

**Ecoimmunological Investigations of the Cottonmouth (*Agkistrodon piscivorus*)**

by

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## Abstract

Despite the benefits gained from studying the immune system of non-traditional model organisms, including the discovery of key components of the human immune system, squamates remain the most poorly-understood major vertebrate group in terms of immune system components and function. This bias is more striking given that squamates may play important roles in disease transmission cycles; without even a rudimentary understanding of susceptibility and infection clearance in these vertebrates, transmission models will be incomplete. This dissertation explores the following areas: 1) thermal biology of innate immune (complement) function in snakes; 2) seasonal endocrine/immune patterns in free-living snakes; 3) endocrine/immune differences between pregnant and non-pregnant snakes; and 4) the seasonal pattern of exposure to eastern equine encephalitis virus (EEEV) in amphibians and reptiles from Tuskegee National Forest in Alabama. These studies focused on wild-living cottonmouths (*Agkistrodon piscivorus*), a large-bodied snake often found in high densities that experiences infection with arthropod-borne viruses (e.g., EEEV). Complement system function of cottonmouths exhibits a positive correlation with experimental temperature, such that the plasma's ability to lyse bacteria is maximal at temperatures above the range experienced by cottonmouths in the wild. However, seasonal variation in complement performance does not appear to be associated with seasonal variation in cottonmouth body temperatures, body condition, or patterns of steroid hormone secretion and

reproduction. Given the repeated demonstration of immune modulation during pregnancy in unrelated vertebrates, I predicted and confirmed a difference in complement performance between pregnant and non-pregnant females, and this was negatively correlated with the sex steroid progesterone. These findings suggest that, although certain features of vertebrate functional immunity appear to be conserved or convergent among ectotherms (e.g., immune modulation during pregnancy), the overarching influence of body temperature on ectothermic immune systems constitutes a substantial functional difference among vertebrates. As this difference may influence transmission patterns of arthropod-borne viruses with broad host ranges, the seasonal prevalence of EEEV exposure in cottonmouths and other terrestrial ectotherms, and the results support a role for squamates—specifically snakes—as underappreciated arbovirus reservoirs and overwintering hosts.

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## Chapter 1

### General Introduction

Vertebrate physiological systems are remarkably conserved across taxa (Somero, 2000), suggesting that constant selection pressure has maintained the adaptive value of these systems since early geological times. For example, the neuroendocrine system regulates homeostasis, reproduction, and stress responses utilizing similar hormones, receptors, and mechanistic pathways in vertebrates as evolutionarily distant as lampreys and humans (Norris, 1997; Salas et al., 2003; Campbell et al., 2004). Comparative immunological studies (Du Pasquier, 1992; Du Pasquier, 2001; Kasahara et al., 2004) suggest similar trends. However, immunological investigations of non-traditional model organisms are uncommon relative to the large body of work on more typical subjects (e.g., mice), possibly due in part to a perceived lack of importance of these models relative to mammalian ones (Bonnet et al., 2002), an over-reliance on mammalian model organisms (Travis, 1993; Butler, 2006), and lack of species-specific reagents allowing comparative studies (Matson et al., 2006). This situation exists despite the fact that the comparative approach has been shown repeatedly to benefit the general study of organisms and systems, and examples from the field of comparative immunology include the discovery of the adaptive significance of fever, toll-like receptors, interferon, and antimicrobial peptides—key components of the mammalian immune system—in lizards,

fruit flies, chickens, and frogs, respectively (Kluger, 1979; Medzhitov et al., 1997; Isaacs et al., 1957; Csordas and Michl, 1970).

Discoveries in nontraditional model organisms have also illustrated the evolution of the immune system. Important examples include variable gene clusters responsible for T and B cell receptor diversity in sharks (Hinds and Litman, 1986), and a unique, independently derived mechanism for generating lymphocyte receptor diversity in lampreys and hagfish (Alder et al., 2005). Comparative studies have determined that most components of the innate and adaptive immune system are present and function similarly in all major vertebrate groups (Hsu, 1998; Sunyer and Lambris, 1998; Du Pasquier, 2001; Flajnik and DuPasquier, 2004; Roach et al., 2005; Cooper and Alder, 2006). However, turtles, lizards, crocodylians, and snakes (often treated together as an unnatural group due to their similar physiology and morphology—Schwenk, 1994; Fig. 1) are the least studied major vertebrate groups from an immunological perspective. For example, a recent comparative immunology textbook has no section on these groups (Pastoret et al., 1998), despite their occupation of key positions in vertebrate evolution, exhibition of high species diversity (in the case of squamates), and importance to ecosystems (Pough, 1980).

Studies involving the immune system of squamates will elaborate our understanding of the evolution and diversity of the vertebrate immune system, allow interesting comparisons not possible in endothermic models (e.g., the effects of temperature on immune performance *in vivo*), and broaden our understanding of the biology of this understudied group. These studies will also inform our comprehension of the role of squamates in zoonotic disease transmission cycles; recent studies have shown that squamates possibly contribute to pathogen transmission as sources of pathogens (e.g., amplifying hosts) or hosts that do not

contribute to pathogen transmission (e.g., dead end hosts) (Lane and Loye, 1989; Klenk and Komar, 2003; Klenk et al., 2004; Giery and Ostfeld, 2007). Disease transmission models will therefore benefit by inclusion of information about susceptibility and infection latency in squamates, which may differ significantly from birds and mammals in these and other aspects of infection and immune response (Hsu, 1998; Sunyer et al., 1998).

### **The vertebrate immune system: a sketch**

The vertebrate immune system has traditionally been split into two arms, both with apparent strengths and weaknesses, and both of which interact extensively (Table 1; Abbas et al., 2007). Innate immunity, a constitutive defensive system of cells, molecules, and mechanistic pathways, has relatively little capacity to recognize specific molecules derived from pathogens (antigens), but is always present and prepared to respond. Adaptive immunity is made up of lymphocytes (T and B cells) with the capacity to express anticipatory receptors of remarkable specificity for antigens due to the somatic rearrangement of gene segments by RAG gene enzymes and the subsequent proliferation of antigen-specific cloned cell lines. These cells are responsible for the recognition and memory of specific pathogens. However, adaptive immunity is not initiated in the absence of antigen presentation by other immune cells, and requires some time before this induction occurs (Abbas et al., 2007).

Innate immunity is an early warning system, the initiator of inflammation, and often the primary effector of the immune system (Abbas et al., 2007). Innate immunity coordinates with the more specific, adaptive arm of the immune system via cytokines (chemical messengers released locally or systemically). Macrophages and dendritic cells, many of which reside in the skin or other portals of entry (Wikel, 1996), express toll-like receptors

with general specificity for conserved viral, bacterial, or parasitic molecules (i.e., pathogen-associated molecular products; Lien et al., 1999). After recognition, these cells engulf the invader (phagocytosis), process its fragments, and express them on surface antigen-presenting molecules (the major histocompatibility complex—MHC) to cells of the adaptive immune system. Without this immunological “trip wire,” the adaptive immune system responds much more slowly to pathogens (Akira et al., 2001).

The immune system is regulated at several levels by the endocrine system; for example, lymphocytes express receptors for adrenal and sex steroid hormones that can have stimulatory and/or inhibitory effects on immune components, function, and cytokine release (Sapolsky et al., 2000). The most well-known of these interactions is the immunosuppressive and anti-inflammatory actions of glucocorticoids (Harbuz and Lightman, 1992; Chrousos, 1995; Sapolsky et al., 2000). More recently, acute stressors and glucocorticoids have been postulated to enhance the immune response by redirecting leukocytes from circulation to skin, which resulted in faulty assumptions of stress-induced immunosuppression by earlier authors (Dhabhar and McEwen, 1999; Sapolsky et al., 2000). In addition, cytokines and other immune chemical messengers have feedback communication with the endocrine system (Besedovsky et al., 1986).

This simplistic description of the immune system’s recognition of pathogens and interactions with other body systems belies its complexity. In reality, both arms of the immune system have multiple cell types that have compartmentalized roles, produce multiple types of antigen-recognizing receptors and effector molecules, and communicate using multiple cytokines.

### **The vertebrate immune system in an evolutionary context**

Many aspects of innate immunity (e.g., toll-like receptors, phagocytic cells, inflammation, and complement—an innate protein cascade involved in pathogen lysis) are conserved throughout the animal kingdom and some are even shared with plants (Du Pasquier, 2001). Searches for components of the vertebrate adaptive immune system were until recently carried out in vain among invertebrate taxa (Flajnik and Du Pasquier, 2004; Kasahara et al., 2004; Little et al., 2005), as well as in lampreys and hagfish (Flajnik, 2004; Alder et al., 2005), leading to the conclusion that these groups lack an adaptive immune system. However, the lack of components of the vertebrate adaptive immune system does not necessarily indicate lack of any adaptive, specific, or memory response in invertebrates or agnathans (Little et al., 2005; Dooley and Flajnik, 2005).

New discoveries indicate such systems do occur in non-vertebrates, which may have independently derived adaptive-like immunity (Zhang et al., 2004; Watson et al., 2005), or extremely diverse, germline-encoded innate immune receptors (Rast et al., 2006). It is also now clear that lampreys and hagfish have a unique adaptive immune system which differs from that of jawed vertebrates (Pancer et al., 2004; Flajnik, 2004; Alder et al., 2005; Cooper and Alder, 2006). In addition, certain precursors similar to the genes of the adaptive immune system have been identified in non-vertebrate deuterostomes (Rast et al., 2006) and chordates (Yu et al., 2005).

Studies of jawed vertebrates have determined that most, if not all, components of innate and adaptive immunity (e.g., MHC, T and B cells with anticipatory, specific antigen receptors, and RAG genes) are present in such distantly-related taxa as sharks, bony fish, amphibians, birds, and mammals, including humans (Warr et al., 1995; Flajnik, 1996, 1998; Hsu, 1998; Kasahara, 2004; Cooper and Alder, 2006; Fig. 1). Not all components have been

identified in all groups, and certainly their functional role has not been confirmed in every case. Comparative studies have traced the evolutionary development of Ig (antibody) superfamily genes and molecules (Warr et al., 1995), cytokines (Huisling et al., 2006), toll-like receptors (Roach et al., 2005), interferons (Schultz et al., 2004), and complement (Sunyer and Lambris, 1998) within gnathostomes (see Fig. 1).

Three major differences between the immune system of endothermic (mammals and birds) and ectothermic (“fish”, amphibians, and “reptiles”) vertebrates have been identified, and these represent the only major disparities in immune function among gnathostome vertebrates. These include 1) novel specializations peculiar to ectotherms, 2) the inefficient adaptive response of ectotherms, and 3) thermal influences on immune performance in ectotherms. Novel specializations of ectotherms include the Ig NAR (“new antigen receptor”) antibody type of sharks (Dooley and Flajnik, 2005), diverse complement assortments of some fish (Sunyer et al., 1998), abundance of antimicrobial peptides associated with frog skin (Chinchar et al., 2004; Rollins-Smith, 2001; Rollins-Smith and Conlon, 2005), and phagocytic B cell populations in ectotherms (Zimmerman et al., 2010). Often these specializations are suggested to explain how ectotherms compensate for their inefficient adaptive immune response relative to mammals and birds.

Ectotherms are thought to have poor adaptive immune responses, including weaker and less specific antibody production than endotherms, less MHC polymorphism, as well as a weak or nonexistent secondary response on re-exposure to an antigen (Zapata et al., 1992; Flajnik, 1996; Hsu, 1998). These differences exist despite the fact that ectotherms exhibit every component of the adaptive immune response found in endotherms with the exception of germinal centers (localized regions of antigen presentation and lymphocyte proliferation—

see Hsu, 1998). It is assumed that pathogen replication is lower in ectotherms since they are “cold-blooded.” This indicates that terrestrial ectotherms have the capacity to thermally control pathogens, and this may be sufficient to limit infections (Hsu, 1998).

However, in at least one case the apparently inefficient adaptive response of an ectotherm was found to be due to an artifact of earlier approaches that attempted to identify robust humoral and memory responses in sharks (Dooley and Flajnik, 2005). This study concluded that sharks have immunological memory and robust Ig NAR responses that earlier researchers failed to measure (Dooley and Flajnik, 2005). Additional studies that measured the functional response of ectotherms to pathogens (rather than non-infective antigens) suggest that immunological memory may be present and efficient in these vertebrates (Gantress et al., 2003; Desvignes, 2004; Dooley and Flajnik, 2005). Given these differing outcomes, additional research is necessary to confirm whether ectotherms exhibit robust adaptive immune responses.

Finally, and not surprisingly, most aspects of immune function in ectotherms are temperature-dependent (Allen and McDaniel, 1937; Tait, 1969; Pxytycz and Józkwicz, 1994; LeMorvan et al., 1998; Ream et al., 2003). Body temperature also influences immune performance in endotherms (Roberts, 1979). For example, vertebrate complement was originally discovered due to its temperature lability (Bordet and Gengou, 1901; reviewed in Sherwood, 1951). Therefore, thermal immune performance variation is simply more well-known and well-developed in ectotherms since their physiology supports wide fluctuations in body temperatures and allows experimental manipulation (Kluger, 1979).

If the immunological differences between ectotherms and endotherms translate into functional differences in their immune capabilities, then these differences have implications



for the role of ectotherms in disease transmission cycles. Modeling disease transmission cycles often requires knowledge of the immune status of important vertebrate components of the system (Unnasch et al., 2006). Populations of hosts are made up of varying proportions of naïve and immune individuals, and the hypothesized poor adaptive immune response of ectotherms may preclude efficient immunological memory, resulting in large numbers of naïve hosts in a given population. In addition, the viremic period (blood virus levels enabling transmission to vector species) during infection can be different among various vertebrate hosts, and this also influences transmission dynamics (Table 2). Due to interactions among host body temperature, immune function, and pathogen virulence (Roberts, 1979; Thomas and Blanford, 2004), the length of the viremic period is influenced by body temperature in ectotherms (Klenk et al., 2004; White et al., 2011). These factors may result in ectothermic hosts exhibiting a prolonged viremic period and a large naïve host population relative to birds and mammals.

In general, however, the vertebrate immune system is conserved across taxa both in terms of its components and performance, as well as in its interactions with regulatory systems (e.g., the endocrine system). Endocrine and immune interactions have been extensively demonstrated in mammals and birds (Sapolsky et al., 2000; Norris and Evans, 2000), and limited evidence from other taxa, such as fish (Harris and Bird, 2000), amphibians (Rollins-Smith, 2001), and lizards (Zapata et al., 1983; Saad, 1988; Saad and Ridi, 1988; Saad et al., 1990), suggest similar regulatory interactions occur throughout vertebrates. Comparative immunological studies will therefore benefit from an evolutionary approach; it is important to consider that ectothermic taxa do not necessarily represent the ancestral immunological condition to mammals; many groups, including squamates, have traditionally

been presented this way by immunologists, despite evidence to the contrary and the possibility of drawing erroneous conclusions by recognizing non-evolutionary classifications (see Schwenk, 1994).

### **Ecoimmunology**

The growing field of ecoimmunology includes attempts to identify and analyze trade-offs between immune function and other life history components in diverse vertebrates (French et al., 2007; French et al., 2009; Schulenburg et al., 2009). The field grew from studies that attempted to link immune resistance to parasites and sexually selected traits (Hamilton and Zuk, 1982), a view that later become formalized as the “immunocompetence handicap hypothesis” (Folstad and Karter, 1992). This hypothesis identifies several additional layers of interactions between sexually selected traits (e.g., bright colors, adornments, etc.) and heritable resistance to pathogens, which cumulatively result in a hypothesized trade-off between the expression of secondary sexual characteristics and disease resistance. These include links between secondary sexual characteristics and increased testosterone, testosterone and immune performance, and immune performance and susceptibility to disease (Folstad and Karter, 1992). Due to the variable outcomes possible between the interactions among these links, the hypothesis lends itself to diverse outcomes within different study systems (Roberts et al., 2004).

Additional trade-offs between immunity and other life history components also have been identified, and these have led to attempts to quantify the energetic costs of components of immunity (Lochmiller and Deerenberg, 2000). Positive relationships between nutrition/energy (food) intake and immunocompetence have been experimentally identified (Lochmiller et al., 1993; Saino et al., 1997; Uller et al., 2006; French et al., 2007), and trade-

offs between growth and immunocompetence have been described in both laboratory and field settings in birds and lizards (Soler et al., 2003; Brommer, 2004; Uller et al., 2006). Similarly, reproduction and immunity appear to be life history components that are traded off (Ilmonen et al., 2000; Uller et al., 2006; French et al., 2007; French et al., 2009). There is ample evidence that the neuroendocrine system might modulate these trade-offs, if they exist (Nelson et al., 2002; Demas, 2004; Martin et al., 2008).

Various studies have estimated the physiological costs of immunity, and support the view that maintenance of immunity and immune function can be energetically and nutritionally costly (Lochmiller and Deerenberg, 2000). Moreover, different immune components can have different costs and may lead to physiological trade-offs within individuals (Martin et al., 2006). Interspecific comparisons have determined different immunological profiles between closely related species that exhibit divergent life histories (Norris and Evans, 2000; Lee et al., 2005; Lee et al., 2006; Tieleman et al., 2007; Martin et al., 2007; Martin et al., 2008). These studies have led to the development of a model of immune function and its association with life history strategies (e.g., reproductive pace of life; Lee, 2006), and hypotheses about immunity and ecological invasion potential (Lee and Klasing, 2005). These models predict that certain immune components (e.g., inflammation) are more energetically costly than others, and are therefore favored by species exhibiting certain life history strategies. Finally, observations of seasonal variation in immunity have been observed and are consistent with the hypothesis of energetic and reproductive trade-offs with immunity, and support a role for neuroendocrine modulation of seasonal immune variations (Nelson and Demas, 1996; Nelson et al., 2002; Martin et al., 2008). Pregnancy is

viewed as a particularly demanding component of reproduction, and immune changes during pregnancy have potential energetic and fitness consequences (Drazen et al., 2003).

### **Overview**

This dissertation explores several hypotheses in wild populations of the cottonmouth (*Agkistrodon piscivorus*) within the theoretical framework of comparative immunology and ecoimmunology. The cottonmouth is the focus of this research since it is common, large, and easy to capture (facilitating the collection of large samples), a great deal is known about its reproduction and natural history, and this snake is currently the focus of research seeking to determine the contribution of reptiles to the transmission of a medically important arthropod-borne virus with a broad host range. In Chapter 2, I determine the thermal reaction norm of complement performance (e.g., the ability of plasma to lyse bacteria) in cottonmouths, test whether the thermal reaction norm of complement performance is co-adapted with the field body temperatures experienced by cottonmouths, and whether this reaction norm acclimatizes seasonally. In Chapter 3, I further explore seasonal variation in complement performance in cottonmouths, measuring this variable concomitantly with seasonal steroid hormone variation, reproductive parameters, body temperature, and body condition. In Chapter 4, I test the hypothesis that reproductive condition (e.g., pregnant versus non-pregnant) influences complement performance negatively, and that steroid hormones are correlated with the pattern of immunity exhibited by female cottonmouths during reproduction. Finally, in Chapter 5, I take into account the unique characteristics of ectothermic immunity, and describe patterns of exposure to eastern equine encephalitis virus in wild cottonmouths and other terrestrial ectotherms from Tuskegee National Forest, determine if taxonomic or seasonal patterns exist, and explore whether these results support

the role of terrestrial ectotherms as overwintering hosts for arthropod-borne virus transmission.

