

A COMPARATIVE STUDY OF STRESS PHYSIOLOGY IN THE COMMON
WATERSNAKE (*NERODIA SIPEDON*), THE DIAMONDBACK WATERSNAKE
(*NERODIA RHOMBIFER*), AND THE QUEEN SNAKE (*REGINA SEPTENVITTATA*).

by

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ABSTRACT

Vertebrates respond to stressors in part by increasing the secretion of glucocorticoids from the adrenal gland. Both intrinsic and extrinsic factors (e.g., age, sex, season) have been shown to influence glucocorticoid secretion within a species, but understanding of species differences in glucocorticoid secretory patterns is limited. In this study, I examined corticosterone, progesterone, and testosterone levels in the common watersnake (*Nerodia sipedon*), the diamondback watersnake (*Nerodia rhombifer*), and the queen snake (*Regina septemvittata*) at a capture baseline and in response to an acute 30 minute confinement stress. I also examined the same steroids in *Nerodia sipedon* subjected to three weeks of laboratory housing, a known chronic stressor. All three species exhibited elevated corticosterone in response to acute stress but baseline and post-stress levels varied among species. Overall, *Nerodia sipedon* and *Nerodia rhombifer* exhibited similar corticosterone and testosterone levels both at capture baseline and after 30 minutes of confinement stress. *Regina septemvittata* had substantially higher corticosterone levels than the two *Nerodia* species. *Nerodia sipedon* subjected to chronic stress showed no significant changes in corticosterone levels from the baseline to post-lab housing, suggesting downregulation of the hypothalamo-pituitary-adrenocortical axis and glucocorticoid production.

TABLE OF CONTENTS

	Page
LIST OF TABLES	v
LIST OF FIGURES	vi
1. INTRODUCTION	1
1.1 Stress physiology	1
1.2 Reproductive axis	4
1.3 Model organisms	5
1.4 Objectives of study	7
2. METHODS	11
2.1 Comparative study of three species of natricines	11
2.2 <i>Nerodia sipedon</i> chronic stress study	12
2.3 General lab procedure	13
2.4 Statistical analyses	14
3. RESULTS	16
3.1 Acute stress study	16
3.1.1 Comparisons across the three species of natricines	16
3.1.2 Comparison of male <i>Nerodia sipedon</i> and <i>Nerodia rhombifer</i>	17
3.1.3 Comparison of female <i>Nerodia sipedon</i> and <i>Regina septemvittata</i>	18
3.1.4 Individual species data	18
3.1.5 Correlations among the steroids	20

3.1.6 Correlations between body condition and the steroids	20
3.1.7 Sex differences in <i>Nerodia sipedon</i>	21
3.2 Chronic stress of lab housing in <i>Nerodia sipedon</i>	21
4. DISCUSSION	31
REFERENCES	39
APPENDIX	50
Appendix A: IACUC APPROVAL LETTER	51

LIST OF TABLES

Table 1. Comparison of key life history, physiological and ecological characteristics of the three focal species of natricine snakes.	10
Table 2. Descriptive statistics of capture and body size data for <i>N. sipedon</i> , <i>N. rhombifer</i> , and <i>R. septemvittata</i> . Different letters in superscript denote significant differences ($P < 0.05$, post-hoc test).	23

LIST OF FIGURES

Figure 1. Reconstructed phylogeny of Natricinae, with focal species indicated by asterisks (From Pyron et al., 2013).	9
Figure 2. Plasma corticosterone concentrations (Mean \pm SE) upon capture (Baseline) and after 30 min of confinement (Post-stress) in all three species of Natricinae in the field. Different letters above the bars denote significant differences ($P < 0.05$, post-hoc test) in both baseline and post-stress corticosterone among the three species, and different cases of a letter denote differences between baseline and post-stress corticosterone within each species.	24
Figure 3. Mean (\pm SE) hematocrit values for the three species of Natricinae. Different letters above the bars denote significant differences ($P < 0.05$, post-hoc test).	25
Figure 4. Plasma testosterone concentrations (Mean \pm SE) upon capture (Baseline) and after 30 min of confinement (Post-stress) in male <i>Nerodia sipedon</i> and <i>Nerodia rhombifer</i>	26
Figure 5. Plasma progesterone concentrations (Mean \pm SE) upon capture (Baseline) and after 30 min of confinement (Post-stress) for female <i>Nerodia sipedon</i> and <i>Regina septemvittata</i>	27
Figure 6. Plasma corticosterone concentrations (Mean \pm SE) upon capture (Baseline) and after 30 min of confinement (Post-stress) in male and female <i>Nerodia sipedon</i> in the field.	28

Figure 7: Plasma corticosterone concentrations in *Nerodia sipedon* (Mean \pm SE) upon capture (Baseline) and in the same snakes ($n = 6$) after 18-22 days of laboratory housing (Post-lab housing). 29

Figure 8: Linear correlations between body condition and baseline corticosterone for the three species of natricine. Asterisks indicate significance ($P < 0.05$, post-hoc test).30

1. INTRODUCTION

1.1 Stress physiology

Hormonal responses to stressors in vertebrates are highly conserved across evolutionary lineages (Moore and Orchinik, 1994). The main stress axes responsible for hormonal responses are the sympatho-adrenal and hypothalamo-pituitary-adrenocortical (HPA). Upon initial exposure to a stressor (defined as a negative stimulus that requires an energetic response from an organism), the hypothalamus releases corticotrophin-releasing hormone, which stimulates the anterior pituitary to release adrenocorticotrophic hormone (McEwen and Wingfield, 2003; Romero, 2004). Adrenocorticotrophic hormone then stimulates the release of glucocorticoids (GCs) from the adrenocortical cells. As GC concentrations increase in the circulating blood, a negative feedback loop is triggered that downregulates corticotrophin-releasing hormone and adrenocorticotrophic hormone and thereby the release of GCs. The primary GC in birds, reptiles, amphibians, and many rodents is corticosterone (Romero, 2004). Immediately after an acute stressor, cardiovascular tone, immune activation, and energy mobilization increase, and within 1-10 minutes, blood flow and glucose utilization in the cerebrum increase (Sapolsky et al., 2000). These physiological changes are made in preparation for a costly energetic event such as fleeing or defensive behavior.

Both external and internal factors have been shown to influence the GC response. External factors include the type, intensity, and duration of the stressor (Weiss, 1968). The physical and physiological condition of the individual animal has also been shown to

play an important role (Weiss, 1968; Palme et al., 2005). For example, Moore (2000b) found male red-spotted garter snakes with positive body condition to have lower baseline corticosterone levels than snakes in poorer body condition. In terms of the resulting amplitude of the GC response, Romero (2004) argues that the overall health of the animal has a less biologically significant effect than receptor density and contextual conditions like current weather conditions and previous food availability (Moore and Orchinik, 1994; Palme et al., 2005).

Glucocorticoid levels vary both among and within a species. Numerous factors have been shown to influence GC levels including time of day, season, sex, and body condition, among others. For example, among three species of *Thamnophis* the corticosterone response was found to be dependent on diet, more specifically on the degree of bufophagy (Mohammadi et al., 2017). Additionally within a species, GC levels have been tied to variations in geography (e.g., latitude; Eikenaar et al., 2012) and population. Subspecies of the garter snake *Thamnophis sirtalis* may differ in baseline plasma levels of corticosterone (Moore et al., 2000a). A comparative study of white-crowned sparrows (*Zonotrichia leucophrys*) found that the non-migratory Nuttall's subspecies (*Z. l. nuttalli*) had higher baseline and post-stress corticosterone levels than the migratory Gambel's subspecies (*Z. l. gambelii*), which also breeds at higher elevations (Krause et al., 2014). However a similar study on European tree sparrows (*Passer montanus*) found that lowland and highland populations had no significant difference in baseline and post-stressor corticosterone levels (Li et al., 2011).

While comparative studies of GC levels and responses have the potential to advance the understanding of the functions of GCs and to elucidate adaptations among species, it is important to be cautious in labeling differences among subspecies or species as adaptations. Garland and Adolph (1994) argue that by comparing two species and drawing conclusions regarding adaptation, “physiological homogeneity” must be assumed, which is rarely the case unless species are bred and raised in captivity. It may often be inaccurate to look at differences between two populations, subspecies, or even species and brand them as the product of adaptation.

Chronically elevated glucocorticoids can suppress reproduction, immune function, and behaviors such as courtship, locomotion, and foraging (French et al., 2006; Ricciardella et al., 2010). However long-term repetition of a stressor may lead to conditions where the HPA axis becomes altered in order to adjust to the new allostatic load, resulting in a weaker than normal GC response. Such conditions are classified as chronic stress and may be initiated by natural factors like climate, disease, or food shortages, but can also be caused by artificial factors such as laboratory housing. For example, in starlings (*Sturnus vulgaris*) both basal and response levels of corticosterone were reduced after birds were exposed multiple times a day to six chronic stressors for a 20-day period (Rich and Romero, 2005). Tree lizards (*Urosaurus ornatus*) maintained in laboratory cages for up to three weeks had less of an increase in corticosterone levels post-housing than lizards subjected to acute confinement in collection bags (Moore et al., 1991). Arctic-breeding birds displayed variations in sensitivity to capture stress that

decreased with the increasing hostility of their environments (O'Reilly and Wingfield, 2001). Wingfield et al. (1992) hypothesized that downregulation of the stress response allows species to continue with reproduction even in the face of external stressors like storms or food shortages.

