



## Innate immune performance and steroid hormone profiles of pregnant versus nonpregnant cottonmouth snakes (*Agkistrodon piscivorus*)

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### ABSTRACT

Squamates (lizards and snakes) have independently evolved viviparity over 100 times, and exhibit a wide range of maternal investment in developing embryos from the extremes of lecithotrophic oviparity to matrotrophic viviparity. This group therefore provides excellent comparative opportunities for studying endocrine and immune involvement during pregnancy, and their possible interactions. We studied the cottonmouth (*Agkistrodon piscivorus*), since they exhibit limited placentation (e.g., ovoviviparity), allowing comparison with squamate species hypothesized to require considerable maternal immune modulation due to the presence of a more extensive placental connection. Furthermore, the cottonmouth's biennial reproductive cycle provides an opportunity for simultaneously comparing pregnant and non-pregnant females in the wild. We document significantly elevated concentrations of progesterone (P4) and significantly lower concentrations of estradiol (E2) in pregnant females relative to non-pregnant females. Pregnant females had lower plasma bacteria lysis capacity relative to non-pregnant females. This functional measure of innate immunity is a proxy for complement performance, and we also determined significant correlations between P4 and decreased complement performance in pregnant females. These findings are consistent with studies that have determined P4's role in complement modulation during pregnancy in mammals, and thus this study joins a growing number of studies that have demonstrated convergent and/or conserved physiological mechanisms regulating viviparous reproduction in vertebrates.

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### 1. Introduction

Viviparity has evolved numerous times among vertebrates, being found in such diverse clades as sharks, teleosts, amphibians, squamates (lizards and snakes), and mammals. The exact mechanism by which the developing embryo is maintained by the mother differs subtly among these groups [54,7,8], and presumably the conditions that lead to these convergent evolutionary developments also differed [47,59,54,46,56]. Interestingly, physiological systems that support pregnancy often are similar among unrelated groups [54]. For example, the pattern of luteal hormone secretion (increased progesterone) during pregnancy is similar in squamates and mammals [48,12]. This observation suggests that a convergent, physiologically parsimonious pathway has evolved repeatedly during the development of this complex and demonstrably adaptive reproductive strategy [48,12,54,46].

For a developing embryo to develop in the presence of an active maternal immune system, important immunomodulatory mechanisms must be activated to avoid rejection of the fetus. Immune-

privileged sites are those that have a decreased or heavily regulated capacity for certain immune responses, such as inflammation, and develop around key sites that could be damaged irreparably by the body's own immune system during infection or autoimmune episodes (e.g., the brain; see [26]). The fetoplacental interface is another known site of immune privilege, and fetal antigens would be recognized by the mother as allogenic (non-self) and attacked if the developing fetus was not protected from the maternal immune system [3]. Several adaptations have developed in mammals to mitigate possible damage to the fetus, including reduced major histocompatibility complex (MHC) expression and antigen presentation at the fetoplacental interface, active destruction of maternal T cells at the fetoplacental interface, and expression of complement-inactivating molecules by the fetus [35,3,29,16]. Comparative studies have explored changes in maternal immunity during pregnancy in viviparous fish [25], salamanders [2], and lizards [43], and provide support for the prediction that modifications of the maternal immune system are associated with, and are possibly a prerequisite for, the evolution of extensive placentation throughout vertebrates [38,42,25].

Alteration of the immune system during pregnancy is in part modulated by reproductive hormones. Progesterone (P4) is recognized as a key player in this regulation, and P4 is associated with

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altered B cell immunoglobulin secretion, inhibition of natural killer cells, and a general shift from T-helper cell 1 (Th-1; pro-inflammatory) to Th-2 (largely antibody mediated, and non-inflammatory) dominated immune responses in pregnant female mammals [16]. Because innate immune components are potent and nonspecific, they have been increasingly recognized for their role in fetal rejection, and current research confirms that improperly regulated inflammation and complement activity can be responsible for spontaneous abortions [14,29]. P4 has immunomodulatory effects on complement, and has been linked to decreased mRNA transcription of complement protein components [9].

