $n = 7$) or Separated (SE; Y1: $n = 6$; Y2: $n = 9$). Upon parturition, MA females and their neonates were allowed a maternal attendance period, where the litter was left in the mother's cage/enclosure until all neonates in that litter completed ecdysis. Once all neonates of a given litter completed ecdysis, they were removed from the mother's cage/enclosure. Females in the SE group were not allowed a maternal attendance period, but instead were separated from their neonates and associated chemical cues within 24 h of birth. We felt it was important that the SE group not be exposed to residual neonate chemosensory cues, in case those cues affect maternal hormones. We achieved this by either cleaning the maternal cage after neonate removal (Y1) or moving the mother to a new, never-occupied arena (Y2). We chose to move mothers in Y2, because we were concerned that we would not be able to adequately remove all chemical cues due to the porous nature of the arena floors (i.e., concrete); in contrast, Y1 mothers were kept in small plastic cages, which could be adequately cleaned. There was not a significant treatment × year effect for any of the four hormones measured (see Results), which gives us confidence that moving SE females in Y2, but not Y1, was not a significant confounding factor in this study. Although parturition checks were made on a 24-h interval, cottonmouths typically gave birth in the early morning hours (Hoss, pers. obs.); as such, separations likely occurred within 8 h of birth. In Y1, parturition dates ranged from 15 August to 4 September and litters contained 1–5 live neonates (3.15 ± 1.28). In Y2, parturition occurred between 29 August to 17 September and litters contained 2–8 live neonates (4.75 ± 1.44).

A series of five blood samples was collected from all females, the timing of which corresponded to key events related to the maternal attendance period. The first sample, Post-Parturition (PP), was collected within 24 h of parturition, just prior to neonate removal in the SE group. The second sample, Attendance (AT), was collected four days post-parturition; MA mothers were attending neonates, but SE mothers were not. The third sample, Post-Natal Shed (PNS), was collected on the day that all neonates in a given MA litter had completed ecdysis, just prior to separation. Because SE females were not attending neonates, their PNS sample was scheduled to be collected after an equivalent amount of time had passed since collection of the AT sample. The fourth sample, One Week Post-Separation (1 W), was collected one week after the PNS sample; MA mothers had been separated from neonates for one week. The fifth sample, Two Weeks Post-Separation (2 W), was collected two weeks after the PNS sample; MA mothers had been separated from neonates for two weeks. During Y1, we collected additional samples prior to parturition and three and four weeks after the PNS sample. We present these data to show hormonal changes taking place over a longer time period, but because we did not take these additional samples during Y2, we did not include them in analyses. Blood samples (2 ml) were collected via cardiocentesis (within 1–5 min of capture) using a heparinized syringe and centrifuged immediately for 5 min at 9000×*g*. Plasma was separated and stored at −20 °C until assays were conducted.

It is important to note that the length of time it took for all individuals in an MA litter to complete post-natal ecdysis varied greatly between, but not within years (Y1: 8–11 d; mean: 8.67 ± 1.21; Y2: 14–18 d; mean: 16.29 ± 1.60), with Y2 neonates taking twice as long to shed than Y1 neonates. This difference was presumably due to the relatively low humidity that Y2 snakes experienced at the field station. We did take this difference into account when scheduling the PNS blood sample for SE snakes, so the timing of that sample was similar for MA and SE snakes within each year. In other words, the PNS sample for SE snakes was collected approximately 8 d post-parturition in Y1 (8–9 d; mean: 8.00 ± 0.41) and approximately 16 d post-parturition in Y2 (13–18 d; mean: 16.00 ± 2.06).