1.2 Reproductive axis

The relationship between stress and the reproductive axis is highly dependent on context. For example, the newt *Taricha granulosa* experienced rapid (within 8 minutes) stress-induced inhibition of amplexus and amplexic clasping, as well as twenty-four hours later a decrease in circulating plasma androgens (Moore and Miller, 1984; Moore and Orchinik, 1994). Reproductive inhibition following stress has been observed in other amphibians as well as reptiles and birds (Wingfield, 1984; Tokarz, 1987; DeNardo and Licht, 1993; Wingfield et al., 1997) however it is not common in mammals. However in a conflicting study on the red-sided garter snake (*Thamnophis sirtalis concinnus*), Moore (2001) injected corticosterone into males and found no inhibitory effects on courtship behavior. Additionally in males of the common side-blotched lizard (*Uta stansburiana*), Galapagos tortoise (*Geochelone nigra*), and red-spotted garter snake (*Thamnophis sirtalis concinnus*), there was a positive correlation between corticosterone and reproductive activity as well as testosterone levels (Wilson and Wingfield, 1994; Schramm et al., 1999; Moore and Mason, 2001). Both male and female green sea turtles

(*Chelonia mydas*) had increases in corticosterone coinciding with reproduction (Hamann et al., 2002).

In a potential explanation for these results, Romero (2004) discusses issues with artificially increasing corticosterone levels, stating that combining multiple stressors (i.e., captivity and injection) can result in unpredictable responses by the HPA axis.

Additionally, *Thamnophis* engage in large mating balls of hundreds of individuals, where reproductive success is not guaranteed and many individuals are smothered. With such an energetically expensive mating strategy, increased corticosterone levels to mobilize energy stores are beneficial and may outweigh the costs associated with long term elevated glucocorticoids (Moore and Mason, 2001; Moore and Jessop, 2003).

1.3 Model organisms

The common watersnake (*Nerodia sipedon*), diamondback watersnake (*Nerodia rhombifer*), and queen snake (*Regina septemvittata*) are semi-aquatic snakes in the subfamily Natricinae. Recent phylogenetic analysis shows the genus *Nerodia* to be a well-supported monophyletic clade (Pyron et al., 2013). *Regina* is polyphyletic, although the focal species *Regina septemvittata* is in the same clade as *Nerodia* (Pyron et al., 2013). The organization of the “water snake clade” remains unresolved, but the relationship between *Nerodia sipedon* and *Nerodia rhombifer* had an average of 99% bootstrap replicates (Pyron et al., 2013; Fig. 1). While the species are closely related and

may occupy similar environmental niches, several differences in life history, physiology, and ecology serve as an ideal basis for a comparative endocrinology study.

The three species of semi-aquatic snakes utilized in this study differ in age at maturity, body size, litter and offspring size, typical prey eaten, habitat, and docility. For example, *Nerodia rhombifer* mature earlier and grow significantly larger both in snout-vent length (SVL) and mass than either *Nerodia sipedon* or *Regina septemvittata* (Branson and Baker, 1974; Brown and Weatherhead, 1999a; Gibbons and Dorcas, 2004; Table 1). *Nerodia sipedon* has the highest ratio of clutch mass to maternal mass (relative clutch mass) reported of the three species, which is a comparative measure of reproductive investment in squamates (Shine, 1992). *Nerodia sipedon* are reported to have a higher preferred body temperature range than both *N. rhombifer* and *R. septemvittata*, and of the three species *N. rhombifer* has the lowest range reported (Butler, 1978; Gratz, 1978; Brown and Weatherhead, 2000). *Nerodia rhombifer* has the highest size-specific metabolic rate of the three focal species and *R. septemvittata* has the lowest (Gratz and Hutchison, 1977; Butler, 1978; Dean and Gratz, 1983). *Nerodia sipedon* and *R. septemvittata* may be found in similar environments, but while the diets of *N. sipedon* and *N. rhombifer* consist predominantly of fish, *R. septemvittata* is a specialist feeder that only eats molting crayfish (Mushinsky, 1988; Gibbons and Dorcas, 2004; Jackrel and Reinert, 2011). All three species react to pursuit with flight behavior and diving into the water or seeking out hiding places, but *Nerodia* species tend to react to handling with defensive aggression and repeated striking, while *R. septemvittata* is more likely to flee

or musk repeatedly than to bite (Layne and Ford, 1984; Weatherhead and Robertson, 1992; Cooper et al., 2008; Cooper, 2015).

Because GCs influence energetic responses, reproduction, and behavior, these species' differences in life history, physiology, and ecology are likely to influence GC dynamics. Furthermore, comparative studies of stress physiology are uncommon in reptiles (see Dunlap and Wingfield, 1995), and it is difficult to determine differences in GCs among species by reviewing the literature because of differences in methodology (e.g., handling times, assay type). Preliminary work on *N. sipedon* baseline corticosterone levels has been performed by Sykes and Klukowski (2009), and Cordoba (2009) examined the stress response of *N. rhombifer* experiencing salinity changes, but no studies of GCs have been done for *R. septemvittata*. Here I perform a comparative study of three species of semi-aquatic snakes while controlling for methodological differences.

1.4 Objectives of study

The primary question of this study was whether interspecific differences in circulating corticosterone, progesterone, and testosterone occur within closely-related watersnake species. Immediate capture and acute confinement stress were used to measure the hormonal responses of *N. sipedon*, *N. rhombifer*, and *R. septemvittata*. It was hypothesized that *N. sipedon* and *N. rhombifer* would have the most similar results due to their close phylogenetic relationship and life history similarities, and that *R. septemvittata* would exhibit significantly different baseline and stress-induced corticosterone

concentrations. It was also hypothesized that acute stress would cause elevation of plasma corticosterone concentrations in all three species and that there would be a negative relationship between body condition and baseline corticosterone concentrations in all three watersnake species. There were no specific predictions concerning species differences in testosterone or progesterone, but it was hypothesized that levels might decrease due to the effects of an acute stressor on the reproductive axis.

A final objective of this study was to examine the effect of chronic stress on hormone levels in *N. sipedon*. It was hypothesized that *N. sipedon* would exhibit mildly elevated corticosterone levels after 3 weeks of captivity, but that the corticosterone concentrations would be less than the responses observed in snakes subjected to 30 minutes of acute confinement stress. It was hypothesized that this lesser GC response would be a result of acclimation over time to the chronic stressor and downregulation of corticosterone release by the HPA axis (Sapolsky et al., 2000; Romero, 2004; Rich and Romero, 2005). It was also hypothesized that the chronic stress of laboratory housing would result in suppression of the reproductive axis as indicated by a decrease in plasma testosterone and progesterone concentrations in male and female snakes, respectively.

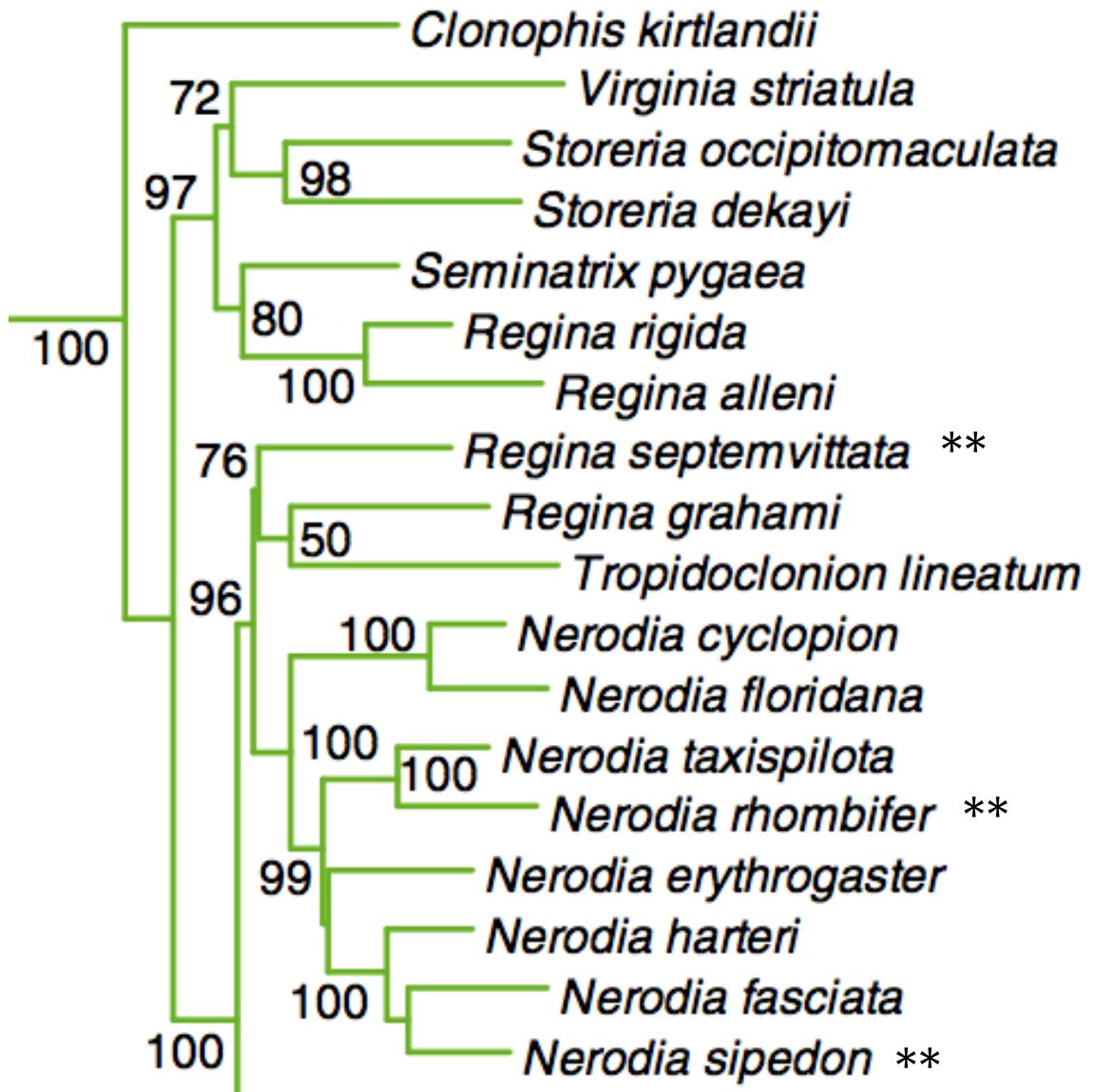


Figure 1: Reconstructed phylogeny of Natricinae, with focal species indicated by asterisks (From Pyron et al., 2013).