Squamates have evolved viviparity independently over 100 times [6,7], and are therefore excellent subjects for comparative reproduction studies [8]. Squamates exhibit a variety of mechanisms that support the developing fetus, from lecithotrophic oviparity, to extensive placentation similar to the pattern exhibited by eutherian mammals (e.g., matrotrophic viviparity [8]). Maternal contribution to the developing fetus (i.e., matrotrophy) is present in all viviparous squamates [7,8], however, the degree of involvement varies greatly. The patterns of viviparity among squamates have been studied in an attempt to reconstruct the evolutionary transition from oviparity to viviparity [7,8]. Investigations of immune involvement in these species may reveal similar transitional patterns and may illuminate evolutionary steps required for extensive placentation to occur in the presence of an active maternal immune system.

For the present study, we chose the cottonmouth (*Agkistrodon piscivorus*), a viviparous snake, as a study animal for several practical reasons. Cottonmouths are large-bodied, facilitating experimentation and sample collection. Much is known about their reproduction (e.g., [22]), including a detailed study of fetal-maternal respiratory dynamics, which determined that placentation is not extensive in this species [4], allowing a comparison between this species and other squamates which have more extensive placentation. Cottonmouths exhibit a biennial reproductive cycle [57] such that in any given year both pregnant and non-pregnant females are available for study at the same time, and confounding seasonal effects thus can be eliminated. We hypothesized steroid hormone immune modulation in pregnant female cottonmouths. Consistent with this hypothesis, we predicted innate immune (complement) performance would differ between pregnant and non-pregnant female cottonmouths, and these differences would correlate with steroid hormones. Specifically, we predicted lower complement bacterial lysis capacity in pregnant cottonmouths versus non-pregnant cottonmouths, and expected hormone profiles in these snakes (e.g., high P4 relative to estradiol in pregnant snakes; increased corticosterone in pregnant females) to be correlated with this innate immune measure. This study is the first of its kind in a squamate with limited lecithotrophic placentation (e.g., 'ovoviviparity').

## 2. Materials and methods

### 2.1. Study animal

The cottonmouth is a heavy-bodied, semi-aquatic pitviper found throughout the southeastern United States of America (USA), sometimes in large population densities [21]. Snakes were collected from a field site in Georgia, USA (see [22] for details). Females exhibit type II vitellogenesis, which is initiated during late summer/fall of the year preceding pregnancy, interrupted by winter, and completed in spring [1]. Only females with enough stored energy initiate follicular yolkling, and therefore only a subset of females is pregnant in any given year [57]. This delay of reproduction to the same initiation point each year results in some females

undergoing pregnancy and parturition at the same time of year that other females are initiating vitellogenesis for the following year's reproduction (e.g., a biennial pattern). Pregnancy lasts from May–June (ovulation) until late August to early September [10,57].

Pregnant animals are easy to recognize morphologically, and embryos can be detected by palpation [17,40]. Pregnant females also are easy to locate because they choose distinct, open microclimates and exhibit gestational thermophily [15]. No detailed study of the degree of matrotrophy exists for this species, although a study of fetal-maternal respiratory dynamics suggested less transfer compared to another snake species known to have more extensive placentation (e.g., *Thamnophis sirtalis*; [4]).