2.3. Hormone extraction and enzyme immunoassays

Plasma samples were shipped frozen to the University of Alabama and were transferred immediately to −20 °C for storage until extraction and assay. Prior to extraction, samples were thawed overnight at 4 °C and centrifuged at 9000×*g* for 5 min at 4 °C. 100 µl of plasma was pipetted into 18 × 150 mm labeled borosilicate vials on ice followed by the addition of 20 ml ultrapure water. One end of Tygon tubing (Saint-Gobain, formulation 2275), precleaned with ethanol and distilled water, was fastened to a Hypersep C18 column (Fisher Catalog #60108–304) fitted to a 24-port vacuum manifold, and the other end of the tubing was inserted into the diluted plasma sample. C18 columns were pre-primed with 2 × 2 ml washes methanol (MeOH) followed by 2 × 2 ml washes of distilled water; columns were kept wet until the vacuum was engaged, drawing the diluted plasma sample over the C18 column. Salts were purged from the column with 2 ml distilled water. Steroids were eluted from the column with 6 ml MeOH into 13 × 100 mm labeled borosilicate vials. Eluted solvent was stored at 4 °C overnight and then evaporated in a 37 °C water bath under a gentle stream of nitrogen (~7 bar) using an evaporating manifold (Evap-O-Rac, Cole-Parmer), resulting in a residue that was immediately re-suspended in 25 µl ethanol and vortexed for 1 min followed by the addition of 475 µl EIA buffer; EIA buffer was provided with the Cayman Chemicals, Inc. enzyme immunoassay kits. Re-suspended samples were stored at −20 °C for 9 days until the assays were conducted. Samples were thawed and 30 µl from each of the samples was combined to form a pool for purposes of running validations and to serve as intra- and inter-assay controls. This pool was initially diluted 1:5 with EIA buffer. Progesterone (P), estradiol (E2), testosterone (T), and corticosterone (CORT) EIA kits were validated for *A. piscivorous* by serially diluting a pool (1:5 to 1:640 for CORT, T, P; 1:5 to 1:160 for E2) and examining parallelism of the serial dilution curve with the standard curve generated using Cayman Chemicals, Inc. EIA kit standards. All serial dilution curves were parallel to the standard curve (comparison of slopes: Zar, 1996; P: $t_{12} = 0.126$, $p = 0.90$; E2: $t_{10} = 0.278$, $p = 0.79$; T: $t_{12} = 0.145$, $p = 0.89$; CORT: $t_{12} = 0.036$, $p = 0.97$). The serial dilution curves allowed us to identify 1:5 as the most appropriate dilution for P, E2, and T, and 1:75 as the most appropriate dilution for CORT. The CORT dilution was accomplished by mixing 50 µl of 1:5 re-suspended sample with 700 µl EIA buffer followed by vortexing for 1 min; intra- and inter-assay controls (pool) also were diluted 1:75 for the CORT assay. Following validation, samples were stored at 4 °C overnight and all assays (10, 96-well plates for each hormone) were conducted on the same day; the manufacturer's proto-

col was followed strictly. All plates were developed for 75–90 min and were read at 405 nm on a BioTek ELx800 spectrophotometer. Samples were assayed in duplicate on the 10, 96-well plates. Intra-assay coefficients of variation were as follows: P (Median: 5.06%, Range: 1.09–7.76%), E2 (Median: 3.78%, Range: 0.96–10.70%), T (Median: 3.01%, Range: 1.79–9.38%), and CORT (Median: 4.16%, Range: 1.49–5.30%). Inter-assay coefficients of variation were as follows: P (6.81%), E2 (7.82%), T (8.38%), and CORT (7.77%). The detection limits (80% B/Bo) for the Cayman Chemicals, Inc. kits were: T (6 pg/ml); P (10 pg/ml); E2 (20 pg/ml); CORT (30 pg/ml). The standard curves encompassed concentrations between 3.9 and 500 pg/ml (T); 7.8–1000 pg/ml (P); 6.6–4000 pg/ml (E2), and 8.2–5000 pg/ml (CORT). All measured concentrations for each hormone, after dilution, were encompassed by the standard curve.

2.4. Statistical analyses

Snakes varied in snout-vent length (range: 570–731 mm; mean ± standard deviation: 638.17 mm ± 39.08 mm), so to ensure that there was no significant difference in body size between MA and SE females in Y1 or Y2, we ran an analysis of variance on log-transformed snout-vent length (SVL), with treatment, year, and treatment × year as factors. SVL did not differ significantly between treatments ($F_{1,25} = 0.035$, $p = 0.853$), year ($F_{1,25} = 3.094$, $p = 0.091$), or treatment × year ($F_{1,25} = 0.989$, $p = 0.330$), so body size was not incorporated into further analyses. For each hormone, we conducted a mixed model repeated measures analysis using PROC MIXED in SAS 9.3 (SAS Institute Inc., Cary, NC). The main effects were treatment (SE or MA) and year (Y1 or Y2), the random effect was snake ID, the repeated measure was sample (PP, AT, PNS, 1W, 2W), and the dependent variable was the plasma concentration of hormone (P, E2, T, or CORT) for each sample. We also included the following interaction terms: treatment × year, sample × treatment, sample × year, and sample × treatment × year. There was not a significant treatment × year or sample × treatment × year interaction for any hormone, so we excluded those interaction terms from final models. To determine the most appropriate covariance structure, we ran each of the four models with 11 different covariance structures and found that the compound symmetry structure minimized the AIC and BIC values across models; thus, we employed this covariance structure in all models. For significant main effects and interactions, pairwise comparisons were conducted using Fisher's LSD. Because we were interested in how hormone levels changed from one sampling time to the next, we limited pairwise comparisons to four a priori contrasts: (1) PP vs. AT, (2) AT vs. PNS, (3) PNS vs. 1 W, and (4) 1 W vs. 2 W. Hormone data were log transformed to meet the assumption of normality. Significance was assessed at $\alpha = 0.05$.

