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# The Reproductive Ecology of the Timber Rattlesnake, Crotalus horridus, in Northwestern Arkansas: Interactions Between Environment, Steroid Hormones, and Life History

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**Abstract:**

In this dissertation I examined the relationship between individual energetic status, hormone production, and life history trait expression in field-active Timber Rattlesnakes, *Crotalus horridus*. In chapter one I reviewed what is known regarding these relationships in snakes and defined major research goals. In chapter two I described the seasonal profile of testosterone (T) and corticosterone (CORT) in relation to the breeding season and to individual energetic status in males. Results showed that the seasonal pattern of T production in *C. horridus* was different than other pit viper species with similar mating patterns. Testosterone was elevated in the months leading up to the breeding season and levels returned to baseline during the months of peak breeding. Testosterone concentrations were positively related to individual energetic status, but only in the months leading to the breeding season when concentrations were elevated. Annual variation was also observed in both the magnitude of T production and the seasonal profile. Corticosterone concentrations were not related to any measured variable. In chapter three I examined the relationship between individual T and CORT concentrations at the onset of the breeding season, energetic status, and male time-energy allocation. Male *C. horridus* with greater reserves of stored energy at the onset of the breeding season had higher T concentrations and allocated more time and energy toward reproduction compared to snakes with lower stored energy reserves (estimated by body condition index). Both mate search area and time allocated to behaviors other than foraging were directly related to both energetic status and T concentrations. My results suggest that male *C. horridus* hedge investment of time and energy towards current reproduction against potential costs in terms of survivorship and future fecundity and that testosterone may play a role in mediating this tradeoff. Results are descriptive and future experiments should be conducted to establish causal links. Annual variation was observed in both T concentrations and male time-energy allocation. In a year with low T and no seasonal

variation in T, snakes did not appear to allocate time and energy towards mate search and reproductive behavior. In chapter four I described the relationship between female body condition and reproductive allocation and behavior. Additionally, a combination of long-term behavioral monitoring and analysis of microsatellite DNA markers was used to describe the mating system of female *C. horridus*. Receptive/attractive females were in better body condition compared to the general population, but maternal snout vent length and not body condition was positively related to litter size and mass. Behavioral and molecular data showed that individual females engaged in mating behaviors with multiple males in a single breeding season and that some litters were sired by multiple males. Behavioral data also showed that females will associate with males even when the likelihood of producing a litter the following year is unlikely. Taken together, my results demonstrate that reproductive life history trait expression varies according to natural variation in energetic status in *C. horridus*, and suggest that the testosterone may be important in mediating this relationship in male snakes.

**Acknowledgments:**

I have been extremely lucky to be surrounded by a group of incredibly talented, helpful, and all-around great people throughout my academic career. I will try to fit them all, in no particular order, into the following paragraphs.

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Lastly, I would like to acknowledge my family for all of their love and support over the years. Mom, Dad, Brett, Jake, Lindsay, Connor, Cooper, and Gams, no words here can express what you all mean to me. I'll just leave it at that and say, thank you.

**Dedication:**

I dedicate this dissertation to my parents, Herb and Terri, for constantly supporting me and my unconventional fascination with the animals that are the subject of this dissertation. Not many parents would have put up with what you put up with raising me, and most certainly would not have been so supportive in every way. Thanks Mom and Dad.

**Table of Contents:**

**Original Article Citations:**

**Chapter 2**:

Lind, C.M., Beaupre, S.J., 2014. Natural variation in steroid hormone profiles of male Timber Rattlesnakes, *Crotalus horridus*, in Northwest Arkansas. General and Comparative Endocrinology. 206. 72-79.

**Chapter 3**:

Lind, C.M., Beaupre, S.J., 2014. Male snakes allocate time and energy according to individual energetic status: body condition, steroid hormones, and reproductive behavior in Timber Rattlesnakes, *Crotalus horridus*. Hormones and Behavior. Submitted for review.

**Chapter 4**:

Lind, C.M., Flack, B.R., Rhoads, D.D., Beaupre, S.J., 2014. The mating system and reproductive life history of female Timber Rattlesnakes in northwestern Arkansas. Journal of Zoology. Submitted for review.

### Introduction:

The expression of life history traits is plastic and modulated by a variety of extrinsic and intrinsic factors (e.g. season or body size). Such factors are integrated by the neuroendocrine system in order to fine tune trait expression according to a variety of environmental and physiological contexts. Potential neuroendocrine mechanisms by which environmental or physiological variables alter life history traits have been identified in model organisms in captive settings. However, far less is known regarding the role of such mechanisms in non-model species in natural settings. The list of intrinsic and extrinsic factors potentially involved in modulating the expression of life history traits is extensive and there are gaps in our understanding of proximate and ultimate causality. This dissertation addresses several of these gaps in field active Timber Rattlesnakes, *Crotalus horridus*.

In particular, the research outlined in the following chapters examines the relationship between the resource environment, endocrine regulatory mechanisms, and time/energy allocation. Human activities have rapidly altered ecosystems, and reptile and amphibian populations have become increasingly imperiled as a result. Anthropogenic disturbances imposed by factors such as habitat fragmentation and climate change can lead to concomitant shifts in resource abundance. Shifts in food resources are predicted to affect organismal life histories (Boutin, 1990), but we know little about the mechanisms by which such effects are manifested. Such regulatory mechanisms can constrain organismal responses to environmental change (Jacobs and Wingfield, 2000). For example, endocrine mechanisms are often involved in regulating suites of correlated traits. Thus, changes in one trait that would constitute an adaptive response to perturbation may be linked to changes in other traits that negatively affect fitness (Ketterson and Nolan Jr, 1999). Therefore, an understanding of underlying endocrine

mechanisms linking energetic status and trait expression (Fig 1) is critical to prediction of how life history traits will respond to disturbance/change (McGlothlin and Ketterson, 2008).

For the purpose of this dissertation, I define the life history as a heritable set of rules that govern the allocation of time among competing behaviors and energy among competing functions (e.g. growth, maintenance, storage, activity, and reproduction; Dunham et al., 1989). Therefore, a reproductive life history trait (RLHT) refers to the amount of time or energy allocated towards reproduction in a given physiological or environmental context (for simplicity we will ignore the nuances of offspring packaging). Allocation can be measured in terms of behavior (time allocation) or in terms of reproductive effort (energy allocation). Snakes, and pit vipers in particular, provide good models for investigating the interplay between environmental variables and time-energy allocation (Beaupre, 2008, Beaupre and Duvall, 1998, Ford and Seigel, 1989). Snakes have also emerged as models for investigating the relationship between sex steroid hormone production and annual reproductive cycles in field settings (Taylor and Denardo, 2010). However, few studies have used these model systems to link environment, endocrine regulatory mechanisms, and RHLT expression in natural settings.

Causal links between the resource environment and RLHTs have been established in a variety of snakes and will be briefly reviewed below. However, most of the literature is biased toward females and little is known regarding the neuroendocrine regulation of RLHT expression in response to energetic cues. Therefore, in this chapter, I will first briefly review the established connections between the food resource environment and RLHT expression in female and male snakes, and then review what is known regarding endocrine pathways linking energy intake and RLHT expression (see Fig 1). I conclude by defining the major research goals of this dissertation

and suggesting future research directions that would potentially fill in the gaps in our understanding of the mechanistic basis of RLHT expression.

### *Food resources and trait expression in female snakes:*

It is well established that food supply can alter female reproductive effort. Links between food resources and RLHTs are typically established via two types of study: Measurement of trait expression in response to experimental food supplementation (e.g. Ruiz et al., 2010, Taylor et al., 2005) or correlational studies relating trait expression with food intake, prey availability, or stored energy (e.g. Beaupre, 2008, Shine and Mason, 2005). Food supplementation studies are more powerful in establishing causal links due to their experimental nature. However, descriptive studies in field settings, particularly those conducted over multiple years, are valuable in establishing relationships in un-manipulated settings and in elucidating how trait expression changes according to natural fluctuation in environmental variables.

Correlational studies that relate energetic status and female reproductive effort are prevalent and reviewed elsewhere (see Ford and Seigel, 1989, Shine and Madsen, 1997). Overall, results show that food availability is often positively correlated with reproductive output (i.e. size or number of offspring). However, in systems where clutch size is fixed, or food resources are not limited, correlations may not exist (Van Noordwijk and de Jong, 1986). A positive relationship between resource acquisition and female RLHT expression can be manifested as either increased clutch/litter size or increased reproductive frequency. In species that breed less than annually, the decision to reproduce in a given year depends upon attainment of a threshold of stored energy in females (i.e. threshold body condition; Aubret et al., 2002, Naulleau and Bonnet, 1996). Studies also show that females are in better postparturient body condition in high prey environments compared to low prey environments, potentially increasing residual

reproductive value (i.e. future reproductive success; Du, 2006, Ford and Seigel, 1989, Taylor et al., 2005).

Several studies have utilized food manipulation to establish causal links between food availability and RLHTs. Boutin (1990) reviewed 138 cases where the response to supplemental feeding was recorded in terrestrial vertebrates. The review included only two studies where reproductive parameters were measured in response to supplemental feeding in reptiles. The results of the two studies were mixed, with one species increasing investment towards reproduction (i.e. increasing clutch size, Guyer, 1988) and the other exhibiting no change (Rose, 1982). In addition, Ford and Seigel (1989) used supplemental feeding to show that clutch size is strongly influenced by food intake under laboratory conditions in Checkered Garter Snakes, and Taylor and DeNardo (2005) showed that food supplementation increased reproductive frequency and postparturient body condition in free-ranging Western Diamondback Rattlesnakes. Finally, James and Whitford (1994) showed that enhancing habitat (i.e. increasing water availability) such that prey abundance is increased, leads to increased reproductive output in a desert dwelling lizard, *Uta stansburiana*.

Taken together, the large body of literature relating prey abundance and or body condition with female reproductive effort supports the existence of a pathway of causation linking food intake and RLHT expression in female snakes (Fig 1). Such connections have been shown in species using the entire range of snake reproductive strategies (e.g. viviparity, oviparity, and annual, multiannual and less than annual reproduction (Ford and Seigel, 1989, Guyer, 1988, Taylor et al., 2005). However, little is known regarding the relationship between individual energetic status and other factors that could potentially affect female fitness (e.g. mating behavior and mate acquisition/attractiveness (but see Aubret et al., 2002). The mating

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systems and reproductive strategies utilized by reptiles are diverse (Seigel and Ford, 1987, Shine, 2003) and relationships between resource availability and female behavior may vary among these diverse systems. For example, different mating systems may be associated with different fecundity-independent costs to reproduction, which could affect the point at which the fitness benefit of current investment in reproduction outweighs the cost in terms of survivorship and future fecundity (i.e. the reproductive threshold; Aubret et al., 2002, Madsen and Shine, 1993). Description of the relationships among environment, behavior, and physiology and their underlying mechanisms in diverse taxa can provide powerful information for comparative analysis of how environmental factors interact with behavior and physiology to produce life history phenotypes.

### *Food resources and trait expression in male snakes:*

Compared to females, less attention has been given to the relationship between the resource environment and male RLHT expression. It is widely assumed that male gamete production is relatively cheap, and therefore, that male reproductive investment will be decoupled from energetic status (Aubret et al., 2002). However, in mating systems where males fight for, defend, or search extensively for receptive females, male reproductive effort can constitute a large portion of an individual's energy budget (Bonnet and Naulleau, 1996, Duvall and Schuett, 1997, Shine and Mason, 2005, Yoccoz et al., 2002). As such, in populations where energy is limited, males could potentially trade off current reproductive effort in favor of increased future fecundity/residual reproductive value. Such a tradeoff requires that male squamates possess the ability to sense their energetic context and adjust RLHT expression accordingly.

There is limited experimental and descriptive support for a link between energy intake and male RLHTs in snakes. Three descriptive studies have evaluated the relationship between body condition and male reproductive investment in snakes (Aubret et al., 2002, Bonnet and Naulleau, 1996, Shine and Mason, 2005). In all three studies male RLHTs were positively related to stored energy at the onset of the breeding season. Experimental studies linking food availability with male RLHTs have not been conducted in snakes. However, Ruiz et al. (2010) showed a positive effect of food supplementation on the frequency of male courtship displays in a lizard, *Sceloporous graciosus*. Their results suggest a causal link between food availability and male reproductive allocation and highlight the importance of considering the male response to shifts in resource abundance.

Whereas studies on female reptiles have largely focused on the effect of the resource environment on egg production and investment in individual offspring and not on reproductive behaviors and mate acquisition, the majority of studies on male reptiles have focused on mating success and reproductive behavior and have ignored potential effects on gametes. Sperm production and quality are dependent upon nutrition in other ectothermic vertebrates (e.g. fish; Izquierdo et al., 2001), however little is known regarding the determinants of sperm quality in squamates. It is becoming increasingly clear that polyandry is common in squamate mating systems and sperm competition likely affects reproductive outcomes (Olsson et al., 1998). Given that gamete production and reproductive behavior are often regulated by the same pleiotropic sex steroids (Moore and Lindzey, 1992, Norris and Lopez, 2010), male gamete production may be influenced by the same factors as behavior. Studies elucidating extrinsic environmental sources of variation in sperm quality/competitive ability in squamates are completely lacking in the literature and warrant further investigation.

***Endocrine regulation of RLHTs in female snakes:***

Reproductive life history traits are regulated by the major hypothalamic-pituitary axes of the endocrine system, particularly the hypothalamic-pituitary-gonadal (HPG) and adrenal (HPA) axes (Hau, 2007, Ricklefs and Wikelski, 2002, Sinervo and Licht, 1991, Wingfield and Sapolsky, 2003). However, the details of these mechanisms are not well defined and may vary within and among species. Recent studies show that the effects of gonadal and adrenal steroids on life history tradeoffs are dependent upon individual context, making clear-cut assertions regarding directional effects of specific hormones difficult (French et al., 2007, French et al., 2011).

Estradiol, the major gonadal product of the female HPG axis, directly stimulates vitellogenesis and can stimulate breeding behavior in squamates (Ho et al., 1982, Whittier and Tokarz, 1992). However, descriptions of female steroid hormone profiles in snakes have revealed that mating behavior can occur when estradiol levels are basal (Taylor and Denardo, 2010). Although estradiol is involved in the decision to reproduce and or the initiation of reproductive investment (i.e. vitellogenesis in most reptiles), the proximate endocrine mechanisms mediating reproductive effort are not well established. Little data exist on snakes. In oviparous lizards follicle stimulating hormone (FSH) regulates the number of follicles recruited and yolked, and exogenous administration during vitellogenesis leads to an increase in clutch size but a decrease in the size of individual offspring (Sinervo and Licht, 1991). However, little is known about the environmental factors that mediate FSH production rendering the ecological relevance of their result unclear.

Corticosterone (CORT) is a downstream product of the HPA axis and is the major circulating glucocorticoid in reptiles. CORT has been suggested to play a role in mediating life history tradeoffs among competing functions (e.g. reproduction and immunity), but its proximate

role in dictating tradeoffs awaits complete description (French et al., 2007, Svensson et al., 2002). CORT can act as an energy mobilizer and may be elevated during reproduction in order to meet increased energetic demands (Moore and Jessop, 2003, Romero, 2002). However, chronic elevation of CORT has been shown to decrease certain RLTHs in vertebrates (Moore and Jessop, 2003, Salvante and Williams, 2003, Svensson et al., 2002). In a review of the correlational evidence for a consistent relationship between baseline CORT concentrations and reproductive success, Bonier et al. (2009) found no consistent pattern (i.e. negative, positive, and no relationship were all reported), and no study to date has experimentally investigated the effect of glucocorticoids on time energy allocation in female snakes. In lizards, exogenous CORT exposure in pregnant females alters offspring size at birth, but it remains unknown as to whether this is the result of maternal allocation or effects on embryonic growth (Vercken et al., 2007). Glucocorticoids likely serve a regulatory function with regard to female RLHT expression that is dependent upon intrinsic and extrinsic context; the precise details of which remain to be described (French et al., 2007, Svensson et al., 2002).

In systems where individuals breed less than annually and reproductive frequency is dictated by stored energy thresholds, some mechanism must connect the HPG axis with peripheral fat and protein stores (Naulleau and Bonnet, 1996, Taylor and Denardo, 2010). In a variety of vertebrates, peptide hormones such as leptin and ghrelin feedback on the hypothalamus and inform the allocation of resources toward reproduction (Finn, 1998, French et al., 2009, Unniappan, 2010, Zieba et al., 2005). Receptors for both leptin and ghrelin have been identified on all three tissues of the HPG axis (i.e. hypothalamus, pituitary, and the gonads) suggesting a regulatory role of these hormones in vertebrate reproduction (Chelikani et al., 2003, Kaiya et al., 2013, Unniappan, 2010, Zieba et al., 2005). Manipulative experiments show that

leptin restores the pulsatile release of luteinizing hormone (LH) in mammals when under nutritional stress (Zieba et al., 2005). In the one experiment that has been conducted on the impact of leptin in female reptiles, mammalian leptin was shown to attenuate the effect of food restriction on follicle size in oviparous lizards (French et al., 2011). The lack of squamate leptin and ghrelin amino acid sequences and homologous (i.e. species-specific) molecular tools for use in manipulative studies has left a hole in our understanding of how reptiles respond to nutritional cues from the environment. Preliminary descriptive and experimental studies in diverse taxa suggest a broadly conserved role of leptin and ghrelin in mediating appetite (energy acquisition) and allocation (energy expenditure; French et al., 2011, Unniappan, 2010). However, until manipulation and measurement of these protein hormones becomes more tractable in non-model species, our understanding of the endocrine pathways connecting the resource environment and RLHTs will remain incomplete.

### *Endocrine regulation of RLHTs in male snakes:*

Similarly to females, reproduction is regulated by the HPG axis in male reptiles. However, in males, the primary gonadal product of the HPG axis is testosterone (T; Moore and Lindzey, 1992). Testosterone stimulates a variety of reproductive traits and behaviors that are energetically costly. For example, testosterone manipulation increases mating displays, territorial behavior, and territory size in male lizards (Marler and Moore, 1988, Marler et al., 1995, Tokarz, 1995). However, studies on the red-sided gartersnake, *Thamnophis sirtalis*, suggest that male courtship behavior is independent of the HPG axis. In fact, male *T. sirtalis* will court females even after hypophysectomy and gonadectomy (Camazine et al., 1980, Crews et al., 1984). Manipulative studies examining the life history and behavioral responses of snakes to testosterone treatment are very rare (but see Frazier, 2012), and, although a causal relationship is

often assumed based on a number of studies on other vertebrate groups, no clear experimental evidence indicates that T regulates RLHTs in male snakes. Most descriptive studies show peaks in testosterone during or just before the breeding season even when the breeding season and gametogenesis are decoupled (Graham et al., 2008, Hoss et al., 2011, Lind et al., 2010, Naulleau et al., 1987, Schuett et al., 1997, Schuett et al., 2002, Schuett et al., 2005, Taylor et al., 2004, Weil and Aldridge, 1981, Zaidan et al., 2003). The large agreement of descriptive studies is consistent with a role of the HPG axis in regulating/stimulating male reproductive behavior in snakes, but attempts to manipulate T experimentally have yet to elucidate proximate causation.

Some recent descriptive and experimental studies indicate that T production is modulated according to energetic context in reptiles. Circulating T concentrations are positively related to body condition in *Thamnophis radix* and *Boa constrictor* (Holding et al., 2014, King and Bowden, 2013). In the only experimental study on a squamate, Ruiz et al. (2010) manipulated food availability in male lizards and showed that fed lizards had significantly higher T concentrations compared to food restricted groups. Just as in females, endocrine mechanisms connecting resource acquisition and the HPG axis exist in male vertebrates. Receptors for leptin and ghrelin are present on the three major tissues of the male HPG axis (Chelikani et al., 2003, Kaiya et al., 2013, Unniappan, 2010, Zieba et al., 2005), and one study suggests that leptin may play a reproductive role in male lizards (Putti et al., 2009). No studies have investigated the role of leptin in male snakes, and little research exists on lizards (but see Niewiarowski et al., 2000, Paolucci et al., 2006, Sciarrillo et al., 2005). Research concerning the connection between energetic/nutritional status and RLHTs in male reptiles suffers from the same lack of amino acid sequences and homologous tools as described for females, and, as such, almost nothing is known

about endocrine integration of nutritional cues from the environment in this large, diverse vertebrate group.

