DEMOGRAPHIC CHARACTERISTICS, INCIDENCE OF *RANAVIRUS* INFECTION, AND SEASONAL CORTICOSTERONE LEVELS IN THE EASTERN BOX TURTLE, *TERRAPENE CAROLINA CAROLINA*, IN A SUBURBAN WETLANDS HABITAT OF MIDDLE TENNESSEE

by

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ABSTRACT

The Eastern Box Turtle, *Terrapene carolina carolina*, is a species of concern in Tennessee because of population decline. Decline is due mainly to anthropogenic causes including, but not limited to, road mortality, disease, and habitat fragmentation. The purpose of this study was to assess the general health of the *T. c. carolina* population in a suburban wetlands habitat in Murfreesboro, TN, USA (Nickajack) by measuring demographic characteristics, infection status, and physiological characteristics. Demographic characteristics recorded include population density, age class, sex ratio, and several body size measurements (e.g., carapace length and carapace height). A small blood sample (≤0.2 ml) was drawn to measure corticosterone levels, triglycerides, uric acid, innate immunity, and to determine *Ranavirus* infection status. The population density was estimated to be approximately 14-15.5 turtles per hectare. The age class structure had a normal distribution with most turtles falling within the middle age class (10-14 years). The sex ratio was 1.26 male:1 female. For body size, the only variable with a significant difference between males and females was straight-line carapace length in which males were longer than females. A single turtle (LPW) was positive for *Ranavirus* infection out of the 102 turtles sampled and tested, accounting for 1% prevalence in the sampled population. Females had higher body condition indices than males. In 2013, corticosterone levels were significantly higher in summer than in spring. In 2014,

corticosterone levels were significantly higher in fall than in both spring and summer. The difference in the seasonal timing of peak corticosterone levels between the years may be related to weather conditions or associated with opportunistic mating. Corticosterone levels were positively correlated with hemolysis titer, possibly because of an immunoenhancing effect of stress hormones. In both 2013 and 2014, triglyceride levels were significantly higher in females than in males. The higher triglyceride levels of females were most likely associated with the energetic demands of egg production, carrying eggs, and nesting. Triglycerides were positively correlated with body condition, indicating greater lipid reserves in individuals with a better body condition. All other physiological measures, including total leukocytes and heterophil counts, failed to exhibit significant seasonal or sex related differences. To my knowledge, this is the first report of baseline corticosterone values and innate immunity in free-ranging *T. c. carolina*. The results obtained will aid in the conservation and protection of this species of concern.

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CHAPTER ONE

INTRODUCTION

Background

The Eastern Box Turtle, *Terrapene carolina carolina*, is one of six subspecies of box turtles belonging to the family Emydidae. *Terrapene c. carolina* has a range extending throughout much of the eastern United States from Mississippi to Georgia and northward into Michigan and Massachusetts (Conant and Collins 1991). *Terrapene c. carolina* is currently in decline across its range and is considered to be vulnerable (IUCN, van Dijk 2013) and is a species of concern in Tennessee (Brian Flock, TWRA pers. comm.).

A substantial amount of research has been conducted on *T. c. carolina*, in topics such as home range (Stickel 1950, Stickel 1989, Cook 2004, Donaldson and Echternacht 2005), nesting (Wilson 1998, Flitz and Mullin 2006, Willey and Sievert 2012), hibernation and issues with global warming that affect hibernation (Dolbeer 1971, Costanzo and Claussen 1990, Savva et al. 2010), demographics such as body size (Weiss 2009) and reproduction (Ewing 1933, Ewing 1935, Wilson and Ernst 2005), associative learning (Leighty et al. 2013), hematology (Allender et al. 2006, Heatley and Russell 2010, Allender et al. 2011, Kimble and Williams 2012), phylogenetics (Martin et al. 2013), and methods to track turtles such as radio telemetry (Forsythe et al. 2004, Rittenhouse et al. 2005), turtle

dogs (Kapfer et al. 2012), and thread-trailing (Claussen et al. 1997, Iglay et al. 2006).

Although *Terrapene c. carolina* has been studied frequently in many areas, little research has been conducted in Middle Tennessee, and there are still some topics that have had little attention that have important conservation implications such as baseline levels of the major stress hormone (corticosterone) and basic demographic characteristics such as body size and population density. *Terrapene carolina carolina* is a species of concern in Tennessee because of population declines due mainly to habitat fragmentation due to urbanization (Budischak et al. 2006), mortality caused by vehicular traffic (Stickel 1978, Gibbs and Shriver 2002), and disease as a result of ranaviral infection (Allender et al. 2006, Allender et al. 2011). Few studies have analyzed demographic characteristics of *T. c. carolina* populations in Tennessee, and this information is important for monitoring and conservation efforts, not only in Tennessee but for the species range (Weiss 2009). Because of the “concern” status of *T. c. carolina* in Tennessee, it is important to have baseline demographic characteristics (e.g., estimate of population size, age structure) and measures of general health status (e.g., stress hormone levels, disease status) so populations can be monitored.

Demographic characteristics

Demographic measurements recorded in this study included population density, age, sex ratio, and body size. The population density of the turtles at Nickajack should be comparable to other population studies of *T. c. carolina*. It is important to have an estimate of population density so the Nickajack population can be monitored for population decline over subsequent years and appropriate conservation actions can be taken if necessary.

In many studies, most *T. c. carolina* fall within the 20+ year age class (Budischak et al. 2006, Weiss 2009). Age class structure can be an important indicator of many life history traits including recruitment, survivorship, and mortality (Hall et al. 1999, Budischak et al. 2006). Because turtles are a long-lived species (Gibbons 1987), adults typically have a high survival rate (Budischak et al. 2006), and *T. c. carolina* is known to be a long-lived species (Stickel 1978, Hall et al. 1999). However, urbanization can negatively impact adult survival and growth rates (Hall et al. 1999, Budischak et al. 2006). In addition, juveniles are often inconspicuous, possibly associated with their habitat preference (Hall et al. 1999), and they are preyed upon heavily, which may cause a low number of individuals in juvenile age classes. A healthy population of turtles should exhibit a normal distribution of age classes.

Estimation of sex ratio is another important characteristics for understanding population demographics and can be used for monitoring populations, especially those of organisms, such as turtles, that can live to be

very old (Dodd 1997). Sex ratios of box turtles are often skewed toward males (Dolbeer 1969, Dodd 1997, Hall et al. 1999, Weiss 2009). However, in a naturally interbreeding population, the sex ratio should approximate 1:1 (Fisher 1958), which has only been recorded in a few studies involving *T. c. carolina* (Stickel 1950, Williams and Parker 1987). Sex ratios can fluctuate depending on several factors including sampling methods, age and size at which sexual maturity is reached, and temperature dependent sex determination (Dodd 1997). It is important to understand what is influencing the sex ratio in box turtle populations. Several studies have indicated that nesting site is the most influential factor affecting sex ratio, because male box turtles develop in cooler temperature conditions, such as internal forest nests (Dodd 1997, Weiss 2009). This seems a likely scenario for the turtles at Nickajack, because they indeed live in a relatively cool wetland forest (2013 soil temperature: 18.32º C, 2014: 15.45º C, recorded on site).

In addition to sex ratio, body size measurements can shed light on the dynamics of the population in question. Because many box turtle species exhibit sexual size dimorphism (Dodd 1997, Clair 1998), it is important to measure body size to get a better understanding of the population in question. Several body size characteristics were measured to compare males to females including mass, straight-line carapace length, carapace width, carapace height, length of hinge, length anterior to hinge, and length posterior to hinge (Weiss 2009). It is typical for male box turtles to have a longer carapace length but a shorter carapace

height (Legler 1960, Stickel and Bunck 1989, Dodd 1997, Weiss 2009). From these body size measurements, it is also possible to estimate body condition using body mass and straight-line carapace length (Jessop et al. 2004a). Body condition can provide a general estimate of energetic status and overall condition of an individual compared to other individuals in the population (Schulte-Hostedde et al. 2005). Ultimately, all of the aforementioned demographic characteristics are important for understanding population structure, monitoring populations, and implementing conservation and preservation practices when and where necessary. With declining populations in mind, a major objective of this study was to obtain demographic data for *T. c. carolina* in a suburban wetland of Middle Tennessee because it is a species of concern making it of the utmost importance to have baseline demographic data for monitoring.

Ranavirus

Because *T. c. carolina* populations are currently decreasing, it is important to monitor for disease within populations such as Nickajack, because they are at risk of extirpation if disease becomes prevalent. *Ranavirus* is a genus in the family Iridoviridae, and it is a disease that has become quite prevalent in many chelonian species (De Voe et al. 2004, Allender et al. 2006). The most common species to infect reptiles and amphibians is frog virus 3 (called frog virus 3-like virus or FV3) (Allender et al. 2013) which is the only species of *Ranavirus* found

in the turtles of North America (Allender 2012). *Ranavirus* cannot withstand high temperatures and is therefore most prevalent in cold-blooded animals such as fish, reptiles, and amphibians (Allender 2012). Symptoms of *Ranavirus* include, but are not limited to, skin and oral lesions, difficulty breathing associated with mucus and respiratory issues, and lethargy (De Voe et al. 2004, Allender et al. 2006, Allender et al. 2011). In addition, because *Ranavirus* is found in fishes, amphibians, and reptiles, it is possible for the disease to spread between sympatric species (Goodman et al. 2013, Currylow et al. 2014). I tested for the presence of *Ranavirus* at Nickajack due to the high risk of fatality for *T. c. carolina* carrying the disease (Allender et al. 2011). *Ranavirus* has been known to result in mass die-offs of amphibians, fishes, and reptiles, but less is known about the effects of ranaviral disease in *T. c. carolina* (Allender 2012). Allender (2012) proposes that this might be due to the reclusive and hidden nature of *T. c. carolina*, where these die-off events could go unnoticed. Due to the small area of Nickajack and the relatively high density of turtles found there, I believe it would be hard to miss a mass die-off event caused by *Ranavirus*. Therefore, I predicted that *Ranavirus* is not currently prevalent in the Nickajack population, but that it is present in a small number of individuals and could spread over time. Allender (2012) also mentions that *Ranavirus* is more likely to spread in aquatic species that share the same bodies of water and regularly come into contact with each other than in terrestrial turtle species such as *Terrapene c. carolina* that do not come into direct contact very often. However, in the summer, *T. c. carolina* may

congregate in large numbers at small bodies of water, thereby increasing the risk of disease transmission, especially if these bodies of water contain amphibians known to harbor *Ranavirus* (Allender 2012, Currylow et al. 2014). Also, it is possible that increased stress levels are associated with ranaviral infection.

Physiological Measures

In addition to disease status, I also measured seasonal baseline corticosterone levels. Recently, the first study to assess sex steroids (both estradiol 17-β and testosterone) in *Terrapene c. carolina* was published, and until then virtually no work had been done on the natural hormone levels of the species (Currylow et al. 2013). I measured plasma corticosterone levels in *T. c. carolina* at Nickajack to assess the baseline levels of this major stress hormone. Baseline plasma corticosterone levels have not been previously reported for *T. c. carolina*. Case (2003) and Case et al. (2005) reported no effect of enrichment on fecal corticosterone levels in laboratory housed *T. c. carolina*. To my knowledge, Case (2003) is the only known study to report corticosterone levels in *T. c. carolina*. I measured plasma corticosterone levels that are not directly comparable to fecal corticosterone levels. A primary objective of this study was to assess the seasonal variation in baseline corticosterone and to determine if there was a sex difference in baseline corticosterone levels. Corticosterone levels may be associated with body condition (Jessop et al. 2004b) which is a general

measure of energetic status. In addition, corticosterone levels may also be associated with innate immunity.