**Table 1** Comparison between characteristics of adaptive and innate immunity (Abbas et al., 2007).

<b>Innate Immunity</b>	<b>Adaptive Immunity</b>
<b>Characteristics</b>	
Always ready	Inducible
Relatively non-specific	Exceedingly specific
Presents antigens	Induced by antigens
No memory	Memory
<b>Effector Functions</b>	
Inflammation	Apoptosis of infected cells
Phagocytosis	Opsonization of microbes
Triggers adaptive response	Coordinates innate response
<b>Cellular Components</b>	
Dendritic cells	Lymphocytes (B and T cells)
Neutrophils	
NK cells	
Macrophages	
<b>Non-Cellular Components</b>	
Complement	Antibodies
<b>Receptor Types</b>	
Toll-like receptors	TCR, BCR, MHC
Pattern-recognition receptors	
<b>Representative Cytokines</b>	
TNF, IFN	IL-2, IL-4

**Table 2** Comparison of viremia and antibody titer duration among numerous vertebrates after experimental inoculation with arboviruses.

Taxon	Arbovirus	mean Ab max (day of peak)	mean viremia duration (days)	N	Source
<b>Birds</b>					
Canada Goose	WNV	.	4	3	Komar et al., 2003
Mallard	WNV	.	4	2	"
American Kestrel	WNV	.	4.5	2	"
Northern Bobwhite	WNV	.	4	3	"
Japanese Quail	WNV	.	1.3	3	"
Ring-necked Pheasant	WNV	.	3.7	3	"
American Coot	WNV	.	4	1	"
Killdeer	WNV	.	4.5	2	"
Ring-billed Gull	WNV	.	5.5	2	"
Mourning Dove	WNV	.	3.7	3	"
Rock Dove	WNV	.	3.2	6	"
Monk Parakeet	WNV	.	2.7	3	"
Budgerigar	WNV	.	1.7	3	"
Great Horned Owl	WNV	.	6	1	"
Northern Flicker	WNV	.	4	1	"
Blue Jay	WNV	.	4	4	"
Black-billed Magpie	WNV	.	5	3	"
American Crow	WNV	.	3.8	8	"
Fish Crow	WNV	.	5	9	"
American Robin	WNV	.	4.5	2	"
European Starling	WNV	.	3.2	6	"
Red-winged Blackbird	WNV	.	3	3	"
Common Grackle	WNV	.	3.3	6	"
House Finch	WNV	.	6	2	"
House Sparrow	WNV	.	4.5	6	"
Chicken	WNV	19	2.5	20	Langevin et al., 2001
<b>Mammals</b>					
Dog	WNV	.	2.5	4	Austgen et al., 2004
Cat	WNV	.	4	8	"
Cotton Rat	VEEV	18	4.5	12	Young et al., 1969
Spiny Rat	VEEV	18	4.5	10	"
Cotton Rat	EVEV	7	2.5	47	Coffey et al., 2004

Cotton Rat	VEEV	10	3.5	60	Carrara et al., 2007
Horse	WNV	9	3.5	12	Bunning et al., 2002
<b>Amphibians and Reptiles</b>					
Garter snake	WEEV	19.5	6	25	Spalatin et al., 1964
Northern Leopard Frog	WEEV	16.5	.	20	"
Garter snake	WNV	22.5	4.2	18	Steinman et al., 2006
American Alligator, 27°C	WNV	.	10.5	6	Klenk et al., 2004
American Alligator, 32°C	WNV	.	5.5	6	"



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## Chapter 2

### Thermal performance and acclimatization of a component of cottonmouth (*Agkistrodon piscivorus*) innate immunity

**Abstract:** Complement—an immune protein cascade involved in pathogen lysis—was discovered as a temperature-labile component of vertebrate plasma, yet since that time, the thermal performance of complement has not received much attention from a comparative or ecological perspective. We investigated two thermal hypotheses involving the complement system of the cottonmouth snake (*Agkistrodon piscivorus*). We tested whether complement performance would conform to optimal thermal reaction norms commonly observed in ectotherm ecophysiological studies, predicting that complement efficiency would be maximal at or near the cottonmouth's field body temperatures. We also tested thermal acclimatization of complement performance, by comparing temperature/performance curves from samples collected in three different seasons. Complement efficiency exhibited the same significant positive correlation with temperature in all three seasons. This seasonally invariable temperature-performance relationship may allow easy acquisition of behavioral fever, and trade-offs between immune performance and energy balance, ultimately endowing snakes with immunological flexibility not available to endotherms.

## Introduction

Emerging infectious diseases present unique problems to researchers, and seem to have become especially prevalent and linked to precipitous declines in taxa (e.g., amphibians, Daszak et al. 1999; bats, Blehert et al. 2009) which do not receive as much immunological research focus as more typical study species. For example, well documented declines in amphibian species and populations have been convincingly linked to an emerging infectious fungal disease (Lips et al. 2006), and our understanding of the effects of this pathogen has been limited by our lack of knowledge of immune system function in this group (Carey et al. 1999). Functional immune studies in diverse ectothermic vertebrates are therefore crucial not only to determine why some species are highly susceptible to emerging infectious diseases (Carey et al. 1999; Wake and Vrendenburg, 2008), but also to determine ecological and evolutionary patterns. Not surprisingly, evidence suggests that interactions between pathogens, temperature, and the ectothermic host immune response are important in these contexts (Richards-Zawacki, 2009; Ribas et al. 2009).

Most aspects of immune function are strongly influenced by temperature in ectotherms. For example, antibody production and other immune components are strongly temperature-dependant in fish, frogs, and lizards (Allen and McDaniel, 1937; Tait, 1969; Rijkers et al. 1980; Wright and Cooper, 1981; Le Morvan et al. 1998). Seasonal fluctuations in immune function are exhibited by ectothermic vertebrates, although these have not always been linked to temperature *per se* (Zapata, 1992). In addition, the complement system of vertebrates, a phylogenetically conserved protein cascade important for innate immune defense (Ricklin et al. 2010), is temperature-labile, and was initially discovered due to the

exhibition of this characteristic (e.g., Bordet and Gengou, 1901; reviewed in Sherwood, 1951).

The innate immune complement cascade has been divided into three pathways which share common components and function, but differ in their initiation mechanisms (Müller-Eberhard, 1988; Fujita et al. 2004). The classical complement pathway interfaces with the adaptive immune system (antibodies) and can assist the targeting and destruction of specific pathogens (Ricklin et al. 2010). The lectin pathway involves complement proteins interacting with lectin, which targets conserved microbial surface carbohydrates (Fujita et al. 2004). The alternative pathway is triggered by direct adherence to microbial cell membranes, requires no involvement with the adaptive immune system, and can lead directly to the lysis of pathogens (Fujita et al. 2004). The lectin and alternative pathways appear to be among the most ancestral vertebrate immune components, and have even been identified in non-vertebrate taxa (Müller-Eberhard, 1988; Fujita et al. 2004; Zhu et al. 2005). The classical and alternative pathways share C3 and C5 component proteins, and lead to the formation of the membrane attack complex, a potent antimicrobial protein involved in cell membrane disruption (Fujita et al. 2004). Ectotherms may have a more diverse array of complement protein isoforms than those found in mammals, and this has become an active area of comparative immunological research (Sunyer et al. 1998; Sunyer and Lambris, 1998).

However, since the discovery of complement, little research has examined its thermal performance. This is in part due to the lack of experimental work involving complement and temperature in traditional study animals (i.e., mammals), since they are not amenable to large temperature fluctuations *in vivo* (Kluger, 1979), and also due to decreased focus on functional immune measures (Little et al. 2005). In addition, complement activity in

mammals varies thermally, but is heavily restricted by cold temperatures, whereas complement activity in ectotherms exhibits modest antimicrobial performance even at low temperatures (Gigli and Austen, 1971; Koppenheffer, 1987). Studies in crocodylians have confirmed the presence of an active and potent complement system (Merchant et al. 2003; Merchant and Britton, 2006), and components of the complement system have been characterized in snakes (Vogel and Müller-Eberhard, 1985). However, the ecological factors that may modulate complement performance in these vertebrates are still relatively unknown.

Studies of thermal performance of other physiological functions in ectotherms have lead to the development of a useful framework for developing hypotheses (Huey and Kingsolver, 1989; Angilletta et al. 2002). For example, many physiological systems in ectotherms exhibit optimal reaction norms which correspond with preferred body temperatures (e.g., they are coadapted; Rome et al. 1992; Arnold and Peterson, 2002; Angilletta et al. 2002), and these reaction norms often acclimate seasonally (e.g., they acclimatize; Rome et al. 1992). To test if the complement system in the cottonmouth snake (*Agkistrodon piscivorus*) exhibits similar tendencies, we investigated the following ecological hypotheses using a functional immune assay that measures complement performance (Viney et al. 2005): 1) snake complement efficiency exhibits an optimal thermal reaction norm, with a peak of microbe lysis capacity at or near the snake's mean field body temperature; 2) snake complement efficiency shifts seasonally (acclimatizes), with the thermal performance curve shifting toward cooler temperatures during spring months relative to summer months. Samples used in these functional assays were taken from living snakes and tested within ecologically realistic temperatures, and thus this study was conducted within an appropriate ecological context (Arnold and Paterson, 2002). Finally, our



experiments focus on a functional (rather than mechanistic) endpoint (e.g., the ability of plasma to lyse bacteria), an important comparative immunological approach recently advocated by Little et al. (2005) and Tieleman et al. (2005).

## **Materials and Methods**

### *Study animal and field processing*

Cottonmouths are fairly large, semi-aquatic venomous snakes found throughout the southeastern United States, often in large local population densities (Ernst and Ernst, 2004). Snakes were sampled in the field from a study area just south of Atlanta, Georgia, USA (33.47017°N, 84.39116°W). At this site, cottonmouths are active March-October. During March and April, snakes are usually found basking outside hibernacula. Starting in late April-May, cottonmouths are found in characteristic ambush postures, indicating typical foraging behavior and the commencement of the regular activity season. August-September is likely the primary mating season at this study area, and during this time pregnant females can be found basking (Graham et al. 2008). Snakes become scarce after September as they return to hibernacula (S. Graham, unpublished results). Cottonmouths were captured and sampled during the spring basking period when acclimatization was expected (April). Additional groups were sampled well into the feeding period (June), and during the late summer mating/incubation period (August), when regular active season performance was expected. In the metropolitan Atlanta area, local climate is on average ~ 5-8 °C cooler during the spring basking period relative to June and August (NOAA.gov, 2010).

The cloacal temperature of each snake was recorded (nearest 1°C) using a probe thermometer. A blood sample for snakes used in complement experiments was then immediately collected from the caudal sinus using a 26g heparinized needle and 1 ml

syringe, and plasma was immediately separated from whole blood in the field using a battery-operated centrifuge. This sample was stored on dry ice for up to two days, and transported back to the lab for storage at -80°C. Summer samples were obtained in August 2008 (N = 20), and spring samples were obtained April 2009 (N= 10). A second series of summer samples were taken June 2009 (N = 9). August sample sizes were considerably larger due to the acquisition of a larger number of samples for a comparative reproductive study in preparation (S. Graham, unpublished results). Finally, a second series of April samples were collected in 2010 (N = 9).

#### *Immune assay*

Early studies indicate that complement can be frozen for one to two weeks and maintain its viability (Sherwood, 1951). Therefore, a functional bacteria lysis assay of field collected plasma samples (following Tieleman et al. 2005) was employed four days after blood collection to determine complement efficiency. This assay measures a plasma sample's ability to lyse bacteria by comparing control bacteria solutions to those spiked with cottonmouth plasma. Bacteria lysis is calculated as the percentage bacteria lysed compared to controls, with fewer numbers of bacteria colonies indicating higher percentage lysis. Five cottonmouth samples were serially diluted with L-glutamine-CO<sup>2</sup> medium to determine a dilution factor useful for experimentation (e.g., a dilution factor that would allow detection of variation between groups or temperatures). This is the first validation step used in studies utilizing bacterial lysis assays, and different dilution factors have proved useful for different vertebrates (Tieleman et al. 2005; M. Mendonça, unpublished results). Samples were diluted to 1:6.25, 1:12.5, 1:25, 1:5, and 1:100. Commercially available *E. coli* pellets were diluted (1:1000) with phosphate buffered saline (PBS) and incubated at 37°C for 1h. Test solutions

combined 10 $\mu$ l of the bacteria dilution with 140 $\mu$ l growth medium (control), or 140 $\mu$ l growth medium + plasma dilution (plasma treated). These samples were then allowed to react at room temperature simultaneously for 1h. Each sample was then spread in duplicate on agar plates and incubated at 37°C for 12-16h. Colonies on each plate were counted, averaged (across duplicates), and compared to the mean colony counts of four replicated, unchallenged control plates. Percent bacterial killing was calculated as plasma treatment colony counts/mean control colony counts x 100. Results from the serial dilution determined intermediate bacteria lysis for the 1:100 dilution (see section 3.1), therefore, for all subsequent experiments, cottonmouth plasma was first diluted 1:100 since this reduced the amount a plasma needed for assays.

For the temperature experiments, each plasma treated bacteria sample (1:100) was aliquoted and incubated for 1h at eight ecologically-relevant temperatures (12, 16, 18, 20, 22, 24, 26, and 30°C) simultaneously in an aluminum thermal gradient heat block. Samples were then spread on agar plates and incubated as in above. Samples from August, April, and July were tested using the identical temperature gradient experimental design. Within the above temperature range, results were similar (see Results), so we conducted an additional temperature gradient experiment within a wider temperature range (12, 16, 20, 22, 24, 28, 32, 36, and 40°C) collected in April 2010. Samples for this experiment were collected from the same study site, and were otherwise processed and assayed as in above.

To confirm that experimental temperature had no effect on control bacteria colony growth, during the first (August) experiment, control samples were run at each temperature alongside plasma + bacteria samples. The results of this experiment confirmed that

temperature had no association with control colony growth (see results), and we therefore used room temperature for control plates in subsequent experiments.

To confirm that complement is primarily involved in the lysis of bacteria in our assay, July 2009 samples were tested using an additional, complement-inactivating temperature experiment (56°C). This experiment was slightly different from the above assays; samples (N = 9) were not run simultaneously with the thermal gradient experiment. Instead, the samples were either kept at 22°C or 56°C for 30 min, and were then allowed to react with *E. coli* for one h at 22°C (e.g., 30 min at 56°C simultaneously inactivates *E. coli* and complement). Control solutions were run alongside these samples simultaneously. They were then plated, incubated, and calculated identical to the other experimental samples.

#### *Data analysis*

Percent bacteria lysis values were angular transformed to satisfy assumptions of continuousness and normality. Negative percent bacteria lysis values were assumed to indicate no bacteria lysis and were converted to zeroes; this only occurred in a few cases at the lowest experimental temperatures (12-16°C). The relationship between dilution factor and angular-transformed % bacteria lysis of each sample was tested using linear regression. The relationship between temperature and angular-transformed % bacteria lysis were tested similarly. Slopes of the relationship between this immune measure and temperature for the different seasons were compared statistically by comparison of slopes, and the response to the temperature treatment were compared between seasons using an ANCOVA with temperature as the independent variable, % bacteria lysis as the dependent variable, and season as the main effect. For our experiment utilizing a wider temperature range, we tested for differences in angular transformed bacteria lysis between temperatures using an ANOVA.

Samples used in the complement inactivation experiment tested at 22°C and 56°C were compared using a paired sample t test. All statistical tests were performed using SPSS version 11.5 (SPSS Inc., Chicago IL) with  $\alpha = 0.05$ .

## Results

There was a significant negative correlation between bacteria lysis capacity and dilution factor, with more dilute plasma (1:100) resulting in less bacteria lysis than more concentrated plasma ( $R^2 = 0.81$ ;  $p < 0.0001$ ; Fig. 1). There was a significant positive relationship between percent bacterial killing and experimental temperature from samples collected during August ( $R^2 = 0.63$ ;  $F = 345.25$ ;  $p < 0.0001$ ), April ( $R^2 = 0.73$ ;  $F = 214.68$ ;  $p < 0.0001$ ), and June ( $R^2 = 0.66$ ;  $F = 135.20$ ;  $p < 0.0001$ ; Fig. 2). The slopes of the percent bacteria lysis/temperature relationship did not differ significantly between these periods ( $F = 0.14$ ;  $p > 0.05$ ; Fig. 2). There was also no main effect of season on percent bacteria lysis ( $F = 0.51$ ;  $p > 0.05$ ). There was no correlation between temperature and percent bacteria lysis for the control samples (Fig. 2). The mean field body temperature of cottonmouths from this population was 22.73°C (Fig. 3). The observed body temperatures were all within the range of temperatures we tested with the three bacterial lysis experiments for August 2008, and April and June 2009 (12-30°C; Fig. 3). We detected significant differences in bacteria lysis between temperatures in our fourth temperature experiment (overall model:  $F = 7.323$ ;  $p < 0.0001$ ; Fig. 3), such that samples incubated at lower temperatures had lower bacterial lysis capacity than those incubated 24-38°C, and those incubated at the hottest temperature (40°C) did not differ significantly from those incubated at low temperatures (Fig. 3). Treatment at 56°C completely inactivated the antibacterial capacity of cottonmouth plasma ( $N = 9$ ;  $F = 51.97$ ;  $p < 0.0001$ ; Figure 4).

## Discussion

We documented a rigid temperature/functional relationship for the bacteria lysis capacity of cottonmouth plasma, including a significant positive correlation between temperature and performance in all three seasons tested within the range of field body temperatures experienced by wild cottonmouths. This functional relationship is consistent with what is known about the vertebrate complement system; complement is temperature labile and the plasma of ectotherms typically exhibits modest bactericidal properties at low temperatures (Gigli and Austen, 1971). A functional immune study conducted on the American alligator (*Alligator mississippiensis*) determined antimicrobial properties of plasma and used similar techniques as those described here, concluding that an active complement system was primarily involved (Merchant et al. 2003). Specifically, exposure of cottonmouth and alligator plasma to 56°C (a standard complement-inactivation temperature; Vogel and Müller-Eberhard, 1985; Merchant et al. 2003) completely inactivated the plasma's antibacterial activity, as in mammalian (human) complement. However, similar assays have been utilized as a measure of the simultaneous performance of multiple innate immune components (e.g., Tieleman et al. 2005; Matson et al., 2006; Sparkman and Palacios, 2009), and our functional assay may have included some contribution of other innate immunity components (e.g., natural antibodies) in addition to complement.

We hypothesized complement performance would exhibit a co-adapted optimal thermal reaction norm, and predicted that the relationship between complement efficiency and temperature would resemble bell-shaped curves centered at the thermal preferendum of the cottonmouth (22.7°C —this study) within ecologically-relevant field body temperatures (Angilletta et al. 2002; Arnold and Peterson, 2002). Instead, we demonstrate maximum

complement performance at hotter temperatures than those typically experienced by wild cottonmouths (Fig. 3). Complement bacteria lysis continued to perform maximally at temperatures approaching and equaling the lethal body temperature of most snakes (~ 40°C; Brattstrom, 1965). The high variance observed among samples tested at 40°C (Fig. 3) is due to some samples maintaining bacterial lysis capacity at this temperature, and others losing this capacity entirely, probably since this temperature is very near the exact inactivation temperature for key complement proteins in this snake. This pattern is consistent with the hypothesis of ecological trade-offs in thermal performance (Huey and Slatkin, 1976), and indicates that cottonmouths are active at body temperatures lower than what would support a maximal complement response, possibly due to other physiological or ecological constraints.