Just as in females, the proximate role of the HPA axis in regulating/modulating RLHT expression in male reptiles is poorly described. Exogenous CORT manipulation has been shown to suppress reproductive behaviors and T production, indicating that CORT reduces reproductive effort (Moore and Jessop, 2003, Moore and Mason, 2001). However, in other studies CORT is elevated above baseline during reproduction (See Romero, 2002), and CORT and T concentrations are positively correlated in field-active *T. sirtalis* (Moore et al., 2000). The relationship is reversed (i.e. negative) in field-active Timber Rattlesnakes, *Crotalus horridus* (Lutterschmidt et al., 2009). It is unclear whether these seemingly contradictory results are the result of evolutionary or environmental/energetic context. Both baseline levels of CORT and physiological response to CORT have been shown to depend on body condition in reptiles (French et al., 2007, Moore et al., 2000), and in some species baseline concentrations vary seasonally (Graham et al., 2008, Moore et al., 2000). Until a better understanding of the intrinsic and extrinsic factors that affect CORT production and an understanding of the proximate role of CORT in mediating life history tradeoffs is achieved, generalizations regarding the role of the HPA axis in regulating RLHT expression are impossible and must be established on a case by case basis.

**Research goals:**

In the chapters that follow, the relationship between energetic context, steroid hormones, and RLHTs is examined in field-active male and female Timber Rattlesnakes, *C. horridus*. *Crotalus horridus* in northwest Arkansas are a good model species for describing intrinsic and extrinsic sources of variation in time/energy allocation. *Crotalus horridus* are large-bodied,

facilitating the implantation of 2-3 year radiotransmitters and long-term monitoring of energetic status, behavior, and hormone production. Additionally the population studied experiences annual shifts in resource abundance, and large within and among-individual variation in body condition and foraging success has been documented (Beaupre, 2008). Therefore, if individual expression of RLHTs is adjusted according to energetic status in *C. horridus*, the relationship should be evident, and variation in energetic status should be related to variation in time/energy allocation. Additionally, if steroid hormones mediate the relationship between energetic status and RLHT expression, circulating concentration should be related to both energetic status and time/energy allocation. By using a descriptive approach, I aimed to show how reproductive life history traits (RLHTs) vary within and among individuals in relation to natural environmental variation (e.g. variation in resource availability) across years.

In addition to highlighting the relationships between environment, the endocrine system, and RLHTs in field-active males, chapter four describes in detail the reproductive ecology of female *C. horridus,* and how female ecology relates to energetic status. As described above, links between the resource environment and investment in offspring are well-established in female snakes. However, little is known regarding the relationship between female energetic status and female attractiveness or behavior. Chapter four examines these relationships by analyzing female mating behavior, reproductive outcomes, and energetic status in field active snakes.

**Future directions:**

Much is taken for granted or assumed regarding the chain of events linking environmental variation and trait expression in snakes (Fig 1). For example, testosterone's role in mediating sexual displays and territorial behaviors is well documented in other reptiles (Moore and Lindzey, 1992). However, reproductive success in many snake species, including most pit

vipers, is dependent upon a different suite of behaviors (e.g. search patterns and effort; Duvall and Schuett, 1997, Mcgowan and Madison, 2008). No studies have successfully investigated underlying mechanisms regulating mate search (see Frazier, 2012). Also, the role of T in the translation of energetic status into "context appropriate" allocation decisions is little understood in reptiles in general. The research outlined in chapters two and three elucidates many descriptive links between environmental context, steroid hormones, and time energy allocation. However, additional research is required and should focus on experimental investigations of the role of steroid hormones in mediating RLHT expression in snakes. Future research should also take advantage of advancements in molecular technologies in order to describe the mechanistic pathways that link environment and trait expression (Fig 1). An understanding of the entire pathway will allow for a better understanding of organismal responses to environmental change.
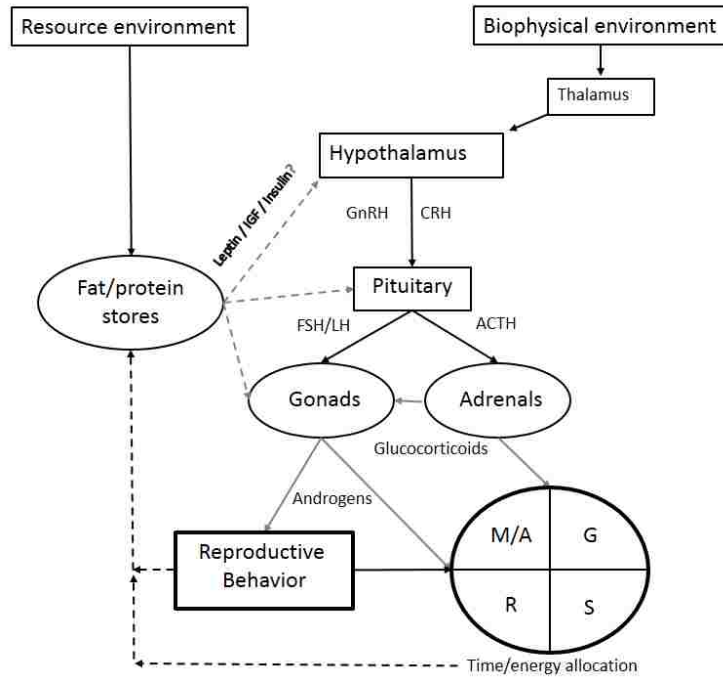
**Figure 1:** Conceptual diagram depicting the translation of information about the resource and biophysical environment into behavioral and energy allocation/life history decisions via the HPG and HPA axes and other peripheral tissues. Gray dashed arrows represent a hypothesized link between energetic status and the HPG and HPA axes. Solid gray arrows indicate hypothesized links between the downstream products of the HPA and HPG axes, reproductive behavior and allocation among competing functions (i.e. Maintenance and activity, M/A; growth, G; reproduction, R; and storage, S; adapted from: Dunham, Grant, and Overall 1989).

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**Chapter II: Natural variation in steroid hormone profiles of male Timber Rattlesnakes, *Crotalus horridus*, in northwest Arkansas**

**Abstract:**

We describe the seasonal profile of circulating steroid hormones (testosterone and corticosterone) in relation to the breeding season in free ranging male Timber Rattlesnakes, *Crotalus horridus,* over the course of three active seasons. In addition, we examine variation in steroid concentrations across years and in relation to body condition. We found that seasonal profiles of plasma testosterone were different compared to other crotalines with similar mating patterns. Concentrations of testosterone were elevated above baseline in the three months leading up to the single late summer breeding season. Testosterone peaked in July at the onset of the breeding season and dropped to baseline during the peak months of breeding (August and September). Testosterone concentrations also varied annually. Although the exact cause of annual variation could not be established, our results indicate that weather patterns may have driven observed differences. Testosterone concentrations were positively related to body condition, indicating that testosterone production is modulated according to energetic status (particularly in the two months prior to the breeding season). Corticosterone did not vary seasonally or with any measured variable, a result similar to other studied crotalines. Our results highlight the importance of long-term descriptive studies of the regulatory mechanisms that underlie behavior and physiology in diverse taxa, as these mechanisms can vary greatly within and among populations and are valuable in elucidating the intrinsic and extrinsic sources of such variation.

## 1. Introduction

Steroid hormones regulate reproductive life history trait expression and mating systems in vertebrates (Hau, 2007, Ketterson and Nolan, 1992). As such, an understanding of how variation in hormone production is related to environmental variables (e.g. season, temperature, or resource availability) is vital to understanding how organisms respond to realized or predicted shifts in environmental conditions. Field studies describing the interplay between environmental variation, regulatory mechanisms (e.g. neuroendocrine axes), and trait expression in diverse taxa contribute valuable data for comparative analysis of the ultimate and proximate forces that drive the evolution of life histories and mating systems in vertebrates (Duvall et al., 1992).

In snakes, life history strategies and mating systems vary widely within and among closely related species (Duvall et al., 1992, Seigel and Ford, 1987, Shine, 2003, Shine, 2005). Due to their diverse mating systems, wide geographical ranges, and large body sizes that facilitate long-term radio tracking, pit vipers (Viperidae, Crotalinae), and North American pit vipers in particular, have emerged as model organisms for investigating the hormonal regulation of reproduction in nature (Almeida-Santos et al., 2004, Beaupre and Duvall, 1998, Schuett et al., 2006). At least 10 studies have described the relationship between steroid hormones and the male reproductive cycles of seven different species of crotaline (Graham et al., 2008, Hoss et al., 2011, Johnson et al., 1982, Lind et al., 2010, Schuett et al., 2002, Schuett et al., 2005, Schuett et al., 1997, Smith et al., 2010, Taylor et al., 2004, Zaidan et al., 2003). The large and growing body of information on hormonal regulation of mating systems in field active crotaline snakes provides the opportunity for comparison of phylogenetic, environmental, and geographical factors that impact steroid hormone production and subsequently drive the expression of traits under steroidal regulation.

Testosterone stimulates seasonal reproductive behavior and spermatogenesis (Moore and

Lindzey, 1992) and mediates life history tradeoffs in male vertebrates (Hau, 2007, Ketterson and

Nolan, 1992). Circulating testosterone concentrations in field active snakes vary according to the

timing of reproductive behaviors (DeNardo and Taylor, 2011, Taylor and Denardo, 2010). North

American pit vipers display either one or two breeding seasons in a given year, and peak

spermatogenesis occurs in the late summer to early fall (Aldridge and Duvall, 2002). To date,

seasonal steroid hormone profiles for four North American pit viper species (i.e. *Crotalus*

*molossus* (Schuett et al., 2005), *Crotalus adamanteus* (Hoss et al., 2011), *Agkistrodon contortrix*

(Smith et al., 2010), and *Agkistrodon piscivorus* (Graham et al., 2008, Johnson et al., 1982,

Zaidan et al., 2003) with male mating patterns similar to *C. horridus* have been described. In all

species studied, a single peak in testosterone coincident with the breeding season was observed

(Graham et al., 2008, Hoss et al., 2011, Schuett et al., 2005, Zaidan et al., 2003). *Crotalus*

*horridus* displays a single breeding season in late summer in most parts of its range (Aldridge

and Brown, 1995, Aldridge and Duvall, 2002, Martin, 1993). However, data presented by

Lutterschmidt et al. (2009) were not consistent with a late summer peak in testosterone

concentrations in *C. horridus* in northern portions of its range (north-central PA). Detailed

description of seasonal testosterone cycles and behavior in populations from other areas of the

wide range of *C. horridus* will either support or refute the broad scale conservation of

hypothalamo-pituitary-gonadal (HPG) axis activity in relation to the breeding season (i.e.

associated) in snakes that breed exclusively in the late summer.

Corticosterone (CORT), a downstream product of the hypothalamo-pituitary-adrenal (HPA)

axis, also plays an important role in vertebrate reproductive cycles. The HPA axis is a key

modulator of physiological and behavioral responses to environmental stress (Moore and Jessop,

2003). Due to its role as an energy mobilizer, CORT is predicted to be elevated at the time of year when energy is most limited (energy mobilization hypothesis, EMH), leading to a positive relationship between CORT and testosterone concentrations and significantly elevated CORT concentrations during the breeding season in most species (Romero, 2002). Studies of many reptile taxa support the EMH (Moore et al., 2000, Romero, 2002, Wilson and Wingfield, 1994). However, studies relating CORT concentrations to the reproductive cycles of pit vipers show no elevation in CORT concentrations during the breeding season (Graham et al., 2008, Lind et al., 2010, Taylor et al., 2004). These studies all speculate that the low metabolic rates and large fat stores of most pit vipers buffer against the need for up-regulation of the HPA axis during reproduction. Additional data from populations where resource availability and body condition are highly variable will aid in elucidating any role of the HPA axis in mobilizing the energy needed for costly behaviors during the breeding season or when under nutritional stress.

In addition to season, steroid hormone concentrations can vary according to a variety of extrinsic factors. Field studies that describe seasonal hormone cycles tend to focus on mean concentrations and provide little explanation for the large and potentially adaptive individual variation observed (Williams, 2008). Environmental factors (e.g. annual weather patterns and resource availability; Knapp et al., 2003, Schuett et al., 2005), and individual characteristics (e.g. body size and body condition; Graham et al., 2008, Lind et al., 2010, Moore et al., 2000, Schuett et al., 2005) have been shown to correlate with steroid hormone concentrations in reptiles. However, results are mixed, and few studies span more than one active season. Multi-year studies utilizing repeated measures on individuals across varying environmental conditions allow for examination of within- and among-individual variation in hormone production. Such studies

are valuable for elucidating the relationships between extrinsic environmental factors and individual variation in hormone concentrations.

We measured steroid hormone concentrations in the plasma of field active Timber Rattlesnakes, *Crotalus horridus*, over the course of three active seasons in order to achieve two goals: (1) to describe the relationship between steroid hormones and the reproductive cycle of *C. horridus* for comparative analysis, and (2) to identify extrinsic sources of individual variation in hormone production in *C. horridus*.


## 2. Methods

### 2.1 Study area and animals

Our study was conducted in Madison County Arkansas (see Beaupre, 2008, for detailed habitat descriptions). The climate data presented (Figure 1) were taken from the NOAA national climactic data center website and were recorded at a weather station approximately 47 km southwest of the field site. Adult male *C. horridus* were captured and implanted with temperature sensitive radiotransmitters (Holohil systems Ltd. Model SI-2T, Carp, Ontario, Canada), following methods described in Reinert and Cundall (1982). Snakes were released at the point of capture and were not sampled for at least one month after surgery. In total 12 males were monitored for varying numbers of years over the three year study period. Individuals ranged from 81.1 – 109.1 cm (mean = 93.46 ± 0.80) in snout vent length (SVL) and from 385-1333 g (mean = 760.16 ± 23.71) mass. Long term study of our population has shown that body condition varies annually in response to shifts in the resource environment, and that individual body condition is highly variable in the population (Beaupre, 2008, Beaupre and Douglas, 2009).

*2.2. Field monitoring*

Snakes were tracked 1-4 times per week during the active season (Apr-Oct) from April, 2011 to October, 2013. Snake handling procedures followed Beaupre and Green (2012). Snout vent length measurements were taken in a squeeze box at the beginning of each active season, and these measurements were used in body condition calculations throughout the year. Because the annual growth rate of adult *C. horridus* in northwest AR is typically lower than the error rate associated with squeeze box measurements (Beaupre et al., In press), repeated SVL measurements were not necessary. Mass was measured using a 2,000 g Pesola® spring scale each time a snake was bled.

The timing and duration of the breeding season were based on long-term observation of specific reproductive behaviors. Reproductive behaviors included; association, courtship, and copulation. Associations were recorded when two snakes of the opposite sex were found with 3 meters of each other. Due to a low and unpredictable annual incidence of observed reproductive behaviors in this population, we used data collected from the long-term study of our population from 1995-2013. Any incidence of reproductive behavior was included, and repeated observations on individuals are included in the graph.

*2.3. Blood collection and RIA*

A blood sample of up to 1 ml was taken monthly from the caudal vein with a 1 ml BD syringe attached to a 27 G needle. To ensure that handling stress did not affect steroid hormone concentrations, all blood samples were taken within 5 minutes of first contact with the snake (Moore et al., 1991, Schuett et al., 2004). Blood was immediately transferred to a 1.5 ml microcentrifuge tube containing 2 drops of EDTA and was stored on ice for no more than 10

hours before centrifugation for 15 min at 13,000 G to separate plasma. Plasma was stored at -20°C until radioimmunoassay (RIA).

Quantification of plasma testosterone and CORT concentrations was conducted using commercially available no-extraction Coat-a-Count® RIA kits (Seimens Healthcare Diagnostics Inc. Los Angeles, CA). Each kit uses a proprietary extraction buffer and is designed to measure total circulating concentrations. Both kits were validated via quantitative recovery of cold spiked plasma (T = 90%, CORT= 94.8%) and parallelism of inhibition curves (T: $p = 0.37$, B: $p = 0.134$).

Testosterone was measured in two separate assays. The inter-assay coefficient of variation was 6.8% and the assay wide coefficients of variation within the range of the standard curve were 12.00% and 10.59%. All kit protocols were followed except that samples were diluted by at least half (1:1) with zero standard to bring elevated concentration within the range of the standard curve (6 samples that read above the curve were diluted 1:2 and re-run). The minimum reportable range of the assay was 0.04 ng/ml. All samples that read below the sensitivity of the assay were reported as 0.08 ng/ml. Cross-reactivity with other steroids is low for the assay (< 0.5% in all cases).

Corticosterone was measured in snakes sampled in two months outside of the breeding season (April and May) and the two months where androgen activity and breeding behavior was predicted to be highest (July and August).  Corticosterone was measured in a single assay. The assay wide coefficient of variation within the range of the standard curve was 13.78%. Kit protocols were only slightly modified. Because circulating CORT concentrations in snakes were often near or below the range of the standard curve and were never near the high end of the curve, the two highest standards were eliminated and the standard curve was diluted out two

extra steps on the low end with zero standard (from 22.0 ng/ml to 5.5 ng/ml). All other assay

procedures followed kit protocol. The minimal detection limit for the assay was 5.7 ng/ml. All

samples that read below the detection limit were recorded as 5.7 ng/ml. About one third of

samples read below the detection limit. However, the detection limit was well below mean

CORT concentrations, and our goal was to elucidate factors associated with elevated CORT

concentrations (e.g. season and body condition) and was not concerned with small variation in

baseline concentrations. Also, readings below the detection limit were distributed among all four

months. For these reasons, the assay was capable of detecting elevation of CORT concentrations

above baseline and relating elevated concentrations to measured variables.

*2.4 Statistical analysis*

Body condition index (BCI) was calculated by dividing the individual residual from a

population-wide nonlinear regression of mass on SVL (Beaupre and Douglas, 2009) by body

mass. We divided individual residuals by mass to correct for the large variation in individual

body size in the study, and we were more interested in quantifying relative deviation above or

below population means than in quantifying overall fat/protein stores.

All data were analyzed for outliers, normality, and homoscedasticity, and transformed as

necessary before interpretation of results. Statistical analyses were conducted in SAS 9.3 and

JMP Pro 11 (SAS Institute, 2013). Data from Apr and Oct were not included in the statistical

analysis of testosterone concentrations due to low sample sizes. Seasonal concentrations were

analyzed using a double repeated measures model in SAS PROC MIXED with month as a

repeated fixed factor nested within year as a repeated fixed factor (full repeated model).

Covariance structures were assigned based on analysis of AIC and BIC scores (Burnham and

Anderson, 2002). Body condition index was included as a covariate in seasonal analyses.

Because testosterone concentrations were different in 2013 compared to 2011 and 2012 (see results), an additional analysis was run using data from 2011 and 2012 only (reduced repeated model). Relationships between BCI and testosterone concentrations within single months were compared in a repeated measures model in PROC MIXED with year as the repeated factor. Only data from 2011 and 2012 were included in single month analyses.

## 3. Results

### 3.1 Seasonal behavior

Reproductive behaviors were observed from July 26th to September 12th (Figure 2). The majority of the 49 reproductive behaviors observed (77.6%) occurred in August followed by September (16.3%) and July (6.1%). One association was recorded in early June and was considered an outlier because no actual reproductive behavior was observed and the two snakes may have simply been in close proximity due to foraging cues or by chance.

### 3.2 Seasonal variation in hormone concentrations

There was a significant effect of sampling month on testosterone concentrations ($F_{4,36} = 38.63$, $p < 0.001$). Post hoc Tukey HSD tests showed that testosterone is elevated above baseline levels as early as May and peaks in July, and that testosterone concentrations were significantly lower in the months of peak breeding behavior (August and September) compared to the months leading up to the breeding season (June and July; Figure 2). There was no significant effect of sampling month on CORT concentrations ($F_{3,20} = 1.46$, $p = 0.26$; Figure 4; Table 1).