3. Results

A composite hormone profile encompassing the longest sampling period (i.e., Y1, MA females only) is provided in Fig. 1, but the following results are from analyses on only those samples collected during both years. Results for all repeated measures analyses are presented in Table 1 and means and standard errors of all hormones for females in each treatment group (years combined) in Table 2. In general, post-parturient P levels dropped markedly by the AT sample (4 d post-parturition), and slowly declined throughout the rest of the sampling period (Fig. 2a). This pattern did not differ significantly between MA and SE females (i.e., no significant sample × treatment interaction; Table 1), but the initial drop in mean P (i.e., PP to AT) was more than twice as large in SE females (0.834 ng/ml to 0.281 ng/ml) as it was in MA females (0.583 ng/ml to 0.316 ng/ml). There was a significant sample × year interaction for P, resulting from Y1 females having significantly higher P than Y2 females during the AT ($p < 0.001$) and PNS ($p = 0.002$) samples, but not the PP ($p = 0.169$), 1 W ($p = 0.153$), or 2 W ($p = 0.484$) samples. This indicates that, although females in both years had similar P levels at the start of sampling, Y2 females showed a stronger post-parturient drop in

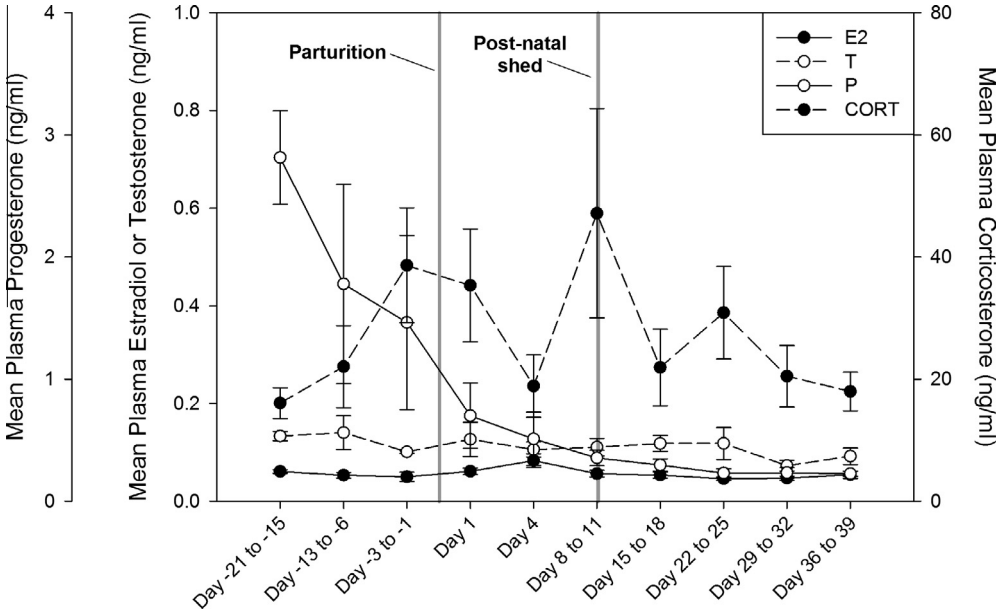

Fig. 1. Composite hormone profile of captive cottonmouths prior to parturition, during maternal attendance, and after neonate removal. Data are mean ± standard error plasma hormone levels for females sampled during 2010 (MA treatment group only; $n = 7$). Labels on the *x*-axis represent how many days before (negative values) or after (positive values) parturition samples were taken (Note: exact parturition times were not known, so Day 1 samples were taken up to 24 h after parturition). Reference lines indicate when parturition occurred (i.e., Day 0) and the day that all neonates in a given litter had completed ecdysis and were removed from the mother (i.e., Day 8 to 11). Thus, mothers were in attendance of young for all samples taken between Day 1 and Day 8 to 11, but were no longer in attendance for the remainder of samples.

Table 1
Results of repeated measures analyses on mean plasma hormone levels of female cottonmouths. Degrees of freedom (*df*) were the same for all analyses. Significant *p*-values are in bold.