We predict that behavioral fever (e.g., an increase in body temperature in response to infection mediated by non-physiological selection of higher ambient temperatures) is likely during infection in this snake, and that the relationship we report may support the snake's ability to achieve an immunologically beneficial behavioral fever. The fever response—a brief increase in the body's physiological thermal set-point (Roberts, 1979)—was once poorly understood due to lack of strict experimental work, and functional studies in lizards eventually demonstrated the adaptive significance of fever (Roberts, 1979; Kluger, 1979). Studies demonstrate behavioral responses to infection in ectotherms (e.g., basking in amphibians, Richards-Zawacki, 2009, and lizards, Kluger, 1979), and here, we describe an immunological mechanism (increased complement efficiency with higher temperatures) that may enable this behavior to clear pathogens.

Furthermore, cottonmouths may increase their immune performance with little additional energetic cost by behavioral thermoregulation. The fever response is largely reliant

on the functional performance of complement (Blatteis et al. 2000), and is one of the most energetically costly components of vertebrate immunity (Nelson, 2004). The ability to behaviorally influence complement performance may result in short-term energetic savings during infection, and also allow for adjustment of annual energy budgets. For example, during summer months, cottonmouths could reduce complement production and rely on enhanced immune performance simply by exploiting warmer ambient temperatures. In this way ectotherms may demonstrate an energy-saving strategy within the context of the immune system (e.g., Martin et al., 2006; French et al., 2007).

We also predicted that complement efficiency would acclimatize, with snakes sampled in spring having higher bacteria lysis capacity at lower temperatures than summer snakes. We found no evidence for such a relationship, suggesting that the complement system of cottonmouths invariably exhibits a fixed temperature relationship throughout the year, and no physiological mechanism appears to be in place to increase performance the cooler season we tested (April). However, it is possible that acclimatization occurs during other months when hibernating snakes are considerably more difficult to locate.

Our results are surprising considering the large body of literature documenting physiological reaction norms which overlap with thermal preferences in ectotherms (Huey and Kingsolver, 1989; Angilletta et al. 2002), as well as those documenting seasonal acclimatization in multiple physiological systems (Rome et al. 1992). However, there are other examples of thermal performance optimum that do not match field body temperatures (e.g., locomotory performance in nocturnal geckos, Huey et al. 1989), and other studies demonstrate decreased immune performance during winter for components of the ectotherm immune system (Zapata et al. 1992). Interestingly, certain enigmatic behaviors of wild



reptiles suggest that cool temperatures may indeed significantly reduce their ability to combat pathogens. For example, prolonged spring and winter basking is commonly observed in many snake species (Prior and Weatherhead, 1996; Olsson et al, 1997; Row and Blouin-Demers, 2006; Schuett et al. 2006), and some researchers have remarked that lethargic and sick reptiles bask, even when basking is an uncommon component of their normal behaviors (Dodd, 1988; Carter et al. 2005). However, little is known about the effects of low temperatures on pathogen growth or virulence in ectotherms, and it has been hypothesized that lower body temperatures are sufficient pathogen control mechanisms (e.g., Hsu, 1998).

The immune system of ectotherms is sometimes considered inefficient and primitive (e.g., Flajnik, 1996; Hsu, 1998; Zarkadis et al. 2001), but the invariable relationship between cottonmouth complement efficiency and temperature we document can instead be viewed as a rigid immunological “seesaw” by which behavioral responses—rather than energetically expensive metabolic and physiological mechanisms—may endow them with flexibility during an immune response. Most physiological systems in ectotherms are similarly streamlined for energy efficiency, and the physiological differences between ectotherms and endotherms are now viewed as two mutually exclusive, yet equally adaptive strategies (Pough, 1983). Growing evidence from studies in the field of ecological immunology confirm that trade-offs exist between immunity and other life history components (e.g., reproduction, energy balance, growth; Martin et al. 2006; French et al. 2009), and with their variable body temperatures, ectotherms may excel at negotiating these trade-offs. Trade-offs must also be balanced with ecological costs associated with behavioral thermoregulation (e.g., increased visibility to predators), costs for which ectotherms have evolved a myriad of solutions (Huey and Slatkin, 1976; Pough, 1983). Furthermore, unlike mammals and birds,

ectotherms still derive some complement protection at fairly low temperatures (Gigli and Austen, 1971; this study).

Ultimately, this temperature/performance relationship may give ectotherms an additional layer of immunological flexibility not available to endotherms. Cottonmouths have two possible ways to increase complement performance: adjusting their body temperatures behaviorally, or by increasing complement protein concentration in their blood. However, given the constrained temperature performance curves we demonstrate, it is also possible that the thermal performance of ectothermic complement (and other immune components) may contribute to their sensitivity to certain (e.g., cold-tolerant) pathogens. For example, the chytrid fungus (*Batrachochytrium dendrobatidis*), which has been linked to worldwide population declines in amphibians (Lips et al. 2006), has a fairly low temperature tolerance range and is inactivated at higher body temperatures (Woodhams et al. 2003). Given the temperature performance we report here for a reptile, researchers focusing on amphibian immunity should consider complement performance and its thermal interactions. In addition, comparative studies in other vertebrates such as reptiles should be undertaken to determine if other ectotherms similarly struggle with cold tolerant pathogens, and to determine if ecological and evolutionary trends exist in the thermal performance of vertebrate complement.

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## **Figure Legends**

**Figure 1** The effect of a serial dilution on angular-transformed percent bacteria lysis capacity of cottonmouth plasma samples.

**Figure 2** The relationship between temperature and angular-transformed percent bacteria lysis capacity from plasma samples collected in three different seasons, as well as controls not treated with cottonmouth plasma.

**Figure 3** Field body temperatures of wild cottonmouths from the study area (frequency = number of cottonmouths observed at each temperature; right y axis), and a comparison of angular-transformed percent bacteria lysis capacity of snake plasma tested across a wide range of temperatures (N = 9) from the same population (left y axis).

**Figure 4** Comparison of angular-transformed percent bacteria lysis capacity for samples treated at room temperature versus 56° C.

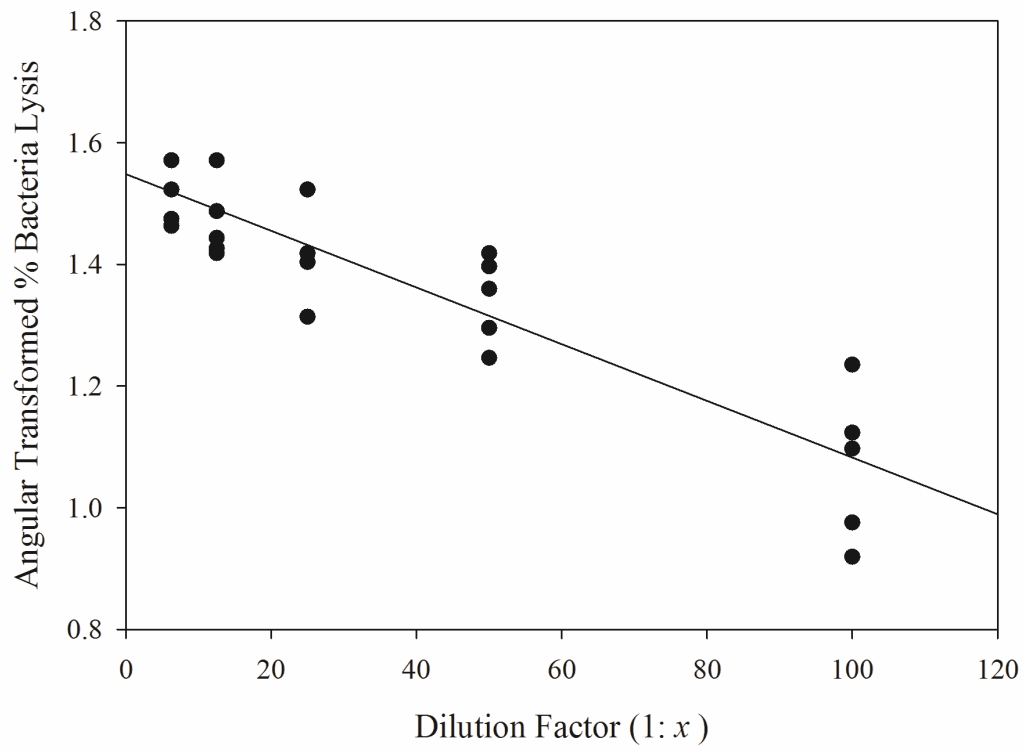


Figure 1

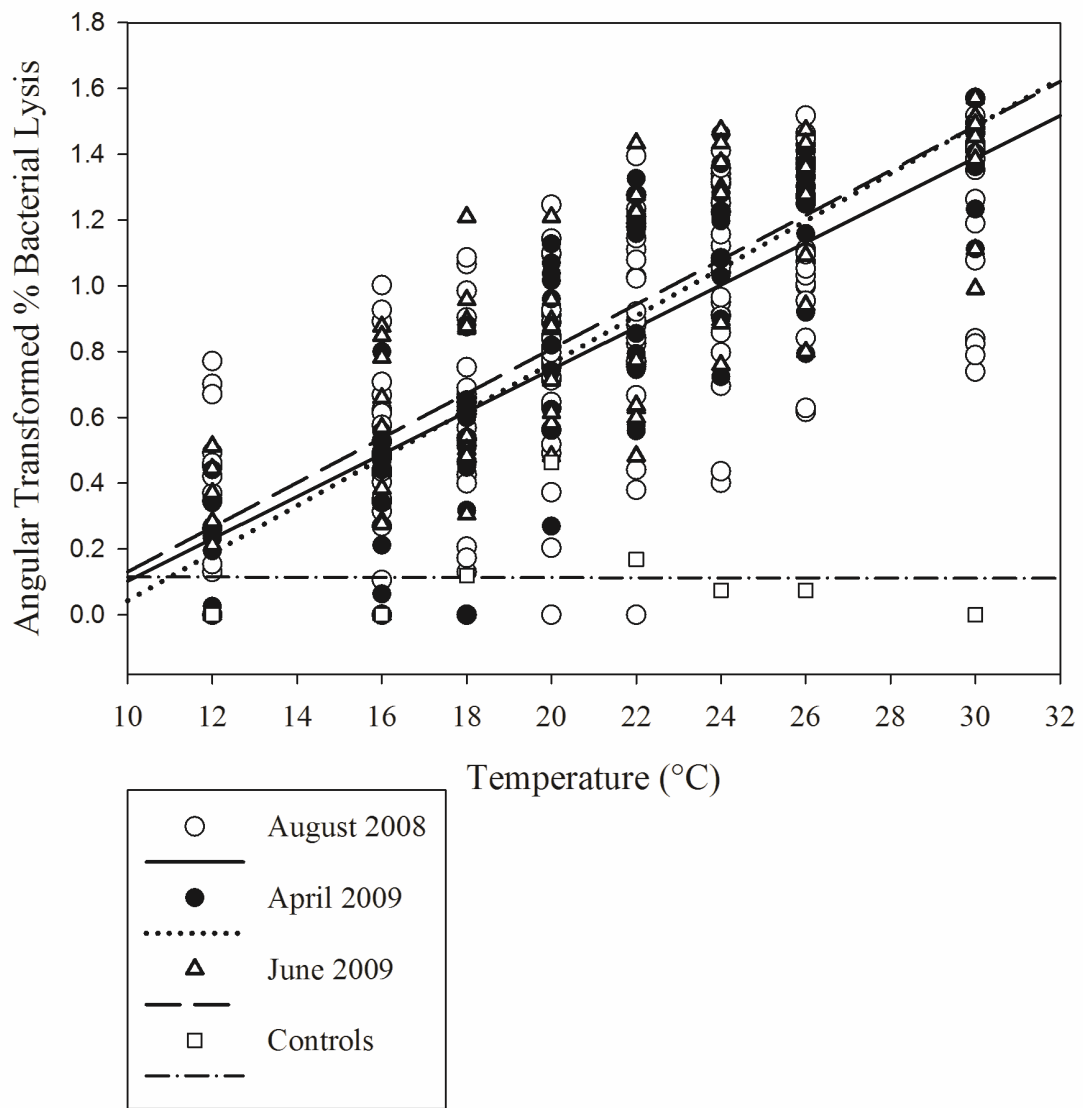


Figure 2

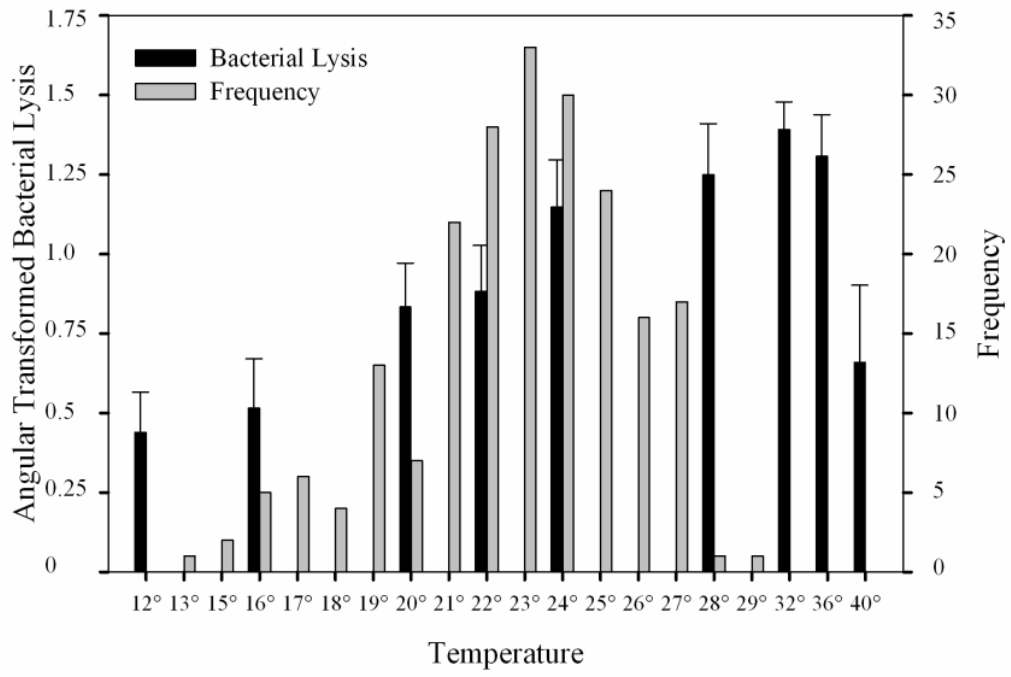


Figure 3

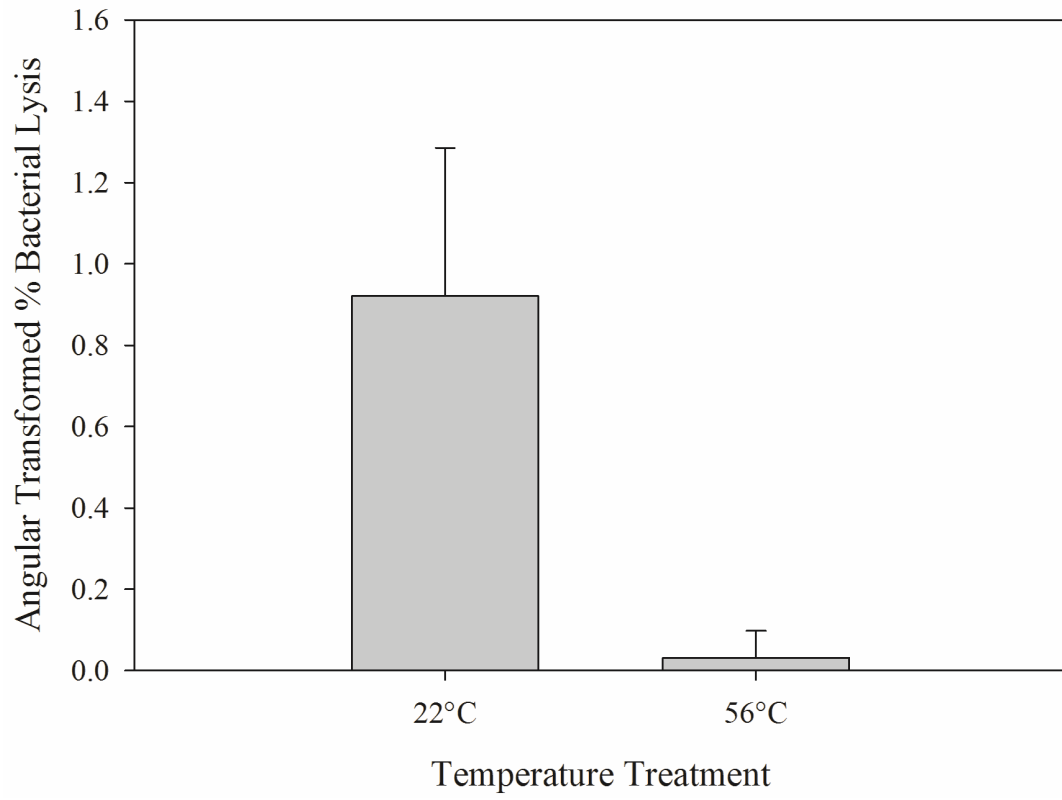


Figure 4

## Chapter 3

### Seasonal changes of steroid hormones and complement performance in the cottonmouth

(*Agkistrodon piscivorus*)

**Abstract:** Due to hypothesized life history trade-offs between reproduction and immunity, concomitant seasonal changes in immune function and hormone levels are often observed in wild vertebrate populations. In ectotherms, immune function is also highly temperature dependent, and thus in temperate zone species seasonal variation in temperature can also influence immune function. In this study I describe reproductive events and the seasonal cycle of steroid hormones in cottonmouths. In addition, seasonal variation in a component of innate immunity was determined and compared to seasonal reproductive events. The reproductive cycle and hormone pattern in cottonmouths were similar to other pitvipers with a type II vitellogenic cycle and biennial reproduction. I determined significant seasonal variation in complement performance, however, this variation did not correspond in any obvious way with annual reproductive events, body temperature, or body condition. It is possible that other components of immunity could correspond with these or other unmeasured factors, or the decline in seasonal innate immune performance observed may instead be due to seasonal variation in pathogen abundance, virulence, or diversity.

## Introduction

Ecological trade-offs between immune function and reproduction and/or energy reserves are hypothesized to occur on a seasonal basis (Nelson and Demas, 1996; Nelson et al., 2002; French et al., 2009). These tradeoffs are in part regulated by the neuroendocrine system, which interfaces with the immune system at various levels (Nelson et al., 2002). Squamates are a good choice for studies attempting to sort varying interacting components of an integrated organism in a seasonal environment, due to their reliance on external energy sources to achieve thermal optima for performance, and lesser reliance on consumed energy for growth, maintenance, and reproduction (Nelson et al., 2002). Seasonal patterns of endocrine/immune interactions have been studied in reptiles (reviewed in Zapata et al., 1992; Nelson et al., 2002), however, recent interest in molecular characterization of immunological components—even in comparative studies—has ignored squamates, probably due to a lack of genomic information, and taxonomic biases (Bonnet et al., 2002). Fortunately, recent discoveries in non-traditional model organisms (Flajnik, 1998; Cooper and Adler, 2006) have paved the way for additional studies in these animals.