*3.4 Annual variation in hormone concentrations*

There was a significant effect of year on testosterone concentrations ($F_{2,12} = 22.74$, p < 0.001) in the full model. Post hoc Tukey HSD tests indicated that July testosterone concentrations in 2013 were significantly lower than in 2011 and 2012, which were not significantly different from each other, and that testosterone concentrations in June of 2012 were significantly higher compared to 2013 (Figure 5). Body condition varied annually ($F_{2,12} = 23.14$, p < 0.001). Post hoc tests showed that snakes were in lower body condition in 2011 compared to 2012 and 2013, which were not significantly different from each other. There was no effect of year on CORT concentrations ($F_{2,11} = 0.68$, p = 0.53).

*3.5 Body condition and SVL*

There was a significant positive relationship between body condition and testosterone concentrations in the full repeated measures model ($F_{1,63} = 9.53$, p = 0.003). There was a significant BCI*month and year*month interaction (BCI*month: $F_{4,63} = 3.75$, p = 0.008, year*month: $F_{7,20} = 3.58$, p = 0.012). There was no significant relationship between SVL and testosterone concentrations ($F_{1,63} = 0.48$, p = 0.49).

There was a significant BCI*month interaction in the reduced repeated measures model for monthly testosterone concentrations (years 2011 and 2012 only; $F_{4,41} = 7.93$, p < 0.001). Within-month repeated measures analyses revealed that BCI was positively related to testosterone concentrations only in the months leading up to the breeding season (Jun: $F_{1,2} = 18.43$, p = 0.05, Jul: $F_{1,9} = 17.01$, p = 0.003; Figure 6). Testosterone concentrations were not related to body condition in the months of May, Aug, and Sep (May: $F_{1,3} = 0.03$, p = 0.88; Aug:

$F_{1,4} = 0.14$, $p = 0.73$; Sep: $F_{1,8} = 1.05$, $p = 0.34$; Figure 6).There was no significant relationship between CORT  concentrations and body condition ($F_{1,37} = 0.01$, $p = 0.94$).

## 4. Discussion

### 4.1 Seasonal variation

*Crotalus horridus* in the Ozark Mountains of northwest Arkansas display a single breeding season in late summer; a pattern of reproductive behavior similar to other unimodally breeding pit vipers (Hoss et al., 2011, Lutterschmidt et al., 2009, Schuett et al., 2005, Smith et al., 2010, Zaidan et al., 2003). The seasonal pattern of circulating testosterone, however, differs from other studied species. Male *C. horridus* display elevated testosterone concentrations indicative of HPG axis up-regulation in May, several months before the breeding season. Testosterone concentrations reach a peak in July near the onset of the breeding season and then drop off sharply during the months of peak breeding (Figures 2 and 3). The pattern we observed supports the hypothesis that the behavioral role of testosterone in *C. horridus* is to condition or prepare males for reproductive behavior, and that mating behavior can and does continue after circulating concentrations have declined (Crews, 1991, Lind et al., 2010, Naulleau et al., 1987, Saint Girons et al., 1993).

Our results show extended up-regulation of the HPG axis from May through July in *C. horridus*. It is likely that the observed up-regulation of the HPG axis is involved in spermatogenesis, sperm maintenance, and the conditioning of reproductive behaviors (Aldridge et al., 2011, DeNardo and Taylor, 2011, Moore and Lindzey, 1992, Norris and Lopez, 2010). Unfortunately, no studies are available on the pattern of spermatogenesis in Arkansas populations of *C. horridus*. A description of male reproduction in *C. horridus* from its northern

range indicated that the sexual segment of the kidney is hypertrophied throughout the active season and that spermatogenesis peaks in July but continues through September (Aldridge and Brown, 1995). Most unimodally breeding crotalines studied to date do not show elevated concentrations as early in the active season as *C. horridus* (with the exception of *Crotalus molossus,* Schuett et al., 2005), and in all studied species, peak concentrations coincide with peak reproductive activity (Hoss et al., 2011, Smith et al., 2010, Zaidan et al., 2003). For example, *Agkistrodon piscivorus* in northwest Arkansas are sympatric with *C. horridus*, and the timing of reproductive behaviors is similar in the two species (there is some evidence of spring opposite sex pairings in *A. piscivorus*, but no spring mating has been observed in either species; Hill and Beaupre, 2008). However, their seasonal testosterone patterns are different. In *A. piscivorus*, testosterone is not elevated in the months leading up to the breeding season and is at its highest level during the peak month of breeding (Zaidan et al., 2003). The only other snake species that shows HPG axis up-regulation in the months leading up to the breeding season is *C. molossus*, an inhabitant of the American southwest (Stebbins, 2003). The observed differences in seasonal testosterone patterns (i.e. differences in the timing and duration of HPG axis up-regulation with regard to the breeding season) between unimodally breeding populations of *Agkistrodon* and the two studied unimodally breeding *Crotalus* could reflect divergence in the regulatory mechanisms that govern seasonal reproductive physiology and or behavior within the Crotalinae. Future comparative and experimental studies exploring how variation in the timing, duration, and magnitude of testosterone up-regulation affects life history and behavior are needed to determine the consequences of and the ultimate reasons for variation in HPG axis up-regulation within and among vertebrate species (Kempenaers et al., 2008).

Our results regarding seasonal CORT concentrations were similar to other studies of crotaline snakes (Graham et al., 2008, Lind et al., 2010). We observed no elevation of CORT concentrations during the breeding season (Figure 4) and no relationship between testosterone and CORT concentrations. CORT concentrations were highly variable, and no measured variable explained the individual differences observed. Our data do not support the EMH or any suppressive effect of CORT on testosterone concentrations. However, the unexplained variance in concentrations within months, likely the result of natural individual variation, coupled with a low sample size in April, makes seasonal interpretation of CORT concentrations difficult.

*4.2. Morphological variation*

Testosterone concentrations in *C. horridus* are positively related to body condition and not to other morphological variables (i.e. SVL or Mass). Testosterone concentrations were not related to body condition in any previous study of temperate zone snakes where the relationship was measured (Moore et al., 2001, Moore et al., 2000, Schuett et al., 2005), including *C. horridus* collected from their northern range (Lutterschmidt et al., 2009). In most studies, SVL or mass is used as a covariate in seasonal analyses of testosterone concentrations, and body condition is dismissed or not considered (Graham et al., 2008, Hoss et al., 2011, Lind et al., 2010, Taylor et al., 2004). Our data show a strong positive relationship between body condition and testosterone concentrations, but the relationship is most evident during the months leading up to the breeding season (Fig 6). We believe that the lack of evidence for a significant relationship between body condition and androgens in other studied snake species may be the product of several factors including; small variation in body condition in studied populations, the seasonal dependence of the relationship itself, and or insufficient sample sizes for season-specific analyses (i.e. before, during, and after the breeding season). *Crotalus horridus* in our

study population are limited in terms of food resources, and body condition varies strongly within and among individuals over time (Beaupre, 2008). Variation in body condition is likely due to variation in foraging success and affects the allocation of time towards reproductive behaviors in *C. horridus* (Beaupre, 2008). Experimental elevation of testosterone has been shown to increase reproductive allocation in male birds and lizards (Ketterson and Nolan, 1992, Olsson et al., 2000). Assuming that high testosterone concentrations lead to increased reproductive allocation in *C. horridus*, some physiological mechanism must exist to monitor available energy (i.e. fat and/or protein stores) and provide feedback to the HPG axis, thus informing allocation decisions in a context specific manner. Whether males of other snake species also possess such a mechanism is not known, however the need for such a mechanism has been suggested many times for females (Taylor and Denardo, 2010). Several hormones (e.g. CORT, leptin, and insulin like growth factor-1) have been proposed to circulate according to energy stores and may play a role in energy monitoring and resource allocation in reptiles (French et al., 2011, Moore and Jessop, 2003, Sparkman et al., 2010). Similar to results from its northern range (Lutterschmidt et al., 2009), baseline CORT concentrations were not related to body condition in *C. horridus*. Our results are in contrast to studies on two garter snake (*Thamnophis*) species where a negative relationship between CORT and body condition was observed (Moore et al., 2001, Moore et al., 2000). The lack of an observed relationship between circulating CORT and body condition indicates that CORT concentrations are not involved in monitoring stored energy levels and that some other mechanism must account for the observed condition-dependent production of testosterone in *C. horridus*. Future descriptive and experimental studies are needed to establish the mechanisms that modulate the HPG axis according to energetic context and, in doing so, regulate life history tradeoffs in reptiles.

*4.3. Annual variation*

By quantifying testosterone concentrations across several active seasons we have shown that testosterone concentrations varied significantly among years (Figure 5). In 2013, early spring temperatures were abnormally low and continued to be lower compared to 2011 and 2012 through the month of September (Figure 1). Concentrations of testosterone in 2013 also showed no significant seasonal variation in post hoc analyses (Figure 5A). No animal handling or assay protocols, with the exception of storage duration, which is not likely to have affected results (Holl et al., 2008), were different between years, and snakes were not in lower body condition in 2013 compared to 2011 and 2012. In fact, snakes were in significantly better body condition in 2013 than in 2011. Three individuals were sampled repeatedly in both 2012 and 2013. Despite similar individual body conditions in the two years, stark within-individual differences in testosterone profiles were observed (figure 5B). Our results are descriptive, and cannot establish a causal link between annual weather patterns and testosterone concentrations. However, the dependence of circulating steroid concentrations on annual weather patterns has been suggested in at least two other studies of seasonal testosterone in field active reptiles (Knapp et al., 2003, Schuett et al., 2005). In both studies variation in environmental stress or energetic status between years is provided as an explanation for the observed annual variation in testosterone concentrations. Our study found that neither body condition nor circulating CORT concentrations explained annual variation in testosterone concentrations. Because differences in the magnitude of HPA up-regulation were observed in May and continued throughout the season, we speculate that abnormally cool spring temperatures in 2013 are the best explanation for the low testosterone concentrations observed in that year. Warming spring temperatures induce male reproductive behaviors in spring breeding populations of the red-sided gartersnake, *Thamnophis*

*sirtalis* (Aleksiuk and Gregory, 1974, Hawley and Aleksiuk, 1975). To our knowledge, thermal dependence of HPG axis up-regulation in reptiles that breed well after emergence from hibernation has not been studied. If such a dependence exists, shifts in climate could potentially have profound effects on fitness related traits and behaviors under HPG axis regulation.

*4.4. Conclusion*

We have shown that circulating testosterone concentrations in *C. horridus* vary seasonally, with individual energetic status, and by year. The seasonal pattern of testosterone concentrations is different compared to some other crotalines (e.g. *Agkistrodon spp.*) with similar mating patterns. The ultimate basis for observed differences is beyond the reach of our study, but our results are a reminder that description of the proximate mechanisms that regulate life histories and behaviors in unstudied populations remains important, as these mechanisms can vary greatly within and among related species. As in other studied crotalines, testosterone concentrations were not related to CORT concentrations, and CORT concentrations did not vary by energetic status, season, or year. We were unable to account for the large individual variation in CORT concentrations. Taken together with past studies (Graham et al., 2008, Lind et al., 2010, Taylor et al., 2004), our results support the conclusion that male crotalines can meet the energetic challenges (if present) of reproduction without activation of the HPA axis, and suggest that some other regulatory mechanism is involved in modulating testosterone production according to energetic context. Stark differences in circulating concentrations were observed between years, and we argue that differences in annual weather patterns were the most likely explanation for observed differences. Whether differences in annual testosterone concentrations translated into annual differences in male reproductive allocation decisions and or behaviors is the subject of future communications. Further experimental study examining the effects of

realized or predicted environmental conditions on modulation of the HPG axis and how such modulation affects trait expression is needed in order to predict how regulatory mechanisms will respond to environmental change and better model the response of fitness related traits under HPG control (Adkins-Regan, 2008).

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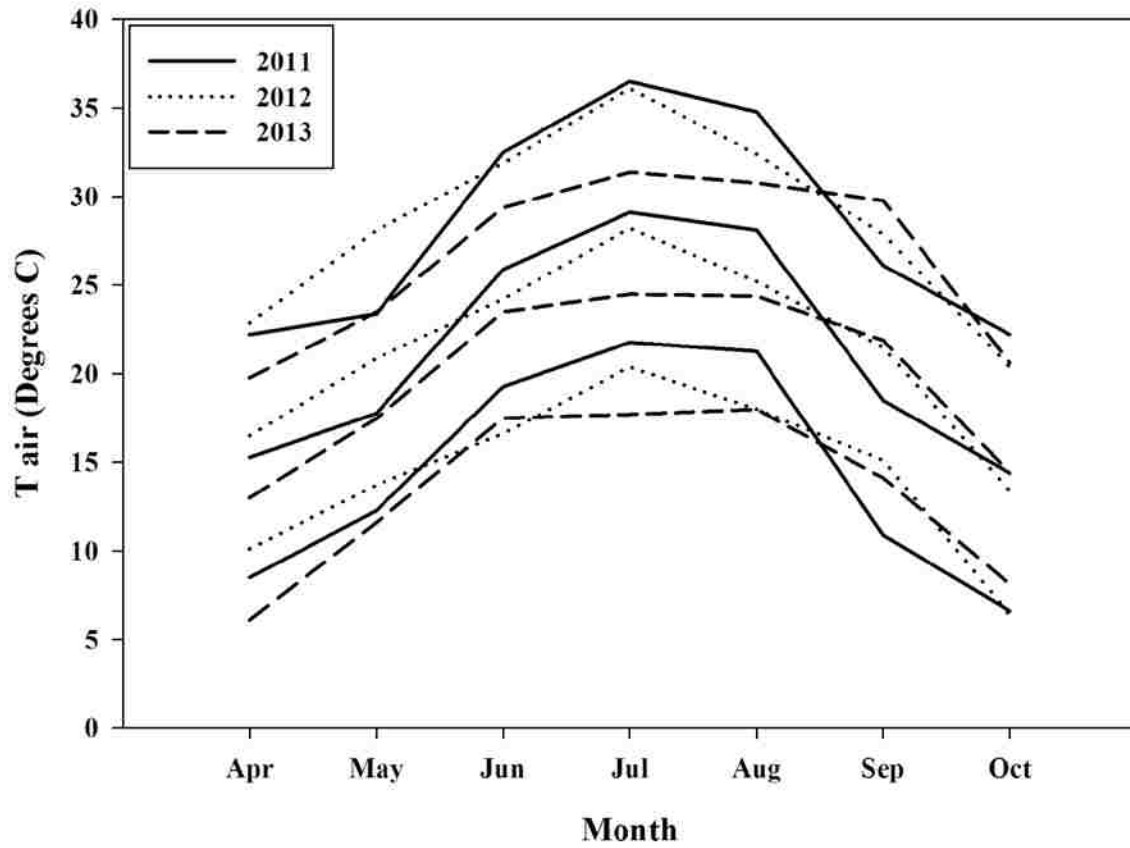
**Figure 1:** Mean minimum, maximum, and average monthly ambient air temperatures taken from a local weather station each year of the study.
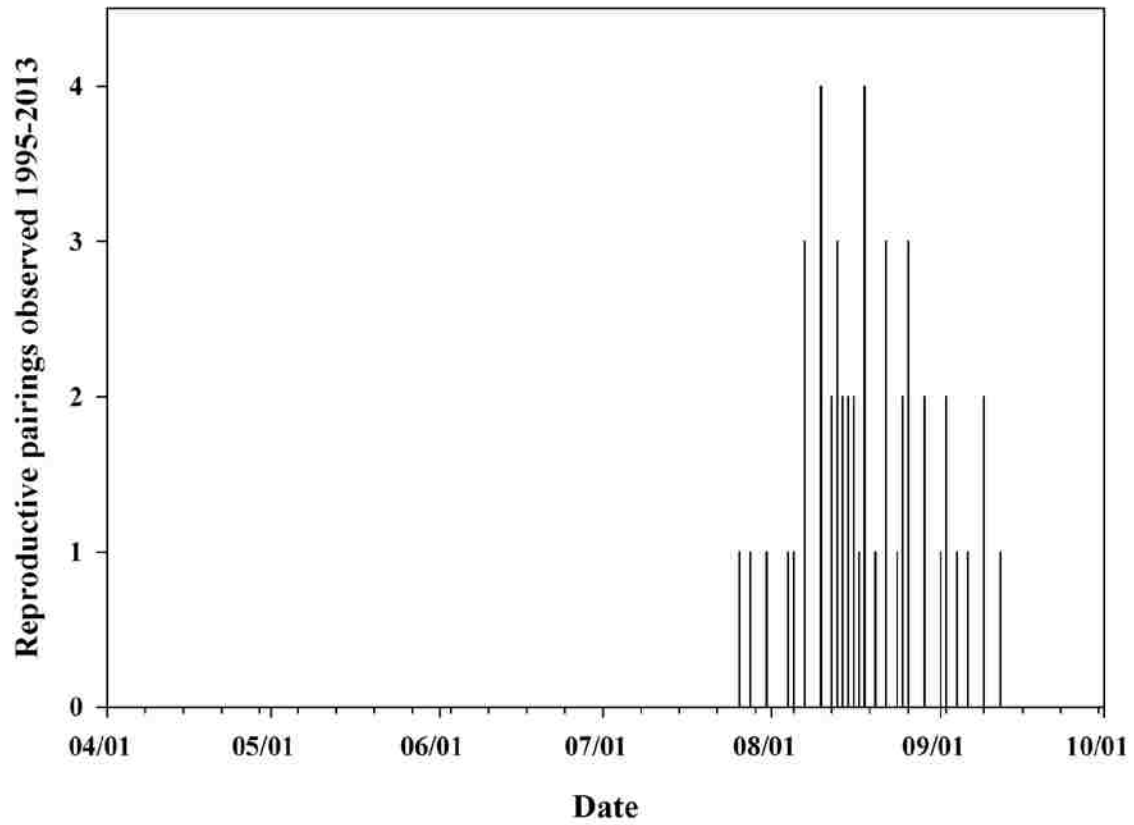
**Figure 2:** Count data for the number of reproductive behaviors observed on each date based on behavioral observation by radiotelemetry from 1995-2013.
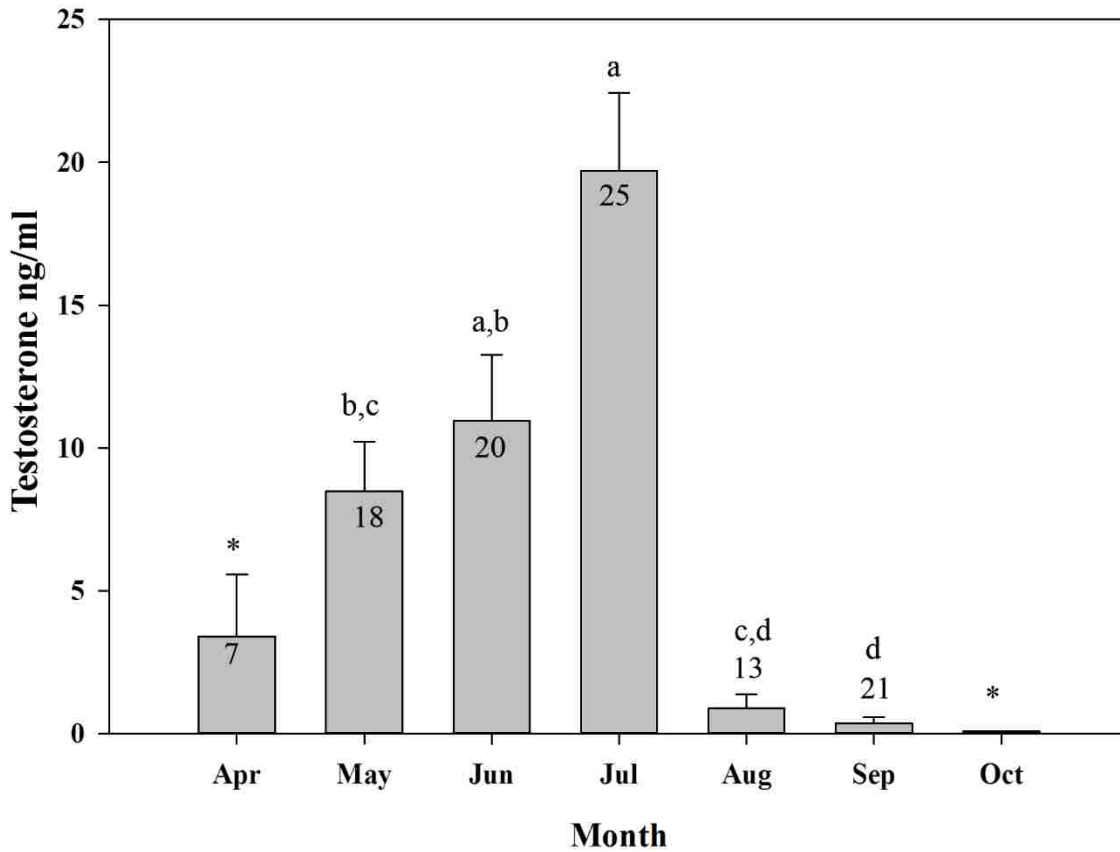
**Figure 3:** Mean monthly testosterone concentrations (2011-2013). Months with significantly different concentrations (full repeated model) do not share letters above their error bars. Numbers on or just above the bars represent sample sizes for each month.