I measured innate immunity of *T. c. carolina* at Nickajack by measuring the ability of turtle plasma to lyse rabbit red blood cells (i.e., hemolysis assays). These assays are indicative of the ability of the turtles' natural antibodies to lyse rabbit erythrocytes. An abnormally low titer score may be indicative of disease or immunosuppression (Matson et al. 2005). Also, seasonality may play a role in the immune response in some reptiles (Leceta and Zapata 1986) which may lead to a seasonal difference in hemolysis titers. No other studies have analyzed innate immunity of *T. c. carolina* to date, and this information will be important in understanding the immune response of *Terrapene c. carolina* to foreign cells. In addition, abnormalities in blood cell counts may be indicative of disease and possibly *Ranavirus* (Allender 2012).

Leukocyte counts were conducted for turtles during the 2013 fall season (the total leukocyte count (per 10,000 RBC), the total heterophil count (per 10,000 RBC), and the differential WBC count (e.g., what proportion of the WBCs are heterophils)). Whether or not WBC counts fall within their normal range may provide indirect evidence on the health of the population (Allender 2012). Also, atypical WBC counts of individuals may be associated with *Ranavirus* infection (Allender 2012). Furthermore, I measured several other physiologic characteristics to determine if there was a seasonal or sex-related difference.

One of these characteristics was plasma triglyceride levels which I measured to determine if there was seasonal variability in fat cycling. Brisbin (1972) stated that many hibernating and migratory animals tend to store fat prior to winter, but reptiles seem to store fat during the summer when reproduction is occurring. However, Brisbin (1972) found no seasonal variation in fat storage of captive *T. c. carolina*. Brisbin's (1972) turtles were captive and fed canned dog food which is not comparable to what free-ranging turtles experience. In comparison, Duggan et al. (2001) found that in the Painted Turtle, *Chrysemys picta*, triglyceride levels were linked with seasonal reproductive patterns and vitellogenesis in females. Duggan et al. (2001) found that triglyceride levels were highest in females during early spring (April), lowest during the summer after depositing eggs, and peaked again during November. I assumed that free-ranging *T. c. carolina* would exhibit seasonal changes in plasma triglycerides, especially females during the mating season.

I also measured uric acid levels in *T. c. carolina* at Nickajack. Uric acid is the primary nitrogenous waste product in terrestrial turtles (Moyle 1949), providing a measure of the degree of protein catabolism. Therefore, uric acid levels can provide an indirect estimate on the nutritional status of *T. c. carolina*. I predicted that levels of uric acid would be highest during a period of starvation as the turtle catabolized its own proteins and lowest when the turtle has larger energetic reserves (e.g., large fat bodies). It has also been found that uric acid levels are significantly higher during hibernation than during the active season

(Hutton and Goodnight 1957) which could cause seasonal fluctuations. Seasonal differences in uric acid could also be associated with food source availability and abundance during each season.

In conclusion, the general purpose of this study was to assess the overall health of *T. c. carolina* in a wetlands habitat (Nickajack) in Middle Tennessee by measuring demographic characteristics, infection status, and several physiological characteristics. Because *T. c. carolina* is a species of concern in Tennessee, it is important to have baseline demographic characteristics for monitoring. In addition, because *Ranavirus* is known to result in massive die-offs (Allender 2012) and can possibly be spread between sympatric herpetofauna (Currylow et al. 2014), it is important to test for its presence in the Nickajack population. The specific primary objectives of this study were to test for seasonal variation and sex differences in baseline corticosterone levels, to record demographic characteristics, and to determine the prevalence of *Ranavirus* infection. Secondary objectives included testing for seasonal and sex differences in innate immunity, white blood cell counts, triglyceride levels, and uric acid levels. Considered together, these physiological and demographical measures can give a better understanding of the health of the population and be used for monitoring over time.

CHAPTER TWO

MATERIALS AND METHODS

Field site, study organism, and demographic characteristics

T. c. carolina can be found in a wide variety of terrestrial environments including both forested and open areas (Stickel 1950, Yahner 1974, Williams and Parker 1987, Todd 2000). Females are often found nesting in open fields or grasslands (Flitz and Mullin 2006). I studied *T. c. carolina* in a wetland habitat, Nickajack Trace and Black Fox Wetlands (Nickajack) in Murfreesboro, TN, USA. Nickajack is a 23.5 hectare (ha) wetland that is cut into two sections by a residential road, and it is also completely surrounded by a suburban housing community. There appeared to be a high density of *T. c. carolina* in this wetland habitat (Rachel Singer, pers. comm.). I captured *T. c. carolina* at Nickajack during the active seasons of 2013 and 2014. I used a modified version of Cagle's (1939) notching system to mark turtles by notching 3 marginal scutes with a triangular file using a unique 3 letter code corresponding to the chart from Somers and Matthews (2006) (Figure 1).

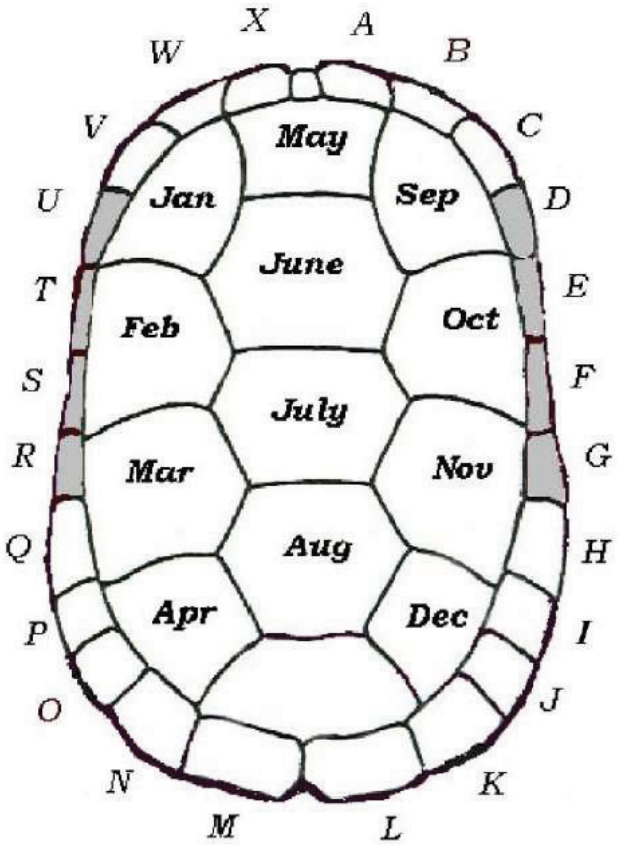

Figure 1. Turtle shell with marginal scutes labelled with corresponding letter of the alphabet for marking (Somers and Matthews 2006).

If a turtle was already marked with a 3 letter code I recorded it as a recapture. When found, I processed each turtle at the site of capture.

Using 2013 and 2014 monthly data, I estimated population size using the POPAN model (variation of Jolly-Seber model) in the program Mark (Gary White, Colorado State University) and using the Lincoln-Petersen Index (Pollock et al. 1990). The POPAN model is used as a submodule in MARK to estimate the entire population or “super population”, and it assumes equal survival and catchability among the individuals of the population in question (Wagner et al.

2011). The equation for the Lincoln-Petersen Index is as follows: $\widehat{N} = \frac{n_1 n_2}{m_2}$, where n stands for the number of individuals captured for each sampling period and m stands for the number of marked individuals from the first sampling period recaptured during the second sampling period (Pollock et al. 1990). The two sampling periods used for this study were 2013 and 2014. After population size was calculated, I then calculated population density by dividing the population size by 23.5 ha to estimate the number of turtles per ha. I used two models (POPAN and Lincoln-Petersen) to get the best estimate of population size and density and present results for both methods.

I estimated age by counting the annular rings on the costal (pleural) scutes (Ewing 1939) and assigning each turtle to an age class (in years) as follows: 0-4, 5-9, 10-14, 15-19, 20+ (Budischak et al. 2006, Weiss 2009). When analyzing age class structure, I combined 2013 and 2014 captures and each turtle was used only once, even if it was recaptured multiple times. I determined sex primarily by eye color and plastron concavity, where males typically have red eyes and a concave plastron and females typically have brown eyes and a flat plastron (Elghammer et al. 1979, Somers and Matthews 2006). When calculating sex ratio, I combined 2013 and 2014 data, and each turtle was used only once, even if it was recaptured.

I measured several body size dimensions. I measured mass to the nearest gram (g) using a 1,000 g Pesola spring scale (Forestry Suppliers, Inc., Jackson, MS) by attaching the clip of the scale to the rear marginal scutes of the turtle and

hanging the turtle upside down. I used digital calipers (200 mm, Neiko Tools, Homewood, IL) to obtain shell measurements to the nearest 0.01 mm, including straight-line carapace length, carapace width, carapace height, length of hinge, length anterior to hinge, and length posterior to hinge. After recording body measurements, I took photographs of shell pattern and any abnormalities of each turtle. In addition, I recorded air temperature (Enviro-safe Thermometer, Forestry Suppliers, Inc.), substrate temperature (Taylor Soil Thermometer, Forestry Suppliers, Inc.), and GPS coordinates (Garmin Etrex 30, Olathe, KS) at the location of each captured turtle.

Blood sampling

I collected blood samples in 2013 and 2014 during the spring (May), summer (July-early August), and fall (late September-October) seasons from approximately 20 turtles each season. Blood collects at the subcarapacial sinus which is situated above the head at the midline of the carapace in between the vertebrae (Heatley and Russell 2010). I drew small samples of blood (~0.2 ml) from the subcarapacial sinus using a 25 gauge sterile needle and a 1.0 ml syringe between the hours of 8:20 AM and 2:30 PM to control for circadian fluctuations in physiological parameters. I recorded the time of day bled and the time required to collect blood (starting from the moment the turtle was first handled). I immediately transferred blood to heparinized microcapillary tubes which I sealed with clay and then stored in a separate test tube labeled with the

appropriate turtle code. I kept samples on an ice pack while in the field for an extended period of time (> 2 hours) on extremely hot days (> 30º C). When in the lab, I transferred whole blood from one microcapillary tube for each turtle to a 0.5 ml Eppendorf tube using a 22 gauge sterile needle and 1.0 ml syringe; this blood was saved and later used to test for *Ranavirus.* I used a new needle and syringe for each turtle to prevent contamination. I sealed each Eppendorf tube with parafilm and then froze them (-80° C). In addition, I broke off the tip of a single microcapillary tube to extract a drop of whole blood used to produce a blood smear for each turtle, and then I recapped the microcapillary tube. I centrifuged each capillary tube to determine hematocrit, extracted the plasma using a 200 µl Hamilton syringe (Fisher Scientific, Pittsburgh, PA) and then I froze (-80º C) the plasma until analysis of physiological parameters.

Ranavirus

I used whole blood from 37 turtles in 2013 and 65 turtles in 2014 to test for the presence of *Ranavirus* through DNA extraction, polymerase chain reaction (PCR), and gel electrophoresis. DNA extraction was completed using an IBI extraction kit and following the provided protocol for whole blood samples (IBI Scientific, Peosta, IA). Step one was cell lysis. I added phosphate-buffered saline (PBS) to each whole blood sample to a volume of 200 µl. PBS consists of 4.0 g NaCl, 0.1 g KCl, 0.575 g Na_2HPO_4, 0.1 g KH_2PO_4, and dH_2O to 500 ml. I transferred the 200 µl solution to a 1.5 ml tube an-d added 15 µl of proteinase K

(20 mg/ml concentration, IBI Scientific). I incubated this solution at 60º C for 15 minutes in a heat block (SciGene, Sunnyvale, CA). Subsequently, I added 200 µl of GB Buffer to the tube and mixed the solution by vortexing (Scientific Industries, Bohemia, NY). I incubated the mixture at 70º C in a heat block (SciGene) for 15 minutes, inverting the tube every 3 minutes during incubation. In addition, I placed 25 µl of Elution Buffer for each sample in a tube and incubated this solution at 70º C.

After incubation, I performed DNA binding, step two, by adding 200 µl of absolute ethanol to the sample lysate and vortexing it immediately for 10 seconds. I placed a GD column in a 2 ml collection tube and transferred the entire mixture (~650 µl) to the column. I centrifuged the solution for 5 minutes at full speed (16,000 x g). I discarded the collection tube and the liquid it contained and then placed the GD column into a new 2 ml collection tube.