Table 1: Comparison of key life history, physiological and ecological characteristics of the three focal species of natricine snakes.

	<i>Nerodia sipedon</i>	<i>Nerodia rhombifer</i>	<i>Regina septemvittata</i>
Age at maturity (years) (♂/♀)	2-3, 3-4 ^{1 2 15 16}	1.2, 2.2 ¹	2, 3 ⁵
Avg size at maturity (SVL in cm) (♂/♀)	43, 60 ^{1 2}	53, 70-80 ¹	45, 51 ⁵
Growth rate of adults	Females faster ²	Females faster ⁴	Females faster ⁵
Degree of sexual dimorphism	Females 40% larger ²	Females 40% larger ¹	Females 30% larger ⁵
Reproduction rate	Annual	Annual	Annual
Relative clutch mass (RCM)	0.350 ¹¹	0.289 ¹¹	0.320 ¹⁰
Litter size	Mean: 22.7 Range: 4-99 ²	Mean: 25 Range: 4 - >50 ^{1 3}	Mean: 12.8 Range: 8-17 ⁵
Offspring size Average weight (g) Average SVL (cm)	3.35 16.7 ⁶	10.3 24.6 ⁷	3.28 16.1 ⁵
Preferred body temperature range (°C)	25-30 ¹²	23-25 ¹³	24-27 ¹⁴
Metabolic rate (mL O ₂ /g/hr)	0.06 at 25°C ¹⁷	.137 at 25°C ¹⁸	0.09 at 30°C ¹⁴
Prey	Opportunistic; fish and amphibians ⁹	Predominantly fish ⁹	Molting crayfish ⁸
Habitat	Largely any body of water within their range (man-made, natural, wetlands) ¹	Largely any body of water within range but less often in clear-flowing streams ¹	Small/medium permanent streams with mild current and rocky bottoms ¹
Behavior upon handling	Defensive aggression	Defensive aggression	Relatively docile

¹ Gibbons and Dorcas, 2004; ² Brown and Weatherhead, 1999a; ³ Gibbons and Dorcas, 2005; ⁴ Preston, 1970; ⁵ Branson and Baker, 1974; ⁶ Weatherhead et al., 1994; ⁷ Plummer, 1992; ⁸ Jackrel and Reinert, 2011; ⁹ Mushinsky, 1987; ¹⁰ Shine, 1992; ¹¹ Seigel and Fitch, 1984; ¹² Brown and Weatherhead, 2000; ¹³ Gratz, 1978; ¹⁴ Butler, 1978; ¹⁵ King, 1986; ¹⁶ Bauman and Metter, 1975; ¹⁷ Dean and Gratz, 1983; ¹⁸ Gratz and Hutchison, 1977

2. METHODS

2.1 Comparative study of three species of natricines

Twenty-five adult *Nerodia sipedon* ($n = 16$ males, $n = 9$ females) and 16 *Regina septemvittata* ($n = 4$ males, $n = 12$ females) were collected from sites in Rutherford County and Hamilton County, Tennessee, in the spring and summer of 2016 and 2017 (May 1, 2016 – July 1, 2017). Twelve adult *Nerodia rhombifer* ($n = 9$ males, $n = 3$ females) were collected from Reelfoot Lake in Lake County, Tennessee, in the spring and summer of 2017 (May 1, 2017 – July 1, 2017). Snout-vent lengths used to assess reproductive maturity in *N. sipedon* were 42.5 cm for males and 55.0 cm for females, in *N. rhombifer*, 47.5 cm for males and 67.0 cm for females, and in *R. septemvittata*, 39.5 cm for males and 41.5 cm for females (Fitch, 1981; Aldridge et al., 1995; Weatherhead et al., 1995; Brown and Weatherhead, 1999b). Snakes that failed to meet these minimum sizes were considered juveniles and not included in this study.

Snakes were hand-captured between the hours of 1000 and 1530 (CST) to control for potential circadian rhythms in hormone concentrations. Approximately 100 μ L of blood was collected immediately upon capture into heparinized microcapillary tubes after puncturing the palatine vein with a 25 ga needle (Sykes and Klukowski, 2009). The average time to bleed was 3.29 min (range: 1.35 - 7.58 min). Snout-vent length and tail length were measured to the nearest 0.5 cm, and body mass was measured to the nearest 0.1 g using a spring scale. Immediately after blood collection and taking body measurements, snakes were confined individually in cloth bags until 30 minutes had

passed from the time of initial capture. After the 30 minutes had elapsed, a second blood sample was drawn, and a ventral scale was clipped to prevent resampling individuals. Snakes were then released back into their environment within 10 m of their initial capture site.

2.2 Nerodia sipedon chronic stress study

Adult *Nerodia sipedon* were collected during May of 2016 from sites in Rutherford County, Tennessee ($n = 4$ males, $n = 5$ females) and bled immediately upon capture, as described above, to determine baseline hormone concentrations. These snakes were bled between the hours of 1200 and 1450 (CST), and the average time to bleed was 2.88 min (range: 1.50 – 5.50 min). Snout-vent, tail length, and mass were recorded. Captured snakes were transported to the laboratory and housed individually in either 61 x 33 x 33 cm or 122 x 33 x 43 cm aquaria, depending on snake size, with aspen bedding, available water, a 25°C housing temperature, and a photoperiod simulating spring in middle Tennessee (13L:11D). Snakes were fed minnows (*Fundulus* spp.) approximating 20% of their body weights once per week. Snakes were housed between 18 and 22 days in captivity. At the end of their captive period, body mass was re-measured and blood was drawn from the palatine vein to determine the effect of laboratory housing, a presumed chronic stressor, on plasma corticosterone concentrations. Post-lab housing blood samples were collected between the hours of 1023 and 1457 (CST), and the average time to bleed was 3.45 min (range: 2.17 – 5.75).

2.3. General lab procedure

After return from the field or blood collection in the lab, blood samples were centrifuged at 1000 g for 10 minutes, and hematocrit was recorded. Plasma was stored at -80°C.

Enzyme-linked immunosorbant assays (ELISAs) were used to measure plasma concentrations of corticosterone (Arbor Assays K014-H1, Ann Arbor, MI), progesterone (Enzo ADI-900-011, Farmingdale, NY), and testosterone (Enzo ADI-900-065, Farmingdale, NY). For the corticosterone assays, 20 µL of raw plasma was treated with steroid displacement reagent and then diluted to 30-fold in assay buffer. Steroid concentrations were measured via competitive binding of endogenous steroid and a phosphatase-labeled steroid to antibody binding sites (Sykes and Klukowski, 2009). The plate was shaken at room temperature for one hour, then excess reagents were washed away. After the addition of substrate and an additional hour of incubation, the optical density generated by each well was read by a SpectraMax M5 plate reader at 450 nm. The concentration of corticosterone was determined via comparison to a standard curve. The progesterone and testosterone assays were ran similarly except the plasma was diluted to 25-fold or 40-fold, respectively. These assay kits have previously been validated for use with water snake plasma (Webb et al., 2016). The intra- and interassay values were 5% and 12% for corticosterone, 3% and 12% for progesterone, and 7% and 14% for testosterone.

2.4. Statistical analyses

For the acute stress study of the three natricine species, plasma corticosterone concentrations and body size variables were compared among the three species with a one-way ANOVA using species as a factor, followed by a Tukey HSD multiple pairwise comparison test to determine differences among the means. Corticosterone values were log-transformed prior to analysis to normalize the data. A Kruskal-Wallis rank sum test was used to determine differences among the three species in baseline hematocrit, followed by a Dunn's test of multiple comparisons.

Linear regressions of snake mass on SVL were run within each of the three species to determine residuals for individual snakes, which were then used as estimates of body condition. Pearson's product-moment correlations were run to identify associations between body condition and steroid concentrations within each species. Where data were not normally distributed even after transformation (determined by Shapiro-Wilk normality tests), Spearman's rank correlations were run instead.

Two sample *t*-tests were used to compare baseline corticosterone, post-stressor corticosterone, baseline testosterone, baseline hematocrit, day of year captured, time of day bled, and initial time to bleed between *N. rhombifer* and *N. sipedon* males. Only males of these two species were used because very few male *R. septemvittata* were captured ($n = 4$). Similarly, only females of *N. sipedon* and *R. septemvittata* were compared because very few female *N. rhombifer* were sampled ($n = 3$). For females, the same variables were compared with the exception of comparing baseline progesterone

instead of testosterone. Two sample *t*-tests were also used to determine sex differences, but only in *N. sipedon*, since there were too few female *N. rhombifer* and too few male *R. septemvittata*. A Mann-Whitney rank sum test was used to evaluate sex differences in *N. sipedon* in baseline hematocrit.

The paired *t*-test was used to test for changes in corticosterone (e.g. from baseline to 30 min sample), testosterone, and progesterone within each of the three species, as well as in the chronic stress experiment that utilized *N. sipedon* only. All statistical processes were performed using RStudio (Gandrud, 2014; RStudio Team, 2015).

3. RESULTS

3.1 Acute stress study

3.1.1 Comparison across the three species of natricines

Baseline plasma corticosterone concentrations differed significantly among the three species (one-way ANOVA $F = 6.50$, $df = 2$, $P < 0.01$; Fig. 2). Baseline corticosterone was significantly higher in *R. septemvittata* than in *N. rhombifer* (Tukey HSD, $P < 0.01$). There was not a significant difference in baseline corticosterone between *N. sipedon* and *N. rhombifer* (Tukey HSD, $P = 0.10$) nor between *N. sipedon* and *R. septemvittata* (Tukey HSD, $P = 0.07$).

There was also a significant difference in post-stressor corticosterone (e.g., after 30 min of confinement) among the three species (one-way ANOVA $F = 9.61$, $df = 2$, $P < 0.001$; Fig. 2). Post-stressor corticosterone was significantly higher in *R. septemvittata* than in both *N. sipedon* and *N. rhombifer* (Tukey HSD $P = 0.01$ and $P < 0.001$, respectively). Post-stressor corticosterone did not differ between *N. sipedon* and *N. rhombifer* (Tukey HSD, $P = 0.19$).