		Progesterone		Estradiol		Testosterone		Corticosterone	
Model parameters	df	*F*-value	*p*-value	*F*-value	*p*-value	*F*-value	*p*-value	*F*-value	*p*-value
Treatment	1,26	1.12	0.301	1.32	0.260	0.03	0.566	0.59	0.451
Year	1,26	13.64	**0.001**	4.57	**0.042**	80.74	**<0.001**	4.05	0.055
Sample	4,104	21.54	**<0.001**	2.89	**0.026**	2.49	**0.048**	2.34	0.060
Sample * treatment	4,104	2.09	0.087	1.59	0.182	1.60	0.181	2.55	**0.043**
Sample * year	4,104	3.11	**0.019**	1.27	0.286	0.05	0.995	4.44	**0.002**

Table 2
Mean ± standard error of plasma hormone levels in adult female cottonmouths taken at five time points. The timing of samples was as follows: PP – within 24 h of parturition; AT – 4 d after parturition; PNS – the day that all neonates in a litter completed post-natal ecdysis; 1 W – one week after PNS sample; 2 W – two weeks after PNS sample. Females in the SE treatment group ($n = 15$) had their litter permanently removed immediately after the PP sample was collected. Females in the MA group ($n = 14$) did not have their litter removed until after the PNS sample was collected.

Sample	Progesterone (ng/ml)		Estradiol (ng/ml)		Testosterone (ng/ml)		Corticosterone (ng/ml)	
ID	MA	SE	MA	SE	MA	SE	MA	SE
PP	0.583 ± 0.163	0.834 ± 0.128	0.065 ± 0.004	0.052 ± 0.006	0.092 ± 0.023	0.065 ± 0.012	26.05 ± 7.40	34.89 ± 5.01
AT	0.317 ± 0.119	0.282 ± 0.058	0.068 ± 0.008	0.052 ± 0.004	0.069 ± 0.013	0.064 ± 0.008	16.48 ± 3.22	18.33 ± 2.74
PNS	0.276 ± 0.047	0.260 ± 0.039	0.055 ± 0.005	0.048 ± 0.005	0.081 ± 0.014	0.087 ± 0.020	32.31 ± 10.36	19.12 ± 2.99
1W	0.242 ± 0.037	0.216 ± 0.025	0.051 ± 0.004	0.054 ± 0.004	0.079 ± 0.014	0.055 ± 0.008	23.46 ± 3.86	21.05 ± 3.91
2W	0.195 ± 0.019	0.178 ± 0.024	0.048 ± 0.003	0.047 ± 0.003	0.080 ± 0.022	0.050 ± 0.009	35.51 ± 5.66	30.38 ± 4.50