Step three of washing involved adding 400 µl of W1 Buffer to the column and centrifuging for 30 seconds at full speed. I discarded the flow-through and placed the column back into the emptied collection tube. Next, I added 600 µl of Wash Buffer (containing ethanol according to the manufacturer instructions) to the column and centrifuged it at full speed (16,000 x g) for 30 seconds. I discarded the flow-through and centrifuged the column for 30 seconds to dry the column.

Finally, I completed step four, or DNA elution, by transferring the dried GD column to a new 1.5 ml tube and adding 25 µl of the preheated Elution Buffer to

the center of the column. After standing for 3-5 minutes to allow for absorption into the column matrix, I then centrifuged the solution for 30 seconds at full speed (16,000 x g) to elute the purified DNA. I removed the 25 µl of eluted DNA from the 1.5 ml tube using a micropipet and replaced it to the center of the column to allow adequate elution. I then centrifuged the tube again for 30 seconds at full speed (16,000 x g), then I discarded the column and stored the eluted DNA at 4º C until PCR. I performed PCR by adding 34.5 µl dH_2O, 37.5 µl Master Mix (Promega, Madison, WI), 0.5 µl of both forward (sense) and reverse (antisense) primer (Integrated DNA Technologies, Coralville, IA), and 2 µl of the extracted DNA sample to a 0.5 ml tube, and I ran the samples in a thermocycler (Eppendorf Mastercycler Personal, Brinkmann Instruments Inc., Westbury, NY) using the program FLU (Figure 2).

1. 93º C, 2 min
2. 93º C, 30 sec
3. 46º C, 45 sec
4. 72º C, 1 min
5. Go to step #2, 5 times
6. 93º C, 30 sec
7. 48º C, 45 sec
8. 72º C, 1 min
9. Go to step #6, 10 times
10. 93º C, 30 sec
11. 50º C, 45 sec
12. 72º C, 1 min
13. Go to step # 10, 15 times
14. 72º C, 5 min
15. 4º C, forever
16. End

Figure 2. Program for PCR (named FLU) that was used for all turtle blood samples.

Master Mix is a mixture of buffer, deoxynucleotide triphosphates (dNTPs), and *Taq* polymerase. The forward and reverse primers I used are part of the 531 base pair sequence of the *Ranavirus* major capsid protein (MCP) commonly used for detection of *Ranavirus* (Mao et al. 1997) and are as follows: forward: 5'-GACTTGGCCACTTATGACA-3', reverse: 5'-GTCTCTGGAGAAGAAGAA-3'. In addition, I made a negative control by adding 2 µl of dH_2O instead of extracted DNA and a positive control by using 2 µl of a positive *Ranavirus* sample (cultured from a *Ranavirus* positive frog with Frog Virus 3 or FV3) obtained from Dr. Deb Miller of University of Tennessee Knoxville. After completion, I stored samples at 4º C until gel electrophoresis.

I evaluated amplified samples by gel electrophoresis. I made a gel using 0.5 g NuSeive agarose (FMC Bioproducts, Rockland, ME) and 40 ml 1X TAE solution. I made the 1X TAE solution by diluting 10X TAE by 1:10 using dH_2O. 10X TAE consists of 9.6 g of Tris base, 10 mL of 0.2 M EDTA (pH 8.0), 2.3 mL of glacial acetic acid, and enough dH_2O to make 200 ml. I added the mixture of agarose and 1X TAE to a glass beaker and microwaved it for approximately 30-60 seconds, while removing and swirling the liquid every 10 seconds for proper mixing. I allowed the liquid to cool to approximately 50º C, and taped each gel tray around the edges to seal the tray. I applied a small amount of the gel liquid to each side of the gel tray using a Pasteur pipette to seal the taped ends. Finally, I inserted an 8-well comb into the tray, and poured the liquid agarose into the gel tray. It was then allowed to solidify for approximately 15 minutes.

In the meantime, I prepared samples to load by adding 1.5 µl of blue sample buffer (BioVentures, Murfreesboro, TN) and 8.5 µl of the PCR product (including the positive and negative controls) to newly labeled 1.5 ml tubes. I made a separate tube containing 1.5 µl of the blue sample buffer (BioVentures) along with 8.5 µl of ladder (BioVentures). This ladder is used as a standard of known base pair sizes to analyze any bands that showed up on the gel, and to estimate the number of base pairs the band shows at. I then placed the gel tray with solidified gel into a gel box (BioRad Minisub Cell GT, BioRad Hercules, CA). Subsequently, I added approximately 250 ml of 1X TAE with approximately 80 µl of Ethidium Bromide (10 mg/ml concentration, Curtin Matheson Scientific, Inc., Houston, TX) to the gel box to cover the gel completely. Finally, I then loaded each 10 µl sample into each of the 8 wells using a 10 µl micropipette, and the gel box was attached to the voltage machine (PowerPac Basic, BioRad, Hercules, CA). I set the voltage to 75 volts and allowed the gel to run for approximately 1 hour and 15 minutes. After time had lapsed, I used a UV transilluminator (Foto/Phoresis, Fotodyne Inc. Hartland, WI) to read the gel, and I recorded any bands that appeared. If needed, a photograph was taken using the UV transilluminator and Kodak camera system (Kodak DC290, Eastman Kodak, Rochester, NY) and saved to file using the Kodak ID 3.6 software. I sent any samples that showed up as a potential positive on a gel to SeqWright (Houston, TX) to be sequenced. The sequence of each sample was then subjected to a Basic Local Alignment Search Tool (BLAST) in GenBank (Bethesda, MD).

Physiological measures

I measured plasma corticosterone levels using an enzyme linked immunosorbant assay (ELISA) (Enzo Life Sciences, Inc., Farmingdale, NY, product no. ADI 900-097). Briefly, in the corticosterone ELISA, competitive binding occurs between corticosterone labeled with alkaline phosphatase and the corticosterone from the turtle plasma samples. The two are competing to bind to sheep polyclonal antibody which has a strong affinity for corticosterone, and the sheep antibody is actively binding to the plate which is coated with donkey antibody specific to the sheep antibody. To begin, I thawed all plasma samples and vortexed (Fisher Scientific). I then diluted each sample 1:2 with the stock steroid displacement reagent (SDR). Subsequently, I added 560 ul of Assay Buffer to each sample, diluting each sample by 30. I ran all samples in duplicate on a 96-well plate. I then added the sheep polyclonal antibody to each sample, and I incubated the plate at room temperature for two hours on a plate shaker (Barnstead International, Dubuque, Iowa). After incubation, I emptied the plate of its contents, and I washed the plate with wash solution. Finally, I added the substrate p-nitrophenyl phosphate (p-Npp) to each well where it reacted with the alkaline phosphatase to create a yellow product. The less yellow the contents of the well, the more corticosterone in the turtle plasma sample. I then read the plates on a plate reader (SpectraMax M5, Molecular Devices LLC, Sunnyvale, CA) at 405 nm. I then based levels on a standard curve with known amounts of

corticosterone. The intraassay coefficient of variation was 8% and the interassay coefficient of variation was 12%.

I estimated innate immunity by measuring the ability of serially diluted turtle plasma to lyse rabbit erythrocytes in a 96-well plate following a protocol similar to Matson et al. (2005). The positive control consisted of rabbit red blood cells (RBCs) added to deionized water. The negative control consisted of rabbit erythrocytes added to phosphate buffered saline (PBS). Turtle plasma was serially diluted from 1:2 to 1:2,048. After overnight incubation, the plates were read visually to determine the maximum titer that caused lysis ($\log_2$ of the dilution factor).

I made blood smears in the lab for each of the 17 turtles from fall 2013 and labeled them with date, turtle code, and sex, and they were allowed to air dry before fixing with methanol for 60 seconds. At the end of the active season, I stained all slides using a Quik Diff Stain Kit (Thermo Shandon, Pittsburgh, PA). I took photographs (100) of each slide at 1,000X with oil immersion using a microscope with a built-in camera (Olympus BX51, Waltham, Massachusetts) using the Olympus DP Controller software. I counted erythrocytes either manually or using ImageJ (National Institutes of Health, Bethesda, MD) and identified leukocytes as either heterophil or not a heterophil. I calculated both the total leukocyte count (per 10,000 RBCs), total heterophil count (per 10,000 RBCs), and the differential heterophil count (percent heterophils). I identified heterophils as cells with rod-shaped granules and a dark-brownish stain, and

with the nucleus typically round in shape and usually at the periphery of the cell (Heatley and Russell 2010, Allender 2012).

I measured plasma triglyceride and uric acid concentrations using commercial enzymatic kits (Pointe Scientific, Canton, MI, product nos. T7532 and U7581, respectively). I measured triglycerides by adding reagent to each turtle plasma sample in a cuvette. After a series of reactions involving lipase, glycerol kinase, glycerol phosphate oxidase, and peroxidase, the solution produced a quinoneimine dye in a shade of red. The more intense the red color, the higher the level of triglycerides. I read cuvettes at 500 nm in a spectrophotometer (Spectronic Genesys 5, Milton Roy, Warminster, PA) and compared them to a standard.

Similarly, I measured uric acid by adding reagent to each turtle plasma sample, but this was done in a 96-well plate rather than cuvettes. After a series of reactions involving uricase and peroxidase, the solution produced a red chromogen that if more intense indicates a higher level of uric acid. I read the plate at 520 nm (SpectraMax M5, Molecular Devices LLC, Sunnyvale, CA) and compared them to a standard.

Statistical analyses

I combined 2013 and 2014 data for body size measurements and calculation of body condition. I compared male and female body size measurements and mass using the Welch t-test to control for unequal variances

and unequal sample sizes. I determined body condition by regressing body mass on straight-line carapace length to obtain the residuals. I initially analyzed seasonal and sex-related differences of corticosterone, innate immunity, triglycerides, and uric acid for 2013 and 2014 separately using a two-way ANOVA with sex and season as factors. Due to unequal variances and unequal sample sizes, I used a one-way ANOVA with complex comparisons assuming unequal variances to further test any significant seasonal differences. I used a two-way ANOVA with season and year as factors to analyze corticosterone. I used simple effects tests with Games-Howell pairwise comparisons when there was a significant interaction. I adjusted the alpha of 0.05 for complex and pairwise comparisons accordingly.

I analyzed fall 2013 WBC counts using a Welch t-test to compare sexes and a one-way ANOVA to compare age classes. I calculated Pearson Correlation Coefficients for corticosterone, hemolysis titer, triglycerides, uric acid, average hematocrit, and body condition. Also, I conducted Pearson Correlation Coefficients for WBCs, corticosterone, body condition, and hemolysis.

For recaptures that were only captured on two occasions, I tossed a coin to indicate which data point would be used for analyses. For any recaptures that were captured on more than two occasions, I used the RANDBETWEEN function in Microsoft© Excel to randomly select which data point would be used for analyses. I did not include any turtles with undetermined sex in analyses involving sex as a factor. Also, I did not include juveniles (< 180 grams) in

analyses. I transformed all percentages for analysis using the arcsine square root transformation. For all statistical tests, I set alpha to 0.05. I presented data in the text as means ± standard deviations, but figures show the means ± standard errors. I completed all statistical analyses in IBM SPSS Statistics (Armonk, NY).

CHAPTER THREE

RESULTS

Demographic characteristics

Using the program MARK, I estimated the population size of turtles at Nickajack to be 363 turtles (SE = 56.4, 95% CI: 268.1, 491.3). Using the Lincoln-Peterson Index, I estimated population size to be 329 turtles. This equates to 15.45 and 14 turtles per hectare, respectively.

For age class, there were 3 turtles in the 0-4 age class, 16 in the 5-9 age class, 84 in the 10-14 age class, 52 in the 15-19 age class, and 4 in the 20+ age class. Age class structure produced a symmetric (skewness = -1.97) and a mesokurtic (kurtosis = 1.67) distribution, with most of the turtles falling within the middle age class (10-14 years) (Figure 3).