Baseline hematocrit differed significantly among the species (Kruskal-Wallis chi-squared = 18.2, $df = 2$, $P < 0.001$, Fig. 3). *Regina septemvittata* had a significantly higher hematocrit than both *N. rhombifer* (Dunn $Z = -3.84$, $P < 0.001$) and *N. sipedon* (Dunn $Z = -3.61$, $P < 0.001$). There was not a significant difference in hematocrit between *Nerodia sipedon* and *Nerodia rhombifer* (Dunn $Z = -1.21$, $P = 0.11$).

There was a significant difference among the three species in the average day of

the year captured (one-way ANOVA $F = 3.74$, $df = 2$, $P = 0.03$; Table 2). *Nerodia rhombifer* were sampled later in the active season than *N. sipedon* (Tukey HSD, $P = 0.02$; Table 2). The remaining pairwise comparisons were not significantly different (*R. septemvittata*/*N. rhombifer* Tukey HSD, $P = 0.13$, *R. septemvittata*/*N. sipedon*, $P = 0.82$). There was not a significant difference in time of day captured among the three species (one-way ANOVA $F = 2.37$, $df = 2$, $P = 0.10$). *Regina septemvittata* and *N. rhombifer* differed in the time needed to collect the blood samples, with *N. rhombifer* having a shorter average time to bleed (one-way ANOVA $F = 4.10$, $df = 2$, $P = 0.02$; Tukey HSD, $P = 0.02$). The remaining pairwise comparisons were not significantly different (*R. septemvittata*/*N. sipedon* Tukey HSD, $P = 0.56$; *N. sipedon*/*N. rhombifer*, $P = 0.09$; Table 2).

3.1.2 Comparison of male *Nerodia sipedon* and *Nerodia rhombifer*

Neither baseline nor post-stressor testosterone concentrations differed significantly between male *N. sipedon* and *N. rhombifer*, although *N. sipedon* tended to have higher concentrations (two sample $t = -2.01$, $df = 24$, $P = 0.06$; $t = 1.68$, $df = 23$, $P = 0.11$, respectively; Fig. 4). Baseline hematocrit was significantly lower in male *N. rhombifer* than in *N. sipedon* (two sample $t = -2.10$, $df = 23$, $P < 0.05$; Mean \pm SE = $31.9 \pm 1.3\%$, $34.4 \pm 1.1\%$, respectively). Although blood samples for *N. sipedon* were collected on average 28 days earlier than for *N. rhombifer*, the time to bleed and time of day were comparable (Table 2). Average baseline testosterone for the four male *R. septemvittata*

was 10.9 ng/mL (range: 5.9 - 30.7 ng/mL), which was higher than most *N. rhombifer* and similar to *N. sipedon* males (see Fig. 4).

3.1.3 Comparison of female *Nerodia sipedon* and *Regina septemvittata*

Baseline progesterone concentrations were significantly higher in female *N. sipedon* than in *R. septemvittata*; however, there was not a significant difference in progesterone concentrations after 30 min of confinement stress (two sample $t = 1.90$, $df = 18$, $P = 0.01$ and $t = 0.86$, $df = 17$, $P = 0.40$, respectively; Fig. 5). There was also a significant difference in baseline hematocrit (two sample $t = 3.93$, $df = 23$, $P < 0.001$) with *R. septemvittata* having higher values (Mean \pm SE, 40.4 ng/mL \pm 1.2%) than *N. sipedon* (34.4 ng/mL \pm 1.1%). Females of the two species did not significantly differ in day of the year captured or initial time to bleed (two sample $t = -0.03$, $df = 19$, $P = 0.98$ and $t = 1.40$, $df = 19$, $P = 0.18$, respectively). The baseline values of progesterone for the three *N. rhombifer* females were 0.90, 2.75, and 25 ng/mL, which were similar to the other two species except for the value of 25 ng/mL which was unusually high (see Fig. 5).

3.1.4 Individual species data

All 25 *N. sipedon* individuals experienced an increase in corticosterone after 30 minutes of confinement stress with the average increase being 6.1 fold (range: 5 - 1890%). The increase in corticosterone was significant (paired $t = -9.15$, $df = 25$, $P << 0.0001$, Fig. 1). Changes in testosterone and progesterone after 30 minutes of stress were not significant

in *N. sipedon* males and females, respectively (paired $t = -1.31$, $df = 16$, $P = 0.21$; $t = 0.03$, $df = 11$, $P = 0.98$, respectively; Figs 4 and 5). Post-acute stress, nine male *N. sipedon* experienced increases in testosterone (range: 5.6 - 77.3%), and six experienced decreases (range: 2-28%). One female experienced a decrease in progesterone after 30 minutes of confinement stress (26.5%), while the eight others had increased progesterone (range: 6.7 – 60%).

In *N. rhombifer* as well, plasma corticosterone after 30 minutes of confinement stress increased significantly (paired $t = -7.2$, $df = 11$, $P \ll 0.01$; Fig. 1) and all 12 individuals exhibited an increase in corticosterone from baseline values. Changes in testosterone in male *N. rhombifer* after 30 minutes of confinement stress were not significant (paired $t = -0.99$, $df = 9$, $P = 0.35$; Fig. 4). Eight of the 11 males sampled had higher testosterone post-stressor with an average increase of 12.8% (range: 1.77 - 27.8%), and only three individuals experienced decreases (range: 1.5 - 26%).

Corticosterone levels in *R. septemvittata* after 30 minutes of capture stress were significantly higher than at baseline (paired $t = -11.7$, $df = 14$, $P \ll 0.01$; Fig. 1). There was an average change in corticosterone of 4.8 fold, and all 16 individuals experienced an increase in corticosterone after 30 minutes of capture stress. All four male *R. septemvittata* sampled experienced a modest increase in testosterone after 30 minutes of capture stress (range: 6 – 10%). Progesterone levels in female *R. septemvittata* were increased significantly after 30 minutes of capture stress (paired $t = -2.46$, $df = 9$, $P = 0.04$; Fig. 5). Eleven female *R. septemvittata* experienced increases in progesterone in

response to 30 min of confinement with an average increase of 59.7% (range: 1.6 – 228%), yet two females sampled experienced decreases in progesterone.

3.1.5 Correlations among the steroids

In female *N. sipedon* there was a significant positive correlation between baseline corticosterone concentrations and baseline progesterone levels (Pearson $R = +0.99$, $n = 11$, $P \ll 0.01$) but no significant correlation post-confinement stress (Pearson $R = +0.25$, $n = 7$, $P = 0.51$). There were no significant correlations in male *N. sipedon* between corticosterone and testosterone concentrations at baseline (Pearson $R = -0.34$, $n = 15$, $P = 0.18$) or post-confinement stress (Pearson $R = +0.17$, $n = 13$, $P = 0.95$). There were no significant correlations in male *N. rhombifer* between corticosterone and testosterone concentrations either at baseline (Pearson $R = -0.09$, $n = 7$, $P = 0.82$) or post-confinement stress (Pearson $R = +0.09$, $n = 7$, $P = 0.82$). There were no significant correlations in *R. septemvittata* females between corticosterone and progesterone concentrations either at baseline (Pearson $R = -0.03$, $n = 9$, $P = 0.94$) or post-confinement stress (Pearson $R = +0.17$, $n = 9$, $P = 0.63$).

3.1.6 Correlations between body condition and the steroids

Body condition was not significantly correlated with baseline corticosterone in either *N. sipedon* (Pearson $R = -0.25$, $n = 29$, $P = 0.17$) or *N. rhombifer* (Pearson $R = -0.31$, $n = 10$, $P = 0.33$; Figure 8); however, the correlation coefficient was negative in both species.

Body condition of *R. septemvittata* was significantly negatively correlated with baseline corticosterone (Pearson $R = -0.73$, $n = 13$, $P < 0.01$; Figure 8). Male body condition was negatively but not significantly correlated with baseline testosterone for either *N. sipedon* (Pearson $R = -0.24$, $n = 15$, $P = 0.36$) or *N. rhombifer* (Pearson $R = +0.23$, $n = 8$, $P = 0.53$). Female body condition was positively but not significantly correlated with baseline progesterone in both *N. sipedon* (Pearson $R = +0.48$, $n = 11$, $P = 0.10$) and *R. septemvittata* (Pearson $R = +0.46$, $n = 9$, $P = 0.15$).

3.1.7 Sex differences in *Nerodia sipedon*

There was not a significant sex difference in baseline corticosterone (two sample $t = -0.06$, $df = 29$, $P = 0.95$) or post-stressor corticosterone (two sample $t = -0.26$, $df = 23$, $P = 0.80$; Fig. 6). However male *N. sipedon* had significantly larger hematocrit values than females (Mean \pm SE, males: $35.9 \pm 1.1\%$; females: $32.3 \pm 2\%$; Mann-Whitney $W = 163$, $P = 0.03$) and significantly longer tails (two sample $t = 2.4$, $df = 25$, $P = 0.03$).

3.2 Chronic stress of lab housing in *Nerodia sipedon*

Corticosterone levels of *N. sipedon* upon initial capture in the field were not significantly different from corticosterone levels post-lab housing (paired $t = -2.02$, $df = 5$, $P = 0.10$; Fig. 7). Average baseline corticosterone concentrations at initial capture was 30.1 ng/mL (range: 10.9 - 64.7 ng/mL) and the average post-lab housing corticosterone was 41.9 ng/mL (range: 19.1-92.6 ng/mL). Baseline body mass decreased on average 9.6 g (range:

1.5 g – 24 g) and was significantly different post-captivity (paired $t = 2.32$, $df = 5$, $P = 0.049$) in all snakes except for one female *N. sipedon* which gained 13.1 g. Nevertheless, snake body condition did not change significantly (paired $t = -0.18$, $df = 5$, $P = 0.87$).