### 2.2. Blood sampling

Seven pregnant and 10 non-pregnant females were sampled August–September 2008. These snakes were captured in the field with tongs, and the head and neck of the snakes were coaxed into a clear plastic tube so that the anterior part of the body could be restrained within the tube while the rest of the body was available for manipulation. We used heparinized, 26 gauge needles to collect blood (within approximately 2 min) from the caudal sinus, and this was pipetted into a sterile, labelled 1.5 ml centrifuge tube. The total amount of time required to obtain a blood sample from the moment a snake is noticed in the field to the point of capture, restraint, placement in secure tube, and total blood collection varies from approximately 3–10 min; unfortunately, the exact amount of time required for each of these processes was not recorded. We separated plasma using a battery operated centrifuge, and equal volumes were aliquoted into two new, sterile, labeled 1.5 ml centrifuge tubes before being fast frozen on dry ice. These samples were transported to the laboratory for storage (–80 °C) until immune (4 days later) or hormone assays (fall 2008) were performed. This sample collection protocol has been used in other studies of snake reproductive endocrinology [51,44,22]. We transported snakes to the lab in a secure transport device [5] in an air-conditioned vehicle. Pregnant females and non-pregnant females were housed in separate sweater-box shelving units at 25 °C (local light:dark light cycle) on clean newsprint substrate. Water was provided continuously and snakes were offered a 100 g mouse every 2 weeks while in captivity. Snakes were kept until parturition to confirm that the snakes were pregnant at the time of blood collection. We then transported the snakes to the study site and released them at their exact capture location after they received unique marks (scale clip).

### 2.3. Bacteria lysis assay

Complement is a potent innate immune component of vertebrate plasma, which can directly adhere to pathogen cell membranes and lyse them [19]. Studies indicate that plasma can be frozen for one to two weeks and maintain its complement viability [45]. Therefore, a functional bacteria lysis assay of field-collected plasma samples (following [53,33]) was employed 4 days after blood collection to determine complement efficiency. This assay was developed for immune studies in birds and has become common in comparative immunological research because it requires no species-specific reagents [53,33]. The assay measures the ability of a plasma sample to lyse bacteria by comparing the number of bacteria colonies resulting from control bacteria solutions (no plasma added) to those spiked with plasma. Bacteria lysis is calculated as the percentage of bacteria lysed compared to controls, with fewer numbers of bacteria colonies indicating higher percentage lysis. Plasma samples were diluted (1:100; 6 µl plasma + 994 µl CO<sub>2</sub> L-glutamine growth medium) based upon a previous study that determined this as an appropriate dilution (i.e., a dilution allowing

detection of variation and comparison among individuals) for this species [Graham et al., in review]. A previous study also determined this assay primarily assesses complement performance; alligator and cottonmouth plasma samples treated at 56 °C, a standard complement-inactivation temperature, lose their bactericidal activity completely ([32], Graham et al., in review). However, others have used this method as a proxy for constitutive innate immunity in birds and snakes [53,33,49], and thus other innate immune components may have contributed to bacteria lysis as well.

Commercially available *Escherichia coli* pellets (Microbiologics Inc., Saint Cloud, Minnesota) were diluted (1:1000) with phosphate buffered saline (PBS) and incubated at 37 °C for 1 h. Test solutions combined 10 µl of the bacteria dilution with 140 µl CO<sub>2</sub> L-glutamine growth medium (control), or 140 µl of the 1:100 plasma + growth medium dilution (plasma treated). These samples were then allowed to react at room temperature simultaneously for 1 h. Fifty microliters of each dilution (sample or control) was then spread in duplicate on agar plates and incubated at 37 °C for 12–16 h. Colonies on each plate were counted, averaged (across duplicates), and compared to the mean colony counts of four replicated, unchallenged negative control plates (200 CFUs). No obvious contamination was present in agar plates during this experiment. Percent bacterial killing was calculated as [(plasma treatment colony counts)/mean control colony counts] × 100.

#### 2.4. Hormone assays

Steroid hormones were extracted from plasma using a column extraction protocol [58,28]. Extraction using this method yields results similar to ether extraction methodologies [58]. Plasma progesterone (P4), corticosterone (CORT), and estradiol (E2) concentrations were measured using EIA kits (Cayman Chemicals, Ann Arbor, MI). Samples were processed and analyzed as in Graham et al. [22], Wong et al. [58], Hoss et al. [28]. In brief, 200–350 µl plasma was transferred into sterile, labeled 18 × 150 mm borosilicate vials, and diluted with 1000 µl ultrapure water. Sep-Pak® Plus C18 columns (500 mg, 4.0 ml; Waters, USA) were primed with two washes of HPLC-grade methanol (MeOH), followed by two washes of ultrapure water. Water-dilute samples were then transferred through Tygon® tubing (Saint-Gobain, formulation 2275, ID = 1/16, OD = 3/16, Wall = 1/16) and the columns using a vacuum manifold. Steroids were eluted with two additional 2 ml MeOH washes from the columns into sterile, labeled 12 × 75 mm borosilicate vials, placed in a water bath at 37 °C, and MeOH was evaporated under a gentle nitrogen stream (~0.7 bar).