Figure 3. Age class structure of the *T. c. carolina* population at Nickajack.

There were a total of 77 males and 61 females found across the 2013 and 2014 active season which is a sex ratio of 1.26 male:1 female. This is indicative of a male-skewed sex ratio in the Nickajack *T. c. carolina* population.

Body mass did not differ between males and females ($p = 0.747$), but males had a significantly longer straight-line carapace length than did females ($p = 0.005$) (Table 1). There were no significant sex differences in carapace width, carapace height, length of hinge, length anterior to hinge, or length posterior to hinge (Table 1).

Table 1

Descriptive Statistics and t Test Results for Body Size Measurements

	Male	n	Female	n	*t* value	*p* value
Body Mass	383.6 ± 72.1	77	388.3 ± 92.9	59	-0.32	0.747
SLCL	131.4 ± 8.8	74	126.4 ± 10.9	54	2.89	0.005
Carapace Width	99.2 ± 6.1	77	97.8 ± 7.6	59	1.16	0.250
Carapace Height	62.1 ± 3.9	77	63.6 ± 6.0	59	-1.65	0.102
Length of Hinge	69.0 ± 4.5	77	67.6 ± 5.8	58	1.55	0.125
LAH	52.0 ± 4.3	76	50.8 ± 5.9	59	1.25	0.215
LPH	72.3 ± 5.5	76	72.9 ± 7.5	59	0.57	0.572

Note: Shown are means ± standard deviations. Body mass is in grams and all other body size measurements are in millimeters. SLCL = straight-line carapace length, LAH = length anterior to hinge, LPH = length posterior to hinge.

Ranavirus

Of the 102 whole blood samples analyzed for *Ranavirus* infection, one sample (turtle code: LPW) was positive (Figures 4 and 5). This equates to a detected 1% prevalence of the virus in the sampled Nickajack population. This positive sample matched 99% with *Terrapene carolina Ranavirus* viral core protein gene (Figure 6) (Accession No.: U82553) (Mao et al. 1997). Turtle LPW was a 16 year old female turtle found in the fall of 2014 weighing 452 grams. There were no abnormalities or indications of sickness noted at the time of her capture. Turtle LPW had a total WBC count of 283.95 WBCs/10,000 RBCs. Her heterophil count was 94.65 heterophils/10,000 RBCs and percent heterophils

was 33.33%. Her total lymphocyte count was also 94.65 lymphocytes/10,000 RBCs and percent lymphocytes was 33.33%. Her total eosinophil count was 98.29 eosinophils/10,000 RBCs and percent eosinophils was 34.62%. There were no basophils or monocytes found in LPW's WBC count. It seems that turtle LPW's total WBC count was very close to the fall 2013 average of 285.52, while her total heterophils and percent heterophils were both above the average of 78.04 and 24.98%, respectively. Turtle LPW's corticosterone level was 5.86 ng/ml which was close to the average of 5.34 ng/ml for the fall 2014 season. Her hemolysis titer was 4 which was below the average of 5.78 for fall 2014. Turtle LPW's triglyceride level was 58.57 mg/dl which was below the average of fall 2014 females of 167.20 mg/dl, and turtle LPW had a low hematocrit of 7.87% while the average for fall 2014 was 15.66%. Finally, turtle LPW had a body condition residual of 1.54 which was higher than the average of 0.79 for fall 2014 females. The other 101 samples were considered negative for *Ranavirus* because they either did not show a band on a gel or if they did, after sequencing and running a BLAST there were no matches with *Ranavirus* sequences.

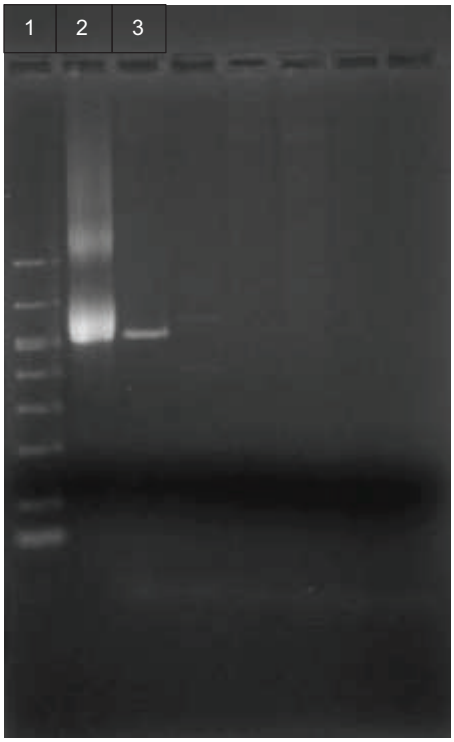

Figure 4. A gel showing the positive band for LPW in lane 3 with positive control in lane 2 and ladder in lane 1 for comparison.

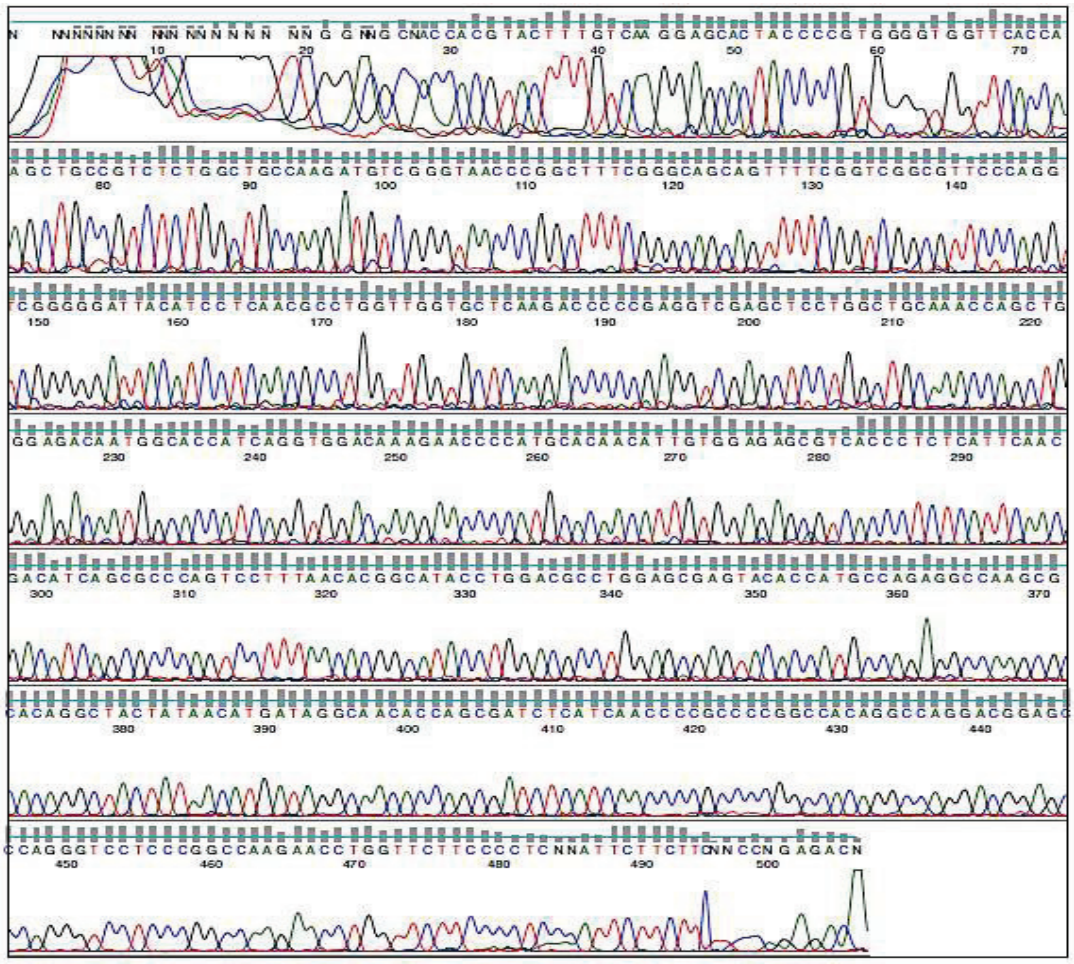

Figure 5. Chromatogram of turtle LPW's sequenced blood sample positive for *Ranavirus*.

Figure 6. Sequence of positive *Ranavirus* sample from turtle LPW aligned with a previously published and sequenced positive *Ranavirus* sample from *Terrapene carolina* (Sequence ID: gb|U82553.1|TCU82553) (Mao et al. 1997). Subject is the turtle from Mao et al. (1997) and query is turtle LPW.

Body condition

Body mass was significantly correlated with straight-line carapace length (R = 0.831, p < 0.001) (Figure 7). I predicted a 6.75 g increase in body mass per 1 mm increase in length. I predicted 69% of the differences in mass based on differences in length (R^2 = 0.69). Body condition residuals were higher in females (0.46 ± 0.96 g) than males (-0.35 ± 0.88 g) (t = -4.95, p < 0.001).

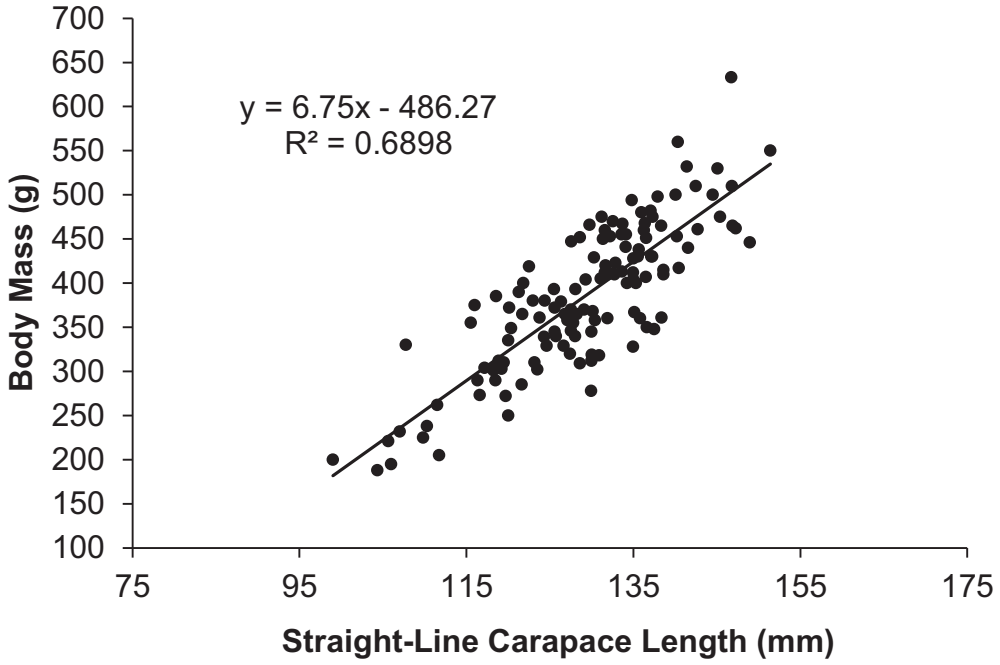

Figure 7. Linear regression of body mass on straight-line carapace length in Eastern Box Turtles, *Terrapene c. carolina* (n = 130).

Physiological measures

The mean ± standard deviation corticosterone level for the 2013 active season was 4.8 ± 4.5 ng/ml. Corticosterone levels differed seasonally ($F = 4.0$, $p = 0.025$). The Dunn/Bonferroni adjustment was used to adjust the familywise alpha of 0.05 to 0.0167 for complex comparisons on season ($\alpha = 0.05/3 = 0.0167$). Summer corticosterone levels (mean = 6.9 ± 5.0 ng/ml) were significantly greater than spring corticosterone levels (mean = 2.5 ± 3.6 ng/ml) based on the complex comparisons ($p = 0.008$) (Figure 8). There was no significant sex difference ($F = 0.9$, $p = 0.351$) or interaction of season and sex ($F = 1.5$, $p = 0.240$).