Baseline testosterone levels in the two males for which there was enough plasma to assay testosterone were 4.3 and 8.6 ng/mL, and post-lab housing testosterone levels were higher in each male (5.8 and 10.1 ng/mL, respectively). The average baseline progesterone level in females was 2.6 ng/mL (range: 1.5 - 3.8 ng/mL, $n = 4$ females), and the average post-lab housing progesterone level decreased to 1.6 ng/mL (range: 0.55-3.0 ng/mL, $n = 3$). Progesterone concentrations decreased post-lab housing in all three female *N. sipedon* for which there were both baseline and post-lab housing data.

Table 2: Descriptive statistics of capture and body size data for *N. sipedon*, *N. rhombifer*, and *R. septemvittata*. Different letters in superscript denote significant differences ($P < 0.05$, post-hoc test).

	<i>Nerodia sipedon</i> Mean (Range)	<i>Nerodia rhombifer</i> Mean (Range)	<i>Regina septemvittata</i> Mean (Range)
Day of year captured ¹	138 (92-197) ^a	164 (135-173) ^{bc}	144 (93-190) ^{ac}
Time of day captured	11:58 (10:03-15:28) ^a	12:41 (10:42-14:55) ^a	12:54 (10:50-14:39) ^a
Initial time to bleed	3:27 (1:21-6:49) ^{ab}	2:36 (1:17-3:36) ^b	3:50 (2:16-7:35) ^a
Mass (g)	212.7 (70.0-710) ^a	358.7 (170-793.5) ^b	113.7 (50.0-193.5) ^c
SVL (cm)	62.3 (46.0-90.0) ^a	72.5 (62.0-91.0) ^b	56.9 (44.0-69.5) ^a
Tail length (cm)	19.8 (14.0-24.0) ^a	22.7 (16.6-28.0) ^b	16.0 (11.9-20.0) ^a
Sample size (♂/♀)	16/9	9/3	4/12

¹ Numerical day of year; 1 = January 1, 32 = February 1, etc.

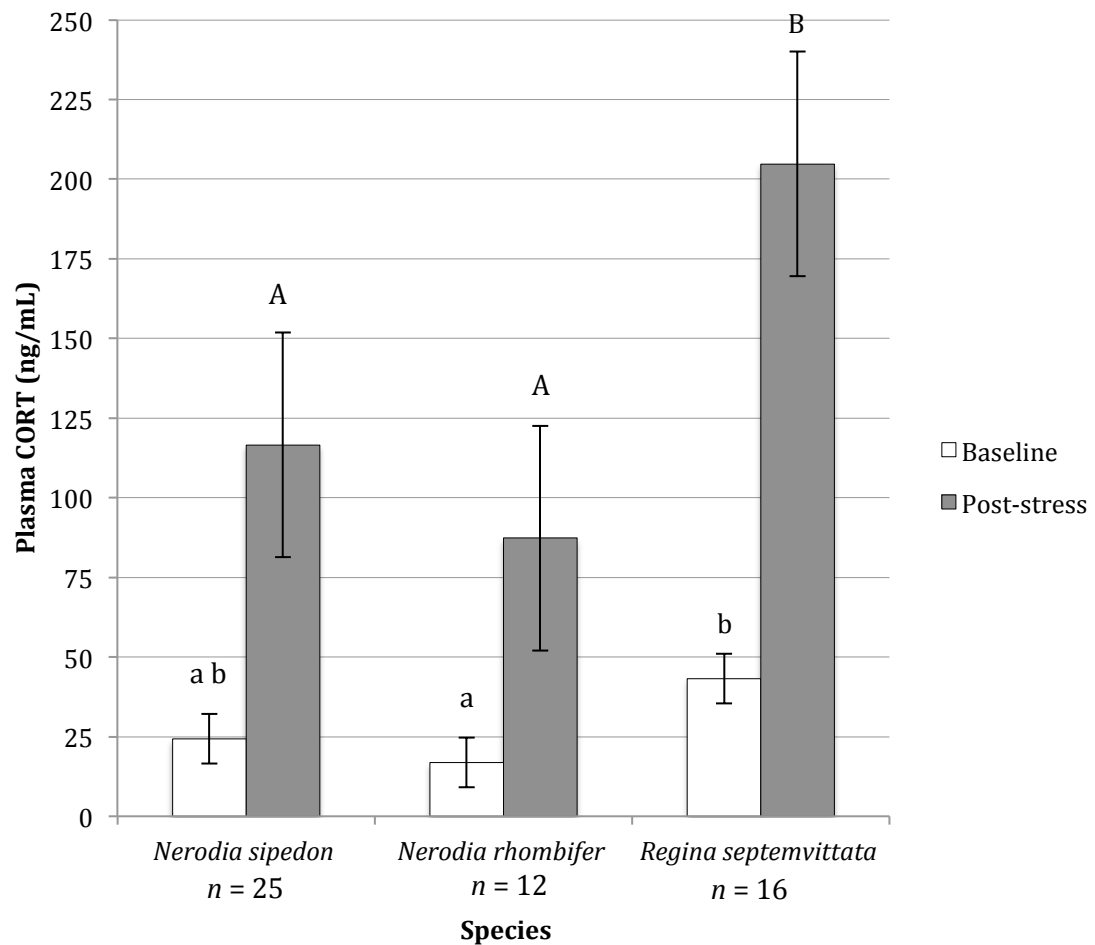


Figure 2: Plasma corticosterone concentrations (Mean \pm SE) upon field capture (Baseline) and after 30 min of confinement (Post-stress) in three species of Naticinae. Different letters above the bars denote significant differences ($P < 0.05$, post-hoc test) in both baseline and post-stress corticosterone among the three species, and different cases of a letter denote differences between baseline and post-stress corticosterone within each species.

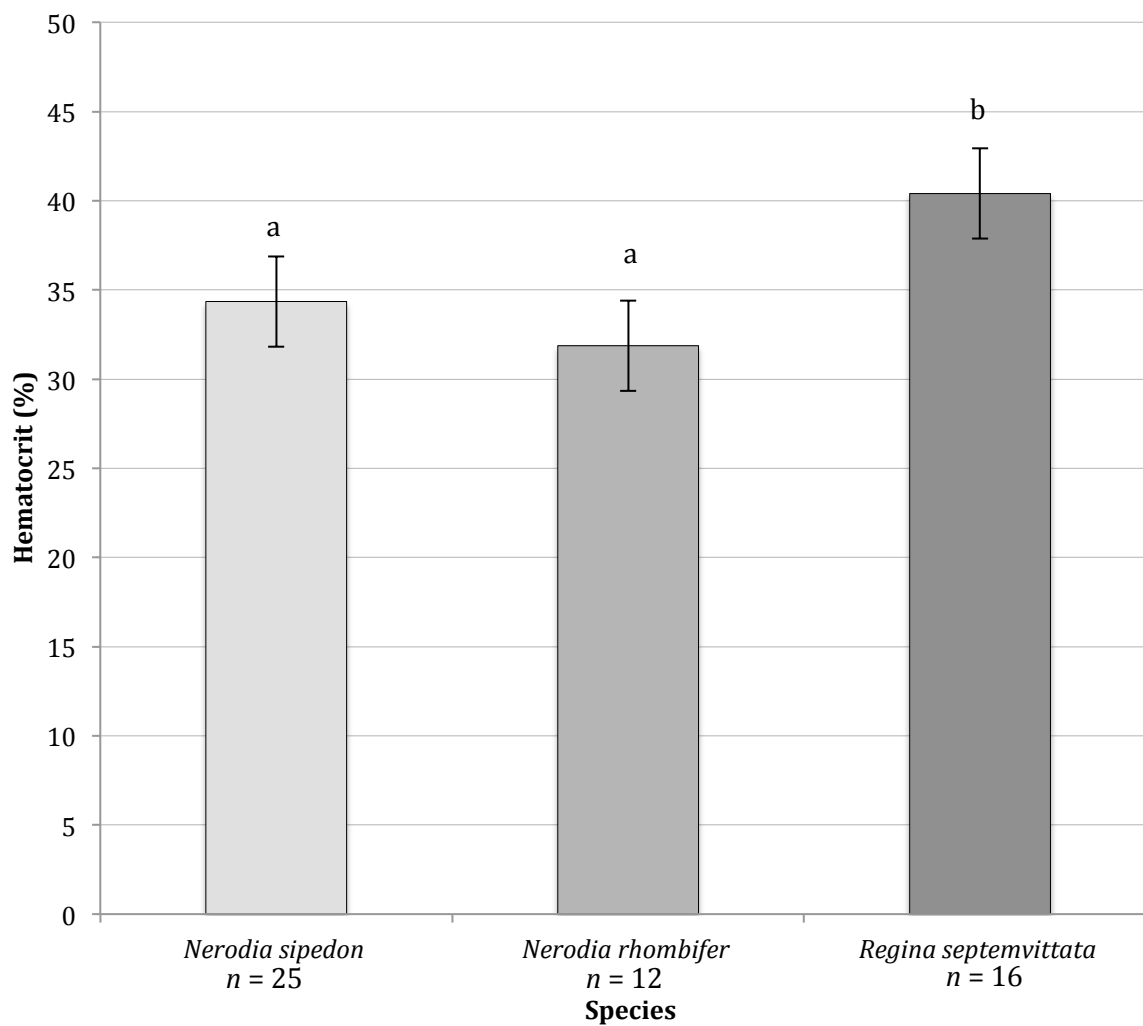


Figure 3: Mean (\pm SE) hematocrit values for the three species of Natricinae. Different letters above the bars denote significant differences ($P < 0.05$, post-hoc test).