Understanding the seasonal patterns of immunity and susceptibility in squamates has a practical benefit; most models of zoonotic disease transmission do not consider ectotherms despite emerging evidence of their possible status as pathogen reservoirs and amplifiers (Lane and Loye, 1989; Klenk and Komar, 2003; Klenk et al., 2004; Giery and Ostfeld, 2007). Ectotherms can potentially maintain pathogens at higher blood titers for a longer period of time due to possession of an apparently inefficient adaptive immune response (Zapata et al., 1992; Hsu, 1998). Squamates also may play a role in overwintering of pathogens in zoonotic disease cycles (Gebhardt and Hill, 1960; Spalatin et al., 1964). At lower body temperatures

associated with hibernation, the capacity to clear infections is compromised (Tait, 1969; Lock et al., 2003), and immune function is potentially depressed during winter in reptiles independent of body temperature (Zapata et al., 1992). Therefore, determining basic aspects of infection duration in reptiles—including seasonal cycles of immune readiness or decline—will facilitate our understanding of zoonotic disease transmission, since these factors are key components of transmission modeling (Unnasch et al., 2006).

Seasonal variation of immune function in many species has been determined using various measures, from histological characterization of immune tissues (seasonal involution and recrudescence of thymus and spleen), to more direct measures of antibodies and T-cell proliferation in response to novel antigens (Zapata et al., 1992). Most of these studies have suggested depressed immune function in winter and during the breeding season in squamates, and this appears to be partially independent of ambient temperature, since lizards held at constant temperatures in the laboratory still exhibit seasonal changes (Zapata et al., 1992). Steroid hormone parameters are correlated with immune measures in a lizard (*Chalcides ocellatus*); the stress-associated hormone corticosterone (CORT) appears to have permissive effects on these immune measures at low levels, and inhibitory effects at high levels (Saad and Ridi, 1988). These findings are consistent with studies in other study organisms (Sapolsky et al., 2000). Sex steroids show similar effects; high levels of testosterone (T) during the breeding season are associated with thymic involution and lowered lymphocyte function in *C. ocellatus* (Saad et al., 1990).

The goal of this study is to describe the seasonal patterns of immune measures in a wild population of the cottonmouth (*Agkistrodon piscivorus*) and their relationship to seasonal levels of plasma sex steroids. The cottonmouth is an excellent study animal since it



is large (typically ~ 300g—facilitating sample collection and immune challenges), abundant, easy to collect in appropriate habitats, and well known from the perspectives of information on its natural history (Gloyd and Conant, 1990) and reproductive cycle (Burkett, 1966; Johnson et al., 1982; Zaidan et al., 2003; Graham et al., 2008). I hypothesized that a component of innate immunity—complement bacterial killing ability—would vary seasonally and will be associated with seasonal patterns reproduction and steroid hormones. Specifically, and consistent with previous studies that have investigated seasonal immune variation in reptiles (Zapata et al., 1992), I predicted seasonal patterns of corticosterone (CORT), estradiol (E2), progesterone (P4) testosterone (T) would show inverse associations with complement performance. For this variation in immune function to be linked to seasonal levels of steroid hormones, I also expect it to exist while also taking into account snake body temperature and body condition.

## **Materials and Methods**

### *Field Sampling*

Four large beaver ponds in Tuskegee National Forest (32.43282°N, 85.64590°W) were sampled one night and the following day on an alternating basis such that each pond was sampled once per month. Sampling cottonmouths entailed walking slowly along the pond edge searching for individual snakes, capturing them with metal tongs, and securing their head and body in clear plastic tubes. Immediately upon capture, time of capture and ambient/body temperature (e.g., cloacal temperature, nearest 1° C, using a temperature probe) were recorded.

A blood sample (up to 1ml) was taken from the caudal sinus using heparinized, 0.26g tuberculin syringes. Time until blood collection was then recorded (mean 6.8 min; range 2-19

min from capture). Blood samples for use in hormone analyses were collected April-October 2007-2008 and were stored in labeled 1.5ml centrifuge tubes, placed on ice packs for no more than 24 h, centrifuged (5 min at 5000 rpm), and plasma stored at -20° C until hormone assays were performed (November 2008). Blood samples for use in immune assays were collected April-October 2009, and were centrifuged in the field using a battery-powered centrifuge, stored in labeled 1.5 ml centrifuge tubes, placed on dry ice, and transported from the field and stored at -80° C for four days until immune assays were performed.

After blood collection, snakes (snout-vent-length—SVL; tail length—TL) were measured to the nearest 1.0 mm by stretching flexible measuring tape along their side. Snakes were categorized as to sex by probing the cloaca for the presence of hemipenes. To determine the breeding season, cloacal flushes were performed by injecting ca. 1ml of saline into the cloacal cavity, extracting the cloacal fluid into the syringe, storing the extract for no more than three days (3-5° C) in a 1.5 ml microcentrifuge tube, placing the extract on a slide, staining it (Hematoxylin and Eosin), protecting it with a cover slip, and examining the slide for the presence or absence of spermatozoa.

Each snake received a unique passive integrated transponder (PIT) tag subcutaneously, and recaptured snakes had their PIT tags read in the field with a portable PIT tag scanner. The location of each snake was recorded using a handheld GPS unit. The snakes were then placed in a cloth bag and weighed (nearest 1-5 g) using a Pesola spring scale. After processing, all snakes used for this study were released at their exact point of capture.

To reconstruct the female reproductive cycle of follicular development, snakes were dissected from the Auburn University Museum (AUM) and the University of Alabama Herpetological Collections (UAHC). The body cavity of each specimen was viewed by

making a ventral incision, and the diameter of all follicles were measured and averaged per snake (nearest mm). All females of mature body size (> 60 cm SVL; Ernst and Ernst, 2003) available at the museums were sampled across all collection months for which they were available.

### *Hormone Assays*

Testosterone (T), Corticosterone (CORT), Progesterone (P), and Estradiol (E2) were measured from field-collected plasma samples using commercially-available enzyme immunoassay (EIA) kits (Cayman Chemicals, Ann Arbor, MI). For T, only samples from one field season were used to establish the seasonal androgen cycle in males; three other studies (Johnson et al., 1982; Zaidan et al., 2003; Graham et al., in press) have established a similar seasonal pattern in this species, and only the timing of peak T in this population was germane to this study. The annual P4, E2, and CORT cycle of females were determined from both years.

Hormones were extracted from plasma using a column extraction protocol (Wong et al. 2008; Hoss et al., 2011) that is as efficient as ether extraction (Wong et al., 2008). Samples were processed and analyzed as in Graham et al. (2008), Wong et al., (2008), and Hoss et al., (2011). In brief, 200-350 µl of plasma was transferred into sterile, labeled 18 x 150 mm borosilicate vials, and diluted with 20 ml ultrapure water. Sep-Pak<sup>®</sup> 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. Diluted samples were then transferred through Tygon<sup>®</sup> 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 2 ml MeOH washes from the columns into 13 x 100 mm borosilicate vials that were then placed in

a water bath at 37°C so that MeOH was evaporated under a gentle nitrogen stream (~ 0.7 bar).

Samples were then re-suspended to an initial 1:2 dilution using EIA buffer provided in the EIA kits and adding 400 µl EIA buffer to each original 200 µl sample, and 700 µl buffer to each 350 µl sample, followed by vortexing for 30 min.

E2 and P4 assays were validated by assessing parallelism. A pooled cottonmouth sample from eight individuals not used in this study was serially diluted (1:1 through 1:64), and the standard curve produced from this dilution series was compared (comparison of slopes; Zar, 1996) to standard curves using P4 and E2 standards provided from the EIA kits. The slopes of the P4 standard and pooled sample dilution series were identical ( $t_{10} = 0.0$ ;  $p > 0.05$ ), and slopes of the E2 standard and pooled sample dilution series were nearly so ( $t_{11} = 0.02$ ;  $p > 0.05$ ). Thus, parallelism was achieved, and the kits therefore measured cottonmouth hormones in an identical fashion to that measured from standards provided in the kits. T and CORT kits were validated previously using identical methods (Graham et al., 2008). Quantitative recovery was assessed using the pooled sample. The sample was equally distributed (110 µl) in eight micro-centrifuge tubes, and mixed with an equal volume of standards provided in the kit (500, 250, 125, 62.5, 61.3, 15.6, and 7.8 pg/ml concentrations). Recovery was based on the known P4/E2 concentrations present in the sample. For E2 and P4, slopes were indistinguishable from 1, suggesting excellent recovery. Minimum estimated recovery was 158% for E2 and 138% for P4. Quantitative recovery of CORT and T kits was assessed previously using identical methods (Graham et al., 2008). Assays were performed in duplicate using instructions provided in the kits. Intra-assay coefficients of variation ranged between 1.28-3.97% (E2), 3.0-3.9% (CORT), 2.4% (T), and 1.3-2.5% (P4). Inter-assay

coefficients of variation ranged between 3.0-10% (E2), 4.4-18.6% (CORT), and 2.1-14.6% (P4). All T samples were run on the same plate and thus inter-assay coefficient of variation was not calculated for this hormone.

### *Immune Assay*

Indirect estimates of complement activity were measured on field-collected plasma samples using a functional bacterial killing assay (BKA). Complement is a potent innate immune component of vertebrate plasma, which can directly adhere and lyse pathogen cell membranes (Fujita et al., 2004). Early studies indicate that plasma can be frozen for one to two weeks and maintain its complement viability (Sherwood, 1951). Therefore, field collected plasma samples were assayed (following Tieleman et al., 2005; Millet et al., 2007) four days after blood collection.

This assay was developed for immune studies in birds and has become common in comparative immunological research because it requires no species-specific reagents (Tieleman et al., 2005; Millet et al., 2007). 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) based upon a previous study that determined this as an appropriate dilution for this species (Graham et al., submitted). 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 (Merchant et al., 2003; Graham et al., submitted). However, other studies have adopted this assay as a

general measure of constitutive immunity (Teileman et al., 2005; Millet et al., 2007; Sparkman and Palacios, 2009), and therefore other plasma immune components may also be responsible for the effects observed.

Commercially available *E. coli* pellets were diluted (1:1000) with phosphate buffered saline (PBS) and incubated at 37°C for 1h. Test solutions combined 10µl of the bacteria dilution with 140µl growth medium (control), or 140µl of the 1:100 plasma + growth medium dilution (plasma treated). These samples were then allowed to react at room temperature simultaneously for 1h. Each sample was then spread in duplicate on agar plates and incubated at 37°C for 12-16h. Colonies on each plate were counted, averaged (across duplicates), and compared to the mean colony counts of four replicated, unchallenged control plates. Percent bacterial killing was calculated as [(plasma treatment colony counts) / mean control colony counts] x 100.

#### *Data Analysis*

Bacteria lysis values were angular transformed (arcsine square root), and plasma steroid hormone values were log transformed to meet assumptions of continuousness and normality. However, untransformed values are presented to assist interpretation. To determine if there was an effect of body size on hormones or bacteria lysis capacity, I regressed hormone and bacteria lysis values on SVL using linear regression. I examined differences in bacteria lysis values between males, females, and juveniles using ANOVA, found none ( $p > 0.05$ ), and thus combined these groups across months for analysis. Seasonal differences in sex steroids and innate immunity were determined by comparing mean monthly variation in plasma levels of CORT, E2, P (adult females), T (adult males) and percent bacterial killing capacity (adults and juveniles) using ANOVA. If SVL was significantly correlated with the response variable,

I used SVL as a covariate in the analysis. The relationship between bacteria lysis and body temperature was compared using linear regression. Mean body temperature of cottonmouths sampled for bacteria lysis capacity was compared across months using ANOVA. Percentage of males and females with sperm present in the cloaca was calculated and compared qualitatively across months. The mean follicle diameter for each female cottonmouth museum specimen was also compared across months. Statistics were analyzed using Statview 3.0, with  $\alpha = 0.05$ .

### Results

I found no significant monthly variation in CORT ( $F_{6,60} = 1.953$ ;  $p > 0.05$ ) or P4 ( $F_{6,65} = 0.633$ ;  $p > 0.05$ ) in female cottonmouths (Fig. 1); however, E2 was significantly positively correlated with SVL and varied significantly by month when SVL was included as a covariate ( $F_{6,47} = 4.398$   $p = 0.0013$ ; Fig. 1). Female follicle diameters from museum specimens exhibited a pattern similar to that described by Burkett (1966) and Wharton (1966); diameters were largest in spring prior to ovulation, and diameters were small during summer in non-reproductive females. Only one summer/fall vitellogenic female was detected in the dataset (Fig. 2). I detected no significant seasonal variation in T from male cottonmouths, however, sample sizes were low ( $T_{4,23} = 0.559$ ;  $p > 0.05$ ; Fig. 3), and the highest T values were detected Jul-Aug. Sperm smears indicated presence of sperm in males from April-August, and females with cloacal sperm present were detected in June and September (Table 1).

Angular transformed bacterial lysis varied significantly by month ( $F_{6,49} = 9.193$   $p < 0.0001$ ; Fig. 4), with highest percentage lysis in April-May, followed by a progressive decline in late summer-fall. Mean monthly body temperature varied significantly ( $F_{6,49} =$

2.372  $p = 0.04$ ; Fig. 4) due to slightly lower body temperatures recorded from snakes in June. However, there was no association between body temperature and angular transformed bacterial lysis ( $F_{1,54} = 0.021$ ;  $p > 0.05$ ; Fig. 5). Body condition did not vary by month in the snakes tested for bacteria lysis ( $F_{6,47} = p < 0.611$ ;  $p > 0.05$ ), or when snakes sampled for hormones and bacteria lysis were combined and analyzed together ( $F_{7,156} = 1.227$ ;  $p > 0.05$ ; Fig. 6).

### Discussion

This study provides evidence for seasonal variation in an innate immune component of cottonmouth plasma, which peaks in April-May and steadily declines throughout the rest of the active season into fall. Other studies have demonstrated seasonal variation in a diversity of immune components in reptiles, although results are inconsistent and often species-specific (Zapata et al., 1992). For example, thymic involution typically occurs in winter in most species (Zapata et al., 1992), however, in the snake *Psammophis schokari* and turtle *Mauremys caspica*, involution also occurs during summer (El Ridi et al., 1981; Laceta et al., 1985). Immune responses to antigens are usually most robust during warm months, especially spring, and lowest during winter (Zapata et al., 1992). However, low response seasons differ among species (Hussein et al., 1979; El Deeb et al., 1980; El Ridi et al., 1980; Laceta and Zapata, 1985; El Ridi et al., 1987; Saad and El Ridi, 1988), and for certain species responses to certain antigens were low in summer (El Ridi et al., 1981). Even within the same snake species (*Psammophis schokari*), the immune response to different antigens had different seasonal patterns (El Ridi., et al. 1981).

In many cases seasonal variation in immune function corresponds with seasonal changes in reproductive parameters, including steroid hormones, which led to formulation of



a model of seasonal endocrine modulation of immune performance (Zapata et al., 1992). This model is consistent with the view that seasonal reproduction and immune function represent a life-history trade-off (French et al., 2007; French et al., 2009). Studies have also determined sexually dimorphic immune responses in snakes, and have attributed these differences to differing levels of circulating sex steroids (Saad and Shoukrey, 1988; Saad, 1989). On the contrary, this study suggests complement function (percent bacteria lysis) does not track seasonal variation in hormone levels or reproductive events in cottonmouths, and was similar in males, females, and juveniles. Complement bacteria lysis capacity was lower July-October than April-June, and no obvious steroid hormone peak was confined to these months.

Female cottonmouths show a reproductive hormone pattern consistent with Taylor et al.'s (2004) study of western diamond-backed rattlesnakes (*Crotalus atrox*), which demonstrated increased E2 during vitellogenesis (late summer and spring). For CORT and P4, results differed from Taylor et al. (2004), who demonstrated seasonal increases in P4 and CORT coincident with pregnancy. However, pregnant individuals were rarely collected within my study site, precluding a thorough comparison between pregnant and non-pregnant females seasonally. Instead, baseline CORT and P4 levels did not statistically vary seasonally among the snakes sampled, most likely because the individuals sampled were either non-reproductive or undergoing vitellogenesis, rather than being pregnant. Similar findings were reported from a study of *C. oreganus* (Lind et al., 2010), and these authors also concluded that a lack of reproductive females in their dataset was responsible for the lack of seasonal variation observed. For a thorough comparison between pregnant and non-pregnant females from another study area, see Chapter 4.

The general pattern in males appeared similar to three previous studies that have demonstrated a late summer/fall peak of T in cottonmouths (Johnson et al., 1982; Zaidan et al., 2003; Graham et al., 2008); however, low sample sizes precluded a thorough comparison. Based upon microscopic analysis of sperm smears, male sperm presence preceded female sperm presence by two months, and mating is assumed to have occurred in midsummer through September, consistent with studies of seasonal cycles of testosterone, spermiogenesis, and mating in this species (Graham et al., 2008; Graham and Sorrell, 2010) in this species. Previous studies have determined presence of sperm in the vas deferens of male snakes year round (Aldridge and Duval, 2002; Graham et al., 2008). This study further indicates that male cottonmouths can have sperm present in the distal portion of the vas deferens/cloaca throughout the active season.

Glucocorticoids have demonstrated immunomodulatory effects (Harbuz and Lightman, 1992; Chrousos 1995; Dhabhar and McEwen, 1999; Sapolsky 2000), and associations between seasonal variation in circulating CORT with seasonal troughs of immune performance have been demonstrated in the skink *Chalcides ocellatus* (Saad and El Ridi, 1988). In this study, there appeared to be no seasonal variation in baseline CORT in the population of cottonmouths used in this study, and therefore no association between seasonal CORT and complement performance. Likewise, seasonal variation in immune performance was not explained by variation in body temperature; cottonmouths appear to maintain a relatively constant body temperature (within 2-4°C) across months, despite seasonal changes in ambient temperature. There was no correlation between complement performance and body temperature at the time of blood collection. These results are consistent with Graham et

al. (submitted; Chapter 2), who demonstrated a rigid thermal performance gradient for this innate immune function in this species.

Many more studies are required in multiple reptile species, incorporating numerous immune components, to determine if any clear ecophysiological or evolutionary trends occur in seasonal patterns of immune function. One potential limitation of this study was that it attempted to describe the seasonal pattern of reproduction, steroid hormone secretion, and innate immune function in cottonmouths using a composite of information from museum specimens and wild caught individuals sampled over several years. Although a better approach would have been to sample all variables from the same individual snakes, this proved to be logistically difficult. However, seasonal patterns of sex steroids and corticosterone (CORT) were similar to previous studies in cottonmouths (Graham et al., 2008) and other pitvipers (Taylor and DeNardo, 2004; Lind et al., 2010), and studies have shown that patterns of sex steroid secretion often are similar from year to year within this species (e.g., Johnson et al., 1982; Zaidan et al., 2003; Graham et al., 2008).