The mean corticosterone level for the 2014 active season was 2.9 ± 3.2 ng/ml. In 2014, corticosterone levels differed seasonally ($F = 7.0$, $p = 0.002$) with fall corticosterone levels (mean = 5.3 ± 4.6 ng/ml) being significantly greater than spring (mean = 1.8 ± 1.2 ng/ml) and summer (mean = 1.9 ± 1.4 ng/ml) corticosterone levels based on the complex comparisons ($p = 0.003$ and 0.002, respectively) (Figure 9). There was no significant sex difference ($F = 2.1$, $p = 0.151$) or interaction of season and sex ($F = 1.1$, $p = 0.333$).

When observing season and year as factors, corticosterone levels differed both seasonally ($F = 7.9$, $p = 0.001$) and yearly ($F = 7.6$, $p = 0.007$), and there was a significant interaction of season and year ($F = 5.3$, $p = 0.007$). Simple effects tests with Games-Howell pairwise comparisons ($\alpha = 0.05/2 = 0.025$) to assess seasonal differences within each year showed that there were seasonal

differences in 2013 (F = 4.7, p = 0.017), with summer being greater than spring (p = 0.021). In 2014, there were also seasonal differences (F = 5.0, p = 0.013), with fall being greater than spring (p = 0.013) and summer (p = 0.016). For seasonal differences between the years, simple effects tests with Games-Howell comparisons (α = 0.05/3 = 0.0167) were used again, and there was no significant difference in the spring season between 2013 and 2014 (F = 0.6, p = 0.439). However, there was a significant difference in the summer season between the years (F = 14.8, p = 0.001), with corticosterone levels in summer 2013 (mean = 6.9 ± 5.0 ng/ml) being higher than corticosterone levels in summer 2014 (mean = 1.9 ± 1.4 ng/ml). Finally, there was no significant difference of corticosterone levels in the fall season between 2013 and 2014 (F = 0.01, p = 0.928)

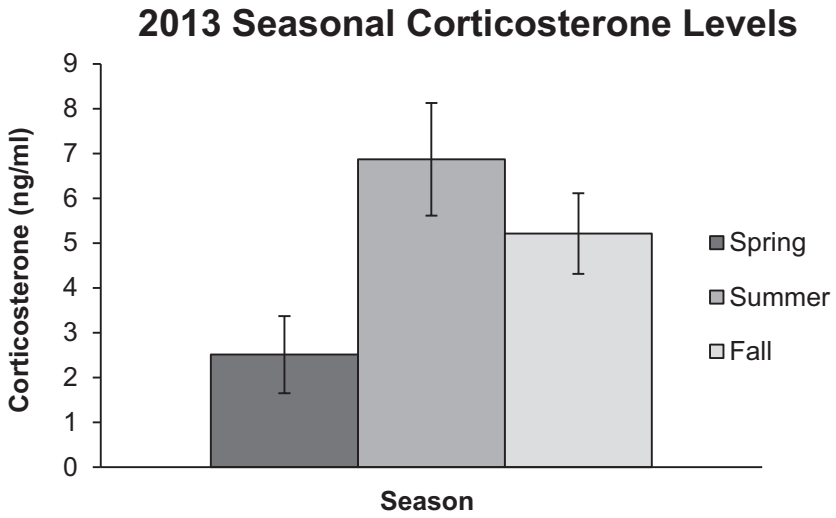

Figure 8. Seasonal corticosterone levels in *T. c. carolina* for the 2013 active season (n's: 18, 16, and 19 for spring, summer, and fall, respectively). Shown are the means and standard errors.

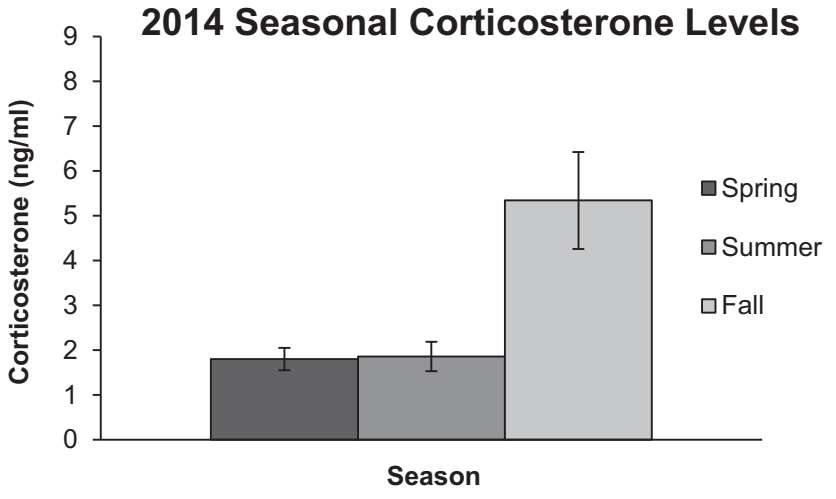

Figure 9. Seasonal corticosterone levels in *T. c. carolina* for the 2014 active season (n's: 22, 17, and 18 for spring, summer, and fall, respectively). Shown are the means and standard errors.

The mean hemolysis titer for the 2013 active season was 5.8 ± 1.2. There were no seasonal (F = 1.7, p = 0.192) or sex related (F = 0.2, p = 0.642) differences, and there was not a significant interaction of season and sex (F = 0.7, p = 0.510) (Figure 10).

The mean hemolysis titer for the 2014 active season was 5.3 ± 1.7. Again, there were no seasonal (F = 1.0, p = .358) or sex related (F = 0.01, p = 0.913) differences, and there was not a significant interaction of season and sex (F = 2.1, p = 0.134) (Figure 11). In addition, hemolysis titer was positively correlated with plasma corticosterone concentrations in both 2013 (R = 0.32, p = 0.031) and 2014 (R = 0.36, p = 0.007) (Figures 12 and 13, Tables 2 and 3).

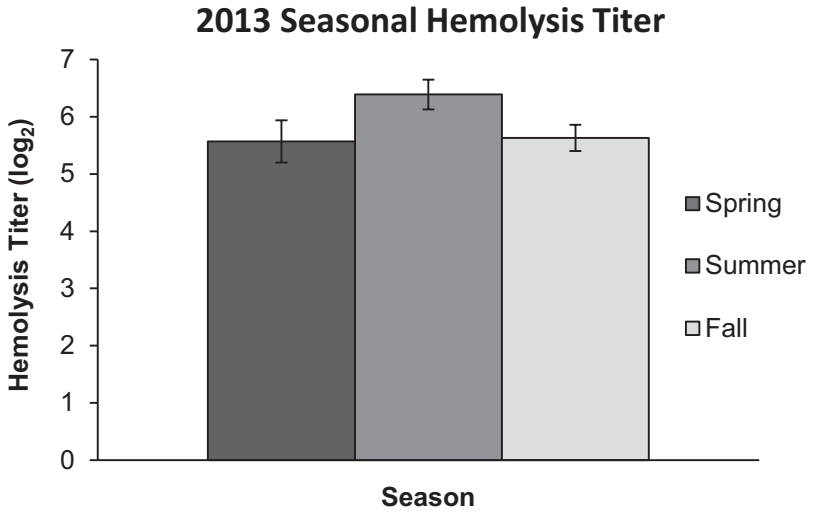

Figure 10. Seasonal hemolysis titer for *T. c. carolina* for the 2013 active season (n's: 15, 14, and 18 for spring, summer, and fall, respectively). Shown are the means and standard errors.

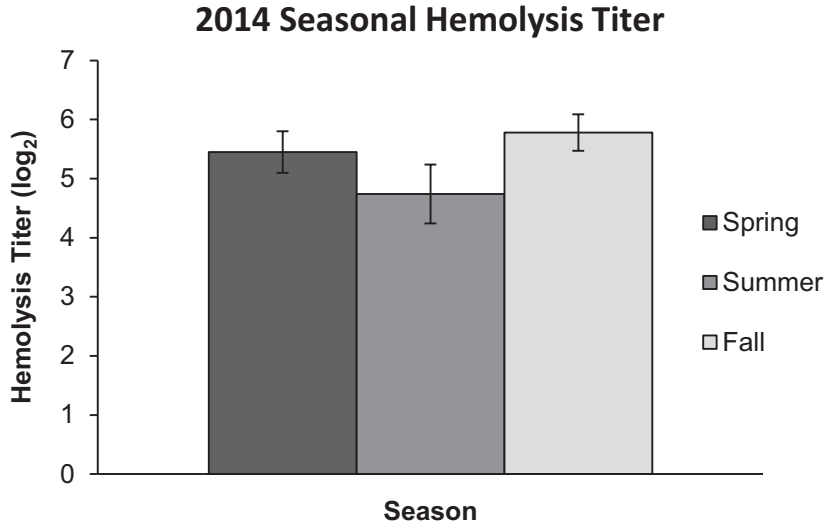

Figure 11. Seasonal hemolysis titer for *T. c. carolina* for the 2014 active season (n's: 22, 17, and 18 for spring, summer, and fall, respectively). Shown are the means and standard errors.

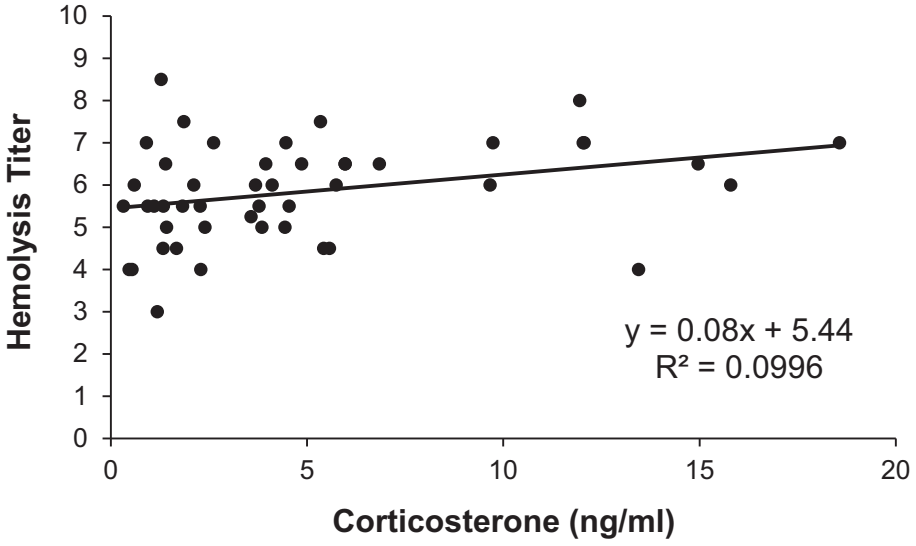

Figure 12. Linear regression of hemolysis titer on corticosterone in Eastern Box Turtles, *Terrapene c. carolina*, in 2013 (n = 47).

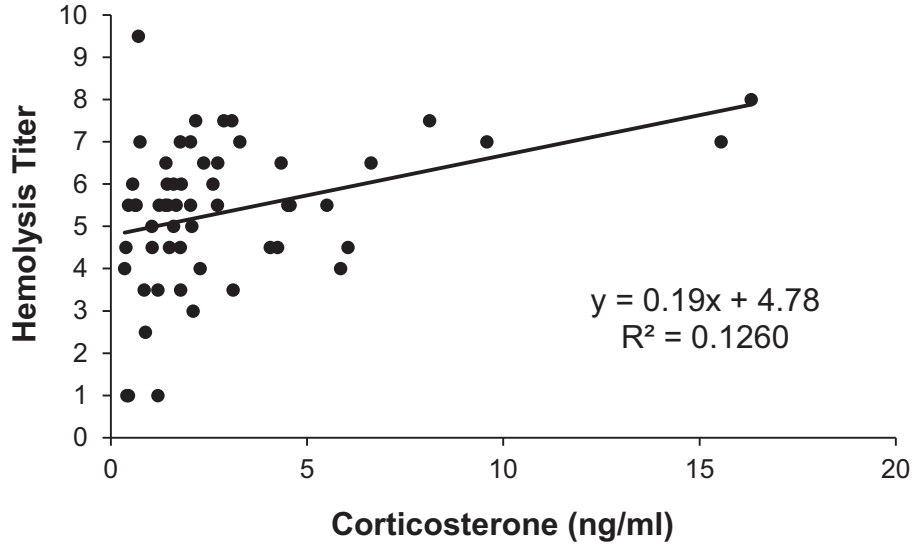

Figure 13. Linear regression of hemolysis titer on corticosterone in Eastern Box Turtles, *Terrapene c. carolina*, in 2014 (n = 57).