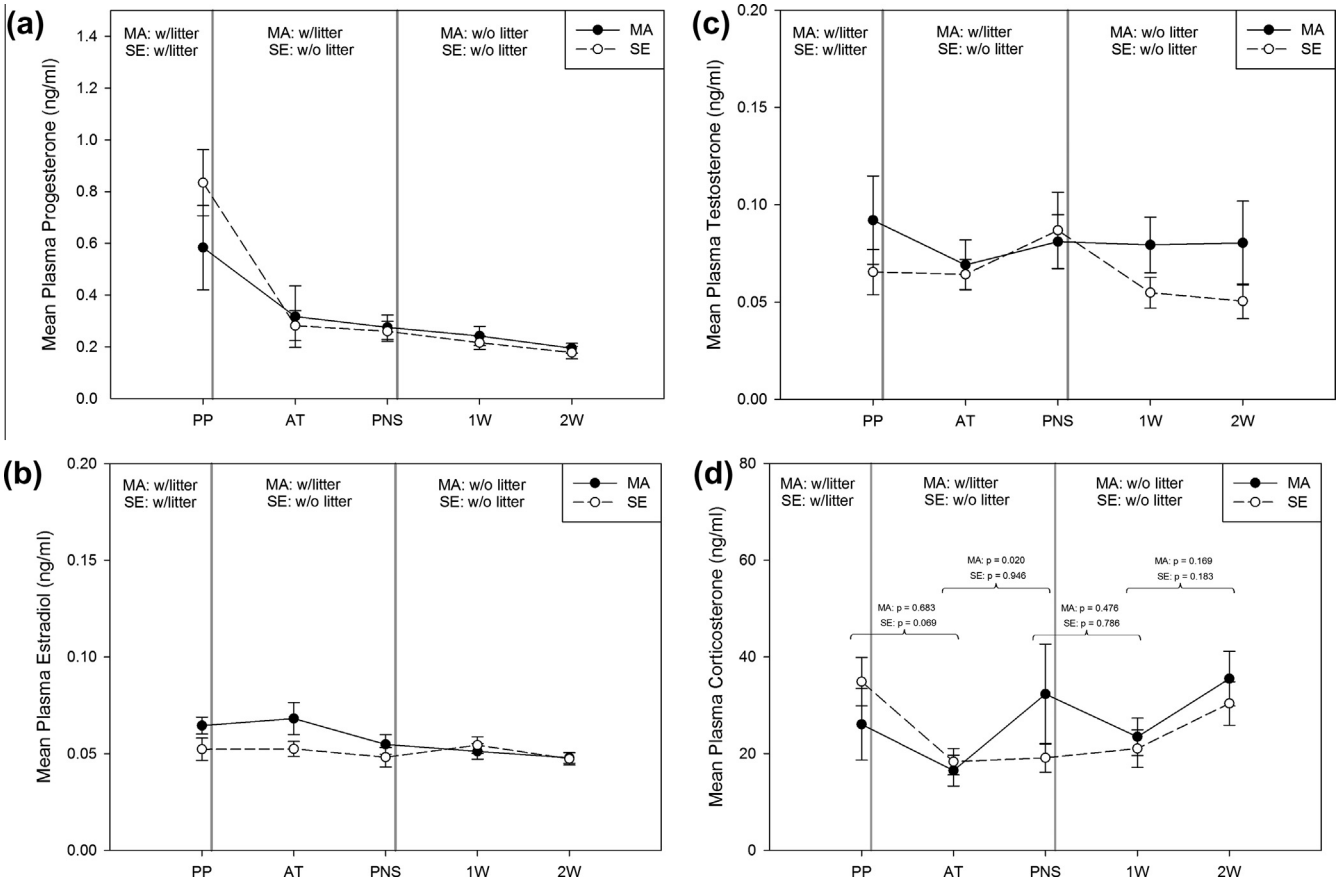

Fig. 2. Mean ± standard error of plasma progesterone (a), estradiol (b), testosterone (c), and corticosterone (d) of adult female cottonmouths taken at five time points during 2010 and 2011. The timing of samples was as follows: PP – within 24 h of parturition; AT – 4 d after parturition; PNS – the day that all neonates in a litter completed post-natal ecdysis; 1 W – one week after PNS sample; 2 W – two weeks after PNS sample. Females in the SE treatment group ($n = 15$) had their litter permanently removed immediately after the PP sample was collected. Females in the MA group ($n = 14$) did not have their litter removed until after the PNS sample was collected. Vertical reference lines indicate the point at which litters were removed from MA or SE females and, thus, whether females were with or without their litter when a sample was collected. Corticosterone (d) was the only hormone to exhibit a significant treatment × sample interaction, so p-values associated with the four a priori pairwise comparisons of consecutive samples (i.e., PP vs. AT, AT vs. PNS, PNS vs. 1 W, 1 W vs. 2 W) are provided, with significant *p*-values in bold.

P than Y1 females. Regardless, during both years, females showed a significant decrease in P between the PP and AT samples (Y1: $p = 0.007$; Y2: $p < 0.001$), but no significant changes between the AT and PNS (Y1: $p = 0.636$; Y2: $p = 0.248$), PNS and 1 W (Y1: $p = 0.112$; Y2: $p = 0.819$), or 1 W and 2 W (Y1: $p = 0.229$; Y2: $p = 0.644$) samples.

Estradiol levels changed very little throughout the sampling period (Fig. 2b), and there was not a significant sample × treatment or sample × year interaction (Table 1). When treatment groups were combined (i.e., main effect of sample), females showed a significant decrease in E2 between the AT and PNS samples ($p = 0.021$), but no significant changes between the PP and AT ($p = 0.673$), PNS and 1 W ($p = 0.372$), or 1 W and 2 W ($p = 0.177$) samples. The main effect of year was significant, with Y1 females having E2 levels that were significantly higher than Y2 females ($p = 0.042$). Similar to E2, T levels did not show large changes between sampling periods (Fig. 2c), and there was not a significant sample × treatment or sample × year interaction (Table 1). There was a significant main effect of sample, where females showed a marginally significant increase in T between the AT and PNS samples ($p = 0.052$), a significant decrease between the PNS and 1 W samples ($p = 0.049$), and no significant change between the PP and AT ($p = 0.230$) or 1 W and 2 W ($p = 0.416$) samples. Although the repeated measures analysis failed to find a significant sample × treatment interaction, the significant increase and subsequent decrease in T during the middle of the sampling period (i.e., AT to PNS to 1 W) was mostly attributable to SE females (see Fig. 2c). The main effect of year was significant, with Y1 females exhibiting higher levels of T than Y2 females ($p < 0.001$).