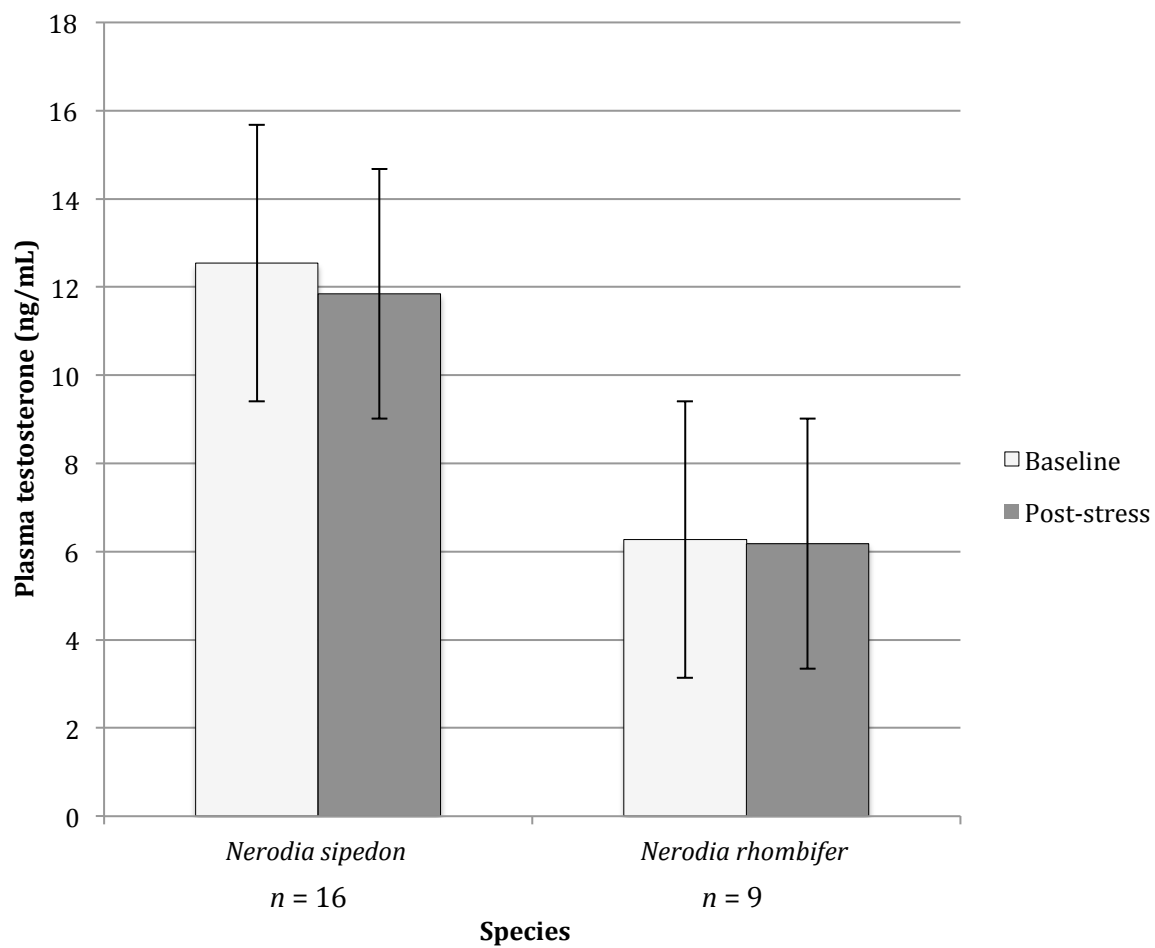


Figure 4: Plasma testosterone concentrations (Mean \pm SE) upon capture (Baseline) and after 30 min of confinement (Post-stress) in male *Nerodia sipedon* and *Nerodia rhombifer*.

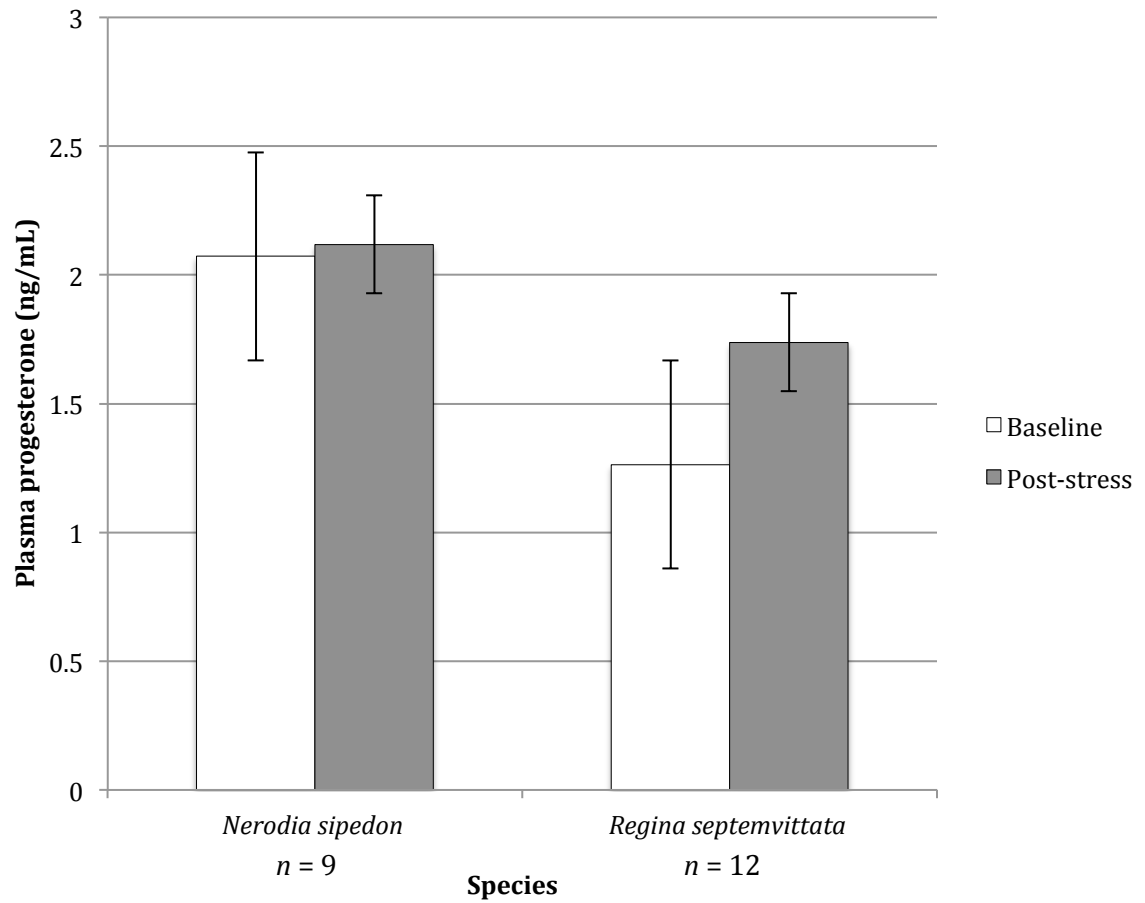


Figure 5: Plasma progesterone concentrations (Mean \pm SE) upon capture (Baseline) and after 30 min of confinement (Post-stress) for female *Nerodia sipedon* and *Regina septemvittata*.

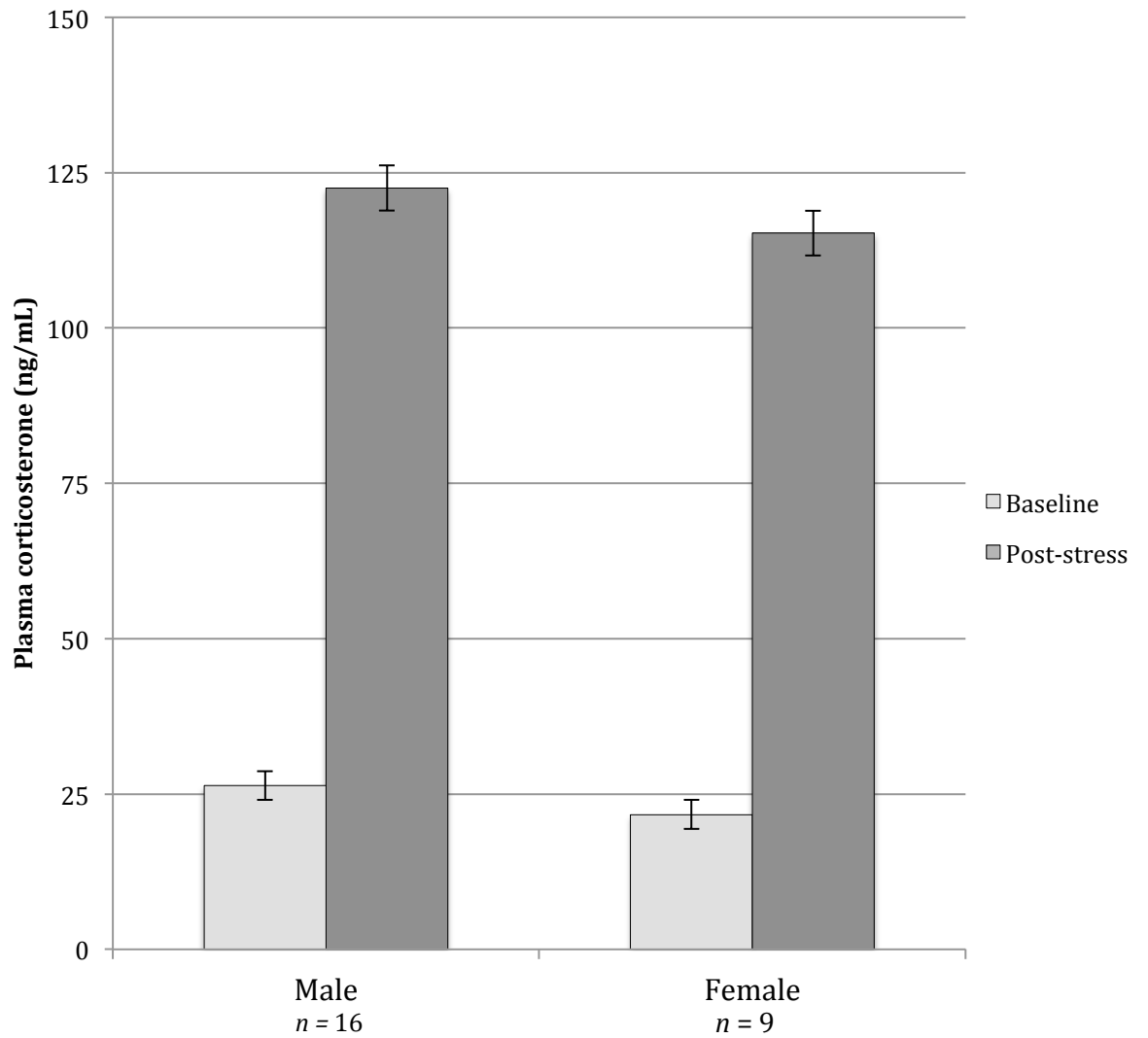


Figure 6: Plasma corticosterone concentrations (Mean \pm SE) upon capture (Baseline) and after 30 min of confinement (Post-stress) in male and female *Nerodia sipedon* in the field.