The mean number of total WBCs per 10,000 RBCs for fall 2013 was 285.52 ± 176.23. Total WBCs per 10,000 RBCs did not differ between males and females (t = -1.7, p = 0.130). Total WBCs also did not differ by age class (F = 0.5, p = 0.611). The mean number of heterophils per 10,000 RBCs for fall 2013 was 78.04 ± 96.21. Total number of heterophils did not differ by sex (t = -2.2, p = 0.069) or by age class (F = 0.5, p = 0.637). The mean percent heterophils for fall 2013 was 24.98 ± 24.56%. The percent heterophils did not differ by sex (t = -2.0, p = 0.086) or by age class (F = 0.7, p = 0.527). There were no significant correlations of total WBCs per 10,000 RBCs with corticosterone (R = -0.3, p = 0.273), body condition (R = 0.3, p = 0.273), or hemolysis (R = -0.04, p = 0.879), during fall 2013. There were no significant correlations of total heterophils per 10,000 RBCs with corticosterone (R = -0.1, p = 0.790), body condition (R = 0.1, p = 0.731), or hemolysis (R = -0.3, p = 0.237), during fall 2013. Finally, there were no significant correlations of percent heterophils with corticosterone (R = 0.1, p = 0.692), body condition (R = 0.2, p = 0.557), or hemolysis (R = -0.5, p = 0.064), during fall 2013.

The mean triglyceride level for the 2013 active season was 66.5 ± 78.2 mg/dl. There was a seasonal (F = 4.8, p = 0.013) and sex related (F = 11.8, p = 0.001) difference in triglyceride levels with females (mean = 96.7 ± 93.4 mg/dl) having higher levels than males (mean = 44.6 ± 57.2 mg/dl). However, the complex comparisons for season were not significant (spring vs. summer p =

0.108, spring vs. fall $p = 0.208$, summer vs. fall $p = 0.294$). The interaction between season and sex was also not significant ($F = 1.9$, $p = 0.161$) (Figure 14).

The mean triglyceride level for the 2014 active season was 92.4 ± 125.9 mg/dl. There was a significant sex related difference ($F = 12.6$, $p = 0.001$) in triglyceride levels with females (mean = 176.2 ± 166.4 mg/dl) having higher levels than males (mean = 43.6 ± 53.7 mg/dl) (Figure 15). There was not a significant seasonal difference ($F = 1.6$, $p = 0.219$) or interaction ($F = 1.0$, $p = 0.382$). Also, plasma triglyceride levels were positively correlated with body condition in both 2013 ($R = 0.41$, $p = 0.012$) and 2014 ($R = 0.48$, $p = 0.001$) (Figures 16 and 17, Tables 2 and 3).

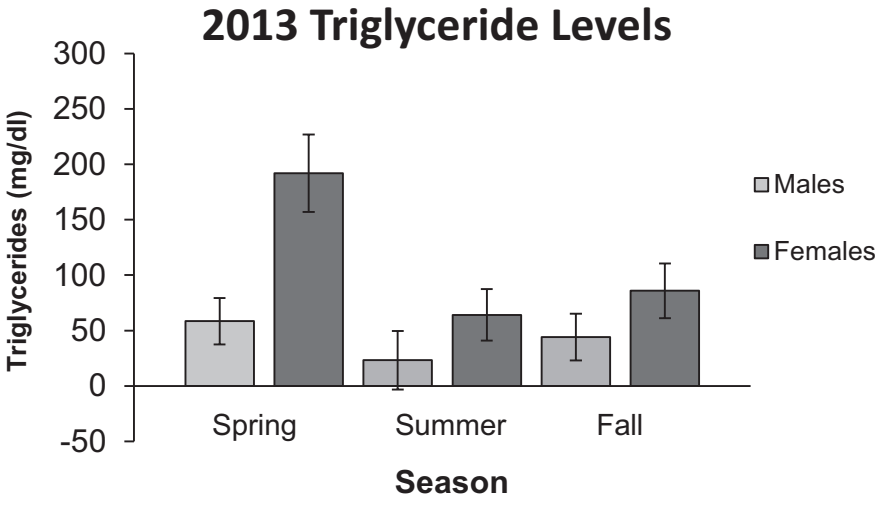

Figure 14. Plasma triglyceride levels in male and female *T. c. carolina* for the 2013 active season (n's: 11, 7, and 11 for spring, summer, and fall males, respectively, n's: 4, 9, and 8 for spring, summer, and fall females, respectively). Shown are the means and standard errors.

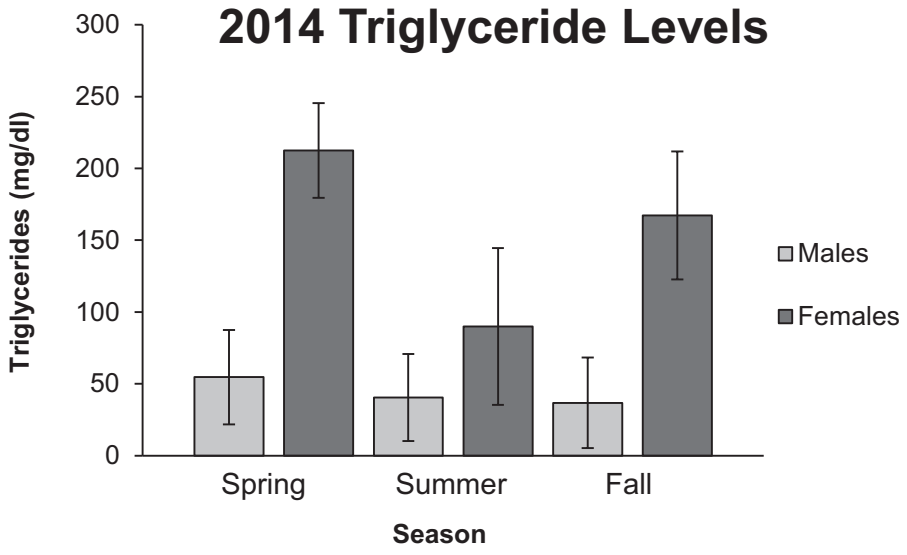

Figure 15. Plasma triglyceride levels in male and *T. c. carolina* for the 2014 active season (n's: 11, 13, and 12 for spring, summer, and fall males, respectively, n's: 11, 4, and 6 for spring, summer, and fall females, respectively). Shown are the means and standard errors.

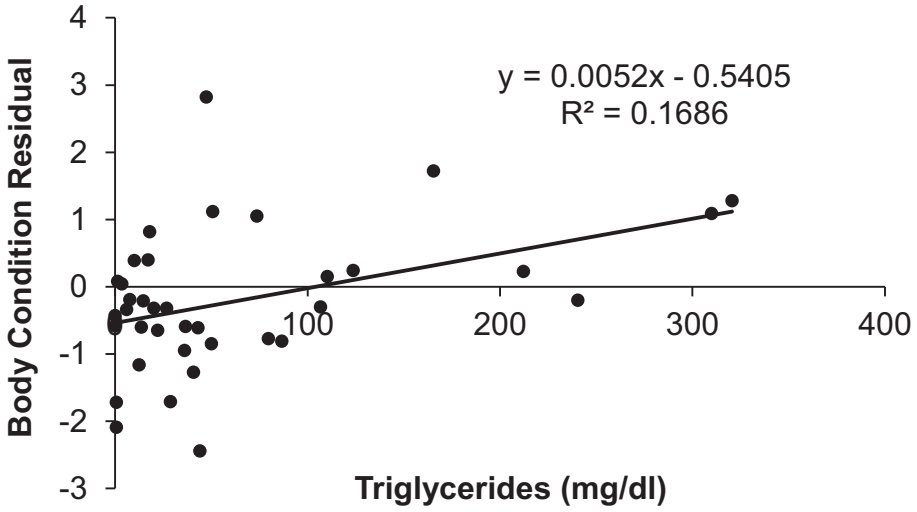

Figure 16. Linear regression of body condition residuals on plasma triglycerides in Eastern Box Turtles, *Terrapene c. carolina*, in 2013 (n = 37).

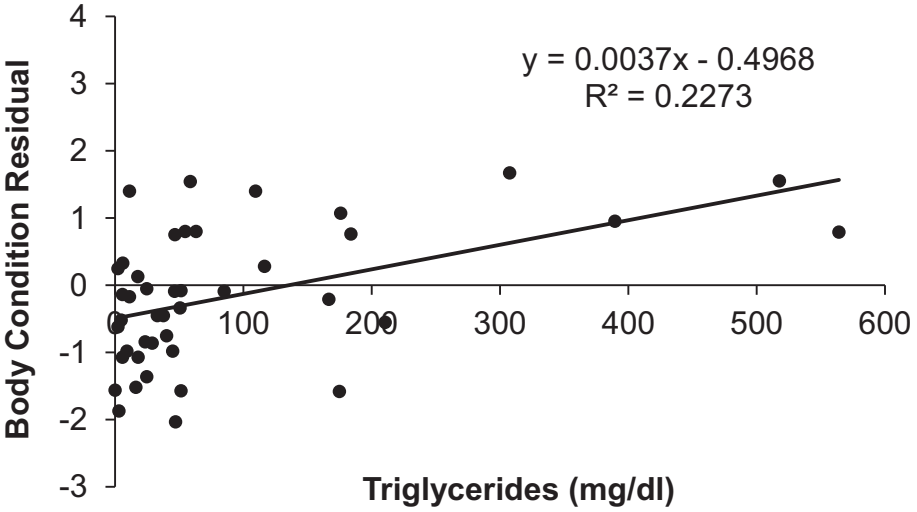

Figure 17. Linear regression of body condition residuals on plasma triglycerides in Eastern Box Turtles, *Terrapene c. carolina*, in 2014 (n = 43).

The mean uric acid level for the 2013 active season was 1.5 ± 1.2 mg/dl. There were no seasonal (F = 0.3, p = 0.766) or sex related (F = 0.2, p = 0.681) differences and there was not a significant interaction of season and sex (F = 0.4, p = 0.659) (Figure 18).

The mean uric acid level for the 2014 active season was 1.6 ± 1.2 mg/dl. There were no seasonal (F = 0.6, p = 0.436) or sex related (F = 0.7, p = 0.877) differences and there was not a significant interaction of season and sex (F = 1.2, p = 0.737) (Figure 19).

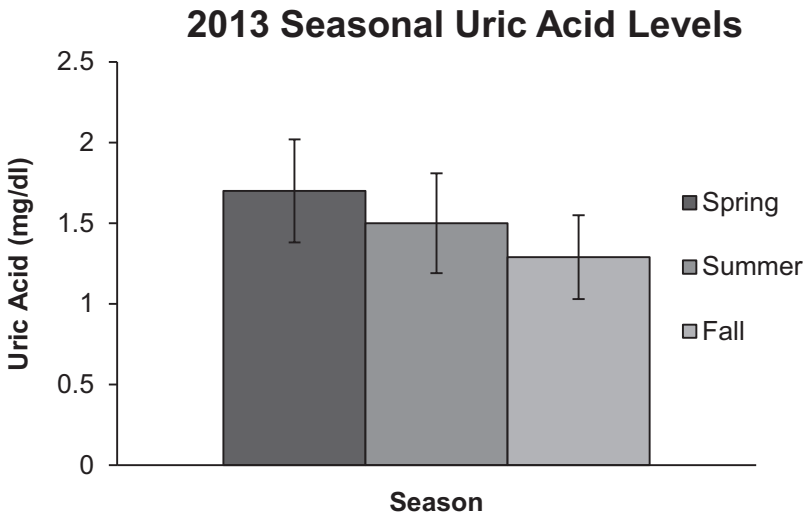

Figure 18. Seasonal plasma uric acid levels in *T. c. carolina* for the 2013 active season (n's: 15, 16, and 18 for spring, summer, and fall, respectively). Shown are the means and standard errors.