Females in both treatment groups showed a general pattern of a post-parturient decline in CORT, with levels recovering by the final sampling point (Fig. 2d), but there was a significant sample × treatment effect (Table 1). This treatment effect was mostly due to a significant increase in CORT between the AT and PNS samples in mothers attending neonates ($p = 0.020$) that was not observed in non-attending mothers ($p = 0.946$). Also, though the initial drop in CORT between the PP and AT samples was not significant for either treatment group (MA: $p = 0.683$; SE: $p = 0.069$), the difference in mean levels between the two samples in MA females (9.57 ng/ml) was almost half that of SE females (16.56 ng/ml), indicating that CORT did not drop as drastically in mothers attending neonates. Corticosterone levels did not change significantly between the final three sampling points in MA (PNS vs. 1 W: $p = 0.476$; 1 W vs. 2 W: $p = 0.169$) or SE (PNS vs. 1 W: $p = 0.786$; 1 W vs. 2 W: $p = 0.183$) females. There was a significant sample × year interaction, resulting from Y1 females (treatment groups combined) having significantly higher CORT levels for the PP ($p < 0.001$) and AT ($p = 0.038$) samples, but not the PNS ($p = 0.207$), 1 W ($p = 0.352$) or 2 W ($p = 0.194$) samples.

4. Discussion

4.1. General hormone patterns

Female cottonmouths showed a marked drop in progesterone (P) four days after parturition, with a steady, but very small decline over the following weeks. This pattern is similar to what has been shown in other pitviper species (*Agkistrodon contortrix*: Smith et al., 2012; *Crotalus atrox*: Schuett et al., 2004; Taylor et al., 2004; *C. durissus*: Almeida-Santos et al., 2004), as well as distantly related viviparous snakes (Bonnet et al., 2001; Gorman et al., 1981; Tsai and Tu, 2001). Progesterone is secreted by the corpora lutea of the ovaries and is associated with the maintenance of pregnancy through its effect on oviductal vascularity (Callard et al., 1992; Custodia-Lora and Callard, 2002; Mead et al., 1981). There is limited evidence that the characteristic pre-parturient decline in P observed in reptiles might be required for parturition to occur (e.g., Guillette et al., 1991; but see Bonnet et al., 2001). Although the sampling period analyzed in the current study began after parturition, we did measure hormones in some females (Y1) up to 21 d prior to parturition. Earlier in pregnancy, P levels were sometimes five times higher than those observed within 24 h after parturition (4.9 ng/ml vs. <1 ng/ml), and similarly elevated P levels in pregnant females have been documented in free-ranging cottonmouths (Graham et al., 2011).

Females in this study exhibited very low estradiol (E2) levels (<0.1 ng/ml) that did not vary substantially throughout the sampling period. There was a significant decline in E2 between the AT and PNS samples (i.e., 4 d to ~8–16 d post-parturition), but it is difficult to say whether a difference of ~0.008 ng/ml is biologically meaningful. The low levels of E2 were expected, as E2 promotes vitellogenin production by the liver (Callard et al., 1990; Ho et al., 1982) and studies of other pitvipers have found E2 to be elevated only during vitellogenesis (reviewed in DeNardo and Taylor, 2011). Cottonmouths appear to follow the 'type 2' pattern of vitellogenesis (Aldridge, 1979), where it is initiated in the fall, suspended during winter, and resumed and completed the following spring (Siegel et al., 2009). Because cottonmouths are purported to reproduce on a less-than-annual cycle (Scott et al., 1995; Wharton, 1966; Zaidan III et al., 2003), post-parturient females most likely delay vitellogenesis until the subsequent fall (Siegel et al., 2009), and thus, should not have elevated E2. Our finding that E2 remained low up to 40 d after parturition supports the hypothesis of delayed vitellogenesis in post-parturient cottonmouths. Similar to E2, T remained low throughout the study, and the statistically significant difference between the PNS and 1 W samples was very small (~0.02 ng/ml). These low T levels correspond to the results of Smith et al. (2012), in which copperheads (*A. contortix*) – the sister taxon to cottonmouths – had undetectable T levels during the two weeks following parturition. Few other studies have measured T in female pitvipers, and all of them sampled T on a monthly basis; T was slightly elevated only during the mating season in reproductive females (Lind et al., 2010; Taylor et al., 2004). The exact role of elevated T in female snakes is unknown, but hypothesized to function in E2 synthesis (i.e., T is a precursor to E; Staub and De Beer, 1997) and/or mating behavior (reviewed in DeNardo and Taylor, 2011).