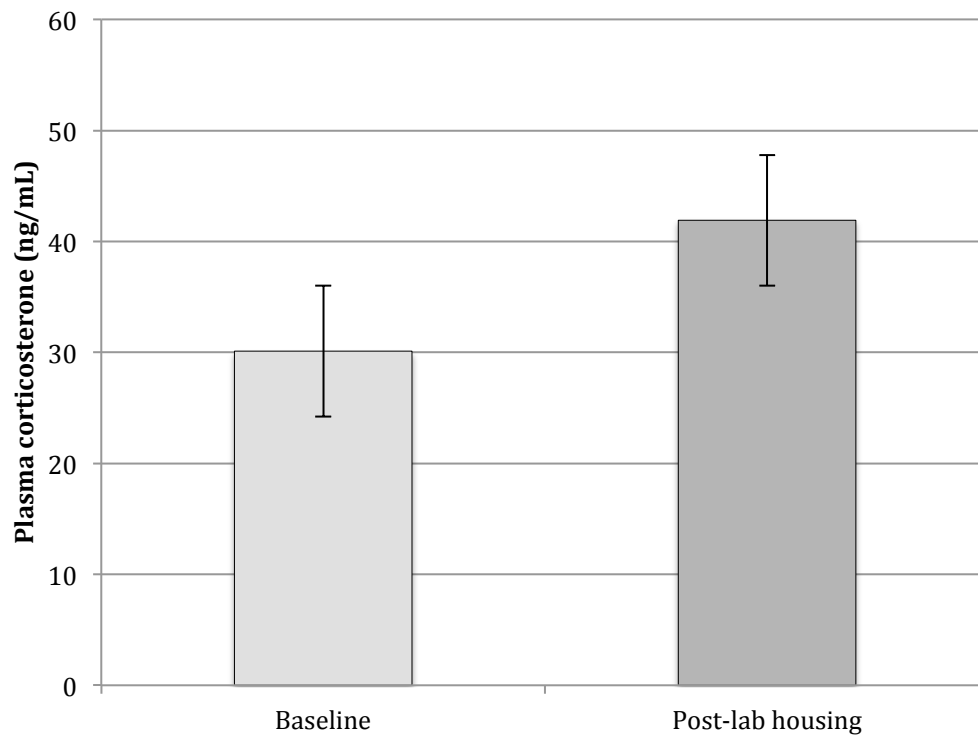


Figure 7: Plasma corticosterone concentrations in six *Nerodia sipedon* (Mean \pm SE) upon capture (Baseline) and after 18-22 days of laboratory housing (Post-lab housing).

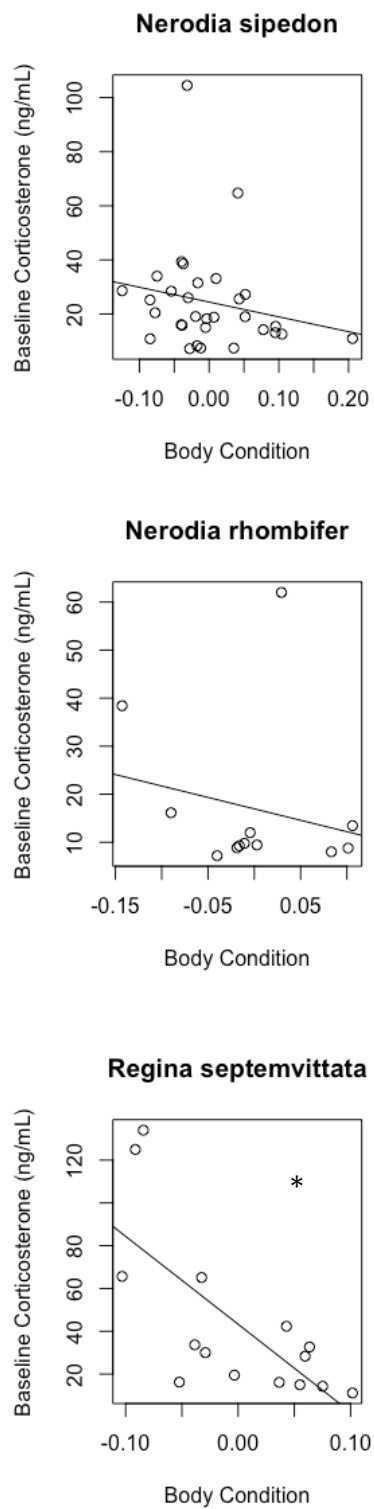


Figure 8: Linear correlations between body condition and baseline corticosterone for the three species of naticine. Asterisks indicate significance ($P < 0.05$, post-hoc test).

4. DISCUSSION

The initial prediction was that *R. septemvittata* would have different plasma corticosterone concentrations than the two *Nerodia* species, but *N. sipedon* and *N. rhombifer* would have similar corticosterone concentrations because of their greater phylogenetic and ecological similarity. In partial agreement with the first prediction, *R. septemvittata* had higher baseline corticosterone than *N. rhombifer*, but not higher than *N. sipedon*, post-stress corticosterone was significantly higher in *R. septemvittata* than in both *Nerodia* species. The second prediction was that acute stress would cause a significant elevation of plasma corticosterone levels and that baseline corticosterone concentrations would have a negative relationship with individual body condition. All three species did experience significant increases in corticosterone levels after thirty minutes of confinement stress, supporting the second prediction. In partial agreement with the third prediction, *R. septemvittata* exhibited a negative relationship between baseline corticosterone and body condition, but the correlation, while negative, was not significant in either of the *Nerodia* species.

Baseline corticosterone levels are linked with the present physiological state of the animal and can be affected by environmental stressors such as fluctuations in food availability (Lewanzik et al., 2012). Black-legged kittiwakes (*Rissa tridactyla*) experiencing declines in food availability have higher baseline corticosterone levels than those with abundant food (Lancot et al., 2003). Baseline cortisol levels (the primary mammalian glucocorticoid) in the vampire bat (*Desmodus rotundus*), a sanguivore that

experiences seasonal fluctuations in food availability, are significantly higher than the short-tailed fruit bat (*Carollia perspicillata*), a frugivore with a more reliable food supply (Lewanzik et al., 2012). *Regina septemvittata* is a dietary specialist that eats crayfish in molt, but prey can be scarce, in part because adult crayfish molt only approximately twice per year (St. John, 1976; Jackrel and Reinert, 2011). Populations of *N. sipedon* and *R. septemvittata* often overlap spatially, which may provide a similar range of stressors that play a larger role in their HPA axis adaptations than food availability and contributed to their similar baseline corticosterone concentrations.

The maximum corticosterone levels that an animal reaches post-stressor tend to be indicative of energetic requirements, as well as the severity and duration of environmental stressors. For example, desert-dwelling birds under chronic environmental stress experienced a lesser degree of corticosterone elevation post-acute stressor than similar species dwelling in more resource-rich riparian habitats (Wingfield et al., 1992). Wingfield et al. (1992) suggests that this modulation of the stress response ensures that breeding behaviors are less likely to be disrupted, which is crucial for species living in more severe environments or that have shorter breeding seasons. For this study, all three species surveyed breed in the late spring and give birth in the fall, and differences in post-stressor corticosterone levels may indicate differences in allostatic load, or the amount of activity by hormone mediators like corticosterone that is required to maintain homeostasis (McEwen and Wingfield, 2003; Gibbons and Dorcas, 2004). For instance, *R. septemvittata* may have had higher circulating corticosterone levels because they were in

a more energetically demanding phase of the reproductive cycle when sampled and therefore required extra corticosterone-mediated mobilization of energetic resources. This has been documented in anurans, where corticosterone levels will peak alongside androgens during the breeding season, presumably to accommodate the energetic demands of courtship behaviors (Emerson, 2001; Moore and Jessop, 2003).

A few sources of sampling bias may have skewed the results. For example, because the sample of *R. septemvittata* was predominantly female, there also may have been an effect of sex on the response to acute stress in the present study. However, results on sex and stress sensitivity in reptiles vary by species. Female brown tree snakes (*Boiga irregularis*) and six-lined racerunners (*Cnemidophorus sexlineatus*) had significantly higher corticosterone after confinement and capture stress than males, but in green sea turtles (*Chelonia mydas*), hawksbill sea turtles (*Eretmochelys imbricata*), and olive Ridley sea turtles (*Lepidochelys olivacea*) males had higher stress-induced corticosterone than females (Grassman and Hess, 1992; Valverde et al., 1999; Jessop, 2001; Mathies et al., 2001; Moore and Jessop, 2003). The reasons for the inconsistent effect of sex on corticosterone are presently unclear. In the present study, in addition to the possible confounding effect of sampling an inadequate number of snakes of each sex, there was also a significant difference in time required to bleed and day of the year captured between *R. septemvittata* and *N. rhombifer* but not between *R. septemvittata* and *N. sipedon*, which mimics the post-stress corticosterone levels and could potentially explain the higher corticosterone observed in *R. septemvittata*. Among the three species there

were no differences in body condition, which indicates that body condition did not account for the species differences in baseline or stress-induced corticosterone.