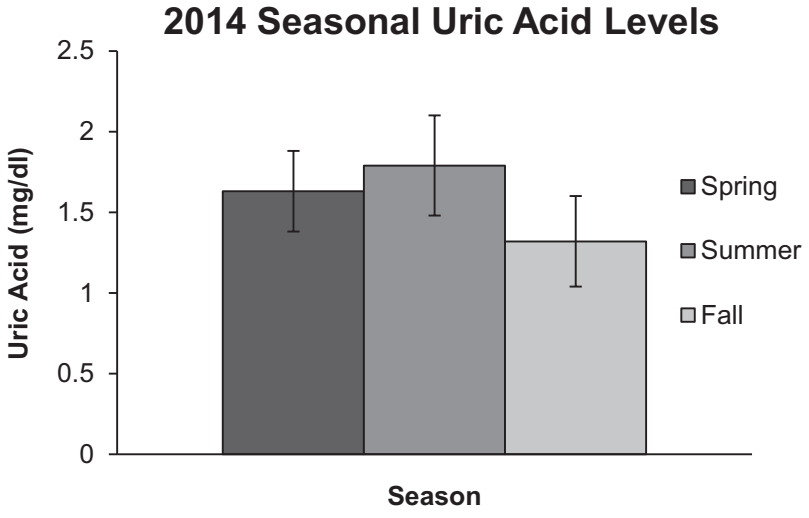

Figure 19. Seasonal plasma uric acid levels in *T. c. carolina* for the 2014 active season (n's: 22, 17, and 18 for spring, summer, and fall, respectively). Shown are the means and standard errors.

Potentially confounding variables

The mean time bled for the 2013 active season was 11:24 AM ± 1 hour and 7 minutes. There were no seasonal ($F = 2.5$, $p = 0.097$) or sex related ($F = 1.5$, $p = 0.222$) differences and there was not a significant interaction of season and sex on the time of day that turtles were bled ($F = 0.4$, $p = 0.699$).

The mean time bled for the 2014 active season was 11:01 AM ± 53 minutes. There was a significant seasonal difference ($F = 4.3$, $p = 0.020$) with spring time bled being at a later time than summer (spring mean = 11:21 AM ± 38 minutes, summer mean = 10:39 AM ± 42 minutes.). The seasonal difference was further verified by complex comparisons ($p < 0.001$). There was no sex related difference ($F = 1.0$, $p = 0.325$) and no significant interaction of season and sex ($F = 1.2$, $p = 0.317$).

The mean time to collect blood for the 2013 active season was 4 minutes and 49 seconds (4:49) ± 2:15. There was a significant sex difference ($F = 4.2$, $p = 0.046$) with males (mean = 5:16 ± 2:24 min) taking longer to bleed than females (mean = 4:08 ± 1:51 min). There was no significant seasonal difference ($F = 2.0$, $p = 0.149$), and there was no interaction of season and sex on the time needed to collect blood samples ($F = 2.6$, $p = 0.082$).

The mean time to bleed for the 2014 active season was 3:10 ± 1:15 min. There was no significant seasonal ($F = 0.5$, $p = 0.626$) or sex-related ($F = 0.2$, $p = 0.658$) differences and there was no significant interaction ($F = 0.1$, $p = 0.876$).

The mean hematocrit level for the 2013 active season was 17.8 ± 9.3%. There was no seasonal (F = 0.1, p = 0.896) or sex-related (F = 0.001, p = 0.973) differences and there was no significant interaction of season and sex on hematocrit (F = 2.8, p = 0.074).

The mean hematocrit level for the 2014 active season was 13.5 ± 8.2%. There was no seasonal (F = 1.1, p = 0.329) or sex-related (F = 0.1, p = 0.806) differences and there was no significant interaction of season and sex on hematocrit in 2014 (F = 0.4, p = 0.699).

In 2013, hematocrit and hemolysis titer were positively correlated (R = 0.55 p < 0.001, Table 2). Hematocrit and uric acid were positively correlated (R = 0.44, p = 0.001, Table 2).

Similarly, in 2014, hematocrit and hemolysis titer were positively correlated (R = 0.61, p < 0.001, Table 3). Hematocrit and uric acid were also positively correlated (R = 0.30, p = 0.024, Table 3).

Table 2

Pearson Correlations among the 2013 Variables for T. c. carolina

	Pearson Correlation Coefficients (*R*)				
	2	3	4	5	6
1. Corticosterone	0.32*	-0.15	0.01	0.22	0.11
2. Hemolysis Titer		-0.02	0.38*	0.55*	0.04
3. Triglycerides			0.19	0.21	0.41*
4. Uric Acid				0.44*	-0.03
5. Avg. Hematocrit					0.22
6. Body Condition					

*$p < .05$

Table 3

Pearson Correlations among the 2014 Variables for T. c. carolina

	Pearson Correlation Coefficients (*R*)				
	2	3	4	5	6
1. Corticosterone	0.36*	0.12	0.14	0.11	-0.10
2. Hemolysis Titer		0.28*	0.31*	0.61*	-0.06
3. Triglycerides			0.28*	0.28*	0.48*
4. Uric Acid				0.30*	0.08
5. Avg. Hematocrit					0.15
6. Body Condition					

*$p < .05$

CHAPTER FOUR

DISCUSSION

Demographic characteristics

Overall, the demographic data collected in this study are consistent with a healthy, growing population of *T. c. carolina* at Nickajack. The population density at Nickajack of 14-15.5 turtles per hectare is comparable to other studies and may be slightly higher than what is normally found. For example, Stickel (1950) estimated a population of approximately 130 individual *T. c. carolina* in her 11.8 ha study site in Maryland (about 11 turtles/ha). Also, Williams (1961) estimated a population density of 10.5 *T. c. carolina* per ha in a study area in Indiana. A study done on a 36.4 ha island in Florida estimated that 544 (approximately 15/ha) Florida Box Turtles (*Terrapene carolina bauri*) resided there (Langtimm et al. 1996), which is quite similar to my study. Williams and Parker (1987) studied a population of *T. c. carolina* in a 72.9 ha Indiana forest over the course of 26 years and found that the population density decreased over time. Their population estimates seemed to be somewhat lower than other study areas and ranged from 162 to 251 individuals over the course of their study with population density dropping from approximately 5 individuals per ha in the 1960s to 3.7/ha in 1970 and 2.7/ha in 1983 (Williams and Parker 1987). It seems the *T. c. carolina* population at Nickajack has a similar population density to *T. c. bauri* found on an island (Langtimm et al. 1996), which makes sense because it is a smaller study

area and is essentially isolated (similar to an island) due to the surrounding suburban housing community.

Age class structure for the turtles at Nickajack had a normal distribution with most of the individuals falling into the middle (10-14 years) age class. However, many other studies have found that most box turtles are 20+ years in age (Budischak et al. 2006, Weiss 2009). It is uncertain why there were so few turtles in the 20+ age class found at Nickajack. However, finding few individuals within the 0-4 age class is not unusual (Weiss 2009), especially because juveniles are often reclusive and hard to find. In addition, Jennings (2007) found that juveniles prefer habitat with more vegetation cover and leaf litter, which makes them even more difficult to find.

It is possible that counting annular rings is not an appropriate measure for age as stated by Wilson et al. (2003). Wilson et al. (2003) found that most authors do not actually test the growth of rings to make certain that it is yearly growth occurring. However, many researchers have used this technique and believe it to be accurate (Ewing 1939, Legler 1960, Stickel and Bunck 1989, Budischak et al. 2006, Weiss 2009), as it is the only way to approximate age of a living turtle. In addition, it is common for “pseudoannual growth zones” to be deposited due to different weather and activity conditions (Ewing 1939, Legler 1960). Perhaps, many previous studies counted these smaller pseudoannual growth rings and considered them as a year of growth which would have caused an overestimation of age. In this study, only the major, prominent growth rings

were counted. However, there may be problems of underestimation as well. This is something that should be explored further, by, e.g., tracking several turtles over a long period of time to analyze their growth and the deposition of growth rings.

The male-skewed sex ratio recorded at Nickajack (1.26 male:1 female) has also been commonly reported in studies of box turtles. For example, Weiss (2009) had a male-skewed sex ratio of 1.19:1 and 1.20:1 in two separate study areas in West Virginia. Dolbeer (1969) found a 1.61:1 male skewed sex ratio in an east Tennessee (Knoxville) population of *T. c. carolina*. However, Stickel (1950) found a 1:1 sex ratio in Maryland. The proposed reason for this male-skewed sex ratio observed at Nickajack is temperature-dependent sex determination (Bull and Vogt 1979). Several other studies have suggested that the male-skewed sex ratio is because male box turtles develop at cooler temperatures (Ewert and Nelson 1991), and the habitats of box turtles are typically in cooler, inner forests (Dodd 1997, Weiss 2009) which would cause more male births in the population.

In addition, Dodd (1997) found a 1.6 male: 1 female sex ratio in a population of Florida Box Turtles, *Terrapene carolina bauri*, and determined that the male-skewed sex ratio did not result from earlier development (males develop earlier) of the smaller sex because males are actually the larger sex. Dodd (1997) also suggested that sampling bias may occur if sampling occurs during certain time frames or in certain microhabitats where one sex may be

more abundant or active than the other. However, in this study, all microhabitats were searched at varying times of the day, so it is unlikely that there was significant sampling bias. Also, Hall et al. (1999) suggested that females must travel and move around a lot to find a proper nesting site which makes them more vulnerable to predation and human-caused deaths such as mowing or vehicular death. Dodd (2001) suggested that a sex ratio is really only indicative of the population at the time it was measured because sex ratios are constantly fluctuating and changing in the population over the years. It would be beneficial to continue to monitor the sex ratio at Nickajack long-term to evaluate any fluctuations or patterns that may occur over time.

Brisbin (1972) and Dodd (1997) found no differences in body mass between the sexes, which is consistent with this study, but sexual size dimorphism has been observed in box turtles (Dodd 2001). In most populations, males are larger than females in terms of shell size (Dodd 2001). In Maryland, Stickel and Bunck (1989) found that male *T. c. carolina* were larger in all shell measurements except height. Likewise, in West Virginia, Weiss (2009) found that males had a longer carapace length whereas females had a taller carapace height. Stickel and Bunck (1989) measured *T. c. carolina* in their Maryland population for nearly 40 years allowing them to calculate growth rates at all different ages. Stickel and Bunck (1989) showed that *T. c. carolina* grow quickly during the first few years of life, but growth slows after maturation and eventually ceases once the turtle is 20+ years old. Clair (1998) mentioned that growth and

maturation both affect sexual size dimorphism. However, Clair (1998) found that male Three-toed Box Turtles (*T. carolina triunguis*) were actually smaller than females, which is inconsistent with other studies. Despite nearly all studies finding both a difference in length and height in *T. c. carolina*, this study only found a significant difference in straight-line carapace length, with males being longer. The most plausible reason for sexual size dimorphism is reproduction; larger males are better equipped to hold on to females and successfully reproduce (Dodd 2001). My results are consistent with other studies on *T. c. carolina*, with males having a longer carapace length than females. A longer term study would help determine the extent of sexual size dimorphism in other characteristics.