*The months of April and October were removed from statistical analysis due to low sample sizes and a lack of data from all years.
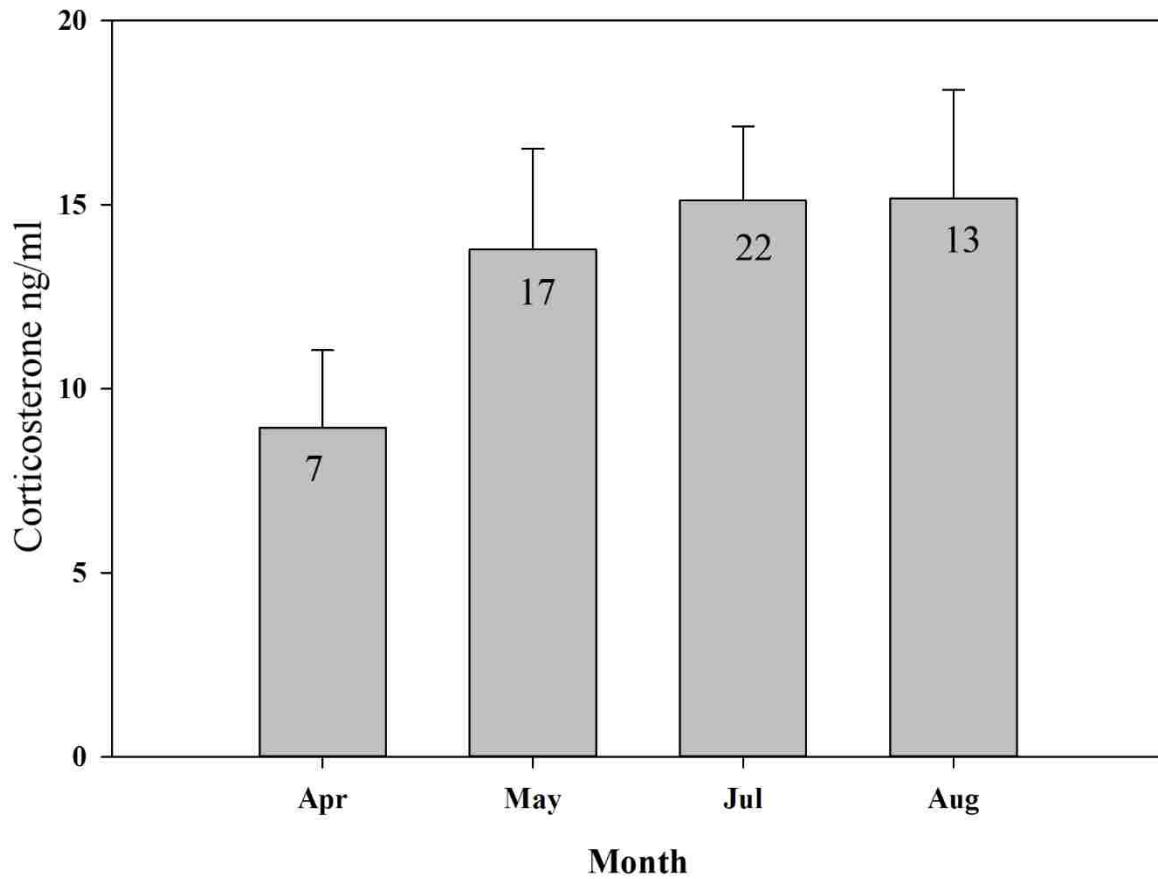
**Figure 4:** Mean monthly CORT concentrations compiled for all years (2011-2013). The lack of letters above the standard error bars indicates no significant relationship between month and CORT concentrations. The numbers provided in the bars indicate sample size.

5 A:
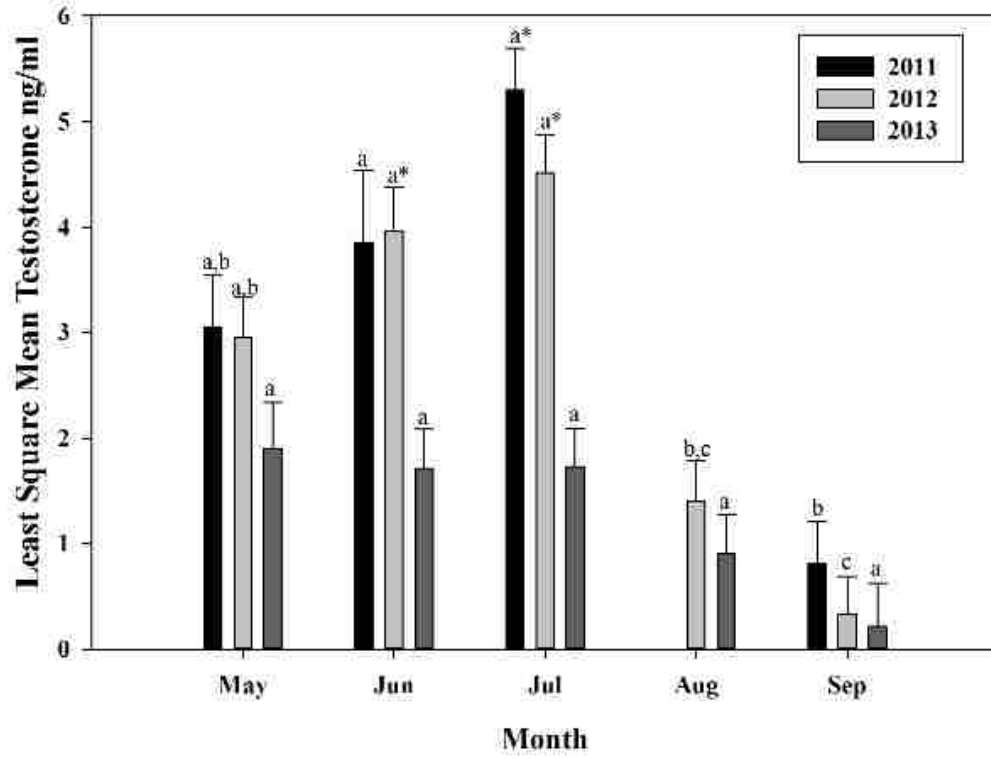
5 B:
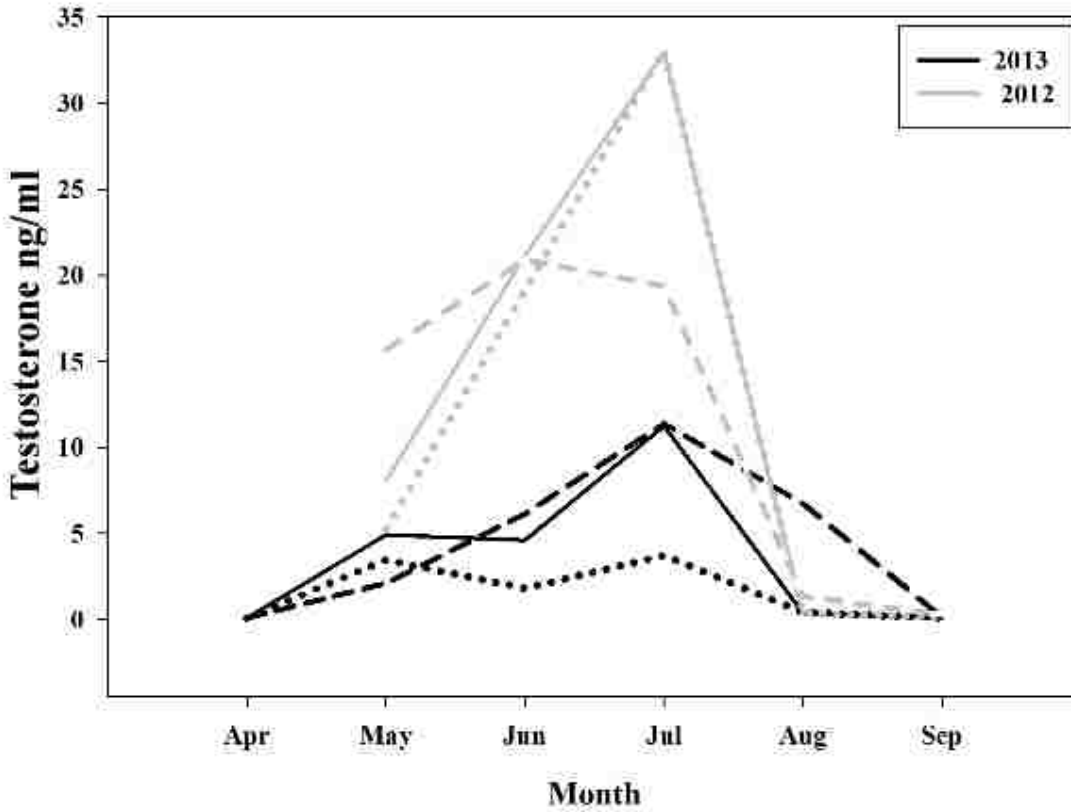


**Figure 5:** (A) Least square means for each combination of month and year in the full model with year included as a fixed factor. Bars of the same color (i.e. representing the same year) that do not share letters were significantly different in post hoc analyses (i.e. show seasonal variation within each year). Bars with a * indicate month-year combinations that were significantly different compared to 2013 in post hoc analyses (i.e. show annual differences by month). (B) Individual testosterone profiles for three snakes measured repeatedly in both 2012 and 2013. Lines with the same style (e.g. dashed) represent the same individual.
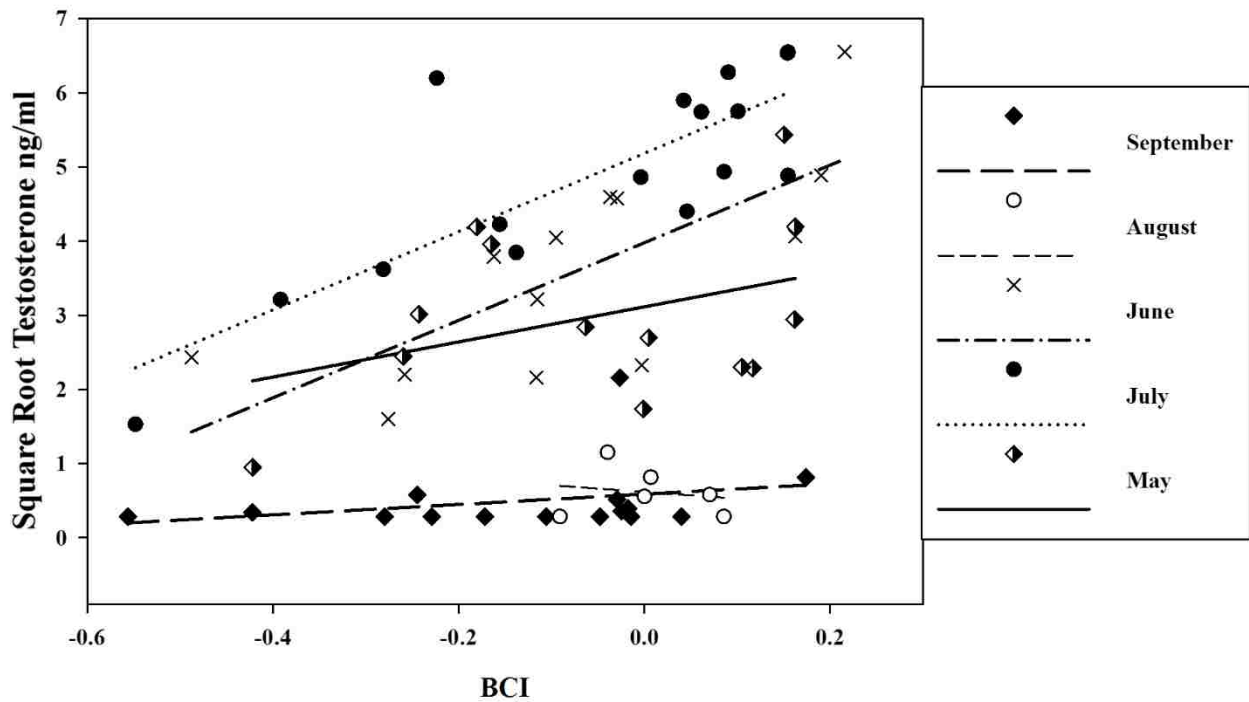
**Figure 6:** Scatterplot describing the relationship between body condition and square root transformed testosterone concentrations in 2011 and 2012. Regression lines were generated from simple linear regression analyses. The relationship between BCI and testosterone titer was only significant in June and July when analyzed in a repeated measures model.

**Chapter III: Male snakes allocate time and energy according to individual energetic status: body condition, steroid hormones, and reproductive behavior in Timber Rattlesnakes, *Crotalus horridus*.**

**Abstract:**

Life history theory predicts that organisms will hedge current reproductive investment against potential costs in terms of survivorship and future fecundity. However, little is known regarding the endocrine mechanisms underlying bet hedging strategies in free-ranging male vertebrates. We examined the relationships among individual energetic status, steroid hormones, mate search, and reproductive behavior in free-ranging male Timber Rattlesnakes. Snakes were monitored over four active seasons in order to test two hypotheses: (1) Males adjust the amount of time and energy allocated toward reproduction according to the level of individual energy stores, and (2) observed condition-dependent reproductive allocation is associated with circulating concentrations of steroid hormones (testosterone and corticosterone) thought to regulate reproductive behaviors in vertebrates. A positive relationship between body condition and testosterone was observed in both the field and in the laboratory. Male mate search effort was positively correlated with both body condition and testosterone. Body condition and testosterone concentrations were negatively related to time allocated toward foraging during the breeding season. A strong effect of year was observed in the analysis of testosterone and search effort suggesting that multiple environmental factors impact hormone production and reproductive investment. Corticosterone was not related to any measured variable, indicating that the relationships observed between energetic status and allocation are not mediated by corticosterone. Observed relationships are consistent with the hypothesis that males allocate time and energy towards reproduction according to individual energetic status and that testosterone

plays a role in mediating the tradeoff between current reproductive investment and residual reproductive value.

**Introduction:**

Life history trait expression is modulated by a variety of environmental factors including: the past or current resource environment, thermal conditions, and social context (Angilletta et al. 2004, Oliveira, 2009, Reznick and Yang, 1993). The ability to modulate trait expression according to past or prevailing environmental conditions likely maximizes fitness across a range of environmental contexts (Gotthard and Nylin, 1995). As the impact of the growing human population continues to rapidly alter ecosystems, researchers struggle to understand how organisms integrate environmental cues and trait expression at a mechanistic level. Little is known about how such integration is achieved in vertebrates in general (Hill et al. 2008, Ricklefs and Wikelski, 2002), and far less is known in male ectotherms (but see Ruiz, et al. 2010). A better understanding of the interplay between environment, regulatory mechanisms, and trait expression is required in order to accurately predict how organisms will respond to environmental change.

Life history theory predicts that long-lived vertebrates will trade off current reproductive success in favor of future fecundity in order to maximize lifetime fitness (Stearns, 1989), and that such tradeoffs will be most evident when energy is limited (Van Noordwijk and de Jong, 1986). In vertebrates, energy available for production (i.e. growth and reproduction) varies with the availability of food resources (Congdon, 1989). Key life history traits such as reproductive effort are therefore expected to shift directionally with changes in the resource environment (Zera and Harshman, 2001). The mechanisms by which vertebrates translate information about past or current resource levels into fitness-optimizing phenotypic expression are poorly

understood, particularly in males (Hill et al. 2008). In order to make appropriate allocation decisions, organisms require a mechanism to assess energy available either in the environment or stored in body reserves. Studies often allude to a physiological need for a mechanism relaying energetic context to the hormonal axes that regulate reproductive effort in female reptiles, particularly those that rely largely on capital to fuel reproduction and breed only when a threshold of stored resources is achieved (Aubret, et al. 2002, Stearns, 1989). However, very little is known about how shifts in the resource environment affect the allocation of time and energy toward reproduction in males, and even less is known regarding underlying endocrine mechanisms.

The paucity of studies that relate available energy to reproductive effort in males is likely due to the widely held assumption that gamete production is inexpensive for males relative to females (Olsson, et al. 1997). Regardless of the veracity of such assumptions, in mating systems where males fight for, defend, or search extensively for receptive females, male reproductive effort can constitute a large portion of an individual's energy budget (Bonnet and Naulleau, 1996, Shine and Mason, 2005, Yoccoz, et al. 2002). The mating system of many temperate zone snakes may require males to fight for, defend, or search widely for females during discrete breeding seasons (Aldridge and Duvall, 2002, Clark, et al. 2014, Duvall, et al. 1992, Glaudas and Rodríguez-robles, 2011). Male snakes allocate time and energy towards mating behaviors (from here on referred to as reproductive effort) and away from competing behaviors (e.g. foraging) during the breeding season (Bonnet and Naulleau, 1996, King and Duvall, 1990, Martin, 1992), and female vipers commonly reproduce less than annually as a consequence of their inability to replenish energy reserves to a threshold level in a single year (Blem, 1982, Naulleau and Bonnet, 1996). Given that male mating success in pit vipers likely requires significant energetic

investment (estimation of the energetic cost of mate search and predicting the consequences of behavioral allocation away from foraging in terms of kilojoules is difficult and has never been accomplished), males are also likely to tradeoff that effort in favor of conserving and acquiring energy (i.e. increasing residual reproductive value) when food resources are low. However, empirical evidence of such tradeoffs and any underlying mechanisms are rare in field active male vertebrates.

In the present study we aimed to test the hypotheses that: (1) Male snakes invest time and energy towards reproduction in direct relation to stored energy levels (i.e. body condition), and (2) condition dependent reproductive allocation is related to circulating concentrations of steroid hormones known to regulate reproductive behaviors in vertebrates (i.e. Testosterone, T and corticosterone, CORT). To do so, we monitored the body condition, steroid hormone concentrations, movement, and behavior of male Timber Rattlesnakes, *Crotalus horridus*, from a population in northwestern Arkansas over the course of four active seasons. Additionally, we measured body condition and steroid concentrations in laboratory snakes in order to test the link between stored energy and hormone production under controlled conditions. As long-lived, food limited, infrequent feeders that rely largely on capital to fuel reproduction, *C. horridus* is an excellent model species for examining life history tradeoffs and their underlying mechanisms (Beaupre, 2008). We predicted a significant relationship between estimates of stored energy (body condition), steroid hormones, and proxies for reproductive effort (i.e. search effort and time allocated away from foraging). Such a result would be consistent with the existence of a mechanistic pathway linking the resource environment, hypothalamic axes, and male reproductive allocation (Fig. 1).