Samples were then resuspended to an initial 1:2 dilution using EIA buffer provided in the EIA kits (Cayman Chemicals, Ann Arbor, MI), by adding 400 µl EIA buffer to each original 200 µl sample, and 700 µl buffer to each 350 µl sample. Plasma P4, CORT, and E2 concentrations were measured using EIA kits following the instructions in the kits as in Graham et al. [22].

We validated P4 and E2 kits for *A. piscivorus* plasma by assessing parallelism and quantitative recovery. To assess parallelism, ten 200 µl *A. piscivorus* plasma samples not used in this study were pooled and extracted as in above, and serially diluted from 1:1 to 1:64 in EIA buffer. The dilution curve was log-logit transformed, and compared to the standard curve developed from standards provided in the kit. The slopes of the P4 and E2 standards (provided with the kits) and pooled sample serial dilutions were statistically indistinguishable (slope comparison test [24]; P4:  $t_{12} = 0.27$ ;  $p = 0.79$ , E2:  $t_{10} = 0.03$ ;  $p = 0.97$ ). Thus, parallelism was achieved, and the kits therefore measure cottonmouth hormones in an identical fashion as they measure standards provided in the kits. We previously validated a kit's ability to measure *A. piscivorus* CORT using identical methods [22]. Recovery of P4 and E2 was estimated by spiking equal amounts of the pooled sample with an equal vol-

ume of the kit standards. Recovery was based on the known P4/E2 concentrations present in the sample. Regression slopes (expected vs. observed concentrations) were close to 1 for both P4 and E2, suggesting excellent recovery. Minimum estimated recovery was 91.7% (range 91.7–115%; mean ± SEM: 104 ± 3.5%) for E2 and 72.7% (range: 72.7–114%; mean ± SEM: 98.8 ± 6.3%) for P4. CORT recovery was assessed previously in this snake using identical methods [22]. Intrassay coefficients of variation were 3% for CORT, 2% for E2, and 1.9% for P4. For this study, CORT, E2, and P4 samples were run on the same EIA plate and therefore it was not necessary to calculate inter-assay coefficient of variation.

#### 2.5. Data analysis

Plasma hormone results were log transformed and plasma percent bacteria lysis data were angular transformed to meet assumptions of normality and continuousness. Angular transformation involves arcsine transformation of the square root of proportion values, enabling utilization of percent values for parametric statistics [23]. However, untransformed data are presented in figures to assist interpretation. Mean log P4, E2, and CORT were compared between pregnant and non-pregnant groups using ANOVA. Mean angular transformed percent bacteria lysis was similarly compared between the same treatment groups. To eliminate body size as a confounding factor, we compared snout-vent-length (SVL) between pregnant and non-pregnant females. We chose SVL as a measure of size since determining body condition in pregnant females (e.g., mass, SVL/mass, or residual body mass calculations) would be highly inaccurate; a significant and unknown proportion of each female's mass during pregnancy is made up of developing embryos. SVL was not significantly different between groups, and SVL did not correlate with any of the response variables. The relationship between mean log plasma P4, E2, and CORT and mean angular transformed percent bacteria lysis was determined by combining pregnant and nonpregnant females into one group using linear regression. All analyses were conducted using Stat-View with  $\alpha = 0.05$ .