In the case of the cottonmouth, three non-mutually exclusive hypotheses can be proposed to explain the seasonal pattern of innate immune performance observed. First, sperm smear data appear to indicate a prolonged mating season in cottonmouths from this area, and this period corresponds generally with the observed trough of complement performance, suggesting a possible trade off between reproduction and immune function in this species (e.g., French et al., 2007; French et al., 2009). However, mating activity is rarely observed in snakes, especially cottonmouths (Graham et al., 2008; Graham and Sorrell, 2010), making this a difficult hypothesis to test. In addition, juveniles showed similar patterns to adults, making this explanation unlikely. Next, at the study area, annual drying of

the ponds occupied by cottonmouths is seasonally pronounced, and the timing of pond drying (June-October; unpubl. obs.) corresponds well with the timing of reduced complement performance. However, if this is a stressor for cottonmouths, it does not appear to affect body condition or activate the glucocorticoid stress response (e.g., CORT is not elevated at this time), although other physiological mechanisms (e.g., sympatho-adrenal axis stress response) I did not measure could be involved. Comparisons between different populations of cottonmouths, and across different years, may provide support for this hypothesis. Finally, given the inconsistent and species-specific trends observed thus far, studies of seasonal trends in immunity should consider seasonal patterns of infection and parasites in wild vertebrate populations; the diversity of pathogen life histories, phenology, virulence, and ecology impacting different vertebrate populations perhaps best explain the observed patterns. Simultaneous consideration of seasonal changes in hormones, immunity, and infection in numerous vertebrates will ultimately unravel this problem.

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**Table 1** Results from sperm smear analysis of male and female cottonmouths.

<b>Month</b>	<b>N (males)</b>	<b>N (females)</b>	<b>% males with sperm</b>	<b>% females with sperm</b>
April	12	14	8	0
May	6	14	17	0
June	4	9	25	11
July	4	7	25	0
August	4	4	50	0
September	0	6	N/A	17
<b>total tested</b>	<b>30</b>	<b>54</b>		

## **Figure Legends**

**Figure 1** Seasonal variation in log transformed mean plasma CORT, P4, and E2 in female cottonmouths. Error bars indicate standard error.

**Figure 2** Seasonal variation in mean follicle diameter of female cottonmouths derived from museum specimens. Each open circle indicates the mean from an individual snake.

**Figure 3** Seasonal variation in log transformed mean plasma T in male cottonmouths. Error bars indicate standard error.

**Figure 4** Seasonal variation in angular transformed mean percent bacteria lysis (bar graph; standard error indicated) and mean body temperature (line plot; standard error indicated) in cottonmouths.

**Figure 5** Relationship between body temperature and angular transformed bacteria lysis in cottonmouths.

**Figure 6** Seasonal variation in body condition (SVL/mass) in cottonmouths. Error bars indicate standard error.

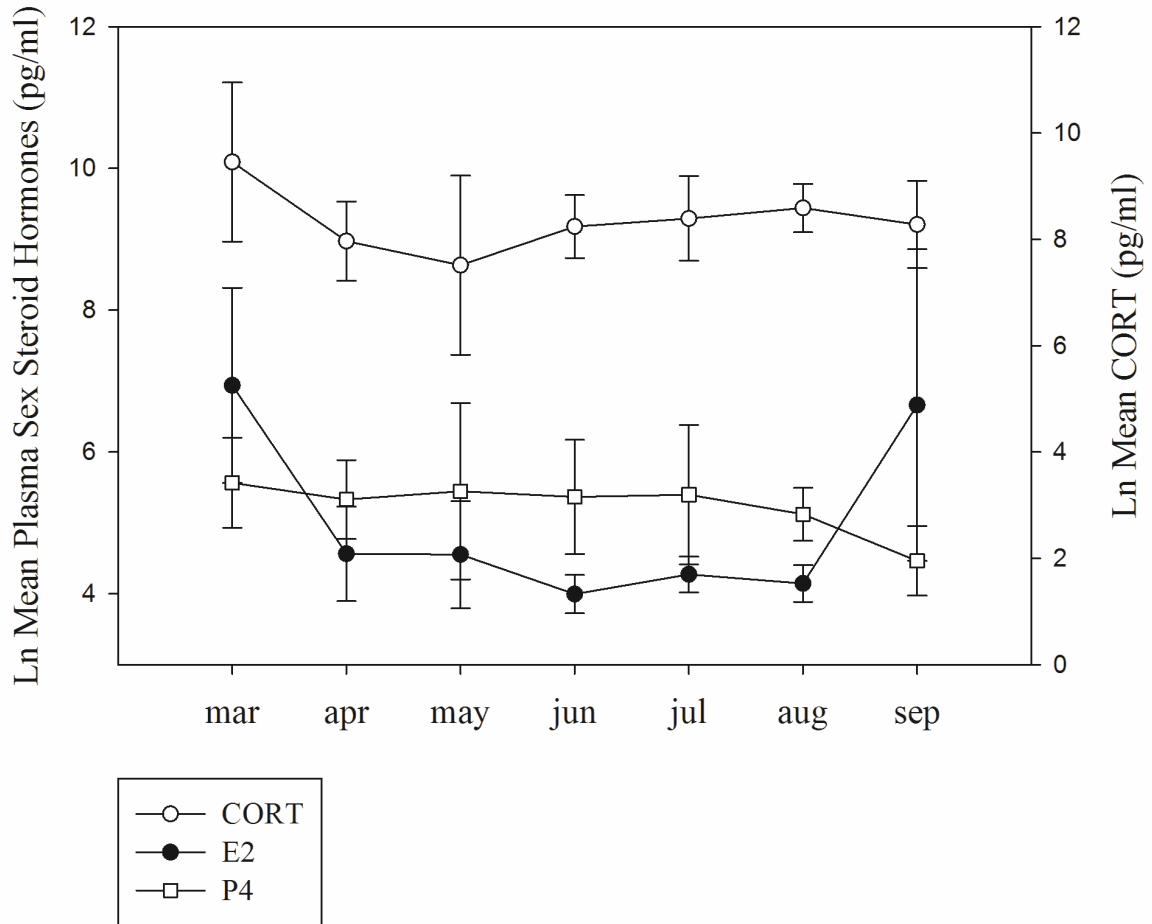


Figure 1

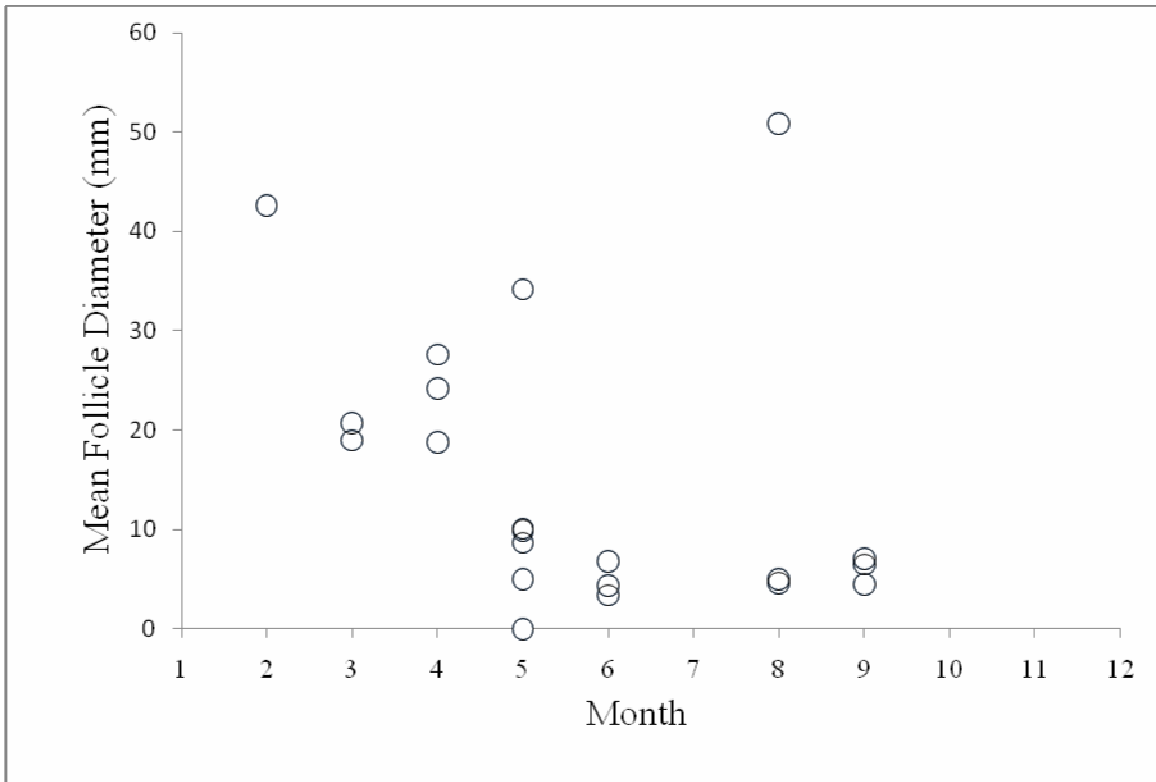


Figure 2

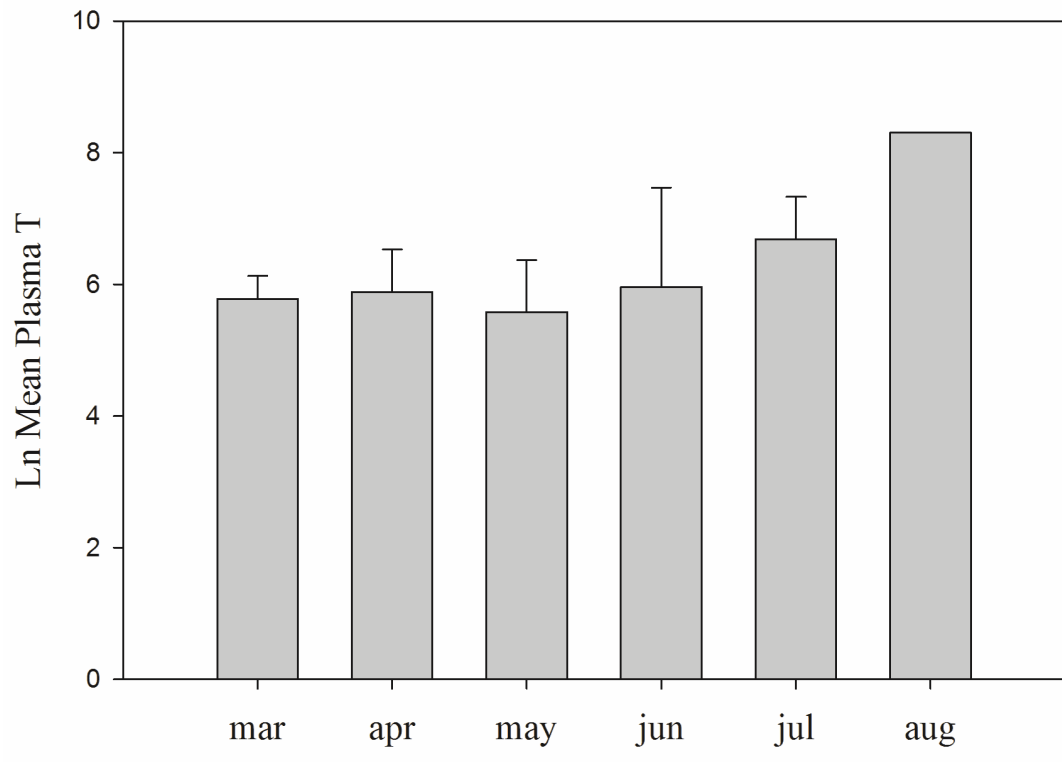


Figure 3

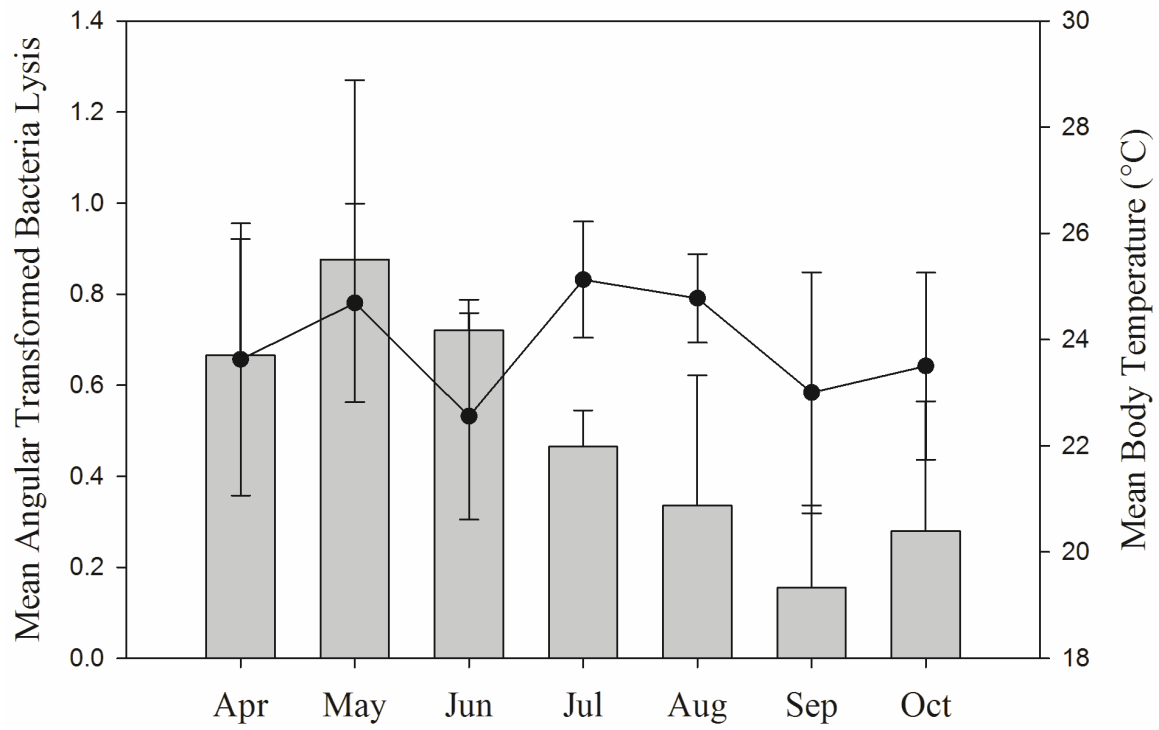


Figure 4



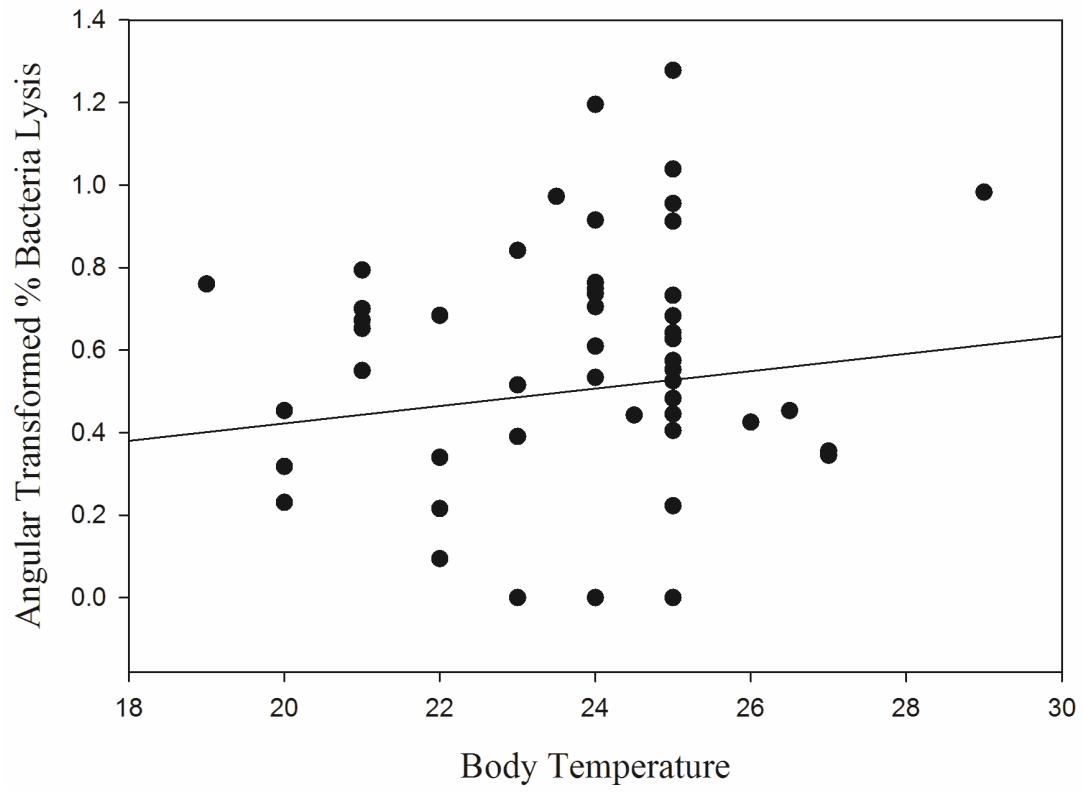


Figure 5

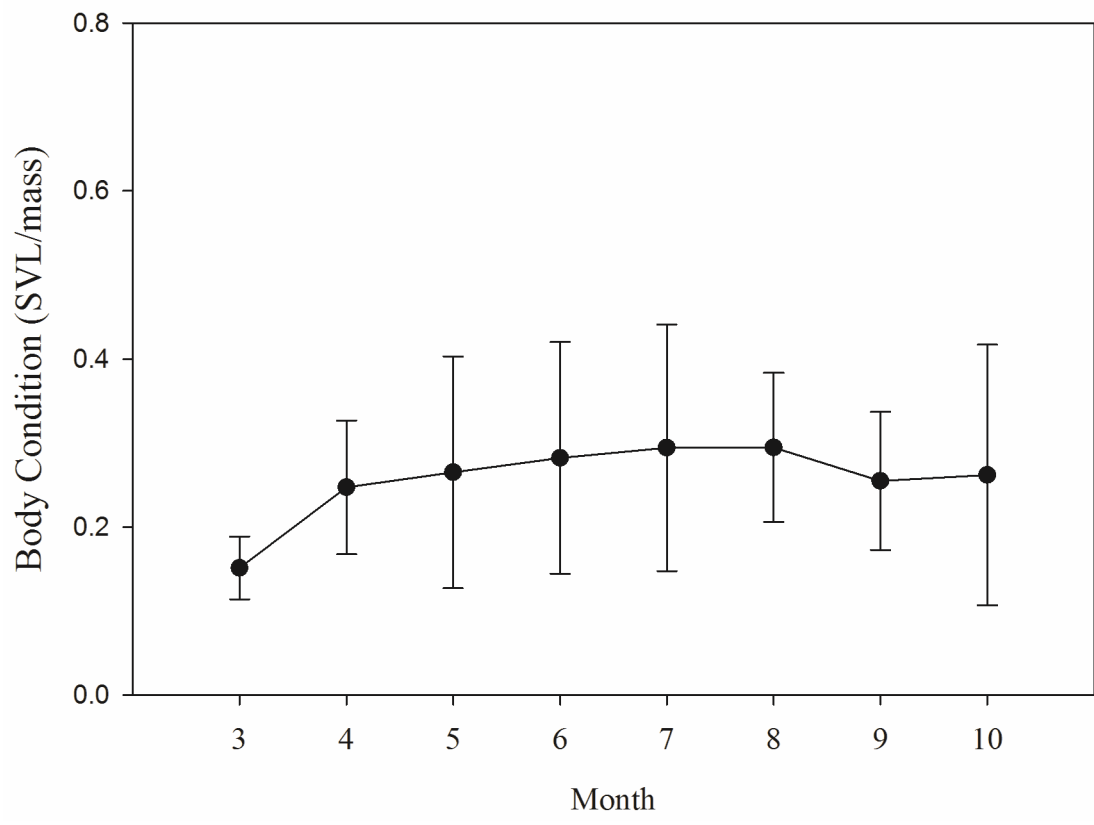


Figure 6

## Chapter 4

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

**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 levels of progesterone (P4) and significantly lower levels 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 surrogate 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.