Corticosterone (CORT) levels rose during late gestation, peaked prior to parturition, then declined over the weeks following parturition (see below for discussion of CORT peak unique to MA females). This same pattern was exhibited by *A. contortrix* (Smith et al., 2012) and *C. atrox* (Schuett et al., 2004), and included the parturition-related 3–4-fold increase in CORT observed in this study. Other studies of pitvipers found CORT to be elevated in pregnant females relative to those that were non-reproductive or post-parturient (Lutterschmidt et al., 2009; Taylor et al., 2004). The functions of CORT in reptilian gestation and parturition are not known, but they are likely similar to those documented in mammals, such as mobilizing the large amount of energy required during this stage of reproduction, suppressing the mother's immune reaction to fetuses, and controlling the timing of parturition (reviewed in Brunton et al., 2008). Also, it is important to note that the high circulating levels of CORT experienced by females during late gestation and parturition might be a result of additional CORT being secreted from the placentae and fetuses (Wada, 2008; Weiss, 2000).

4.2. Hormone changes associated with maternal attendance of young

Mother cottonmouths attending young (MA group) did not show significantly different patterns of P relative to mothers

deprived of young (SE group). There was a tendency for MA females to show a smaller drop in P between the first two samples (i.e., PP and AT), but this might have been a result of SE females having higher P levels when the PP sample was taken, due to random factors. In other words, both groups had been treated identically when the PP sample was taken, so treatment group cannot account for the difference in P levels in that first sample. The cottonmouths gave birth at various times throughout the night, but the PP sample always was taken the following morning; thus, if P was rapidly dropping after parturition and SE females happened to give birth closer to when the PP sample was taken, this would explain the higher P levels in the SE group. Levels of P in the AT sample were similar in both groups, suggesting that P levels drop to baseline within 4 days of parturition.

The pattern of P in post-parturient cottonmouths corresponds to what has been observed in species with obligate parental care (i.e., birds and mammals). In general, elevated P prior to oviposition or parturition with a subsequent decline is required for the onset of maternal behaviors, but continued parental care requires somatosensory input from the young and is disrupted if P is artificially elevated during this time (reviewed in Bridges, 1996; Buntin, 1996; Gonźalez-Mariscal and Poindron, 2002). However, viviparous snakes that do not exhibit parental behavior show the same rise and fall of P during gestation and parturition (Bonnet et al., 2001; Chan et al., 1973; Gorman et al., 1981; Highfill and Mead, 1975; Tsai and Tu, 2001), so it was not surprising that the pattern of P in SE females did not significantly differ from that of MA females. There are no comparable studies investigating the effects of the presence of young on maternal P levels in other species that exhibit relatively simple forms of parental behavior, but the results of our study suggest that P does not play a unique role in this particular system.

The presence of young did not significantly affect patterns of E2 or T in female cottonmouths, and both of these hormones were very low throughout the post-partum period. Similar to P, the other gonadal hormones (e.g., E2 and T) typically are low during parental care in birds (reviewed in Buntin, 1996) and mammals (reviewed in Bridges, 1996; Gonźalez-Mariscal and Poindron, 2002). This pattern is thought to reflect the general incompatibility of sexual behavior – governed by gonadal hormones – with parental behavior, and consequently, one of the primary costs of parental care in most organisms (Stearns, 1992; Trivers, 1972; Williams, 1966; but see Stiver and Alonso, 2009). For example, numerous studies have shown that male birds with artificially elevated T allocated more time to sexual behaviors than parental behaviors, but males with low T showed the opposite behavioral pattern (De Ridder et al., 2000; Hegner and Wingfield, 1987; Ketterson et al., 1992). Much less is known about the effects of T or E2 on female parental behavior in birds, and while some species show a negative effect of E2 (Lehrman, 1958), others show no effect of artificially elevated E2 (Hunt and Wingfield, 2004; Wingfield et al., 1989) or T (Clotfelter et al., 2004). However, there are many avian studies that have shown large increases in maternal gonadal hormones after the removal of eggs or chicks, and a consequent reinstatement of sexual behavior (reviewed in Buntin, 1996). We did not find a similar effect of neonate removal on E2 or T levels in female cottonmouths, which can be explained by the low frequency of reproduction in cottonmouths. Post-parturient cottonmouths are unlikely to initiate vitellogenesis and become sexually receptive until the year following parturition, and indeed, this lack of a trade-off between sexual and parental behavior in pitvipers could have been a factor in the evolution of maternal attendance of young in this group.