### 3. Results

Angular transformed mean percent bacteria lysis differed significantly between groups, with pregnant females demonstrating reduced capacity for bacteria lysis ( $F_{1,15} = 5.27$ ,  $p = 0.037$ ; Fig. 1). Log-transformed mean plasma P4 and E2 values differed between the non-pregnant and pregnant groups (P4:  $F_{1,15} = 24.581$ ,  $p = 0.0002$ ;

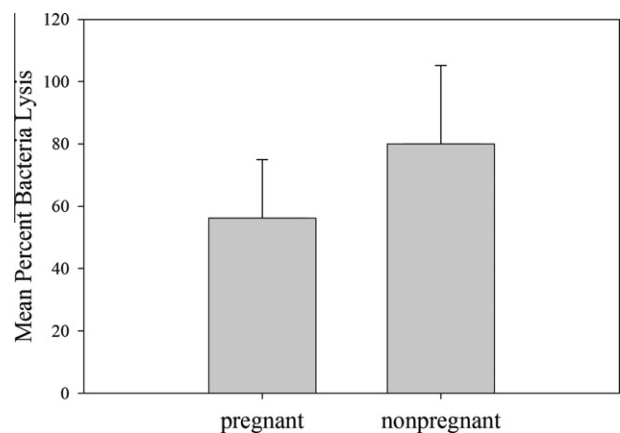


Fig. 1. Comparison between mean plasma percent bacteria lysis in pregnant ( $n = 7$ ) versus non-pregnant ( $n = 11$ ) female cottonmouths ( $F_{1,15} = 5.27$ ,  $p = 0.037$ ). Bars represent one standard error.

Fig. 2a; E2:  $F_{1,15} = 7.646$ ,  $p = 0.014$ ; Fig. 2b), with pregnant females exhibiting higher mean concentrations (mean 1114.58 pg/ml; range 290.28–2675.35 pg/ml) of P4 than non-pregnant females (mean 205.16 pg/ml; range 122.76–341.01 pg/ml). Pregnant females had lower mean concentrations of E2 (mean 41.26 pg/ml; range 37.41–44.55 pg/ml) relative to non-pregnant females (mean 52.56, range 38.70–69.85 pg/ml). Log transformed plasma corticosterone did not differ between these groups (mean pregnant: 16273.67 pg/ml, range 2638.08–48393.50 pg/ml; mean non-pregnant: 14221.68 pg/ml, range 1093.47–66862.07;  $F_{1,15} = 0.666$ ,  $p = 0.43$ ; Fig. 2c). Angular transformed mean bacteria lysis was sig-

nificantly negatively correlated with log mean P4 ( $R^2 = 0.29$ ,  $p = 0.027$ ; Fig. 3a) but not E2 ( $R^2 = 0.021$ ,  $p = 0.58$ ; Fig. 3b) or CORT ( $R^2 = 0.013$ ,  $p = 0.66$ ; Fig. 3c). This relationship appeared to be driven by variation in P4 exhibited by pregnant females, especially two individual females with higher P4 values (Fig. 3a); when these individuals were removed from the analysis, the relationship became non-significant ( $R^2 = 0.14$ ,  $p = 0.17$ ). In addition, when pregnant and non-pregnant females were analyzed separately, there was no significant correlation between log P4 and angular transformed bacteria lysis in either group; however, there was a negative trend exhibited by pregnant females ( $R^2 = 0.25$ ,  $p = 0.25$ ), but not non-pregnant females ( $R^2 = 0.002$ ,  $p = 0.79$ ).