## 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 (Wake, 1992; Blackburn, 1992; Blackburn, 2006), and presumably the conditions that lead to these convergent evolutionary developments also differed (Shine and Bull, 1979; 58, Wake, 1992; Shine, 2002; Webb et al., 2006). Interestingly, physiological systems that support pregnancy often are similar among unrelated groups (Wake, 1992). For example, the pattern of luteal hormone secretion (increased progesterone) during pregnancy is similar in squamates and mammals (Shine and Guillette, 1988; Callard et al., 1992). This observation suggests that a convergent, physiologically parsimonious pathway has evolved repeatedly during the development of this complex and demonstrably adaptive reproductive strategy (Shine and Guillette, 1988; Callard et al., 1992, Wake, 1992, Shine, 2002).

Another possible convergent mechanism that appears to support the developing embryo is immune privilege at the fetal-maternal connection. 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 Hauwel et al., 2004). The developing fetus is protected from the maternal immune system; fetal antigens should be recognized by the mother as allogenic (non-self) and attacked (Bainbridge, 2000). Several adaptations have developed in mammals to mitigate possible damage to the fetus, including reduced MHC expression and antigen presentation at the fet-

placental interface, active destruction of maternal T cells at the feto-placental interface, and expression of complement-inactivating molecules by the fetus (Mossman and Sad, 1996; Bainbridge, 2000; Kanellopoulos-Langevin et al., 2003; Druckman and Druckman, 2005). Comparative studies have explored changes in maternal immunity during pregnancy in viviparous fish (Haines et al., 2006), salamanders (Badet, 1984), and lizards (Saad and Deeb, 1990), 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 (Paulesu et al., 1995; Romagnoli et al., 2003; Haines et al., 2006).

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 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 (Druckman and Druckman, 2005) 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 (Cauchteaux et al., 2003; Kanellopoulos-Langevin et al., 2003). P4 has immunomodulatory effects on complement, and has been linked to decreased mRNA transcription of complement protein components (Brown et al., 1990).

Squamates have evolved viviparity independently over 100 times (Blackburn, 1985; Blackburn, 1992), and are therefore excellent subjects for comparative reproduction studies

(Blackburn, 2006), and for understanding the evolution of hormone-immune interactions in viviparous vertebrates. Squamates exhibit a range of maternal involvement in fetal development from none (lecithotrophic oviparity) to extensive placentation similar to the pattern exhibited by eutherian mammals (matrotrophic viviparity; Blackburn, 2006). At least some form of maternal contribution to the developing fetus (i.e., matrotrophy) is present in all viviparous squamates (Blackburn, 1992; Blackburn, 2006). 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 following 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., Graham et al., 2008), including a detailed study of fetal-maternal respiratory dynamics, which determined that placentation is not extensive in this species (Birchard et al., 1984), allowing a comparison between this species to previous studies in squamates having more extensive placentation. Cottonmouths exhibit a biennial reproductive cycle (Wharton, 1966) 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 differences between pregnant and non-pregnant females in innate immune (complement) performance, predicting that 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’), and the first of its kind in any snake.

## **Materials and Methods**

### *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 (Gloyd and Conant, 1990). Snakes were collected from a field site in Georgia, USA (see Graham et al., 2008 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 (Aldridge and Duval, 2002). Only females with enough stored energy initiate follicular yolk, and therefore only a subset of females is pregnant in any given year (Wharton, 1966). 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 (Burkett, 1966; Wharton, 1966).

Pregnant animals are easy to recognize morphologically and embryos can be detected by palpation (Farr and Gregory, 1991; Plummer, 1997). Pregnant females also are easy to locate because they choose distinct, open microclimates and exhibit gestational thermophily (Crane and Greene, 2008). 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*; Birchard et al., 1984).

### *Blood Sampling*

Seven pregnant and 10 non-pregnant females were sampled Aug-Sep 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 a heparinized, 26 gauge needles to collect blood (within two min) from the caudal sinus, and this was pipetted into a sterile, labelled 1.5 ml centrifuge tube. We separated plasma using a battery operated centrifuge, and equal volumes were aliquotted into two new, sterile, labelled 1.5ml centrifuge tubes before being fast frozen on dry ice. These samples were transported to the laboratory for storage (-80°C) until immune (four days later) or hormone assays (fall 2008) were performed. This sample collection protocol has been used in other studies of snake reproductive endocrinology (Taylor and Schuett, 2004; Schuett et al., 2005; Graham et al., 2008). We transported snakes to the lab in a secure transport device (Birckhead et al., 2004) 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 two 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).

### *Bacteria Lysis Assay*

Complement is a potent innate immune component of vertebrate plasma, which can directly adhere to pathogen cell membranes and lyse them (Fujita et al., 2004). Early studies indicate that plasma can be frozen for one to two weeks and maintain its complement viability (Sherwood, 1951). Therefore, a functional bacteria lysis assay of field-collected plasma samples (following Tieleman et al., 2007; Millet et al., 2007) was employed four 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 (Tieleman et al., 2007; Millet et al., 2007). 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) 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 (Merchant et al., 2003, Graham et al., in review). However, others have used this method as a proxy for constitutive innate immunity in birds and snakes (Tieleman et al., 2007; Millet et al., 2007; Sparkman and Palacios, 2009), and thus other innate immune components may have contributed to bacteria lysis as well.

Commercially available *E. coli* pellets were diluted (1:1000) with phosphate buffered saline (PBS) and incubated at 37°C for 1h. Test solutions combined 10µl of the bacteria

dilution with 140µl growth medium (control), or 140µl of the 1:100 plasma + growth medium dilution (plasma treated). These samples were then allowed to react at room temperature simultaneously for 1h. Each sample was then spread in duplicate on agar plates and incubated at 37°C for 12-16h. Colonies on each plate were counted, averaged (across duplicates), and compared to the mean colony counts of four replicated, unchallenged control plates. Percent bacterial killing was calculated as [(plasma treatment colony counts) / mean control colony counts] x 100.

### *Hormone Assays*

Steroid hormones were extracted from plasma using a column extraction protocol (Wong et al., 2008; Hoss et al., 2011). Extraction using this method yields results similar to ether extraction methodologies (Wong et al., 2008) 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., 2008; Wong et al., 2008; Hoss et al., 2011). In brief, 200-350 µl plasma was transferred into sterile, labeled 18 x 150 mm borosilicate vials, and diluted with 1000 µl ultrapure water. Sep-Pak<sup>®</sup> 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<sup>®</sup> 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 x 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  $\mu$ l EIA buffer to each original 200  $\mu$ l sample, and 700  $\mu$ l buffer to each 350  $\mu$ l sample. Plasma P4, CORT, and E2 concentrations were measured using EIA kits following the instructions in the kits as in Graham et al. (2008).

We validated P4 and E2 kits for *A. piscivorus* plasma by assessing parallelism and quantitative recovery. To assess parallelism, ten 200  $\mu$ l *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 standard and pooled sample dilution were identical ( $t_{10} = 0.0$ ;  $p > 0.05$ ), and slopes of the E2 standard and pooled sample dilution were nearly so ( $t_{11} = 0.02$ ;  $p > 0.05$ ). 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 *Agkistrodon piscivorus* CORT using identical methods (Graham et al., 2008). Recovery of P4 and E2 was estimated by spiking equal amounts of the pooled sample with an equal volume of the kit standards. Recovery was based on the known P4/E2 concentrations present in the sample. For E2 and P4, slopes were close to 1, suggesting excellent recovery. Minimum estimated recovery was 158% for E2 and 138% for P4. CORT recovery was assessed previously in this snake using identical methods (Graham et al., 2008). 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.

### *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 the square root of proportion values, enabling utilization of percent values for parametric statistics (Graham et al., 2009). 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. Body size (snout-vent-length, 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 StatView with  $\alpha = 0.05$ .

### **Results**

Angular transformed mean percent bacteria lysis differed significantly between these 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:  $F_{1,15} = 24.581$ ,  $p = 0.0002$ ; E2:  $F_{1,15} = 7.646$ ,  $p = 0.014$ ), with pregnant females exhibiting higher mean concentrations of P4, and lower mean concentrations of E2 relative to non-pregnant females (Figs. 2-3). Log transformed plasma corticosterone did not differ between these groups ( $p > 0.05$ ; Fig. 4). Angular transformed mean bacteria lysis was significantly negatively correlated with log mean P4 ( $R^2 = 0.29$ ;  $p = 0.027$ ; Fig. 5a) but not or E2 ( $p > 0.05$ ; Fig. 5b) or CORT ( $R^2 = 0.013$ ,  $p > 0.05$ ; Fig. 5c).

This relationship appeared to be driven by variation in P4 exhibited by pregnant females; when pregnant and non-pregnant females were analyzed separately, there was no significant correlation between log P4 and angular transformed bacteria lysis ( $p > 0.05$ ) in either group; however, there was a negative trend exhibited by pregnant females ( $R^2 = 0.25$ ), but not non-pregnant females ( $R^2 = 0.002$ ).

## 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 (Norris, 1997; Campbell et al., 2004). Progesterone is recognized for its role in maintaining uterine tissue, ova, and embryos in sharks, amphibians, squamates, and mammals (Callard et al., 1991; Callard et al., 1992; Wake, 1993; Norris, 1997), 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 (Aldridge and Duval, 2002). The role of E2 in initiating vitellogenesis and ovulation is well known and appears to be conserved throughout vertebrates (Callard et al., 1991, Norris, 1997). We found no difference in corticosterone (CORT) levels 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 (Taylor et al., 2004). Other than this, our hormone results for *Agkistrodon piscivorus* in late gestation are very similar to late gestation values documented in *C. atrox* (Taylor et al., 2004). 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 (Taylor et al., 2004), when mean CORT values in pregnant female rattlesnakes dropped from an August peak and became more similar to levels exhibited by non-pregnant females. Taylor et al. (Taylor et al., 2004) 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% less 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 (Hirsch, 1982; Gasque, 2004). Three major complement cascade pathways have been categorized, based upon their pathogen recognition mechanisms. The classical pathway (so named since it was discovered first) is initiated by antibodies, which link the nonspecific, innate complement system with the specificity of the adaptive immune system (Ricklin et al., 2010). The alternative complement pathway requires no recognition molecule and can lead directly to the formation of the membrane attack complex (MAC), which perforates pathogen cell membranes (Fujita et al., 2004). The lectin pathway is triggered by conserved microbial

surface molecules interacting with lectin, a pattern recognition receptor (Müller-Eberhard, 1998; Zarkadis et al., 2001; Fujita et al., 2004; Zhu et al., 2005). These pathways share common protein components (C3 and C5 proteins and the MAC) and are tightly regulated, especially at the level of C3 cleavage, which is considered the most important regulatory point in the cascade (Fujita et al., 2004) Improper regulation can lead to self-tissue damage (Mollnes and Fosse, 1994) and rejection of developing embryos by complement (Cauchteaux et al., 2003; Kanellopoulos-Langevin et al., 2003).

Progesterone has documented regulatory effects on immune components during pregnancy in mammals (Szekeres-Bartho et al., 2001, Druckman and Druckman, 2005), and has been shown to reduce complement production by modulating mRNA transcription of the C3 complement protein in rats (Brown et al., 1990). 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 (1990) 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 (Saad and Deeb, 1990), but unfortunately, they did not measure P4. Others have documented cytokine involvement in uterine development in a viviparous skink (Paulesu et



al., 1995; Paulesu et al., 2005). 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 feto-placental 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., *Thamnophis sirtalis*; Birchard et al., 1984). 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., Saad and Deeb, 1990).

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 (Martin et al., 2006; French et al., 2009). During pregnancy many viviparous snakes reduce feeding or avoid eating entirely, and post parturient snakes are often extremely emaciated (Madsen and Shine, 1993; 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., Shine, 2002) 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 (Shine and Guillette, 1988; Callard et al., 1992). 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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## **Figure Legends**

**Figure 1** Comparison between mean plasma percent bacteria lysis in pregnant versus non-pregnant female cottonmouths. Bars represent standard error.

**Figure 2** Comparison between mean plasma progesterone in pregnant versus non-pregnant female cottonmouths. Bars represent standard error.

**Figure 3** Comparison between mean plasma estradiol in pregnant versus non-pregnant female cottonmouths. Bars represent standard error.

**Figure 4** Comparison between mean plasma corticosterone in pregnant versus non-pregnant female cottonmouths. Bars represent standard error.

**Figure 5** Relationship between mean plasma progesterone (Fig. 5a) estradiol (Fig 5b) and corticosterone (Fig. 5c) and mean plasma percent bacteria lysis in female cottonmouths.