**Methods**:

*Study area and animals*:

Our study was conducted in Madison County Arkansas (see Beaupre, 2008, for detailed habitat descriptions). Adult male *C. horridus* were implanted with temperature sensitive radiotransmitters (Holohil systems Ltd. Model S1-2T, Carp, Ontario, Canada) according to the methods of Reinert and Cundall (1982). Snakes were released at the point of capture. No snake was implanted fewer than two months before the onset of the breeding season. In total, 13 males were monitored for various numbers of years over the four year study period resulting in 27 total snake years of data. Individuals ranged from 81.1 – 109.6 cm (Mean ± SE = 93.31 ± 1.62) in snout vent length (SVL) and from 385-1333 g mass (Mean ± SE = 794.37 ± 51.89).

Lab samples were taken from twelve long-term captives that had been maintained in the laboratory for at least two years on a simulated northwest AR photoperiod and hibernation regime. Snakes were maintained in ventilated plastic containers with a hide box. Individuals were hibernated in an environmental chamber at 10°C from November through April in complete darkness. Natural light signals outside of hibernation were achieved by housing snakes in rooms with large south facing windows. The 12 males used in the study ranged from 80.0-116.9 cm SVL (mean ± SE = 98.85 ± 2.24) and from 340.2 -2000.0 g in mass (mean ± SE = 868.5 ± 93.96). In order to manipulate body condition, 6 of the 12 snakes were randomly assigned to a food restriction treatment group and taken off food from May until after a final blood sample was taken in August. The remaining six were fed an appropriately sized (10-40% of body mass) thawed rodent about every three weeks for the duration of the study (May-Sep, 2012).

*Field monitoring:*

Radiotagged snakes were tracked approximately every third day beginning just before the breeding season of *C. horridus* in northwest AR (Jul, 15) and ending just after the breeding season (Sep, 15; Lind and Beaupre, 2014). Snakes were tracked at least once per week outside of the breeding season. Each time a snake was tracked GPS coordinates were recorded to an accuracy of less than 10 m using an Etrex® legend GPS unit (Garmin Ltd., Olathe, Kansas). Behaviors were classified as one of eight behaviors known to be displayed by timber rattlesnakes (Beaupre, 2008).

Snake handling procedures followed Beaupre and Greene (2012). The length of each snake was measured in a squeeze box in late July in 2010 and at the start of each field season in 2011-2013. Because the seasonal growth rate of adult timber rattlesnakes in our population is lower than the error associated with squeeze box measurements, repeated SVL measurements each month were not required (Beaupre et al. in press). A blood sample of up to 1 ml was taken from each accessible male snake in each month of the active season (Apr-Nov) from 2011-2013. Samples from outside of the breeding season were taken as part of another study (Lind and Beaupre, 2014). Snakes were only sampled at the onset of the breeding season in 2010. Blood samples were taken from the caudal vein using a 1 ml BD syringe with a 27 G needle. Each time a snake was bled weight was measured in the field using a pesola® spring scale.

*Blood collection and RIA*:

In order to ensure that handling had no effect on steroid hormone concentrations, all blood samples were taken within 5 min of first contact with the snake (Moore, et al. 1991, Schuett, et al. 2004). Samples were transferred to a 1.5 ml microcentrifuge tube containing 2

drops of EDTA and were stored on ice for no more than 10 h before centrifugation for 15 min at 13,000 rpm to separate plasma. Plasma was stored at -20°C until RIA. Lab samples were taken from all snakes on two occasions in the mid to late summer (June, 18 and Aug, 11, 2012).

Quantification of plasma T and CORT concentrations was conducted using commercially available Coat-a-Count® RIA kits (Seimens Healthcare Diagnostics Inc. Los Angeles, CA). Each kit uses a proprietary extraction buffer and is designed to measure total circulating concentrations. Both kits were validated via quantitative recovery of cold spiked plasma (T = 90%, CORT = 94.8%) and parallelism of inhibition curves (T: $p = 0.37$, CORT: $p = 0.134$).

Field and lab testosterone was measured in two separate assays. The inter-assay coefficient of variation was 6.8% and the assay wide coefficients of variation within the range of the standard curve were 12.00% and 10.59%. All kit protocols were followed except that samples were diluted by at least half (1:1) with zero standard to bring elevated concentrations within the range of the standard curve. The minimum reportable range of the assay was 0.04 ng/ml. All samples that read below the sensitivity of the assay ($n = 2$) were reported as 0.08 ng/ml. Cross-reactivity with other steroids is low for the assay (<0.5% in all cases).

Corticosterone was measured on field samples in a single assay. The assay wide coefficient of variation within the range of the standard curve was 13.78%. Because circulating CORT concentrations in snakes were often near or below the low end of the standard curve and were never near the high end, the two highest standards were eliminated and the standard curve was diluted out two extra steps on the low end (from 22 ng/ml to 5.5 ng/ml). All other assay procedures followed kit protocol. The minimal detection limit for the assay was 5.7 ng/ml, all samples that read below the detection limit were recorded as 5.7 ng/ml. Seven out of 25 samples read below the detection limit. However, the detection limit was well below mean CORT

concentrations (14.91 ± 2.19 ng/ml), and our goal was to elucidate the relationship between CORT concentrations and reproductive physiology and behavior and was not concerned with small variation in baseline concentrations.  For this reason, the assay was capable of detecting elevation of CORT concentrations above baseline if present and allowed for examination of the relationship between CORT concentrations and measured variables.

*Movement and behavior:*

We used minimum convex polygons (MCPs) to estimate male search behavior over a two month period encompassing the breeding season (Lind and Beaupre, 2014). Polygon areas were estimated using the animal movement extension (Hooge and Eichenlaub, 1997) in Arcview 3.2 (ESRI Inc., Redlands, California, USA). We believe that 100% MCP was the best estimator of breeding movement compared to other spatial ecology parameters (e.g. 95% MCP, or Kernel) because it includes all long distance movements made by individuals during the breeding season. Singular, long, directional movements were biologically relevant to the question addressed by our study, making any parameter that throws out such movements inadequate.

Snake behaviors were categorized and recorded each time a snake was tracked. Behavioral categories included foraging, rest, moving, thermoregulation, reproduction, ecdysis, retreat, and arboreal (see Beaupre, 2008). Because the behavioral classifications associated with mate search are often ambiguous (e.g. moving, or at rest), allocation of time towards reproduction was estimated by calculating the frequency of observed foraging behaviors (i.e. the proportion of observations in which a snake's behavior was diagnosed as foraging). Male rattlesnakes engage in functionally designated episodic behaviors. Males that are actively engaged in mate search typically forgo foraging behaviors in favor of mate searching (King and

Duvall, 1990), making foraging frequency a good proxy for allocation of time away from resource acquisition and towards reproduction.

*Statistical Analysis:*

All data were analyzed for outliers, normality, and homoscedasticity and transformed as necessary before analysis and interpretation. Statistical analyses were conducted in SAS 9.3 and JMP Pro 11 (SAS Institute, 2013). Repeated measures analyses were conducted in SAS PROC MIXED. Covariance structures were assigned based on analysis of AICc scores (Burnham and Anderson, 2002).

All field data (2010-2013) were analyzed in a repeated measures model with year as a fixed repeated factor and individual as the subject effect (full model). Due to the fact that results from 2013 were anomalous compared to other years (Fig 2), data from 2010-2012 only were analyzed in a separate repeated measures model with year as a random repeated factor and individual as a subject effect. The dependent variables used were; square root of MCP area, arcsine proportion of time observed foraging, and untransformed T concentration. Body condition index (BCI: the residual of the regression of LnMass on LnSVL) and T concentration were used as predictor variables. Because hormone concentrations and reproductive behavior were different in 2013 compared to the other three years even after accounting for BCI (Figure 2), all analyses relating BCI or hormone concentrations to behavior and search were calculated on data from 2010-2012 only. Simple linear regression was used to analyze relationships among variables in 2013 alone.

Lab data were analyzed using a repeated measures model with sample (bleed date) as the repeated factor and individual as the subject effect. We did not use feeding treatment as a

categorical predictor variable. The goal of the food restriction treatment was to manipulate body condition. However, due to large existing variation in body condition in the population and likely due the low energy life history of *C. horridus*, fed and fasted groups did not differ significantly in BCI ($F_{1,10}= 0.03$, $p = 0.88$). Therefore, BCI was used as a continuous predictor variable in the model.

**Results:**

*Annual variation:*

There was a significant effect of year on T and MCP in the full model (T: $F_{3,10} = 30.68$, $p < 0.001$; MCP: $F_{3,10} = 8.99$, $p = 0.003$). Tukey's post-hoc tests showed that T concentrations were significantly lower in 2013 compared to 2010, 2011, and 2012 which were not different from each other when BCI is accounted for as a covariate (Fig. 2), and that breeding season MCPs in 2013 were significantly lower compared to the other three years which were not significantly different from each other (Fig. 2). The analysis satisfied the additional assumption of slope homogeneity ($F_{3,10} = 1.50$, $p = 0.30$). No effect of year was detected for proportion of time spent foraging ($F_{3,10} = 2.50$, $p = 0.12$). Corticosterone concentrations did not vary significantly by year ($F_{3,9} = 2.42$, $p = 0.13$).

*Morphological variables*:

There was a significant positive relationship between BCI and T concentrations in the full model, the reduced model, and in the laboratory (Field-full: $F_{1,10} = 63.14$, $p < 0.001$: Field-reduced: $F_{1,15}= 54.99$, $p < 0.001$: Lab: $F_{1,10} = 24.76$, $p < 0.001$; Fig. 3). There was no significant relationship between SVL and T concentrations in the field or in the lab (Field: $F_{1,10} = 0.87$, $p = 0.37$; Lab: $F_{1,11}= 2.18$, $p = 0.17$). There was no significant relationship between BCI and CORT

concentrations measured in the field (Full: $F_{1,16} = 2.32$, p = 0.1472; Reduced: $F_{1,14} = 1.74$, p =

0.21). Body condition was significantly positively related to MCP in the 2010-2012 analysis ($F_{1,16} = 25.19$, p < 0.001, Fig. 4), and there was a negative relationship between BCI and proportion

of time spent foraging ($F_{1,16} = 16.06$, p = 0.001, Fig. 4). There was no significant relationship

between SVL and either MCP or time spent foraging in the reduced analysis (MCP: $F_{1,16} <$

0.001, p = 0.971; Forage: $F_{1,16} = 4.18$, p = 0.06). Despite comparatively low T concentrations and

a small sample size in 2013, the positive relationship between T and BCI was detected in a linear

regression analysis ($R^2 = 0.64$, p = 0.03; Fig. 3B).

*Steroid hormones:*

There was no relationship between the two measured steroid hormones, T and CORT ($F_{1,9} = 1.09$, p = 0.33). Corticosterone concentrations were also not significantly related to either

behavioral response variable (MCP: $F_{1,14} = 0.22$, p = 0.65; Forage: $F_{1,14} = 1.45$, p = 0.25).

Testosterone concentrations were positively related to MCP and negatively related to proportion

of time observed foraging in the reduced analysis (MCP: $F_{1,15} = 18.72$, p < 0.001; Forage: $F_{1,15} = 42.98$, p < 0.001, Fig 5). No significant relationship was detected between T concentrations and

response variables in 2013 alone (n = 7, p > 0.05; Figs 5 and 6).

**Discussion:**

Our results show a strong positive relationship between individual energetic status (BCI)

and plasma T concentration. A similar relationship has recently been documented in species

representing two different snake families: Colubridae (King and Bowden, 2013) and Boidae

(Holding, et al. 2014). Additionally, food supplementation studies have established a direct

experimental link between energy intake and T production in other ectotherms (e.g. lizards and

frogs; Cox, et al. 2008, Marler and Ryan, 1996, Ruiz, et al. 2010). Our failure to manipulate body condition in laboratory experimental groups prevented the establishment of a causal link between energy reserves and breeding season T concentrations in *C. horridus*. However, the strong agreement of the field and lab components of the study regarding the relationship between BCI and T is consistent with a causal link.

Modulation of T production according to stored energy levels in male *C. horridus* was hypothesized to lead to modulation of T-dependent behaviors and time-energy allocation (Figure 1). Our results clearly show that males in higher body condition had higher T concentrations, searched more extensively for mates, and spent less time engaging in foraging behaviors (Figs 4 and 5). Ruiz et al. (2010) showed similar relationships among available energy, steroid hormone concentrations, immune function, and reproductive behavior in a lizard, *Sceloporous graciosus*. Their findings support the idea that commonly reported tradeoffs (e.g. between T and immune function) are dependent upon energetic status, and that predicted tradeoffs can disappear when energy is not limited (Van Noordwijk and de Jong, 1986). Such dependence may be the reason for inconsistencies in descriptive reports of the relationships among stored energy, steroid concentrations, and reproductive behavior/investment in reptiles (Lind, et al. 2010, Schuett, et al. 2005, Taylor et al. 2004, Tokarz, et al. 1998). In our energy-limited system, males allocate time and energy towards mate searching behaviors and away from foraging according to their energetic status. Males with a large supply of stored capital at the onset of the breeding season invest heavily in mate searching and invest a very small amount of time towards energy acquisition. Males with little stored capital continue to invest time foraging, and spend little or no time searching for females (Figs 4 and 5). Males that fell along the gradient from rich to poor in terms of energetic capital appeared to invest according to their energetic status and displayed

intermediate T concentrations and reproductive investment. In other words, no all or nothing threshold for reproduction was evident (Aubret, et al. 2002, Naulleau and Bonnet, 1996). However, males in very poor body condition appeared to forgo investment toward reproduction entirely. We interpret the observed condition-dependent reproductive investment as evidence of a tradeoff between current and future reproductive success in male *C. horridus*. *Crotalus horridus* is long-lived, and in our Ozark population, starvation due to a lack of foraging success is not uncommon (McCue, et al. 2012). A mechanism to modulate reproductive investment according to energetic context is likely adaptive and would allow males to hedge the investment of an appropriate amount of available capital towards reproduction against the potential costs in terms of survivorship and future fecundity.

Given the descriptive nature of our study, whether the hypothalamic-pituitary-gonadal (HPG) axis is directly involved in the integration of energetic status and trait expression remains an open question. However, links between both energetic status and T and between T and reproductive behavior have been established experimentally in a variety of vertebrate taxa (Ketterson, et al. 1992, Moore, 1988, Pérez-Rodríguez, et al. 2006, Ruiz, et al. 2010, Stoehr and Hill, 2000), and our multi-year study produced evidence implicating testosterone's role in the integration of energetic context and reproductive behavior/investment. In 2013 no reproductive behavior or mate search behavior was observed in any individual, and T levels were low in all snakes despite being in positive body condition overall (Figs 2 and 3b). We attribute the lack of any discernable breeding season in 2013 to abnormal weather patterns that may have disrupted seasonal cues permissive of reproduction (Lind and Beaupre, 2014). Interestingly, this scenario allowed for comparison of two years in which body condition was relatively high, but where T concentrations were drastically different (i.e. 2012 and 2013). Testosterone concentrations were

positively correlated with BCI in 2013, but snakes in good condition had low T concentrations

compared to other years and invested little in mate search (Fig. 2). We hypothesize that some

aspect of the biophysical environment (e.g. temperature) failed to trigger normal seasonal T

production (abnormally low T was observed as early as May, two months prior to the breeding

season; see Lind and Beaupre, 2014) and resulted in uniformly low concentrations in otherwise

healthy individuals. Regardless of the specific cause of low T in 2013, such a result suggests that

multiple environmental inputs modulate T production in *C. horridus*, and that energetic context

alone does not directly lead to reproductive behaviors and allocation decisions. Comparison

among years supports the conclusion that the HPG axis is likely an intermediary in the

modulation of trait expression according to cues from multiple environmental signals (e.g.

seasonal/thermal cues and resource availability; Fig. 1).

Our results do not support any role for CORT in the integration of resource availability

and trait expression. Studies examining the relationship between the hypothalamic-pituitary-

adrenal (HPA) axis and reproduction in vertebrates and have yielded mixed results (Moore and

Jessop, 2003, Romero, 2002, Wingfield and Sapolsky, 2003). One hypothesis suggests that

CORT is suppressive of reproduction and will act to suppress T production and costly behaviors

under T regulation (e.g. territorial defense and mate acquisition) when conditions are stressful

(Manzo, et al. 1994, Sapolsky et al. 2000). Another suggests that, as an energy mobilizer, CORT

should be elevated when reproductive effort is high and energy is limited (i.e. the energy

mobilization hypothesis; Romero, 2002). Despite large individual variation in body condition, T

concentrations, and reproductive behavior in our population, we did not detect any relationship

between these variables and CORT concentrations. Similar results have been reported in

descriptive studies of other Crotalines (Graham, et al. 2008, Lind, et al. 2010), suggesting that

some other mechanism besides CORT is involved in modulating reproductive effort according to energetic context.

The mechanism by which energetic context is communicated to the HPG axis remains elusive. In mammals, the protein hormone leptin is produced by adipose tissue and plays a key role in informing regions of the hypothalamus as to the level of stored energy (Hill et al. 2008, Ronti, et al. 2006, Zieba, Amstalden and Williams, 2005). Preliminary experiments suggest that a similar mechanism may exist in squamate reptiles (French, et al. 2011). The advent of modern genomics has only recently made the identification of protein hormones potentially involved in energy signaling tractable in non-model species. The establishment of the role of such hormones through classical endocrine techniques utilizing homologous tools has the potential to fill a gaping hole in the understanding of how species interpret and respond to energetic cues from the environment.

In conclusion, we have demonstrated that male reproductive investment is positively related to energetic status in a field-active male vertebrate. We also observed a positive relationship between stored energy and T, a product of the HPG axis that regulates a suite of reproductive life history traits in vertebrates (Hau, 2007). Comparison of results across years suggests that the HPG axis plays an important role the translation of energetic context into appropriate allocation decisions, but that multiple factors are at play in modulating T production and reproductive allocation. We interpret our results as further evidence of a pathway linking energetic status, the HPG axis, and reproductive behavior/effort in male squamates. However, data from 2013 clearly show that the path of causality from energetic status to behavior can be disrupted by annual shifts in environmental conditions. The dearth of knowledge regarding endocrine-environment interactions in male vertebrate ectotherms precludes an explanation of

the causal variables responsible for such disruption. Further investigation of the physiological pathways that integrate environmental cues, regulatory endocrine mechanisms, and trait expression is sorely needed. An understanding of the entire pathway (Fig. 1) will lead to a better perception of how hormonal mechanisms facilitate or constrain trait expression in response to environmental change.