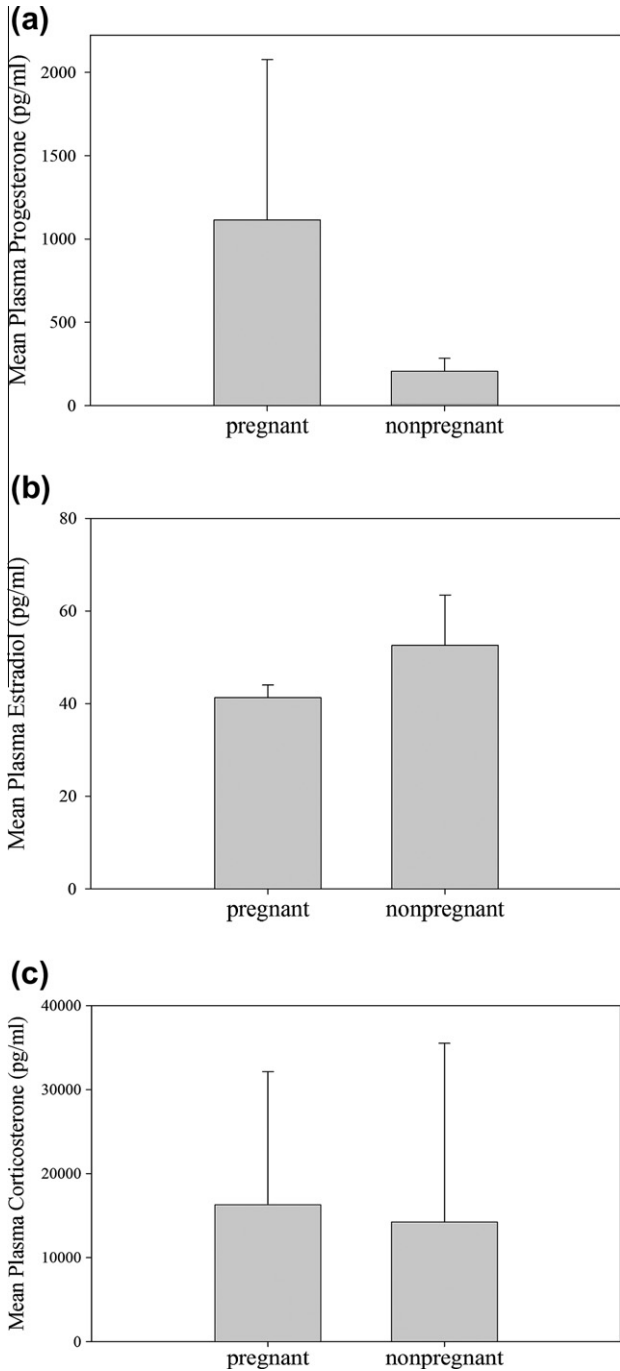


Fig. 2. Comparison between mean plasma progesterone (2a;  $F_{1,15} = 24.581$ ,  $p = 0.0002$ ), estradiol (2b;  $F_{1,15} = 7.646$ ,  $p = 0.01$ ), and corticosterone (2c;  $F_{1,15} = 0.666$ ,  $p = 0.43$ ) in pregnant ( $n = 7$ ) versus non-pregnant ( $n = 11$ ) female cottonmouths. Bars represent one standard error.