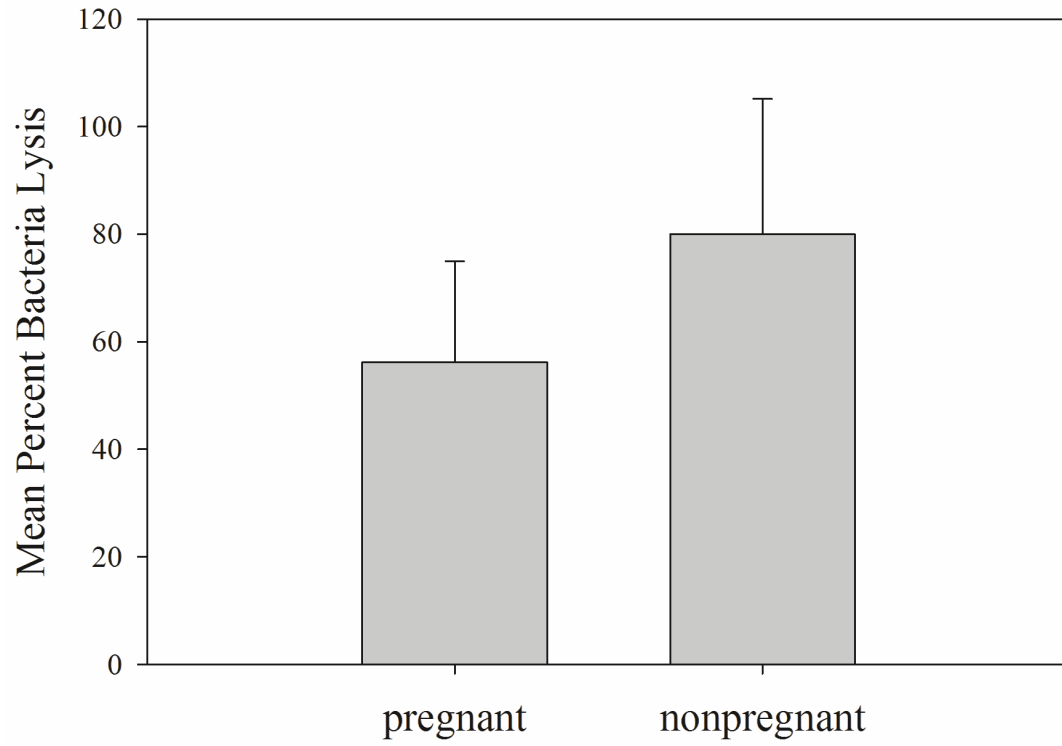


Figure 1

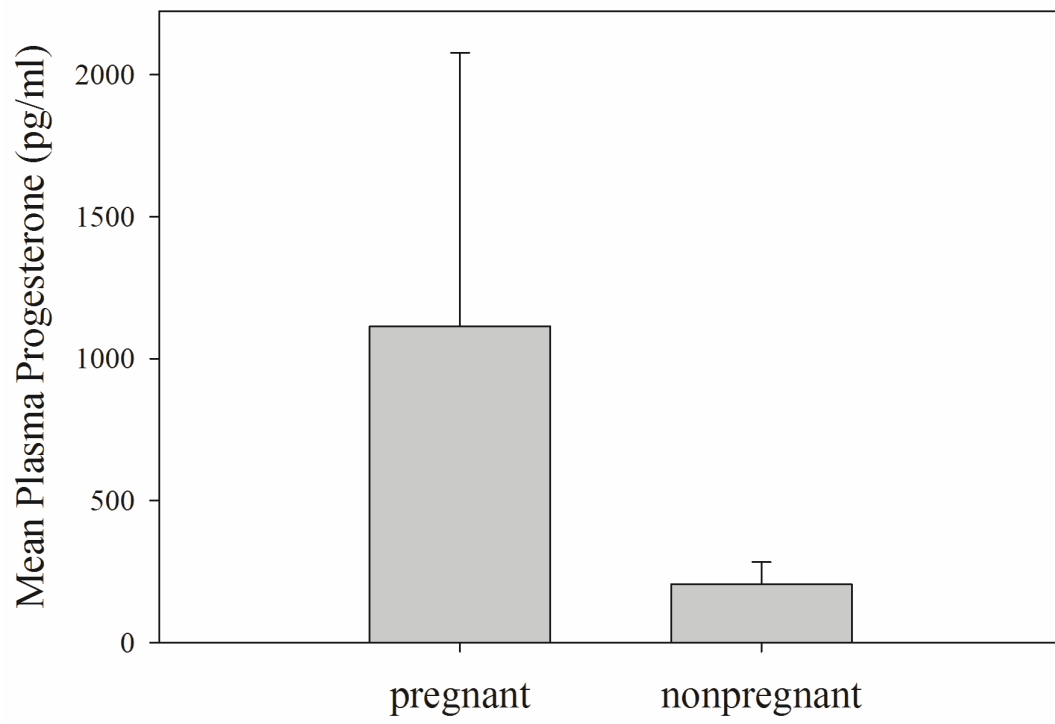


Figure 2

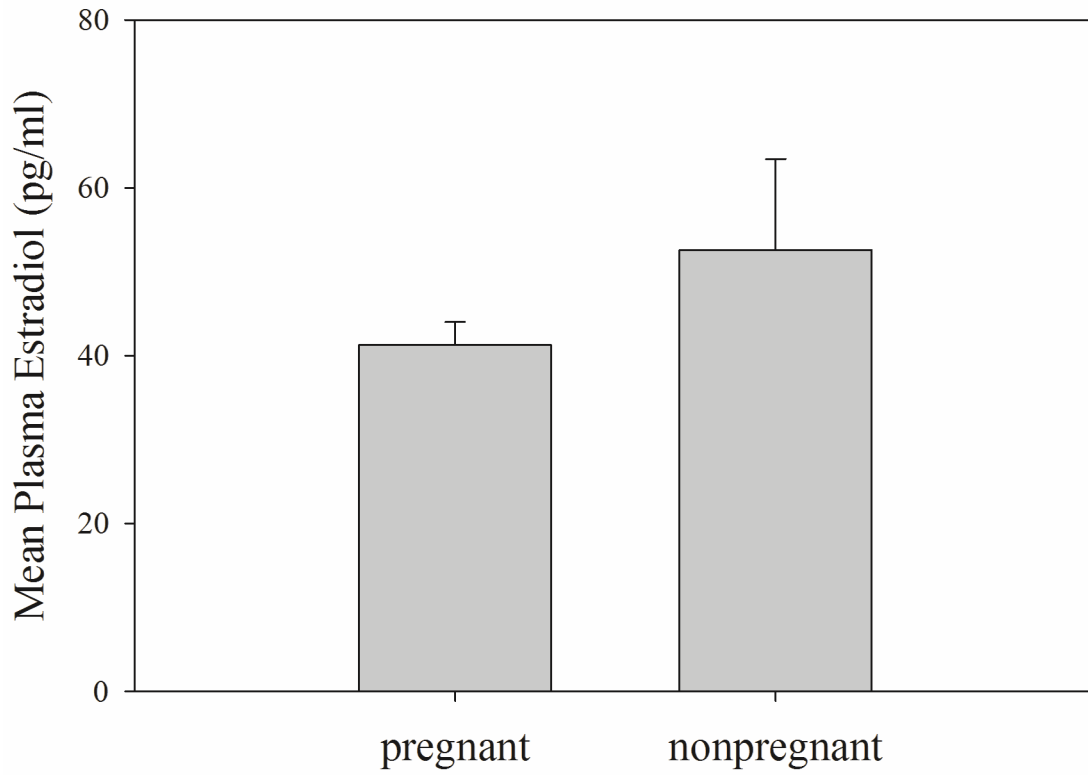


Figure 3

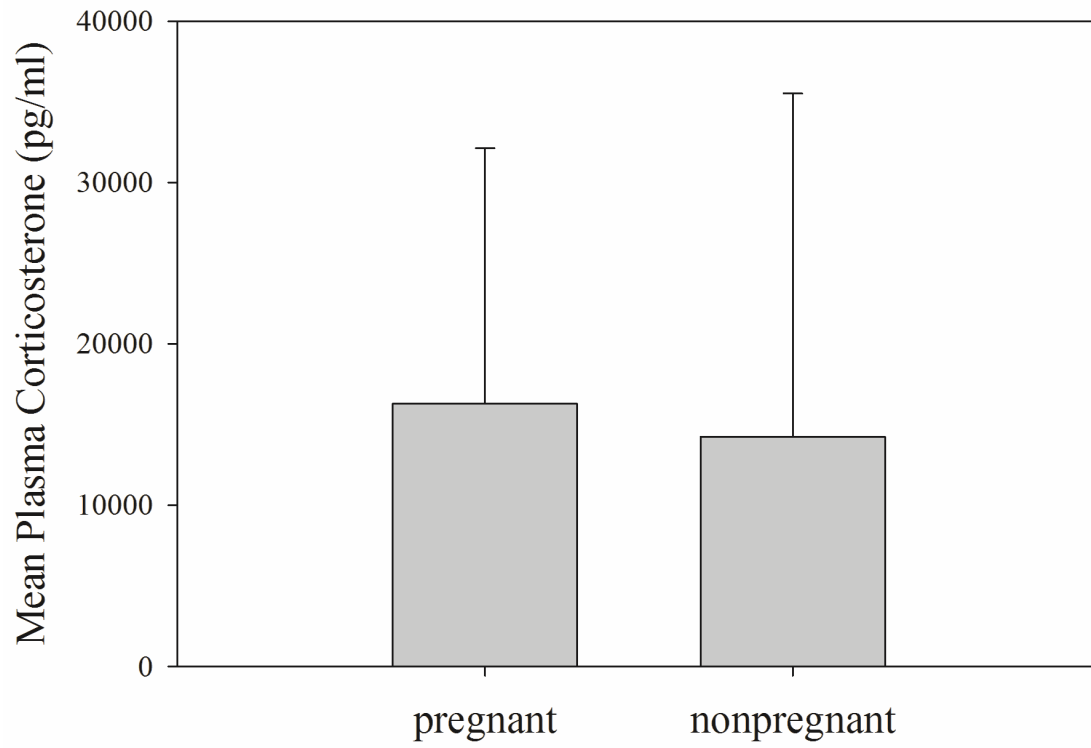


Figure 4

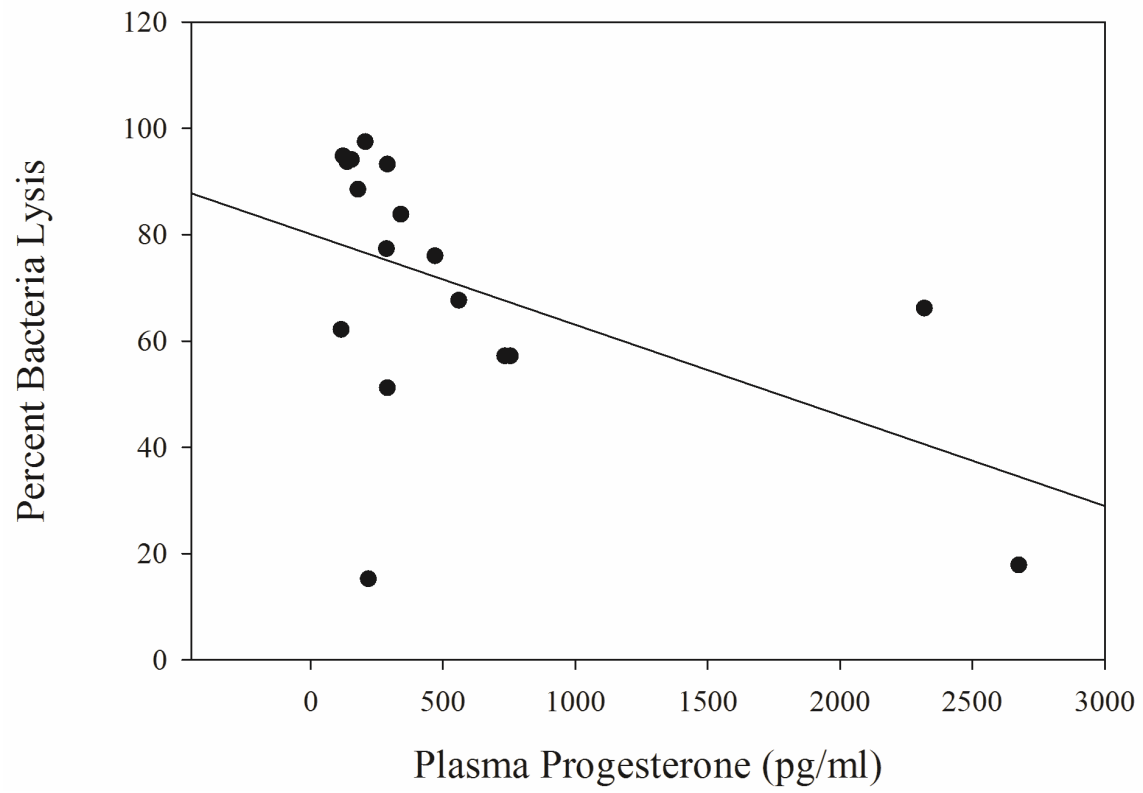


Figure 5a

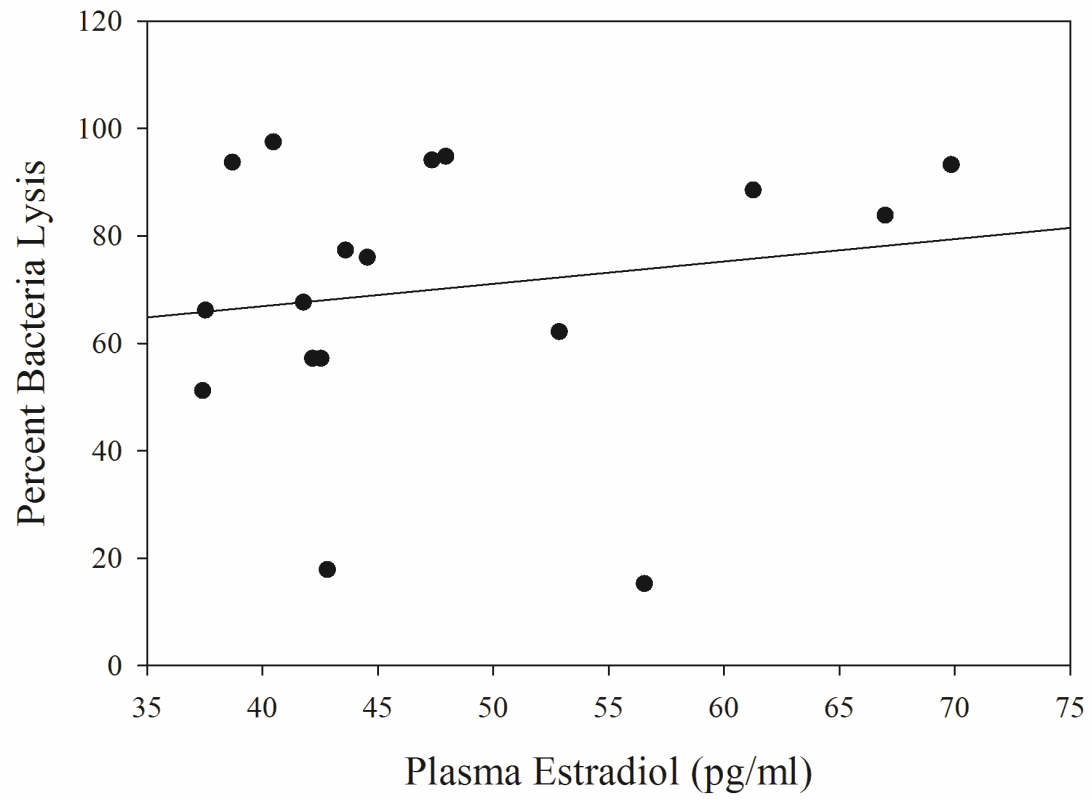


Figure 5b



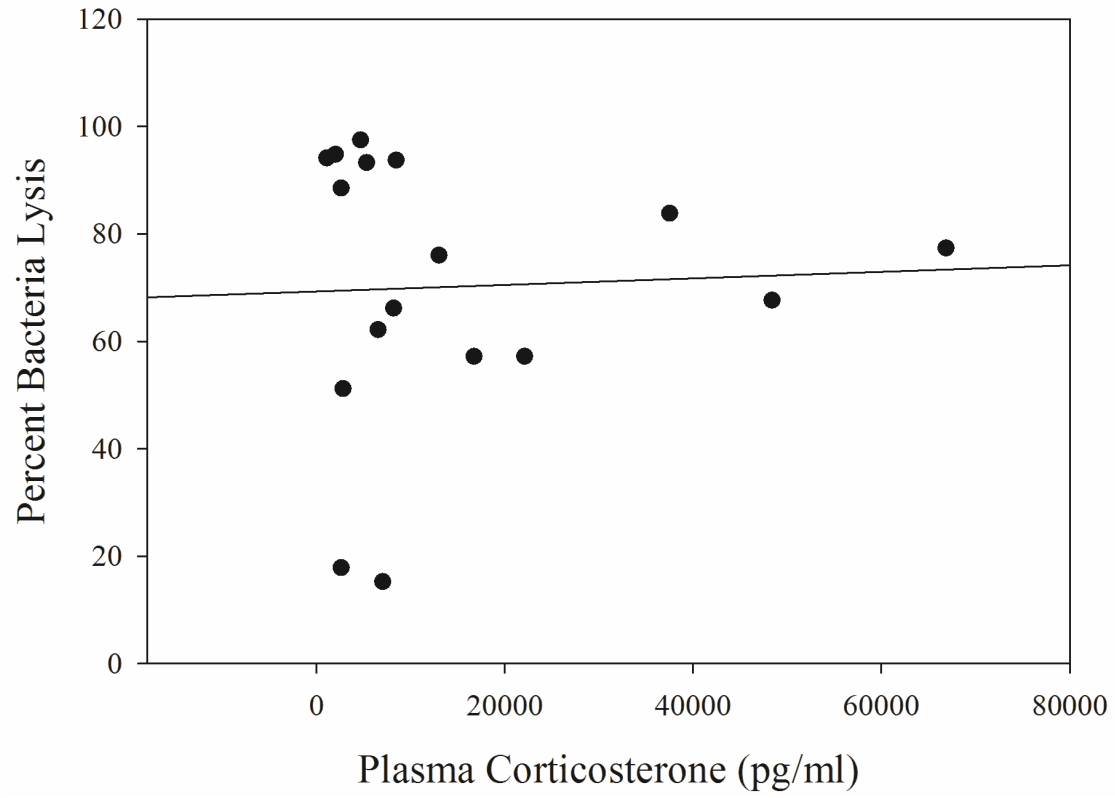


Figure 5c

## Chapter 5

### Serosurveillance of eastern equine encephalitis virus antibodies in amphibians and reptiles from Alabama, U.S.A.

**Abstract:** Eastern equine encephalitis virus (EEEV) is among the most medically important arboviruses in North America, and studies suggest a role for amphibians and reptiles in the transmission cycle of this virus. We tested 351 individual amphibian and reptiles (27 species) from Alabama, U.S.A., for the presence of EEEV antibodies. Frogs, turtles, and lizards showed little or no antibody to EEEV, while snakes had high exposure rates. Most species showing exposure to the virus were preferred or abundant hosts of *Culex* mosquitoes which prefer ectothermic hosts. We predicted high proportions of exposed individuals in spring and fall if these species serve as overwintering hosts for EEEV. Instead, the proportion of exposed individuals peaked in August-September, corresponding to a period of mosquito host shift in the eastern U.S. The cottonmouth—the most abundant ectotherm we sampled—displayed a continual pattern of virus exposure, indicating its possible role as an overwintering reservoir for EEEV.

## Introduction

Due to its high virulence and case fatality rate in humans and horses, Eastern equine encephalitis virus (EEEV; Togaviridae, Genus *Alphavirus*) is considered one of the most medically important viral encephalitides in eastern North America (Scott and Weaver 1989; Villari et al., 1995). Birds are considered the primary reservoir host (e.g., hosts with the capacity to re-infect vectors) for EEEV (Scott and Weaver 1989; Bigler et al., 1976; Crans, 1962; Scott et al., 1984), and the mosquito *Culiseta melanura* its primary vector (Bigler et al., 1976), with other mosquito species serving as bridge vectors for transmission of EEEV to dead end hosts such as horses and humans (Crans and Schulze, 1986; Molaei et al., 2006). A complete understanding of the transmission dynamics of this arbovirus requires knowledge of the availability and abundance of competent and incompetent reservoir hosts, vector mosquito feeding preferences and behavior, and the interactions between these players (Unnasch et al., 2006). To date, most of this research has focused on a few mosquito and host species acknowledged to play a key role in EEEV transmission. However, a more comprehensive understanding will come from the inclusion of understudied taxa in disease transmission models.

For example, amphibians and reptiles may play a larger role in the transmission cycle of arboviruses than previously assumed. Due to their high energy conversion efficiency, ectotherms typically represent a large portion of the vertebrate biomass in many ecosystems (Burton and Likens, 1975; Pough, 1980; Iverson, 1982; Petranka and Murray, 2001; Gibbons et al., 2006), and may therefore greatly influence transmission dynamics either as virus amplifiers or sinks. This may be especially true of EEEV, because it is found in hardwood swamps where amphibian and reptiles are particularly abundant (Scott and Weaver 1989). In

addition, the potentially inefficient adaptive immune response of ectothermic vertebrates (Hsu, 1998) may result in prolonged viremia in amphibians and reptiles relative to mammals and birds, leading to increased probability of transmission to mosquitoes. Scattered studies indicate that some ectothermic hosts serve as potentially competent reservoirs for arboviruses (Bowen, 1977; Karstad, 1961; Hayes et al., 1964; Klenk et al., 2004; White et al., *in press*), and support their possible role as overwintering hosts for arboviruses (Gebhardt and Hill, 1960; Thomas and Eklund, 1962). Certain vector mosquito species appear to prefer ectothermic hosts, and some generalist species feed upon both ectothermic and endothermic taxa (Cupp et al., 2003; Cupp et al., 2004; Hassan et al., 2003; Burkett-Cadena et al., 2008). This indicates that arboviruses can potentially navigate through entire ectoparasite-vertebrate host communities, an observation supported by a network analysis (Graham et al., 2009).

In this paper we report the results of a three year surveillance for EEEV in amphibians and reptiles from our study site in Tuskegee National Forest (TNF), in Alabama, U.S.A. We sampled 351 individuals of nine amphibian and 18 reptile species, testing for the presence of antibodies to EEEV. In general, patterns of virus exposure corroborated results from our previous research on mosquito host preference (Burkett-Cadena et al., 2008), and experimental attempts to induce viremia in amphibian and reptile hosts (White et al., *in press*). In addition, we tested the hypothesis that amphibians and reptiles may serve as overwintering hosts for EEEV by analyzing the seasonal pattern of exposure of ectothermic hosts to EEEV.

## Materials and Methods

### *Study Area*

The study area was the 75.54 km<sup>2</sup> Tuskegee National Forest. Tuskegee National Forest (TNF) is the smallest property in the National Forest system, and was established in 1959. At the time of its designation as a national forest, TNF was composed largely of abandoned farmland. Since this time, ecological succession has proceeded and the forest is now a mosaic of lowland hardwood, mixed pine-hardwood, and wetland habitats. For its size, TNF has a high diversity of amphibian and reptiles, with eighty-one documented species. This study area has been thoroughly investigated by our group as a focus for EEEV zoonotic transmission (Cupp et al., 2003; Cupp et al., 2004; Burkett-Cadena et al., 2008; Hassan et al., 2003; Graham et al., 2009).

### *Field Sampling*

From April-October 2007-2009, a herpetofaunal census was conducted in TNF wetlands (Burkett-Cadena et al., 2008); two to four researchers conducted visual encounter surveys (Heyer et al., 1994) at five TNF wetlands (four beaver marshes and an oxbow lake) twice a week (once during the day and the following night) for each week of the active season (April through October). Visitation to these ponds was rotated such that each was sampled twice per month. During these surveys, observers walked slowly along the wetland margin and counted each individual of each amphibian and reptile species encountered. During 2007-2009, hoop and crayfish traps were set for one night each week at one of the above ponds, such that each pond was trapped once per month. Traps were checked the next day for captured amphibians and reptiles. Crayfish traps were placed in 0.5 m deep water along the pond margin, usually in emergent vegetation. Hoop traps were placed in water 1m

deep, usually along the deep channel of beaver ponds along the dam. Limited minnow trap and drift fence sampling was also conducted once a week in 2008. Minnow traps were placed in emergent vegetation in beaver ponds, and 15m drift fences with two bucket traps and minnow traps were erected within 5m of each beaver pond parallel with its margin.