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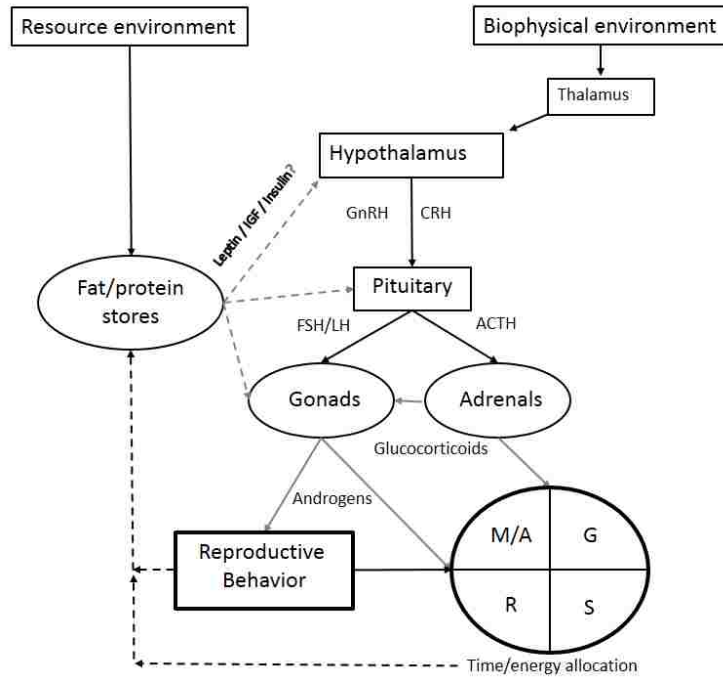
**Figure 1**: Conceptual diagram depicting the translation of information about the resource and biophysical environment into behavioral and energy allocation/life history decisions via the HPG and HPA axes and other peripheral tissues. Gray dashed arrows represent a hypothesized link between energetic status and the HPG and HPA axes. Solid gray arrows indicate hypothesized links between the downstream products of the HPA and HPG axes, reproductive behavior and allocation among competing functions (i.e. Maintenance and activity, M/A; growth, G; reproduction, R; and storage, S; adapted from: Dunham, Grant, and Overall 1989).
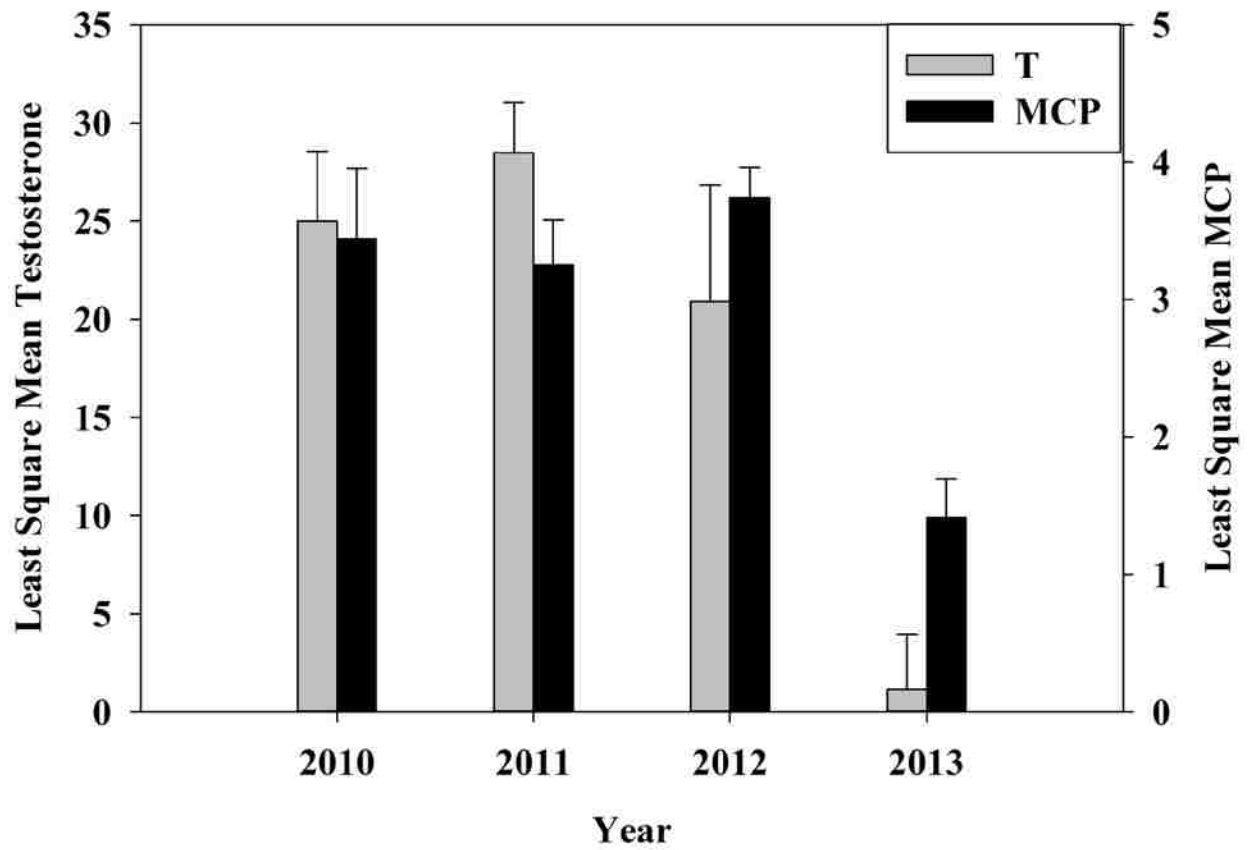
**Figure 2:** Bar graph depicting least square mean (adjusted for individual and BCI) testosterone and MCP in each year. Years that do not share letters above the error bars are significantly different in post hoc analyses.

A.
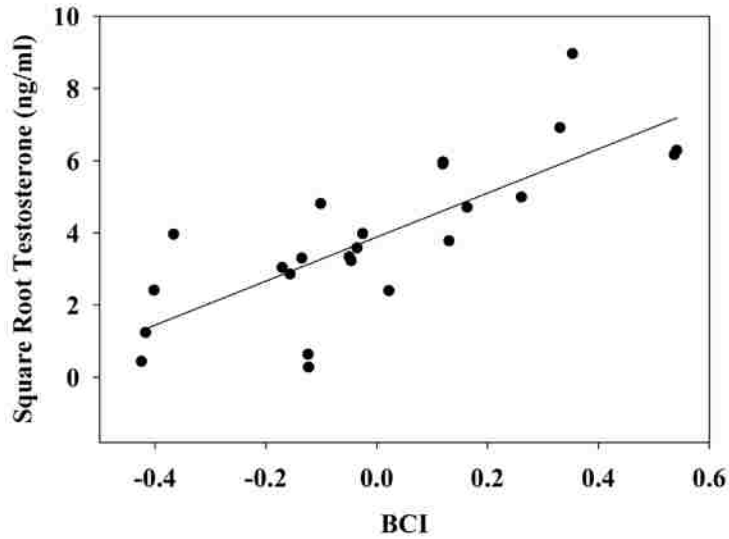


B.
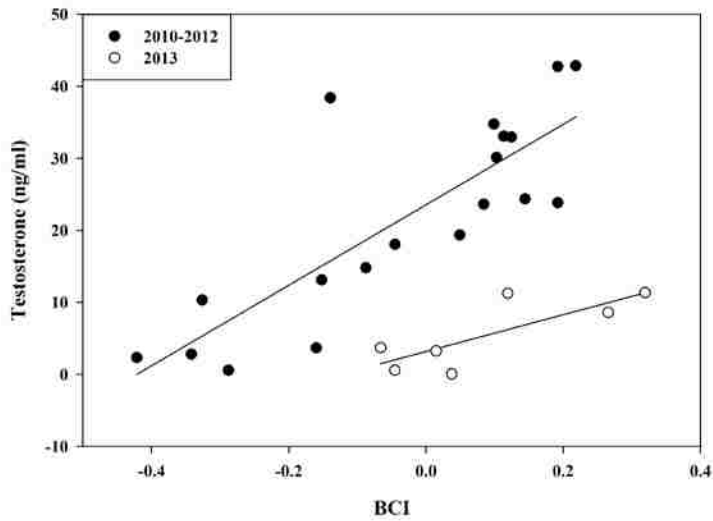


**Figure 3:** (A) Linear regression plots relating BCI and T concentrations in laboratory snakes. (B) Linear regression plots relating BCI and T concentrations in the field in 2010- 2012 together and 2013 separately. Regressions lines represent significant relationships in the regression analyses.

**A.**
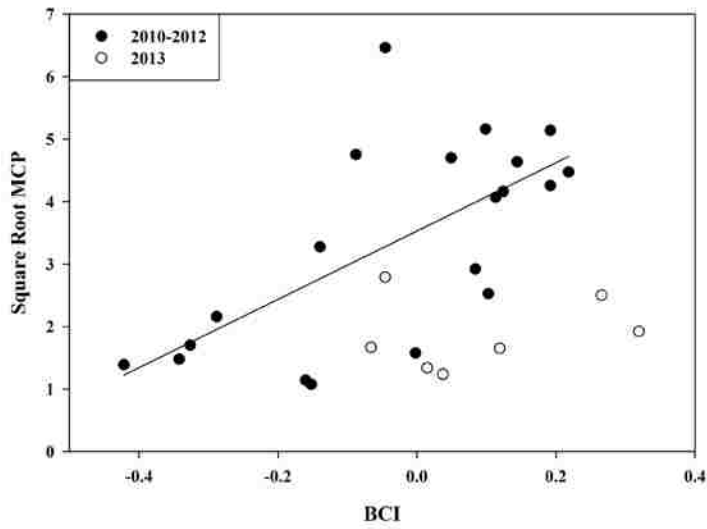


**B.**
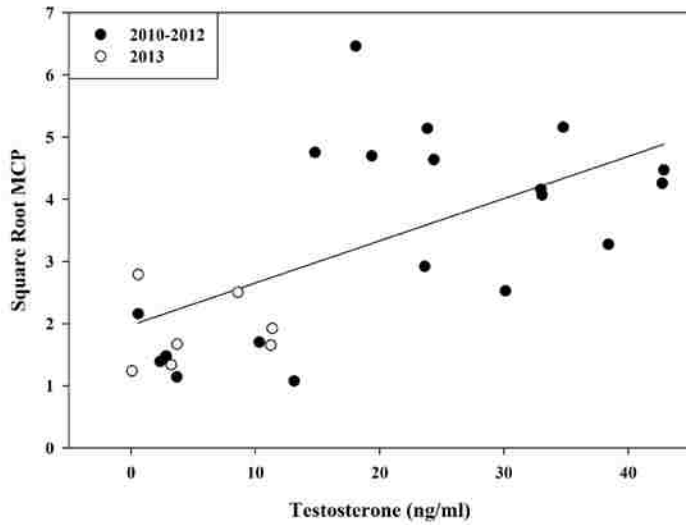


**Figure 4:** (A) Scatterplot with linear regression relating BCI and square root transformed MCP. Trend lines are shown for significant relationships only. (B) Scatterplot with linear regression relating T concentration and transformed MCP. Trend lines are shown for significant relationships only.

**A.**
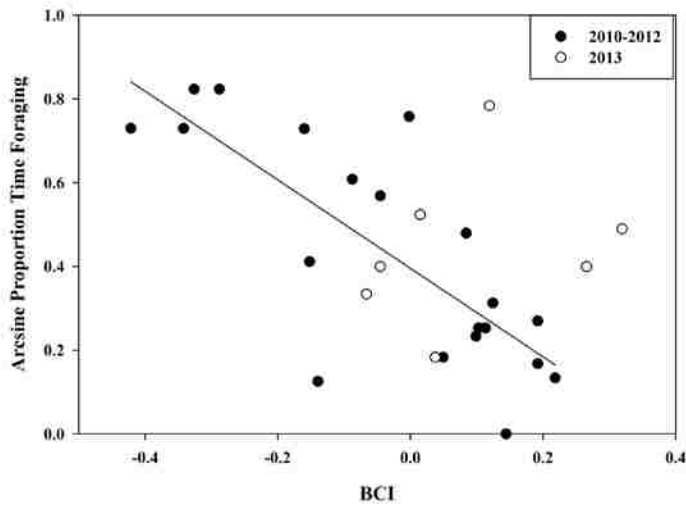


**B.**
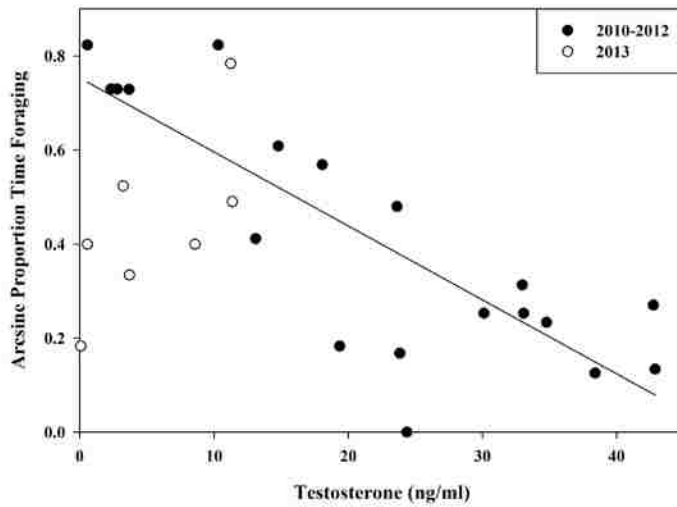


**Figure 5:** (A) Scatterplot with linear regression relating BCI and arcsine transformed proportion of time spent foraging. Trend lines are shown for significant relationships only (B) Scatterplot with linear regression relating T concentration and transformed proportion of time observed foraging. Trend lines are shown for significant relationships only.

**Chapter IV: The mating system and reproductive life history of female Timber Rattlesnakes in northwestern Arkansas.**

**Abstract:**

Vertebrate mating strategies and life history patterns show great variation within and among species. Elucidating phylogenetic and environmental factors that produce variation in reproductive tactics requires detailed natural and life history data on diverse taxa. Collection of such data in secretive species can often only be accomplished through the use of long-term monitoring and molecular tools. We used a combination of long-term (17 years) monitoring via radiotelemetry and molecular tools (microsatellite DNA markers) to describe in detail the reproductive ecology of a population of Timber Rattlesnakes, *Crotalus horridus*, in northwestern Arkansas. Female *C. horridus* are smaller at maturity and produce small litters compared to most reliable estimates for other populations. Female snout vent length and not preparturient body condition is positively related to litter size and total litter mass. Both pregnant and attractive females (individuals found engaged in mating behavior) were in better body condition compared to the general female population, suggesting a threshold body condition for female receptivity and reproduction. Behavioral monitoring of radiotagged females showed that 15% of females observed engaging in mating behavior with a male went on to associate with additional males that year. At least 44% of females found engaged in mating behavior definitely did not go on to produce a litter the following year, indicating that females will associate with males even when their chances of producing a litter the following year are low. Genetic analysis of eight microsatellite markers confirmed multiple paternity in three of the seven litters analyzed. Therefore the mating system of *C. horridus* in northwestern Arkansas is best described as polygynandrous, where both males and females mate multiply.

**Introduction**:

In reptiles, life history patterns and mating strategies vary widely both among and within species (Duvall et al., 1992, Duvall et al., 1993, Shine, 2003, Seigel and Fitch 1984, Seigel et al., 1986, Beaupre, 1995). Such patterns and strategies are the product of phylogenetic and environmental factors that are often unknown, which makes broad theoretical predictions difficult (Taylor and DeNardo 2005, Shine, 2003, Dunham and Miles 1985). Detailed natural history studies on local populations remain important in the identification of geographic and environmental trends and in testing predictions based on theory.

We used a combination of long-term monitoring via radiotelemetry and molecular tools to describe the reproductive ecology of female Timber Rattlesnakes, *Crotalus horridus,* from a population in the Ozark Mountains of northwestern Arkansas. Northwestern Arkansas is near the western edge of the range of *C. horridus* which extends east to the Atlantic coast and north to Minnesota and New York (Conant and Collins 1998). At least 14 studies have investigated some aspect of the reproductive ecology of *C. horridus* from populations ranging throughout the United States (Aldridge and Brown, 1995, Brown, 1991, Clark et al., 2011, Keenlyne, 1978, Martin 1988, Martin 1993, Martin 2002, Sealy 2002, Beaupre 2002, Mcgowan and Madison 2008, Gibbons 1972, Fitch and Pisani 2006, Villarreal et al., 1996, Galligan and Dunson 1979). The extent of published works on populations located throughout the wide range of *C. horridus* provides a unique opportunity for comparison of how reproductive ecology varies geographically and in association with environmental parameters.

Integrative studies incorporating field monitoring with molecular techniques are powerful in elucidating ecological patterns and processes, particularly in secretive species (e.g. most snakes, Clark et al., 2014). Long-term radiotelemetry studies focus on individuals and can

facilitate detailed description of the reproductive ecology of vertebrates, which can provide

valuable insight into the relationship between mating systems, life histories, and the environment

(Beaupre and Duvall 1998, Clark et al., 2014). Our primary objectives in the present study were:

(1) To describe the general life history of female *C. horridus* in northwest AR using data

collected over 17 years of field monitoring, and (2) to use field and molecular data to describe

the mating system of *C. horridus*. We compare our results to other studied populations and to the

patterns and strategies predicted by theory.

## 2. Methods:

### 2.1. Study site and animals.

Our study was conducted at a site in Madison County Arkansas located in the Ozark

Mountains in northwestern portion of the state (see Beaupre, 2008, for detailed habitat

description). Individuals were monitored by radiotelemetry from 1997-2013. Individuals were

captured and processed opportunistically during routine telemetry and on surveys during

emergence from hibernacula. Females in the study ranged from 70.0-98.1 cm snout vent length

(SVL; mean = 81.50 ± 0.37) and from 221.10 to 868.18 g mass (mean = 442.69 ± 5.11).

### 2.2. Field monitoring processing.

All animal handling procedures followed Beaupre and Green (2012). Radiotransmitters

(Holohil systems Ltd. Model SI-2T, Carp, Ontario, Canada) were implanted according to the

methods of Reinert and Cundall (1982). Processing involved measurement of snout vent length

(SVL) and mass, assessment of reproductive status, and the marking of individuals. Snout-vent

length was measured in a squeeze box and mass was measured on a digital scale. Follicles were

evaluated by manual palpation in the field or ultrasound in the lab. Each snake was marked with a unique three color rattle paint code and was implanted with a PIT tag (AVID, Norco, CA).

The mating behaviors of radiotagged snakes were categorized over the course of the study as either association, courtship, or copulation. Associations were recorded when two snakes of the opposite sex were found within 3 m of each other during the breeding season (Jul, 26-Sep, 12 (Lind and Beaupre 2014). Females that were not radiotagged and were engaged in mating behavior (e.g. were found in association with a radiotagged male) were brought to the lab for processing. Radiotagged females were processed periodically over the course of the active season as part of ongoing studies. Only length and mass measurements taken within one month of reproductive behavior were included in analyses. Pregnant females were collected, brought into the lab, and held at the preferred body temperature of pregnant *C. horridus* at our study site (28 C°) for up to one month until parturition and litter analysis (Gardner-Santana and Beaupre 2009). Litters were measured, weighed, and sexed via cloacal probing. Blood samples were taken from the caudal vein using a 1ml BD syringe attached to a 27 G needle. All neonates were released with their mother at the point of capture.

*2.3. Paternity analysis.*

The seven gravid females used in the paternity analysis were collected from throughout northwest AR and were held in the lab until parturition. Five females were collected from the Madison County population and were included in the life history study. Two females were collected from other sites in northwestern AR.

DNA was isolated from blood samples using a simple laboratory extraction procedure (Bailes et al., 2007). Blood samples were thawed and 8 µl of blood was added to 400µl cold STM solution (64 mM sucrose, 20 mM TrisCl pH 7.5, 10mM $MgCl_2$ and 0.5% Triton X-100).

Samples were centrifuged to pellet the nuclei and supernatant was removed. The pellet was resuspended in 200ul TEN (10mM TrisCl pH.7.5, 1mM EDTA, 10mM NaCl) with 10µg/ml pronase enzyme and incubated overnight with shaking at 37°C. The pronase was inactivated at 65°C for 20 min and DNAs were stored at -20°C.

Microsatellite DNA markers used for genotype assay were CwB6, CwA14, CwD15, CwB23, CwA29 (Holycross *et al.* 2002) and 3-155, 5-183, 5-87, 7-150, 7-144 (Villarreal et al. 1996)**.** Primers were synthesized (MWG Operon, Inc) with fluorescent labels on the 5' ends of the forward primers. Primer annealing temperatures varied from 50-55°C; Table 3). PCR was performed in 20µl reactions: 1X Buffer (50mM TrisCl pH8.3,1mM $MgCl_2$, and 3mg/ml BSA), 0.2mM dNTP's, 1µM forward and reverse primer (0.5 µM labeled and 0.5µM unlabeled primer), and 4U Taq polymerase. Cycle conditions were as follows: 90° C for 1 minute for an initial denature followed by 39 cycles of 90° C denature for 20 seconds, 30 seconds annealing, 72° C elongation for 1 minute, and final elongation for 5 minutes at 72° C.

DNA samples were prepared for electrophoresis by mixing 2 µl of the PCR products with 5µl loading buffer (95%formamide, 5% 1X Tris-Borate-EDTA (TBE), 2% bromophenol blue). Samples were denatured at 90° C for 3 min. and immediately placed on ice. Samples were loaded on a 30X40 mm, 0.4mm thick 6% denaturing polyacrylamide gel and electrophoresed at 50 Watts for 2-3.5 hours.

Gels were scanned on a Typhoon fluorescence scanner 8600 (Molecular Dynamics, Amersham Bioscience, Sunnyvale, CA) to detect fluorescently labeled PCR fragments. PCR fragments were sized using a DNA ladder (CxR, Promega Comp.). Genotypes for each snake were determined from gel images with different size fragments designated as different alleles.