Intraspecific differences in hematocrit were evident in this study and have also been shown in two *Thamnophis* species, which are also in subfamily Natricinae (Wack et al., 2012b). Declining habitat and increased environmental pressures were suggested by Wack et al. (2012b) as potential causes of the elevated hematocrit in the giant garter snake (*Thamnophis gigas*) as compared to the valley garter snake (*Thamnophis sirtalis fitchi*). A full hematological panel was not run on the focal species, therefore it is unknown whether the signs of chronic inflammation that Wack et al. (2012b) found in *T. gigas* were present in any of the focal species. The three species have different masses, SVLs, and life history traits, and while *N. sipedon* and *R. septemvittata* may co-occur in the same environment, they seek different prey items (Table 1). *Regina septemvittata* had both the highest hematocrit and plasma corticosterone levels in the present study, which is consistent with a study on male red-legged salamanders (*Plethodon shermani*). Increased plasma corticosterone levels in the salamanders increased their metabolic rates and oxygen consumption, which would likely coincide with a need for greater hematocrit (Wack et al., 2012a).

In the present study, female *N. sipedon* exhibited higher baseline progesterone levels than *R. septemvittata*. *Nerodia sipedon* did not experience changes in progesterone after thirty minutes of confinement stress, yet the progesterone levels for *R. septemvittata* increased significantly. The higher progesterone values in female *N. sipedon* than in *R.*

septemvittata could potentially be due to having a great proportion of *N. sipedon* already being gravid. Pregnant cottonmouths (*Agkistrodon piscivorus*), family Viperidae, had higher baseline progesterone levels than non-pregnant snakes (Graham et al., 2011). Progesterone levels in vertebrates increase during gestation but drop at parturition with the regression of the corpora lutea, then continue to drop back to baseline levels within a few days (Taylor et al., 2004; Smith et al., 2012). *Nerodia sipedon* and *R. septemvittata* both breed in the late spring (April and May) and give birth in the fall, which puts them on a similar reproductive timeline (Ernst, 2003; Gibbons and Dorcas, 2004). In the present study, one of the *N. rhombifer* sampled had a progesterone concentration of 25 ng/mL, which was approximately ten times the level of any other snake sampled. Because this snake was sampled in early June, it is possible that she was gravid and experiencing her highest levels of progesterone at the time.

The lack of testosterone suppression after stress in *N. sipedon* and *N. rhombifer* is in contrast to decreased testosterone reported after acute handling stress for tree lizards (*Urosaurus ornatus*) (Moore et al., 1991). Testosterone levels in *N. sipedon* have been found to be highest in the spring during mating and then to decrease during midsummer and peak again in early fall (Weil and Aldridge, 1981). Weil and Aldridge (1981) observed that all males in their study caught from late April to early June had plasma testosterone levels over 2.5 ng/mL, which was supported in the present study by *N. sipedon* and all but one *N. rhombifer* male. However most *N. rhombifer* were sampled in

late June, which falls outside the six-week window specified by Weil and Aldridge (1981).

Neither the baseline corticosterone nor post-acute stressor corticosterone differed by sex for *N. sipedon*. Sykes and Klukowski (2009) reported that female *N. sipedon* had post-stress corticosterone levels 3-fold higher than males, however Webb et al., (2016) found no statistical difference. Sykes and Klukowski (2009) used one hour of confinement as an acute stressor, whereas Webb et al., (2016) used 15 days of food deprivation, which is a chronic stressor.

Hematocrit values for *N. sipedon* differed by sex, similar to that of viperids and boids. Lower hematocrit values in female snakes have been documented for eastern massasaugas (*Sistrurus catenatus catenatus*) and green anacondas (*Eunectes murinus*) (Calle et al., 1994; Allender et al., 2006). However Birchard et al. (1984) found that in the garter snake (*Thamnophis sirtalis*) pregnancy did not have an effect on hematocrit values. Therefore the sex differences in hematocrit values are likely due to physiological or endocrine factors other than pregnancy, such as higher testosterone levels in males.

Nerodia sipedon appeared to acclimate to the three-week laboratory housing experiment as was indicated by post-captivity corticosterone levels not differing from baseline levels (Romero, 2004). Conversely, Sykes and Klukowski (2009) found that *N. sipedon* experienced an increase in plasma corticosterone levels after 5-8 days of laboratory housing. Potentially additional time is necessary for the HPA axis to adjust to the required allostatic load. Conflicting corticosterone responses to captive stress has

been observed in lizards as well. Male wall lizards (*Podarcis sicula sicula*) housed for eight days had plasma corticosterone levels return to levels resembling samples collected at capture (Manzo et al., 1994). However, male White's skinks (*Egernia whitii*) showed elevated corticosterone levels after one week of laboratory housing, but by four weeks corticosterone levels had dropped to baseline levels measured at capture (Jones and Bell, 2004). Such interspecific variations may be due to differences in housing conditions or reflect physiological differences in the species (Moore et al., 1991).

The corticosterone concentrations measured in *N. sipedon* subject to 3 weeks of laboratory housing in the present study were noticeably lower than the plasma corticosterone levels of snakes experiencing 30 minutes of acute confinement stress (see Figs. 1 and 6). This indicates that corticosterone concentrations rise rapidly in response to acute stressors but then subsequently decrease over the following three weeks of laboratory housing, a presumed chronic stressor. Unfortunately, the sample size was not large enough to draw conclusions regarding the effect of chronic versus acute stressors on testosterone and progesterone levels in *N. sipedon*.

This study provides the first work to date on the hormone levels and hematocrit of *R. septemvittata*. Additionally, there is a paucity of comparative endocrinology work on Serpentes, particularly between closely related species. The magnitude of differences in corticosterone among watersnake species is similar to, but not necessarily greater than, the magnitude of differences in corticosterone reported to occur within a single species of reptile because of such factors as season, sex, or exposure to environmental stressors.

Confirmation of the higher corticosterone observed in *R. septemvittata* in the present study will require replication and even greater control of sampling periods and sex ratios within a species. Nonetheless, if the species differences observed are repeatable, it is interesting that the species with the highest levels of stress hormone was the species that, at least outwardly, appears to respond with the least aggression and physical agitation in response to capture and handling. Expanding the long-term laboratory housing study to include a larger sample of *N. sipedon* as well as *N. rhombifer* and *R. septemvittata* would allow exploration of whether the acclimation of the corticosterone response occurs generally in natricines.

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APPENDIX

APPENDIX A: IACUC APPROVAL LETTER

IACUC**INSTITUTIONAL ANIMAL CARE and USE COMMITTEE**

Office of Research Compliance,
010A Sam Ingram Building,
2269 Middle Tennessee Blvd
Murfreesboro, TN 37129

**IACUCN001: PROTOCOL APPROVAL NOTICE**

Thursday, August 18, 2016

Investigator(s): Louise McCallie (Student PI) and Matt Klukowski (FA)
Investigator Email(s): klm2aw@mtmail.mtsu.edu; matt.klukowski@mtsu.edu
Department/Unit: Biology

Protocol ID: **16-2010**

Protocol Title: **Reproductive behaviors in Nerodia sipedon, the northern water snake**

Dear Investigator(s),

The MTSU Institutional Animal Care and Use Committee has reviewed the animal use proposal identified above under the **Designated Member Review (DMR) mechanism** and has approved your protocol in accordance with PHS policy. A summary of the IACUC action(s) and other particulars in regard to this protocol application is tabulated as below:

IACUC Action	APPROVED for one year	
Date of Expiration	4/27/2017	
Number of Animals	30+20+20 per year for the following species	
Species	Nerodia sipedon, Regina septemvittata; and Nerodia fasciata	
Research Category & Subclassifications	<input type="checkbox"/> Teaching <input type="checkbox"/> Field Study <input type="checkbox"/> Observation <input checked="" type="checkbox"/> Research <input checked="" type="checkbox"/> Field Research <input checked="" type="checkbox"/> Handling or Manipulation <input type="checkbox"/> Classroom <input checked="" type="checkbox"/> Laboratory	
	All animals will be field captured and N sipedon will be housed in SCI 1170K and will be used in SCI1170G	
Restrictions	Euthanasia as shown in the SOP on file	
Comments	"Field Research" box is checked to reflect the approved addendum	
Amendments	<p>Date 08/18/2016</p>	<p>Post-approval Amendments The following amendments were approved through an unanimous vote during a convened FCR on 08.10.2016: (1) Species addition/removal (20 each of Nerodia rhombifer and Nerodia cyclopion) (2) Increase in animal usage (3) Euthanasia of 30 N. sipedon as proposed</p>

This approval is effective for three (3) years from the date of this notice. This protocol **expires on 4/27/2017**. The investigator(s) MUST file a Progress Report annually regarding the status of this study. Refer to the schedule for Continuing Review given below and bear in mind that **NO REMINDERS WILL BE SENT**. A continuation request (progress report) must be approved by the IACUC prior to **4/27/2017** for this protocol to be active for its full term. Once a protocol has expired, it cannot be continued and the investigators must request a fresh protocol.

Continuing Review Schedule:

Reporting Period	Requisition Deadline	IACUC Comments
First year report	3/27/2017	INCOMPLETE
Second year report	3/27/2018	INCOMPLETE
Final report	3/27/2019	INCOMPLETE

MTSU Policy defines an investigator as someone who has contact with animals for research or teaching purposes. Anyone meeting this definition needs to be listed on your protocol and needs to complete IACUC training through the CITI program. Addition of investigators requires submission of an Addendum Approval to the Office of Research Compliance.

The IACUC must be notified of any proposed protocol changes prior to their implementation. Unanticipated harms to subjects or adverse events must be reported within 48 hours to the Office of Compliance at (615) 494-8918.

Also, all research materials must be retained by the MTSU faculty in charge for at least three (3) years AFTER the study is completed. **Be advised that all IACUC approved protocols are subject to audit at any time and all animal facilities are subject to inspections** at least biannually. Furthermore, IACUC reserves the right to change, revoke or modify this approval without prior notice.

Sincerely,

Compliance Office
(On behalf of IACUC)
Middle Tennessee State University
Tel: 615 494 8918
Email: compliance@mtsu.edu