We obtained a blood sample (125-1000  $\mu$ l) via cardiac puncture (frogs), or the caudal sinus (turtles, lizards, and snakes) using a 26 gauge, heparinized syringe from most species and individuals of a size enabling blood sampling. Blood sample volume varied by species, such that blood samples represented no more than 5% of the sampled individual's mass. Samples were placed in a labeled 1.5 ml microcentrifuge tube on ice packs, and transported to the lab. Samples were then centrifuged (5 min at 5000 rpm), and plasma was drawn off and placed in a new labeled tube, frozen, and stored (-20°C) until assay. Processed turtles and snakes received a unique mark and were released at their point of capture. Frogs and lizards did not receive a mark and were released at their point of capture. The procedures utilized in this serosurvey study were approved by the Institutional Review Board for Animal Use and Care (IACUC) of Auburn University.

#### *EEEV Antibody Assay*

Reptile and amphibian serum samples were assayed for the presence of antibodies reacting with EEEV using a Luminex-based species-independent antibody assay (Basile et al., 2010).

#### *Preparation of EEEV Antigen*

Confluent monolayers of Vero cells in a T75 flask were infected with EEEV (Strain M05-316; White et al., *in press*) at a multiplicity of infection of roughly 0.01. The infected monolayers were incubated at 37°C and 5% CO<sub>2</sub> for 1-3 days and monitored daily for

cytopathic effect (CPE). When CPE was evident, the cells were removed from the flask with a rubber policeman, transferred to a 50ml conical centrifuge tube and subjected to centrifugation at 2700 xg for 10 minutes at 4°C to pellet the cells. The cell pellet was resuspended in 10ml ice cold borate saline (0.5M Na<sub>3</sub>BO<sub>4</sub> (pH 9.0)) and the cells pelleted as described above. The cells were washed twice more in 10 ml borate saline, and resuspended in 0.9ml of borate saline containing 0.1% (W/V) SDS and 10% Triton X 100. The cells were homogenized in a glass-glass homogenizer until the solution cleared. The sample was then subject to centrifugation at 12,000 xg for 3 minutes at 4°C to clear the homogenate. The supernatant was recovered and β-propiolactone added to a final concentration of 0.3% (W/V). The antigen preparation was stored at 4°C for 24 hours and viral inactivation confirmed by plaque assay (Beaty et al., 1989). Once viral inactivation was confirmed, the protein concentration in the antigen preparation was determined using the Bradford method (Bradford, 1976). The antigen was stored at -80°C.

#### *Luminex Assay*

In brief, a set of 15 luminex beads coated with monoclonal antibody 2A2C-3 against alphaviruses (Radix Biosolutions, Georgetown TX) was coated with EEEV antigen prepared as described above by mixing 50μ of the beads with 1μg of EEEV antigen in a final volume of 500 μl of PBS. The mixture was placed on a shaker at room temperature for 1 hour. The bead solution was added to 9.5ml of PBS containing 1% BSA (w/v) and stored on ice.

Serum samples (1.5μl per reaction) were biotinylated with approximately a 50 fold molar excess of biotin using the EZ-Link Sulfo-LC-biotin kit (Pierce Biotechnology, Rockford, IL), following the manufacturer's instructions. The biotinylated antibodies were passed through a 100 kDa MW cutoff filter (Acroprep 96 Omega 100K; VWR Scientific, San

Francisco, CA) to remove any components <100 kDa. The retentate was resuspended in 50µl PBS, and the sample passed again through the filter to wash the enriched biotinylated antibodies. The samples were then resuspended in 50µl of PBS, and 12.5µl of PBS 1% BSA was added to each sample, representing a final dilution of 1/50 of the original serum sample.

To bind the biotin labeled antibody preparations, 100µl per well of the antigen coated bead preparation prepared as described above was placed into each well of a 96 well 1.2µm filter plate (MultiScreen-BV, 1.2 µm, Millipore (Billerica, MA)). The beads were washed twice in PBS 1% BSA by vacuum filtration and re-suspended in 50µl of the biotinylated serum sample prepared as described above. The plate was shaken for 45 minutes at room temperature. The plate was removed from the shaker, the supernatant removed by vacuum filtration and the retained beads washed twice PBS, 1% BSA. The beads were re-suspended in 50µl of a solution consisting of 4 µg/ml of streptavidin-phycoerythrin (Jackson Immunoresearch, West Grove, PA) in PBS, 1% BSA and the plate shaken for 15 minutes at room temperature. The solution was removed by vacuum filtration and the beads washed twice PBS, 1% BSA. The beads were re-suspended in 100µl of PBS, 1% BSA and the samples analyzed using a Bio Rad Bio Plex instrument. Results were expressed as a mean fluorescent intensity (MFI) of two replicates per sample tested. All plates tested included a series of three known positive and negative serum samples. Positive serum samples were derived from reptiles and amphibians experimentally inoculated with EEEV, as previously described (White et al., *in press*). Negative samples were obtained from animals not exposed to EEEV. Samples were considered to contain EEEV antibodies if the MFI obtained was greater than the mean plus three standard deviations of the MFI obtained from the negative control serum samples processed in parallel with the experimental sera. The proportion of



infected individuals did not differ appreciably between 2007 (13%), 2008 (22%), or 2009 (22%), so these years were combined and compared across months.

## **Results**

A total of 351 individuals representing 9 amphibian and 18 reptile species were examined during the course of this study (Table 1). EEEV antibodies were detected in approximately 19% of the individual amphibians and reptiles assayed. With the exception of lizards, individuals from each major amphibian and reptile group sampled had detectable levels of EEEV antibodies. Snakes had the highest exposure rate, with 35% of snakes sampled having detectable levels of EEEV antibodies (Table 1). When all amphibian and reptile species were combined and compared across months, the highest proportion of infected individuals were detected in August and September (Fig. 1). Cottonmouths exhibited little seasonal variation in proportion of individuals infected. However, a higher proportion appeared to be infected in April, August, and September (Fig. 2).

## **Discussion**

Evidence of exposure to Eastern equine encephalitis virus was detected in 12/26 amphibian and reptile species sampled, with an overall individual exposure rate of 19%. Frogs, lizards, and turtles had low or no EEEV antibodies, while the prevalence of anti EEEV antibodies in snakes was relatively high. These findings corroborate previous studies on the reservoir competence of these species for EEEV (White et al., *in press*). In these studies, green treefrogs (*Hyla cinerea*) and bullfrogs appeared refractory to EEEV infection. Green anoles (*Anolis carolinensis*) were capable of becoming infected with the virus, but produced low levels of circulating virus (White et al., *in press*). In contrast, garter snakes experimentally infected with EEEV produced relatively high viremias (levels capable of

infecting mosquitoes) which were maintained for a relatively prolonged period (White et al., *in press*). Viremias have also been experimentally induced in garter snakes exposed to Western equine encephalitis virus (WEEV)(Gebhardt and Hill, 1960; Thomas and Eklund, 1962; White et al., *in press*), but not West Nile virus (WNV)( Klenk and Komar, 2003). A previous study in Massachusetts did not identify EEEV antibodies in most amphibians and reptiles sampled, including several of the same amphibian and reptile species that we found to contain EEEV antibodies (e.g., *Coluber constrictor*, *Sternotherus odoratus*, and *Storeria dekayi*)( Hayes et al., 1964). This difference could be due to several factors, including temporal or site effects, differences in the total number of individuals sampled, and the higher assay sensitivity of the Luminex bead approach. The same study determined that many amphibian and reptile species produced antibodies to EEEV when experimentally infected, but not measurable viremias (Hayes et al., 1964). This suggests that our assay successfully detected low levels of circulating antibodies to EEEV present in TNF amphibians and reptiles that may have been below the limit of detection of previous studies.

Host preferences of mosquitoes involved in EEEV transmission at TNF also corroborate the general pattern of exposure observed. Amphibian and reptile species exposed to the virus were often preferred or abundant hosts of the mosquitoes *Culex territans*, *Cx. erraticus* and *Cx. peccator*, which feed upon ectothermic hosts at TNF and which have been found to be infected with EEEV at the TNF site where this study was conducted (Burkett-Cadena et al., 2008). Preferred hosts of these species included the plainbelly watersnake (*Nerodia erythrogaster*), cottonmouth (*Agkistrodon piscivorus*), timber rattlesnake (*Crotalus horridus*) and grey treefrog (*Hyla chrysoscelis*)(Burkett-Cadena et al., 2008). Other species that had detectable EEEV antibodies were not among the species we previously reported to

be commonly feed upon by mosquitoes at TNF (Burkett-Cadena et al., 2008), including the snake *Coluber constrictor*, and the turtles *Trachemys scripta*, *Kinosternon subrubrum*, and *Sternotherus odoratus*. However, since our previous analysis (Burkett-Cadena et al., 2008), we have identified blood meals of *C. constrictor* and *T. scripta* taken from *Cx. erraticus*, and the mosquito species *Ochlerotatus canadensis* has been observed feeding on *T. scripta* and other turtles at TNF (unpubl. data).

Due to their relatively low body temperatures and apparent inefficient antibody responses (Hsu, 1998), ectotherms can support prolonged viremias compared to birds and mammals (Bowen, 1977), and previous laboratory studies have suggested a role for ectotherms as overwintering hosts for arboviruses (Gebhardt and Hill, 1960; Thomas and Eklund, 1962; White et al., *in press*). We therefore hypothesized that amphibians and reptiles would exhibit a bimodal pattern of EEEV antibody presence among amphibians and reptiles at TNF, with the highest proportion of antibody positive individuals detected in spring and late summer/fall. Treated together, amphibians and reptiles did not exhibit this pattern. Instead, the highest proportion of individuals exposed to EEEV was found in August and September. Interestingly, this time period also corresponds to an annual period of mosquito host switching from birds to mammals, and periodic outbreaks of infection in humans and horses (Edman and Taylor, 1968; Dalrymple et al., 1972; Kilpatrick et al., 2006). This suggests that amphibians and reptiles may also be targets of mosquitoes switching from feeding on bird hosts during this time period. Despite low proportions of exposed individuals present in spring, even low levels of infected individuals could still contribute to overwintering of EEEV. Cottonmouths are among the most abundant vertebrates at TNF in terms of biomass (Burkett-Cadena et al., 2008), and since snakes appeared to have high

exposure rates, we sampled large numbers of cottonmouths. When analyzed alone, cottonmouths did not display much seasonal variation in exposure to EEEV. However, the highest proportion of infected individuals was detected in April, August, and September, suggesting that they could possibly be serving as overwintering hosts for EEEV.

The combined findings of this and other studies conducted on TNF amphibians and reptiles suggest that snakes serve as hosts for both specialized and generalist mosquitoes which may transmit EEEV, have the capacity to be reservoir hosts of EEEV, and may serve as overwintering hosts for EEEV in Alabama. Interestingly, the hibernation and emergence habitats of female mosquitoes and cottonmouths are similar enough that during early spring, cottonmouths are probably readily available hosts for emerging mosquitoes. For example, Burkett-Cadena et al (2011) found that several species of *Culex* mosquitoes overwinter in underground burrows and root holes, sites also favored as hibernacula by cottonmouths and other snakes (Ernst and Ernst, 2004). If snakes support viremias at this time, the early emergence period for both mosquitoes and their ectothermic hosts could be a crucial window for the persistence of EEEV from year to year. Although this time period (e.g., March) is considerably earlier than mosquito control programs typically operate, it is possible that the targeted use of pesticides near overwintering sites during this period may succeed in interrupting the annual zoonotic cycle of EEEV.

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**Table 1** Results of serosurveillance for EEEV antibodies in amphibians and reptiles of Tuskegee National Forest, Alabama, USA.

Common Name	Latin Name	N	number positive	% positive ±95% CI
<b>Frogs</b>				
Green Frog	<i>Lithobates clamitans</i>	10		
	<i>Lithobates</i>			
Bullfrog	<i>catesbeianus</i>	6		
Green Treefrog	<i>Hyla cinerea</i>	33	1	3 ± 5.7%
Grey Treefrog	<i>Hyla chrysoscelis</i>	12	1	8 ± 14.5%
Barking Treefrog	<i>Hyla gratiosa</i>	4		
Bird-voiced Treefrog	<i>Hyla avivoca</i>	2		
Fowler's Toad	<i>Anaxyrus fowleri</i>	4		
Southern Toad	<i>Anaxyrus terrestris</i>	7		
Eastern Spadefoot	<i>Scaphiopus holbrookii</i>	4		
			Total	
		82	2	3 ± 3.3%
<b>Turtles</b>				
Pond Slider	<i>Trachemys scripta</i>	67	4	6 ± 5.36%
Stinkpot	<i>Sternotherus odoratus</i>	6	1	16 ± 26%
Southern Painted Turtles	<i>Chrysemys dorsalis</i>	5		
River Cooter	<i>Pseudemys concinna</i>	2		
	<i>Deirochelys</i>			
Chicken Turtle	<i>reticularia</i>	3		
	<i>Kinosternon</i>			
Mud Turtle	<i>subrubrum</i>	7	1	14 ± 22.9%
	<i>Terrapene</i>			
Box Turtle	<i>carolinensis</i>	2		
			Total	
		92	6	7 ± 4.75%
<b>Lizards</b>				
Green Anole	<i>Anolis carolinensis</i>	3		
Five-lined Skink	<i>Plestiodon fasciatus</i>	1		
			Total	
		4	0	0
<b>Snakes</b>				
Ringneck Snake	<i>Diadophis punctatus</i>	1	1	100% ± na
Mud Snake	<i>Farancia abacura</i>	1		
Black Racer	<i>Coluber constrictor</i>	5	3	6 ± 34%

Plainbelly Watersnake	<i>Nerodia erythrogaster</i>	13	2	15 ± 15.07%
Midland Watersnake	<i>Nerodia sipedon</i>	2		
Dekay's Brownsnake	<i>Storeria dekayi</i>	1	1	100% ± na
Rattlesnake	<i>Crotalus horridus</i>	2	1	5 ± 70%
Copperhead	<i>Agkistrodon contortrix</i>	4	1	25 ± 38%
Cottonmouth	<i>Agkistrodon piscivorus</i>	144	51	35 ± 6.17%
			Total	
		173	60	35 ± 5.61%

## **Figure Legends**

**Figure 1** Monthly variation in EEEV-exposed proportion of tested amphibians and reptiles from Tuskegee National Forest, Alabama, U.S.A.

**Figure 2** Monthly variation in EEEV-exposed proportion of tested cottonmouths (*Agkistrodon piscivorus*) from Tuskegee National Forest, Alabama, U.S.A.

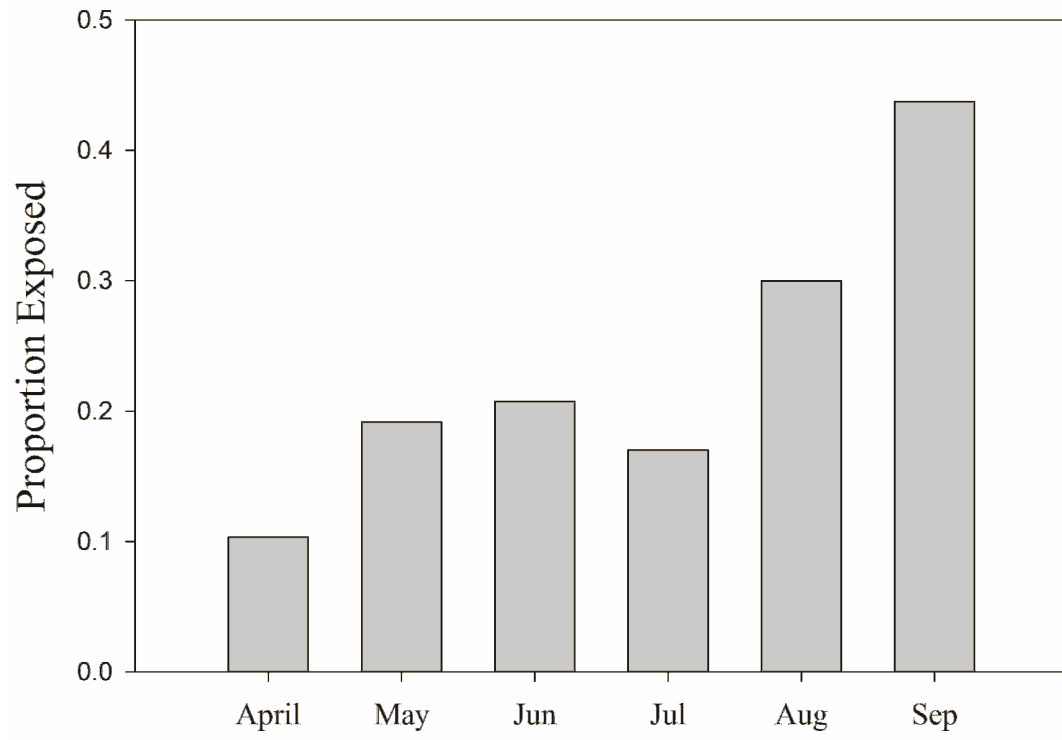


Figure 1

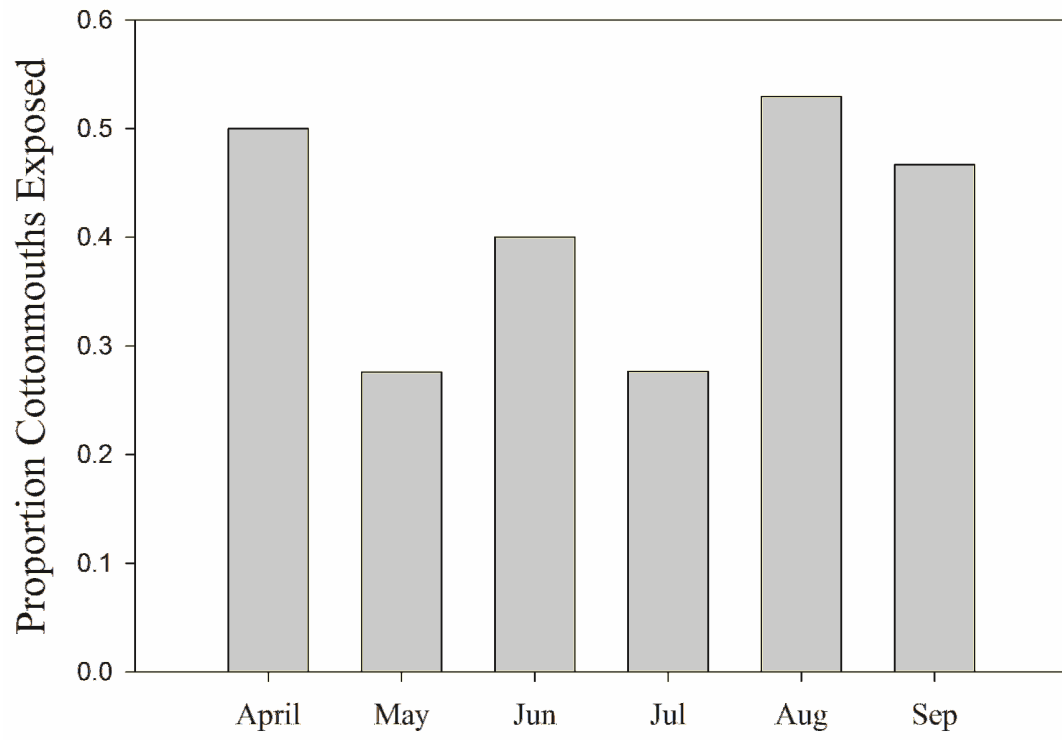


Figure 2