*2.4. Data analysis.*

All data were analyzed for outliers, normality, and homoscedasticity of residuals. Data were transformed as necessary before interpretation of results. All statistical analyses were conducted in SAS 9.3 and JMP Pro 11 (SAS Institute, 2013). Maternal body condition index (BCI) was calculated as the residual of the regression of LnMass on LnSVL. Due to the expected allometric relationship between maternal SVL and litter size, we fit a power function to the regression of these two variables. The power function approach was statistically equivalent to log transformation (i.e. the function was simply the back-transformed linear equation yielded using linear regression on log transformed data), and results were identical (King, 2000). Both the nonlinear fit and log-transformation yielded better fits compared to linear regression on untransformed data. All other litter and maternal characteristics were analyzed using linear Pearson correlations. One female had more than one litter over the course of the study. Data from the second litter were thrown out to satisfy the assumption of independence. Results were not sensitive to the removal. We removed one litter from the analysis of female size on litter size due to high leverage (Cook's D = 0.76). The litter was from a large female and consisted of two male and one stillborn offspring and could have been the result of parthenogenesis (Booth and Schuett 2011), however, we have no way to verify this possibility. The relationships between offspring sex and offspring size and between offspring size and litter size were analyzed separately in mixed models including litter as a random effect.

Adult females processed in the study were separated into one of four life history categories; preparturient/pregnant (n=19), postparturient (n=19), attractive (n=26), and general population (n=229). Females found engaged in mating behaviors with a male were categorized as attractive. Data from the general population likely contained measurements on preparturient and

attractive individuals in addition to non-reproductive females measured throughout the active season. Body condition comparisons among the three different reproductive categories and the general population were conducted by ANCOVA. Log transformed mass was used as the response variable. Reproductive status was included as a fixed factor. Log transformed SVL was used as a covariate, and snake ID was included as a random factor.

**3. Results**

3.1. *Litter characteristics.*

Preparturient females averaged 83.74 ± 1.34 SVL and 572.68 ± 28.00 g mass, and average litter mass was 168.72 ± 11.14 (Table 1). Litter size was positively related to maternal SVL ($R^2 = 0.45$, p = 0.002, Litter Size = 0.000015*$SVL^{2.91}$; Figure 1). Litter size and total litter mass were highly correlated (r = 0.94, p < 0.0001). Maternal preparturient and postparturient BCI were not correlated with litter size or litter mass (p > 0.05 for all comparisons). Male and female offspring did not differ in SVL ($F_{1,108} = 0.19$, p = 0.66) or mass ($F_{1,108} = 0.004$, p = 0.95). Litter size was negatively correlated with the mass of individual offspring ($F_{1,17} = 6.71$, p = 0.02), and was not related to the SVL of individual offspring ($F_{1,17} = 0.63$, p = 0.44).

*3.2. Reproductive female characteristics*

The body conditions of adult females differed according to reproductive state (Fig 2; $F_{3,258} = 26.68$, p < 0.001). Tukey's HSD post hoc tests indicated that; (1) pregnant and attractive females were in significantly higher body condition compared to all other reproductive categories, (2) postparturient females were in lower body condition compared to all other categories, and (3) pregnant and attractive females did not differ significantly in body condition. The analysis satisfied the additional assumption of homogeneity of regression slopes (p = 0.17).

Of the 32 incidences of mating behaviors where the female could be positively identified, 9 (28%) involved females that engaged in mating behavior with more than 1 male in that year. A total of 4 out of 27 (15%) individual females identified in reproductive associations definitely went on to associate with another male in the same breeding season (Table 3). Only five out of the 27 individual attractive (found in association with a male) females (19%) definitely had litters in the year following mating behavior. Ten out of 27 attractive females (37%) definitely did not go on to have a litter the following year. Of those ten females, three (11% of 27) had litters two years after engaging in mating behavior. Two of the 27 (7%) females engaged in mating behaviors, including a confirmed copulation, were pregnant at the time the mating behavior was observed. The remaining nine (33%) attractive females were not tracked in the years after mating behavior was observed and had unknown reproductive outcomes.

*3.3. Paternity analysis:*

The litter sizes of the seven litters used for paternity analysis ranged from four to nine. Between two and ten alleles were detected at the eight microsatellite loci employed (Table 3). Loci 7-144 and 7-150 were removed from the study because of the high number of homozygotes and possible null alleles. The alleles of the seven mothers and their respective neonates were identified at the remaining eight microsatellite markers. Of the seven families analyzed, three contained more than two paternal alleles at a single locus and were likely sired by multiple males (Appendix 1).

**4. Discussion:**

Comparison of our results to other studied populations of *C. horridus* does not reveal any clear geographic trends in litter or offspring size (Brown, 1991, Keenlyne, 1978, Martin, 1988, Martin, 1993, Gibbons, 1972, Fitch and Pisani, 2006, Galligan and Dunson, 1979, Martin, 2002). Overall, mature females in our Ozark population are smaller and produce smaller litters compared to other *C. horridus* populations (but see Keenlyne, 1978, for record of a female with follicles at 67cm and Fitch and Pisani, 2006, for a litter size estimate of 5.75 based on a sample size of 4 litters; Table 4). The average size of reproductive females is also low compared to other reliable reports (Table 4). Average female size at reproduction was only estimated to be lower in females collected from a population in Pennsylvania. However, the reliability of these data are in question as study snakes were of unknown origin and were housed in the lab for extended periods of time (Galligan and Dunson 1979). Our population is frequently energy limited. Females are often found in low body condition and starvation is not uncommon (McCue et al., 2012). Small size at maturity in our Ozark population may be an adaptive life history response to the resource environment and the resultant low growth rates documented in previous studies (Wittenberg and Beaupre, 2014). Theory predicts that relatively low growth rates will be associated with a smaller size at maturity (Stearns and Koella, 1986). Our descriptive data, combined with previous data on growth rates in our Ozark population support this idea. Lastly, no sexual size dimorphism was observed at birth, as has been observed in some other Crotalines (Taylor and DeNardo, 2005, Beaupre et al., 1998).

We observed relationships between maternal size and litter size that are typical, although not universal, in Crotalines. Litter size was positively correlated with maternal SVL, but SVL only explained about half of the variation observed. The allometric scaling exponent of the fitted

power function was not different from three**,** the value expected when litter size is dictated by the size of the body cavity (King, 2000). No relationship was observed between preparturient or postparturient body condition and litter size or mass. Both attractive and pregnant female *C. horridus* were in significantly higher body condition compared to the general population (Figure 2). Body condition thresholds for entering reproductive bouts have been established in other female vipers (Bonnet and Naulleau, 1994, Naulleau and Bonnet, 1996), and at least one study suggests that females in above average body condition are more sexually receptive/attractive compared to females in below average body condition (Aubret et al., 2002). Our results confirm this relationship in a field-active snake, *C. horridus*, suggesting that some level of stored energy is permissive of reproduction (both in terms of attractiveness and pregnancy). However, assuming that BCI is a good estimate of individual energy stores, the lack of relationship between pre and postparturient body condition and litter mass or litter size suggests that once the decision to reproduce has been made, the magnitude of energetic investment towards offspring is not strongly influenced by maternal stored energy levels.

Both field and molecular data demonstrate that female *C. horridus* in the Ozarks are polyandrous. Multiple reproductive pairings by radiotagged females in a single breeding season were observed on several occasions, and three out of the seven litters analyzed were sired by multiple males. Empirical evidence for multiple paternity has been sought in many snake species, including *C. horridus* (Villarreal et al., 1996). In most species except the rattlesnakes, *Sistrurus catenatus* (Gibbs et al., 1998) and *C. horridus,* multiple paternity was confirmed (Uller and Olsson, 2008, Voris et al., 2008, Wusterbarth et al., 2010). However, Villarreal et al. (1996) only analyzed two litters from a northern population of *C. horridus* using four microsatellite markers, and Gibbs et al., 1998, also only analyzed two *S. catenatus* litters. Their results are not

sufficient to rule out multiple paternity in either population. A recent analysis of 24 litters of the Western Diamondback Rattlesnake, *Crotalus atrox*, found that 12 litters (50%) contained multiple sires (Clark et al., 2014). Their results are the first molecular evidence for multiple paternity in rattlesnakes. However, two studies have confirmed multiple paternity in other Crotalines; the Copperhead, *Agkistrodon contortrix* (Schuett and Gillingham, 1986) and Halys Pit Viper, *Gloydius halys,* (Simonov and Wink 2011). Our results confirm that multiple mating by females and multiple paternity also occurs in *C. horridus* in northwestern AR.

Female promiscuity/polyandry appears to be the rule in snake mating systems, and rattlesnakes are likely not an exception. The mating system of temperate zone rattlesnakes has been described as prolonged mate search polygyny where males search widely for relatively sedentary females during discrete breeding seasons (Duvall and Schuett 1997, Duvall et al., 1992). Males may defend females for periods of weeks during the mating season (Mcgowan and Madison, 2008, O'Leile et al., 1994). However, to our knowledge, no additional postcopulatory mechanism for exclusion of additional sires (e.g. a mating plug) has been identified in Crotalines. Females also typically breed less than annually and can store sperm across multiple breeding seasons, thus providing ample opportunity for sperm mixing and competition (Sever and Hamlett, 2002, Booth and Schuett, 2011). In some species, including *C. horridus*, sperm storage is likely obligatory, as the breeding season is dissociated from ovulation by many months (Aldridge and Duvall, 2002, Schuett, 1992). Our finding that almost half of the females that engaged in reproductive behaviors did not go on to reproduce the following year and that 8% of females found engaged in reproductive behaviors were pregnant suggests that a female will mate with a suitable male even when her likelihood of reproducing the following year is unlikely. However, our body condition analysis revealed that attractive females were in better body

condition compared to the general population. If a female has the ability to store sperm over long periods (i.e. years) without any cost in terms of reproductive success, one would expect to observe no relationship between attractiveness and energetic status. These seemingly contradictory results lead to several hypotheses regarding the mating system of *C. horridus,* and snakes in general: (1) There is a cost in terms of fitness to using sperm stored for long time periods to fertilize eggs compared to "fresh" sperm, (2) during the breeding season, females at certain intermediate body conditions do not have the ability to reliably predict reproductive outcomes the following year, and will mate in order to ensure fertilization if future resource availability/acquisition permits reproduction, and (3) males may have the limited ability to assess the likelihood that a female will produce offspring in the near future, and the observed difference in the body condition of attractive females may have to do with male choice rather than female receptivity (i.e. perhaps females of all body conditions are receptive, but only some are chosen as mates).

Assuming that there is a cost to mating in females, there must be some adaptive benefit (e.g. cryptic choice, sperm competition or unpredictable availability of males) to acquiring sperm even when fertilization will likely not occur for several years. Acquisition of sperm from multiple males could protect a female's reproductive investment from potential genetic incompatibility with a single mate (Zeh and Zeh., 1997). At least one study in snakes has shown increased reproductive success in females that mated multiply compared to those that did not, indicating indirect (genetic) fitness benefits to female promiscuity (Madsen et al. 2005). However, Uller and Olsson, 2008, suggest that female promiscuity and multiple paternity in reptiles is more likely driven by "strong selection on multiple male mating, low degree of precopulatory mate choice, high mate-encounter rate, and a relatively low cost of repeated

mating to females," rather than indirect genetic benefits to female multiple mating. We believe that several of these conditions do not apply to *C. horridus*. First, female rattlesnakes are thought to be active participants in reproduction and cannot be "forced" into copulations. Second, mate encounter rates are low in our system. Most females tracked throughout the breeding season are seen with one or no mates despite the fact that male handling time (i.e. courtship and mate guarding) can last for days to weeks (personal observation). Thus, we hypothesize that female polyandry in *C. horridus* is more likely driven by indirect genetic benefits and or a very low mate encounter rate coupled with long-term sperm storage/mixing. Experimental evidence for or against indirect genetic benefits to multiple mating and of the potential fitness costs and benefits of long-term sperm storage is needed in order to establish the adaptive significance of female reproductive patterns and mating systems in reptiles (Clark et al., 2014).

In conclusion, *C. horridus* in northwestern Arkansas mature at small body sizes and have small litters compared to most other studied populations. Litter size is primarily driven by maternal SVL and not maternal body condition. Females do mate multiply within a given breeding season and often engage in mating behavior even when the chances of producing a litter the following year are low. Multiple mating and or sperm storage can result in litters sired by multiple males. Overall, results confirm that, as is the case for many snakes, the mating system of *C. horridus* is best described as "polygynandry" where both males and females mate multiply (Rivas amd Burghardt., 2005). While the benefits to male polygyny are intuitive, the adaptive benefits of polyandry in snakes have not been established. Future studies integrating long-term individual-based field monitoring, molecular techniques, and experimental tests designed to examine the fitness consequences of polyandry are required in order to understand the ecology and evolution of snake mating systems.

**Acknowledgements:**

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**Table 1**: Descriptive statistics for maternal and litter characteristics based on analysis of 19 litters and a total of 112 offspring.

|                      | Mean   | SE    | Maximum | Minimum |
|----------------------|--------|-------|---------|---------|
| SVL                  | 83.74  | 1.34  | 96.20   | 71.10   |
| Preparturient mass   | 572.68 | 28.00 | 852.50  | 371.72  |
| Postparturient mass  | 336.49 | 18.51 | 556.42  | 257.22  |
| Litter size          | 5.90   | 0.44  | 9.00    | 3.00    |
| Total litter mass    | 168.72 | 11.14 | 26.81   | 88.64   |
| Offspring mass       | 29.24  | 0.83  | 37.32   | 22.11   |
| Offspring SVL        | 29.79  | 0.63  | 34.40   | 23.87   |

**Table 2**: Presents the dates of association for females that were observed engaging in reproductive behaviors with multiple males in a given breeding season.

| Pit | Date | Behavior |
|---|---|---|
| 015-780-299 | 2-Sep | Association |
| 015-780-299 | 9-Sep | Association |
| 024-794-083 | 2-Aug | Association |
| 024-794-083 | 7-Aug | Association |
| 025-793-295 | 18-Aug | Association |
| 025-793-295 | 29-Aug | Association |
| 024-853-563 | 7-Aug | Association |
| 024-853-563 | 14-Aug | Association |
| 024-853-563 | 12-Sep | Association |

**Table 3:** Characteristics of the microsatellite DNA markers. Repeat motif, primer sequence, annealing temperatures, and the number of alleles revealed are presented for each marker loci.

| Primer | Repeat Motif | Primer (5'→3') Forward | Reverse | Annealing Temp./°C | Number of Allele |
|---|---|---|---|---|---|
| 3-155 (cy3) | $(CA)_{13}$ | aaaagttaacaactatgtaaccatt | tacatcacttgctgcttcctg | 50 | 5 |
| 5-87 (cy5) | $(CA)_{12}$ | aagacgtggtgctgaagcagt | cctatagtgcagcatattaacaag | 55 | 2 |
| 5-183 (tet) | $(CA)_{11}$ | ttgttgtaaccagtgtgtgtgat | ctgcagacacttattattatacc | 55 | 5 |
| 7-150 (cy3) | $(CA)_{13}$ | gtcaccaacctttctcggttg | catgcaggcagtaactccacc | 55 | 2 |
| 7-144 (cy5) | $(CA)_{13} (GA)_{12}$ | cagagaaaagggaagcatcac | gcatacatgtatgtttgtgtgca | 55 | 2 |
| CwB6 (cy3) | $(GA)_{19}$ | ctcttttacgcccaccacttta | ccccgctaacctttgctcag | 55 | 5 |
| CwA14 (cy3) | $(AC)_{25}$ | ggggaggtagggaggtcag | aggggaaaagatgctgtgag | 55 | 3 |
| CwD15 (cy3) | $(CAT)(TAT)(CAT)_{14}$ | taatgttgtaagcc | ttcttcaaagcacataacacatc | 55 | 3 |
| CwB23 (cy3) | $(TG)_{18} (AG)_{22}$ | tgtgacatatggtgtcatctgg | ccatagcagaagagctgcaa | 55 | 7 |
| CwA29 (cy5) | $(AC)_{13}$ | tccccttccaaccccccaga | cagaggagacgagacagatag | 55 | 5 |

**Table 5:** Summary of life history characteristics available in the literature for other *C. horridus* populations.

| Population | Preparturient Mass (g) | Postparturient Mass (g) | Litter size | Neonate SVL | Neonate mass | Average SVL | SVL at maturity | Source |
|---|---|---|---|---|---|---|---|---|
| New York | 814 | 508 | NA | NA | NA | 91.5 | 84 | Brown, 1991 |
| Virginia | NA | NA | NA | NA | NA | 92.8 | 76 | Martin, 1993 |
| South Carolina | NA | NA | 12.5*1 | 38 | 31 | NA | 100 | Gibbons, 1972 |
| Wisconsin | NA | NA | 7.7*1 | 30 | NA | NA | 67.7 (78)*2 | Keenlyne, 1978 |
| Kansas | NA | NA | 5.75*4 | NA | NA | 89.8 | 80 | Fitch and Pisani |
| Pensylvania*3 | 529.2 | 313.7 | 7 | 27.5 | 21.7 | 82.7 | 77 | Galligan & Dunson,1979 |
| Arkansas | 572.7 | 336.5 | 5.89 | 29.79 | 29.24 | 83.7 | 71.1 | Present study |

*1 estimated by palpating enlarged follicles or embryos

*2 Based on the presence of follicles and no litter was not documented. Shortest SVL at birth was 78cm.