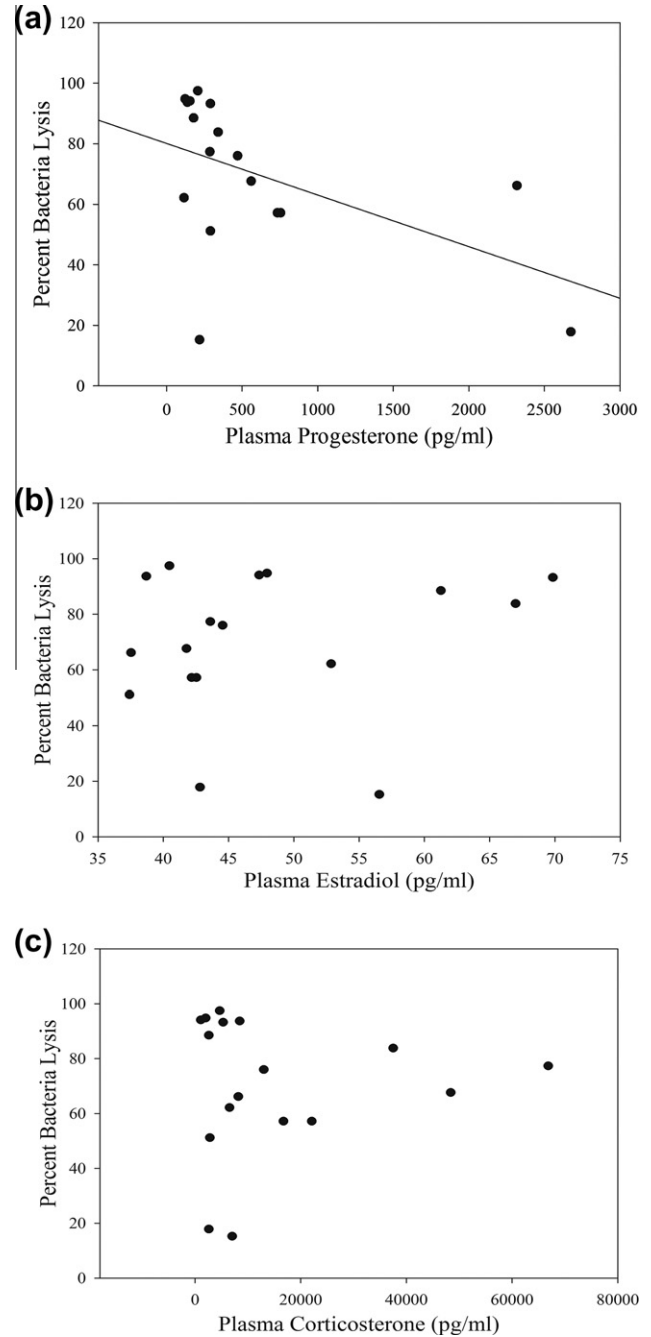


Fig. 3. Relationship between mean plasma progesterone (Fig. 3a;  $R^2 = 0.29$ ;  $p = 0.027$ ); estradiol (Fig. 3b;  $R^2 = 0.021$ ,  $p = 0.58$ ) and corticosterone (Fig. 3c;  $R^2 = 0.013$ ,  $p = 0.66$ ) and mean plasma percent bacteria lysis in female cottonmouths ( $N = 18$ ).

#### 4. Discussion

In this study, we demonstrate a difference in innate immune performance between pregnant and non-pregnant female snakes, and this difference appears to be associated with steroid hormones, specifically progesterone (P4). Steroid hormone profiles in these snakes were similar to those documented in most viviparous vertebrates [37,13]. Progesterone is recognized for its role in maintaining uterine tissue, ova, and embryos in sharks, amphibians, squamates, and mammals [11,12,55,37], and we documented significantly elevated P4 in pregnant snakes relative to non-pregnant snakes. Estradiol (E2) was significantly elevated in non-pregnant females, possibly because some of these snakes were beginning to initiate vitellogenesis for reproduction the following year [1]. The role of E2 in initiating vitellogenesis and ovulation is well known and appears to be conserved throughout vertebrates [11,37]. We found no difference in corticosterone (CORT) concentrations between pregnant and non-pregnant snakes. A previous study in another pitviper, *Crotalus atrox*, demonstrated a surge in CORT in pregnant females immediately prior to parturition [52]. Other than this, the hormone patterns for *A. piscivorus* in late gestation are very similar to late gestation patterns documented in *C. atrox* [52]. The extremely high variance surrounding our mean CORT values may have prevented us from detecting a difference between pregnant and non-pregnant females; the values we report are more similar to those reported for *C. atrox* in September [52], when mean CORT values in pregnant female rattlesnakes dropped from an August peak and became more similar to concentrations exhibited by non-pregnant females. Taylor et al. [52] also mention high variance in CORT values; this may be related to the variance in time required to gather blood samples, since care is required to obtain samples from pitvipers.