Corticosterone was the only hormone that was significantly affected by the presence of neonates in this study, as indicated by a significant treatment × sample interaction. Both MA and SE mothers exhibited a post-parturient drop in CORT, with levels returning to those of the initial sample over the following weeks. However, while SE females showed a gradual increase in CORT throughout the post-parturient period, MA females exhibited a significant transient peak in CORT on the day that neonates completed ecdysis – the point at which free-ranging pitvipers cease attendance behavior and disperse from the birth site. It is important to note that this difference was significant despite the presence of a significant year effect on CORT levels (i.e., Y1 females generally exhibited higher CORT levels than Y2 females throughout the study, but the CORT peak in MA females coincident with neonatal ecdysis was observed in both years). High levels of glucocorticoids have been linked to nest abandonment in fish (e.g., O'Connor et al., 2009) and birds (e.g., Love et al., 2004), and reductions in parental behavior in mammals (e.g., Saltzman and Abbott, 2009). This is presumed to be a result of high glucocorticoids, resulting from acute or chronic stress, signaling the parent to invest more in future reproduction (e.g., self-maintenance) than current reproduction (e.g., offspring care) (Angelier and Chastel, 2009; Sapolsky et al., 2000; Wingfield and Sapolsky, 2003). Supporting this hypothesis is evidence that elevated CORT is associated with increased appetite and foraging behavior in birds (Landys et al., 2006; Sapolsky et al., 2000; Wingfield et al., 1998). Our finding that CORT was elevated significantly in attending females around the time that free-ranging pitvipers terminate neonate attendance (i.e., when neonates complete ecdysis), suggests that CORT might be an important mediator of the shift from attendance to post-attendance behaviors in pitvipers.

Because female cottonmouths (or other pitvipers) do not provision their young and there is no evident reason for attending females to be subject to higher stress than non-attending females, at least in a captive setting, the elevated CORT observed in MA females was likely caused by changes in neonate behavior and/or chemical cues. For example, snakes typically are sedentary in the early stages of ecdysis (King and Turmo, 1997), but must increase activity to remove the shed from the new epidermis. In our study, we witnessed neonates using the mother's body to aid in the shedding process (i.e., rubbing against the mother helped peel back the shed), and neonates became very active once they successfully removed the shed. This large increase in neonate movement and tactile stimulation might have activated the hypothalamic–pituitary–adrenal axis of mothers, resulting in elevated CORT. Alternatively, if maternal CORT levels are modulated by neonate olfactory cues, as they are in rats (Zarrow et al., 1972), then changes in the chemical signatures of neonates as they complete the shed cycle could elevate maternal CORT to a point that it signals mothers to terminate attendance behavior. Of course, changes in neonate activity and chemical cues could act synergistically on maternal CORT, with the ultimate result being maternal dispersal from the birth site.

Overall, we did not find evidence that neonate presence affected maternal P, E2, T, or CORT soon after parturition (i.e., MA and SE females had comparable levels 4 d post-parturition), so it is unlikely that these hormones mediate the maintenance of attendance behavior (i.e., delayed dispersal). This is similar to what has been found in species with obligate parental care, where the onset of parental behavior requires the normal hormonal patterns of reproduction (which we observed in the cottonmouths), but its maintenance depends upon continued somatosensory input from the offspring (reviewed in Bridges, 1996; Buntin, 1996; Gonźalez-Mariscal and Poindron, 2002). We did not measure prolactin (PRL) in attending cottonmouths, but this peptide hormone has been widely implicated in the control of maternal care in mammals (reviewed in Gonźalez-Mariscal and Poindron, 2002) and birds (reviewed in Buntin, 1996). Currently, there are no bioassays available to detect plasma PRL levels in non-avian reptiles, but these

species show remarkable similarities to birds in the molecular structure of PRL and its receptors (Kato et al., 2005; Noso et al., 1992), suggesting similarities in PRL function.

The results of this study provide a much-needed foundation to test further hypotheses concerning the physiological mechanisms mediating maternal attendance of young in pitvipers and other rudimentary forms of parental behavior exhibited by ectothermic vertebrates. For example, if the presence of offspring mediates the maintenance of maternal attendance behavior in pitvipers, we would predict that females deprived of their litter would disperse from the birth site soon after parturition, and if CORT mediates the termination of maternal attendance behavior, we would predict that mothers given exogenous CORT in the middle of attendance would disperse from the birth site prematurely. In light of our results, we suggest that future studies of rudimentary parental behavior focus on the importance of somatosensory input from the offspring to its maintenance, the role of CORT in stimulating its termination, and how PRL functions in these systems.

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