*3 data taken on snakes of unkown history and fed in the laboratory for up to 2 months

*4 Based on only 4 litters
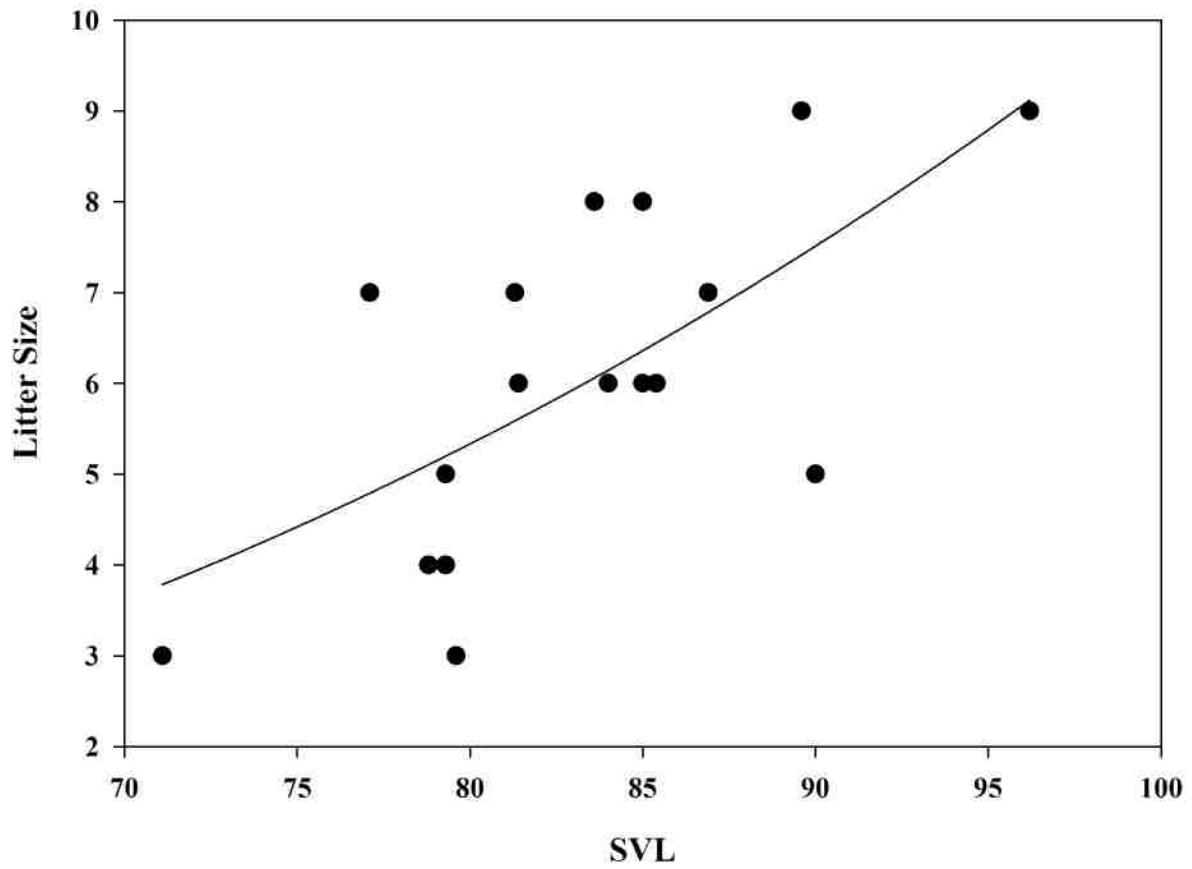
**Figure 1**: Scatterplot and non-linear fit showing the relationship between maternal SVL and litter size (Litter size = $0.00015*SVL^{2.91}$, $R^2 = 0.45$, p = 0.002).
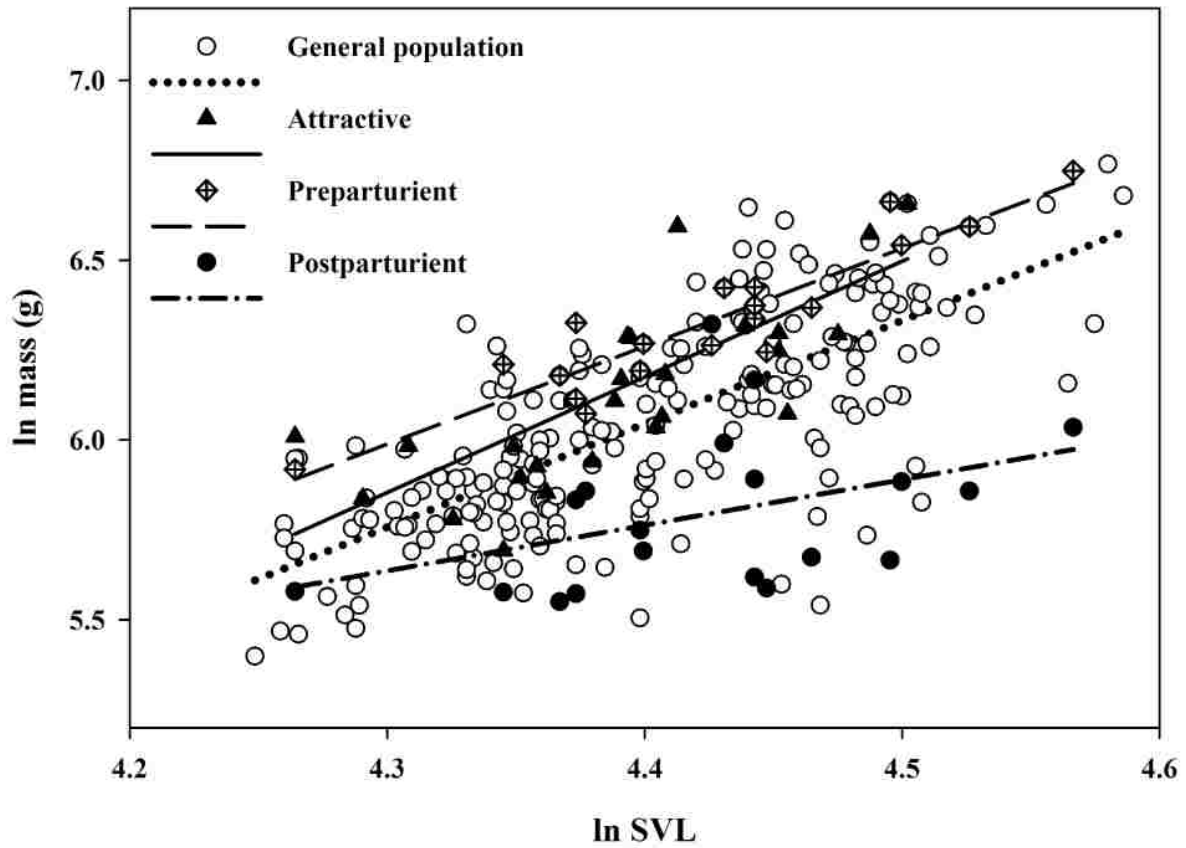
**Figure 2**: Scatterplot with linear regression lines indicating the mass of females in the four life history categories corrected for size (i.e. body condition). ANCOVA results show that both attractive and pregnant females were in better body condition compared to the general population, and that postpartum females were in lower body condition compared to all other categories.

**Appendix 1**: Genotypes from mothers and neonates are listed.  There is a (?) in place of genotypes when alleles could not be determined. Potential paternal (father) alleles are listed for each litter at each locus. More than two paternal alleles suggest multiple sired litters and are highlighted in **Bold**.

| Pit Tag # | CwB6 | | 5-183 | | CwD15 | | CwA14 | | 3-155 | | Cwb23 | | Cw29 | | 5--87 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 027-011-573 (MOM) | 112 | 110 | 142 | 128 | 123 | 123 | 140 | 140 | 142 | 134 | 240 | 218 | 160 | 160 | 150 | 150 |
| 027-293-797 | 112 | 110 | 142 | 120 | 123 | 123 | ? | ? | 142 | 140 | 230 | 218 | 166 | 160 | 150 | 150 |
| 024-630-811 | 112 | 110 | 142 | 120 | 123 | 123 | 140 | 140 | 142 | 140 | 218 | 218 | 166 | 160 | 150 | 150 |
| 027-006-602 | 114 | 112 | 128 | 120 | 123 | 123 | 146 | 140 | 134 | 132 | 230 | 218 | 166 | 160 | 150 | 150 |
| 027-013-072 | 112 | 110 | 128 | 120 | 123 | 123 | 140 | 140 | 140 | 134 | 230 | 218 | 166 | 160 | 150 | 150 |
| 027-028-580 | 112 | 110 | 128 | 120 | 123 | 123 | 146 | 140 | 142 | 140 | 240 | 218 | 166 | 160 | 150 | 150 |
| 027-099-844 | 114 | 112 | 142 | 128 | 123 | 123 | 146 | 140 | 142 | 132 | 230 | 218 | 166 | 160 | 150 | 150 |
| 027-293-008 | 114 | 112 | 142 | 120 | 123 | 123 | 146 | 140 | 142 | 132 | 230 | 218 | 166 | 160 | 150 | 150 |
| Paternal Alleles | 114, (110 or 112) | | 129, (128 or 142) | | 123 | | 140,146 | | 132,140 | | 218, 230 | | 166 | | 150 | |
| 027-089-380 (MOM) | 114 | 112 | 136 | 120 | 123 | 123 | 146 | 146 | 144 | 132 | 236 | 218 | **170** | **166** | 150 | 150 |
| 027-003-524 | 114 | 112 | 142 | 120 | 129 | 123 | 146 | 146 | 144 | 132 | 218 | 218 | **166** | **160** | 150 | 150 |
| 027-076-268 | 114 | 112 | 136 | 120 | 123 | 123 | 146 | 146 | 144 | 144 | 218 | 218 | **172** | **166** | 150 | 150 |
| 027-265-381 | 112 | 108 | 142 | 120 | 129 | 123 | 146 | 140 | 140 | 132 | 218 | 212 | **166** | **166** | 150 | 150 |
| 027-084-859 | 114 | 112 | 136 | 120 | 129 | 123 | 146 | 146 | 132 | 132 | 236 | 218 | **166** | **166** | 150 | 150 |
| 027-056-126 | 114 | 112 | 136 | 136 | 129 | 123 | 146 | 146 | 144 | 132 | 230 | 218 | **170** | **160** | 150 | 150 |
| Paternal Alleles | 108, (114 or 112) | | 136,142 | | 123,129 | | 140,146 | | 144, (132 or 140) | | 212,230,218 | | **160,172,166** | | 150 | |
| 027-014-839 (MOM) | 112 | 110 | 136 | 120 | 123 | 123 | 146 | 140 | 142 | 134 | 218 | 218 | 160 | 160 | 150 | 150 |
| 027-035-769 | 112 | 110 | 136 | 120 | 123 | 123 | 146 | 140 | 142 | 132 | ? | ? | 166 | 160 | 150 | 150 |
| 027-060-802 | 112 | 110 | 136 | 120 | 123 | 123 | 146 | 146 | 142 | 132 | 224 | 218 | 166 | 160 | 150 | 150 |
| 027-072-122 | 114 | 112 | 136 | 120 | 123 | 123 | 146 | 140 | 142 | 132 | 236 | 218 | 160 | 160 | 150 | 150 |
| 027-079-810 | 114 | 112 | 136 | 120 | 123 | 123 | 146 | 140 | 142 | 132 | 236 | 218 | 160 | 160 | 150 | 150 |
| Paternal Alleles | 114, (110 or 112) | | 120 or 136 | | 123 | | 140 or  146 | | 132 | | 224,236 | | 160,166 | | 150 | |
| 036-092-367 (MOM) | 114 | 112 | **140** | **120** | 123 | 123 | 140 | 140 | 140 | 132 | 224 | 212 | 166 | 160 | 150 | 150 |
| 036-038-800 | 112 | 110 | **140** | **120** | 123 | 123 | 146 | 140 | 142 | 132 | 218 | 212 | 170 | 160 | 150 | 150 |
| 036-040-289 | 112 | 110 | **136** | **120** | 123 | 123 | ? | ? | 142 | 132 | 218 | 212 | 160 | 160 | 150 | 150 |
| 036-041-547 | 114 | 112 | **136** | **120** | 123 | 123 | 140 | 140 | 142 | 132 | 224 | 218 | 160 | 160 | 150 | 150 |
| 036-071-083 | 112 | 110 | **128** | **120** | 123 | 123 | 146 | 140 | 142 | 132 | 214 | 212 | 160 | 160 | 150 | 150 |
| 036-107-016 | 114 | 112 | **128** | **120** | 123 | 123 | 146 | 140 | 142 | 140 | 224 | 214 | 160 | 160 | 150 | 150 |
| 036-110-100 | 112 | 110 | **140** | **128** | 123 | 123 | 140 | 140 | 142 | 140 | 224 | 214 | 170 | 166 | 150 | 150 |
| Paternal Alleles | 110,114 | | **128,136 (120 or 140)** | | 123 | | 140,146 | | 142 | | 214,218 | | 160,170 | | 150 | |
| 051-267-860 (MOM) | 112 | 110 | 142 | 120 | 129 | 123 | 146 | 140 | 132 | 132 | ? | ? | 166 | 160 | 150 | 150 |
| 096-591-107 | 112 | 110 | 142 | 120 | 127 | 123 | 146 | 140 | 132 | 132 | ? | ? | 166 | 166 | 150 | 150 |
| 096-599-283 | 112 | 110 | 142 | 136 | 127 | 123 | 146 | 146 | 132 | 132 | ? | ? | 166 | 166 | 150 | 150 |
| 096-618-375 | 112 | 110 | 142 | 136 | 129 | 123 | 146 | 146 | 144 | 132 | ? | ? | 166 | 166 | 150 | 150 |
| 096-804-882 | 110 | 110 | 142 | 136 | 129 | 127 | 146 | 146 | 144 | 132 | ? | ? | 166 | 164 | 150 | 150 |
| 096-805-882 | 110 | 108 | 142 | 136 | 129 | 127 | 146 | 146 | 132 | 132 | ? | ? | 166 | 160 | 150 | 150 |
| Paternal Alleles | 108,110 | | 120,136 | | 120,127 | | 146 | | 132,144 | | | | 164,166 | | 150 | |
| TS1 (MOM) | 112 | 106 | 136 | 120 | ? | ? | 140 | 140 | 132 | 132 | 236 | 218 | 170 | 166 | 150 | 150 |
| TS1:998 | 106 | 106 | 136 | 120 | 123 | 123 | 140 | 136 | 134 | 132 | 236 | 218 | 170 | 160 | 150 | 150 |
| TS1:998#2 | 112 | 106 | 136 | 136 | 123 | 123 | 140 | 136 | 134 | 132 | 236 | 224 | 170 | 166 | 150 | 144 |
| TS1:998#3 | 112 | 110 | 136 | 120 | 123 | 123 | 140 | 136 | 132 | 132 | 236 | 224 | 170 | 160 | 150 | 144 |
| TS1:998#5 | 112 | 110 | 136 | 136 | 123 | 123 | 140 | 140 | 132 | 132 | 236 | 218 | 166 | 166 | 150 | 144 |
| TS1:998#6 | 112 | 110 | 136 | 120 | 123 | 123 | 140 | 136 | 132 | 132 | 218 | 218 | 166 | 160 | 150 | 150 |
| TS1:998#7 | 112 | 106 | 136 | 120 | 123 | 123 | 140 | 136 | 132 | 132 | 236 | 224 | 170 | 160 | 150 | 150 |
| Paternal Alleles | 106,110 | | 120,136 | | 123 | | 136,140 | | 132,134 | | 218,224 | | 160,166 | | 144,150 | |
| TS2 (MOM) | **112** | **106** | 128 | 120 | 123 | 123 | 140 | 140 | 142 | 134 | **240** | **214** | 166 | 166 | 150 | 150 |
| TS2:#1 | **112** | **106** | 128 | 120 | 123 | 123 | 140 | 140 | 134 | 132 | **240** | **214** | 166 | 166 | 150 | 150 |
| TS2:#2 | **112** | **106** | 120 | 120 | 123 | 127 | 140 | 140 | 142 | 142 | **230** | **214** | 166 | 166 | 150 | 150 |
| TS2:#3 | **114** | **112** | 128 | 128 | 123 | 123 | 140 | 140 | 134 | 132 | **240** | **224** | 166 | 166 | 150 | 150 |
| TS2:#4 | **114** | **106** | 128 | 120 | 123 | 123 | 140 | 140 | 142 | 142 | **240** | **240** | 166 | 166 | 150 | 150 |
| TS2:#5 | **114** | **112** | 120 | 120 | 123 | 123 | 140 | 140 | 142 | 132 | **240** | **214** | 166 | 166 | 150 | 150 |
| TS2:#6 | **114** | **112** | 128 | 120 | 123 | 123 | ? | ? | 142 | 134 | **240** | **224** | 166 | 166 | 150 | 150 |
| TS2:#7 | **112** | **106** | 128 | 128 | 127 | 123 | 140 | 140 | 142 | 142 | **240** | **224** | 166 | 166 | 150 | 150 |
| TS2:#8 | **112** | **110** | 120 | 120 | 127 | 123 | 140 | 140 | 142 | 142 | **240** | **214** | 166 | 166 | 150 | 150 |
| TS2:#9 | **112** | **106** | 120 | 120 | 127 | 123 | 140 | 136 | 142 | 134 | **230** | **214** | 166 | 166 | 150 | 150 |
| Paternal Alleles | **110,114,(106 or 112)** | | 120,128 | | 123,127 | | 136,140 | | 132,142 | | **224,230,240** | | 166 | | 150 | |

**Chapter V: Concluding remarks**

Overall, this dissertation reinforces the importance of considering individual, seasonal, and annual variation in studies of vertebrate life histories and underlying endocrine mechanisms. Chapter 2 showed stark annual variation in steroid hormone profiles. Such variation may be the reason that previous studies on Timber Rattlesnakes present conflicting results. Chapters 2 and 3 highlighted the fact that trait expression is variable within and among individuals across years, and that the production of sex steroids shows similar variation. The characteristic that best explained variation in both steroid production and reproductive behavior/investment was energetic status (estimated by body condition). As shown in chapter 4, energetic status also related to female reproduction. Both preparturient and attractive females were in better body condition compared to the general population. Whether this finding is resultant from a female threshold for attractiveness or male choice remains to be elucidated. Chapter 4 also clearly showed that females will engage in reproductive behaviors even when the likelihood of producing a litter in the following year was unlikely. Molecular and behavioral data also showed that females will engage in mating behavior with multiple males in a single breeding season, and litters are often sired by more than one male. The adaptive benefits and ultimate causes of female polygyny in snakes are in need of further study.

# UNIVERSITY OF ARKANSAS

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## *MEMORANDUM*

TO:        Steven Beaupre

FROM:    Craig N. Coon, Chairman
            Institutional Animal Care
               And Use Committee

DATE:    October 7, 2010

SUBJECT:    <u>IACUC PROTOCOL **APPROVAL**</u>
              Expiration date :   **October 3, 2013**

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** Protocol #11007- "
**PHYSIOLOGICAL AND THERMOREGULATORY RESPONSES OF A TOP PREDATOR TO
LARGE-SCALE HABITAT MANIPULATIONS**". You may begin this study immediately.

In granting its approval, the IACUC has approved only the protocol provided.  Should there be any
changes in the protocol during the research, please notify the IACUC in writing **prior** to initiating the
changes.  If the study period is expected to extend beyond **10-03-2013,** you must submit a new protocol.
By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines for
research involving animal subjects.


cnc/car

cc: Animal Welfare Veterinarian

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# UNIVERSITY OF ARKANSAS

## _MEMORANDUM_

TO:      Steven Beaupre

FROM:    Craig N. Coon, Chairman
           Institutional Animal Care
             And Use Committee

DATE:     November 19, 2013

SUBJECT:   IACUC Protocol **APPROVAL**
              Expiration date :  **October 31, 2016**

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** Protocol #14017-
**"Physiological and thermoregulatory responses of a top predator to large-scale habitat
manipulations**". You may begin this study immediately.

The IACUC encourages you to make sure that you are also in compliance with other UAF committees
such as Biosafety, Toxic Substances and/or Radiation Safety if your project has components that fall
under their purview.

In granting its approval, the IACUC has approved only the protocol provided. Should there be any
changes to the protocol during the research, please notify the IACUC in writing [via the Modification
Request form] **prior** to initiating the changes. If the study period is expected to extend beyond **10-31-
2016** you must submit a new protocol. By policy the IACUC cannot approve a study for more than 3
years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines for
research involving animal subjects.


cnc/car

cc: Animal Welfare Veterinarian

## _MEMORANDUM_

TO:       Steven J. Beaupre

FROM:     Wayne J. Kuenzel, Chair
          Institutional Animal Care
            And Use Committee

DATE:     September 11,  2007

SUBJECT:  IACUC PROTOCOL **APPROVAL**
          Expiration date :   09-07-2010

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** Protocol **#08008 -
"PHYSIOLOGICAL AND THERMOREGULATORY RESPONSES OF A TOP PREDATOR TO
LARGE-SCALE HABITAT** _"_ .  You may begin this study immediately.

In granting its approval, the IACUC has approved only the protocol provided.  Should there be any
changes in the protocol during the research, please notify the IACUC in writing prior to initiating the
changes.  If the study period is expected to extend beyond **09-07-2010** you will need to submit a new
protocol. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines in research
involving animal subjects.


wjk/car

cc:   Animal Welfare Veterinarian

MEMORANDUM

TO:        Steven Beaupre

FROM:      Craig N. Coon, Chairman
           Institutional Animal Care
              And Use Committee

DATE:      March 29, 2012

SUBJECT:   IACUC PROTOCOL APPROVAL
           Expiration date :    April 10, 2015


The Institutional Animal Care and Use Committee (IACUC) has APPROVED
Protocol #12031- "THE PHYSIOLOGICAL EFFECT OF LEPTIN IN CROTALINE
SNAKES".   You may begin this study immediately.

The IACUC encourages you to make sure that you are also in compliance with other
UAF committees such as Biosafety, Toxic Substances and/or Radiation Safety if your
project has components that fall under their purview.

In granting its approval, the IACUC has approved only the protocol provided.   Should
there be any changes in the protocol during the research, please notify the IACUC in
writing [Modification Request form] prior to initiating the changes.   If the study period is
expected to extend beyond 04-10-2015, you must submit a new protocol.   By policy the IACUC cannot
approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal
guidelines for research involving animal subjects.


cnc/car


cc: Animal Welfare Veterinarian