We document ~20% lower plasma bactericidal capacity in pregnant snakes relative to non-pregnant snakes collected from the same population at the same time of year, and attribute this difference to lower complement performance in the pregnant snakes. This difference in pregnant females could be due to several, non-mutually exclusive mechanisms: (1) an overall decrease in plasma complement protein concentrations, (2) inhibition of the complement cascade at any of its many regulatory points, or (3) increased complement catabolism and clearance. Complement is a potent protein cascade found throughout vertebrates, and has documented effects against protozoan, helminth, bacterial, and viral pathogens [27,20]. Three major complement cascade pathways have been categorized based upon their pathogen recognition mechanisms, but all three pathways share common protein components (C3 and C5 proteins and the membrane attack complex [36,60,19,61,41]). The three complement pathways are tightly regulated, especially at the level of C3 cleavage, which is considered the most important regulatory point in the cascade [19]. Improper regulation can lead to self-tissue damage [34] and rejection of developing embryos by complement [14,29].

Progesterone has documented regulatory effects on immune components during pregnancy in mammals [50,16], and has been shown to reduce complement production by modulating mRNA transcription of the C3 complement protein in rats [9]. Our results are consistent with this finding; we demonstrate significantly increased P4 in pregnant snakes, and this increase was negatively correlated with plasma complement performance. Although more experimental work is needed to establish a causal link between P4 and complement performance, our study has determined a possible similarity between the endocrine regulation of immunity during pregnancy in a squamate.

Previous studies have demonstrated similar immune/endocrine interactions in other viviparous squamate species. Saad and El

Deeb [43] described less robust immune responses to antigens in pregnant viviparous skinks relative to non-pregnant individuals, and these responses were associated increases in hormones (E2 and T) during pregnancy. The authors hypothesized hormonal regulation of immune function during pregnancy in these skinks [43], but they did not measure P4. Others have documented cytokine involvement in uterine development in a viviparous skink [38,39]. However, in squamates studied thus far, the study species have possessed fairly extensive placentation and an intimate connection between the mother and fetus. Cottonmouths lack an extensive fetoplacental connection and embryos are enveloped by an egg membrane secreted by the mother (e.g., ovoviviparity). The exact extent to which the developing embryo and mother interact in cottonmouths is unknown, although a study that described respiratory dynamics during pregnancy in this species concluded that less nutrient exchange takes place than in snake species with more extensive placentation (e.g., *T. sirtalis*; [4]). Despite exhibiting less extensive placentation, pregnant cottonmouths may exhibit similar changes in immune performance to those reported for species possessing a more intimate placental connection (e.g., [43]).

It is possible that reduced complement function in female cottonmouths may be more related to the costs of reproduction than to immune privilege. For pregnant female pitvipers, the costs of maternal investment in large young (e.g., vitellogenesis, gestation, and gestational anorexia) may tax energy stores of females and lead to a trade-off with immune function. Such trade-offs are becoming increasingly well documented [31,18]. During pregnancy many viviparous snakes reduce feeding or avoid eating entirely, and post parturient snakes are often extremely emaciated ([30]; pers. obs.) suggesting that this heavy investment in developing embryos can greatly tax energy stores. Further experiments are warranted to determine the effects of energy limitation on immunity in snakes, and cottonmouths are an ideal study organism in which to test these ideas because they will accept food during pregnancy (unlike some snakes), which would allow manipulations of food intake to determine if this is responsible for reduced immune performance.

In conclusion, we provide evidence for innate immune changes during pregnancy in a snake, a finding consistent with previous studies conducted on diverse and evolutionarily distant vertebrates. Furthermore, these changes appear to be associated with and possibly modulated by the reproductive hormones typically involved in reproduction in other viviparous vertebrates. Although many more studies need to be conducted to determine evolutionary trends, this study and others that have compared several independently derived cases of viviparity suggest that immune modulation is a key derived evolutionary feature acquired during the evolution of this adaptive (e.g., [46]) reproductive mode. Interestingly, this pattern is consistent with models describing the transitional steps from oviparity to viviparity, which indicate the role of elevated P4 and subsequent prolonged egg retention [48,12]. Additional studies in squamates—the vertebrate group with the most independent lineages exhibiting viviparity—will likely illuminate the ways in which this intricate immune-endocrine interface develops.

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