Ranavirus

Ranavirus is a disease that has resulted in many epizootic events in amphibians and is increasingly becoming more widespread in reptiles, namely chelonians (Johnson et al. 2008, Allender 2012, Allender et al. 2013, Gray and Miller 2013). Allender (2012) used either an oral swab or a whole blood sample from 540 turtles to test for *Ranavirus* in *T. c. carolina* entering rehabilitation centers in Tennessee, Virginia, Alabama, North Carolina, and Georgia and in *T. c. carolina* found in the wild in Knoxville, TN. Allender (2012) found that eight of the turtles were positive with the virus. Three were female *T. c. carolina* and the other five were of unknown sex (Allender 2012). Four samples were identified as

positive from whole blood samples while the other four were identified as positive based on oral swabs (Allender 2012). Seven of the turtles were from rehabilitation centers in Alabama, Tennessee, and Virginia, while only one free-ranging turtle from Tennessee was found positive (Allender 2012). These findings indicated a 1.5% prevalence of the virus in the 540 sampled turtles, while my study found a 1.0% prevalence of the virus out of 102 sampled turtles. Allender (2012) suggested that low prevalence is linked to severe illness and mortality. Gray and Miller (2013) also stated that *Ranavirus* moves very rapidly and results in a quick death in most cases. Dr. Richard Siegel (pers. comm.) suggested that continual monitoring of a large sample size of the same individuals over subsequent years is imperative, otherwise the disease may be overlooked because of quick die-off. Siegel also suggested that turtle dogs (dogs trained to find turtles) are very beneficial for finding high numbers of individuals, and radio telemetry allows for continual monitoring. However, if a mass die-off event was occurring, it would be easy to see the many dead individuals at Nickajack because it is a fairly small area with a fairly high population density. Although only one turtle was found to be infected with *Ranavirus* at Nickajack, the virus is unfortunately easily spread (Gray and Miller 2013) and can be transmitted through water by amphibians (Johnson et al. 2008, Allender 2012, Gray and Miller 2013, Currylow et al. 2014). It is thus imperative that continual monitoring for the disease occur at Nickajack, especially now that it is confirmed that *Ranavirus* is in the population. If the virus spreads, it could be detrimental to

this closed population of *T. c. carolina* and any other reptiles and amphibians in the population that are susceptible to the virus.

Body Condition

Body condition is a measure of an individuals' energetic status in relation to the status of all other sampled individuals (Schulte-Hostedde et al. 2005). In this study, body condition was measured by regressing body mass on straight-line carapace length and calculating residuals for all turtles from 2013 and 2014 (n = 130). Body mass was significantly correlated with straight-line carapace length, with length increasing as mass increased.

Body condition can be used to estimate the general health of an organism, and several studies have looked at the effect of body condition on corticosterone levels. For example, Jessop et al. (2004a) found that body condition had no effect on corticosterone levels in immature Hawksbill Sea Turtles (*Eretmochelys imbricata*) in Australia. However, Jessop et al. (2004b) found that plasma corticosterone levels increased in male Green Sea Turtles (*Chelonia mydas*) in Australia from pre-migratory periods (August) to the mid to late breeding season (October-November). This seasonal increase in corticosterone occurred at the same time body condition was decreasing due to reproduction (Jessop et al. 2004b). My study found no correlation between corticosterone levels and body condition.

Additionally, Polo-Cavia et al. (2010) found that Western Pond Turtles (*Emys marmorata*) living in a water treatment plant in California had higher body condition indices than turtles on an ecological reserve. However, it was determined that the turtles at the treatment plant were less healthy than those at the ecological reserve based on several physiological factors, regardless of the fact that the turtles at the treatment plant had a higher body condition (Polo-Cavia et al. 2010). Body condition is not a good health indicator when geographic and habitat variation occurs, and other physiological measures should be quantified to get a better understanding of health status (Polo-Cavia et al. 2010). In my study, body condition was positively correlated with triglyceride levels in both 2013 and 2014, most likely indicating higher fat stores in turtles with a higher body condition. In addition, females had higher body condition indices, probably because of the energetic demands of reproduction and egg laying efforts.

Physiological measures

The only study that has assessed corticosterone in *T. c. carolina* was a lab study examining the preferences of *T. c. carolina* for an enriched environment versus a barren environment (Case 2003 and Case et al. 2005). The barren environment consisted of a tank with only newspaper on the bottom, while the enriched environment had mulch and shredded paper as the substrate and a small hideaway hut (Case 2003 and Case et al. 2005). Case (2003) and Case et

al. 2005 found no significant difference in fecal corticosterone levels between those turtles in the barren environment versus those in the enriched environment, but the turtles did show a significant preference for the enriched environment. Fecal corticosterone levels averaged about 212 ng/g (Case 2003).

This study at Nickajack is the first study to examine seasonal, baseline corticosterone levels in free-ranging box turtles, and until recently there were no studies on sex steroid levels of free-ranging box turtles. Currylow et al. (2013) analyzed seasonal sex steroids (estradiol-17β and testosterone) in male and female *T. c. carolina* and found that females had a peak of testosterone in April/May while males had two peaks, one in April and a second in August. In addition, females had a significantly higher estradiol level in July than in any other month (Currylow et al. 2013). Although Currylow et al. (2013) did not measure corticosterone levels, the study is still indicative of seasonal fluctuations in hormone levels, and sex steroids may influence adrenal steroids such as corticosterone (Ott et al. 2000).

Several studies have measured seasonal corticosterone levels in reptiles and found varying results (Tyrell and Cree 1998, Munoz et al. 2000, Ott et al. 2000), but, on the whole, glucocorticoids are usually found to be highest during the breeding season for reptiles, amphibians, and birds (Romero 2002). Ott et al. (2000) measured plasma corticosterone levels in Gopher Tortoises (*Gopherus polyphemus*) in Georgia and found no seasonal (monthly: May through October) variation in male or females. Male corticosterone levels ranged from

approximately 3 to 6 ng/ml, while female corticosterone levels ranged from approximately 3.5 to 9 ng/ml (Ott et al. 2000). However, Munoz et al. (2000) found that serum corticosterone levels did fluctuate with season (monthly) in the Spanish Pond Turtle (*Mauremys caspica leprosa*) in Spain. Levels were highest in March (early spring) at approximately 15 ng/ml, decreased during the breeding season (April-May: spring), and rose to a secondary peak in July (summer) of approximately 9.5 ng/ml (Munoz et al. 2000). These turtles were, however, held in the lab until blood sampling which could have biased their results (Munoz et al. 2000).

Tyrell and Cree (1998) studied wild tuataras (*Sphenodon punctatus*) in New Zealand and found that males had significantly higher plasma corticosterone levels in fall (May: 2.03 ng/ml) than in summer (January: 0.11 ng/ml). Females were looked at during two reproductive states, a nesting year and a non-nesting year, and there was a significant difference between reproductive state and season and a significant interaction of reproductive state and season (Tyrell and Cree 1998). Corticosterone levels were highest in spring (November) for both reproductive states (2.48 and 4.45 ng/ml, respectively) and lowest in the winter (August) for both reproductive states (approximately 0.25 and 0.5 ng/ml, respectively) (Tyrell and Cree 1998). In addition, females had higher corticosterone levels during the nesting year (4.45 ng/ml) than during the non-nesting year (2.48 ng/ml), indicating an increase in corticosterone levels during the reproductive period (Tyrell and Cree 1998).

At Nickajack, in 2013, corticosterone levels were significantly higher in the summer than in the spring but in 2014, corticosterone levels were significantly higher in the fall than in both the spring and summer. It is uncertain why the seasonal profile of corticosterone levels differed between the years. Perhaps, as suggested by the work of Tyrell and Cree (1998), the timing of major reproductive events varied between the years. It is also possible that temperature or rainfall could cause fluctuations in the response of the hypothalamic-pituitary-adrenal axis. However, the seasonal temperatures at Nickajack did not fluctuate very much across the 2013 and 2014 active seasons according to air temperature data recorded on site (Means 2013: spring 23.70º C, summer 25.33º C, fall 21.20º C; Means 2014: spring 24.19º C, summer 23.96º C, fall 21.75º C).

Romero (2002) found that 69% of the reptile species examined so far have exhibited seasonal fluctuations in baseline glucocorticoid levels. Romero (2002) gave three possible hypotheses for the seasonal variation that is commonly observed in glucocorticoids: (1) “energetic mobilization” in which glucocorticoids are expected to be highest in times of high energy demand such as the reproductive season, (2) “behavior” in which glucocorticoids allow for expression of certain behaviors at certain times of the year when glucocorticoid levels are at higher levels, and (3) “preparative” in which glucocorticoid levels help prepare the animal for more stressful situations in the future.

T. c. carolina mate opportunistically (Dodd 2001), which would lead one to believe that if corticosterone levels are associated with mating, then

corticosterone levels would most likely fluctuate differently depending on when most matings occur within a given year. It is possible that this is what is happening in the Nickajack population, causing different seasonal fluctuations across subsequent years. Continual monitoring of seasonal corticosterone levels at Nickajack, along with detailed observation on mating frequency, would be beneficial and would possibly shed light on the observed fluctuations.

Innate immunity was measured via hemolysis of foreign rabbit RBCs by natural antibodies and complement proteins (Matson et al. 2005) in turtle plasma. Overall, there were no seasonal or sex-related differences in hemolysis titer in *T. c. carolina* at Nickajack. This is the only known study to date that has analyzed innate immunity in *T. c. carolina*. Leceta and Zapata (1986) found that captive, control (no immunizations) *Mauremys caspica* (Caspian Turtles) in Spain had an average titer of approximately 4 which is somewhat lower than the turtles at Nickajack. In addition, sheep RBCs were used, and there was no difference between the summer (July) and fall (October) seasons (Leceta and Zapata 1986).

My study also found a positive correlation of hemolysis titer and corticosterone levels in 2013 and 2014. Stress has been known to affect the immune system in reptiles (Munoz et al. 2000, French et al. 2007, French et al. 2009, French et al. 2010). In general, chronic stress is associated with immunosuppression. For example, French et al. (2007) found that female Tree Lizards (*Urosaurus ornatus*) in Arizona subjected to corticosterone implants

showed slower wound healing when fed a restricted diet versus control lizards fed *ad libitum* with a blank (corticosterone-free) implant. Also, French et al. (2010) found that corticosterone levels were increased and complement activity and wound healing were decreased in Galapagos Marine Iguanas (*Amblyrhynchus cristatus*) during the nonbreeding season at tourist sites. Understanding innate immunity in reptiles is an important facet that has received negligible attention and deserves further study (Zimmerman et al. 2010) especially in vulnerable species such as *T. c. carolina*.

In this study, there were no significant effects of sex or age on total WBCs/10,000 RBCs, total heterophils/10,000 RBCs, or percent heterophils. Kakizoe et al. (2007) found an average of 149.59 total WBCs/10,000 RBCs and 71.69 total heterophils/10,000 RBCs in adult female Loggerhead Sea Turtles (*Caretta caretta*) in an aquarium in Japan, while Taylor and Jacobson (1982) found 292.21 total WBCs/10,000 RBCs in Gopher Tortoises (*Gopherus polyphemus*) caught wild but held in captivity in Florida for the duration of their study. In addition, Deem et al. (2009) found median levels to be 173.50 WBC/10,000 RBCs and 70.71 heterophils/10,000 RBCs in wild, foraging *Caretta caretta* on the Georgia coast. It seems that 285.52 total WBCs/10,000 RBCs found in this study is very similar to *Gopherus polyphemus* which is another terrestrial chelonian (Taylor and Jacobson 1982). My results of 78.04 total heterophils/10,000 RBCs are very similar to both studies on *Caretta caretta* (Kakizoe et al. 2007, Deem et al. 2009).

Heatley and Russell (2010) reported that heterophils accounted for 44.3%, of the circulating leukocytes in *T. c. carolina*. This study found an average of 25% heterophils which is somewhat lower. However, percent heterophils seem to vary widely depending on the species. Kakizoe et al. (2007) found that heterophils accounted for 48.22% of circulating leukocytes in adult, female *Caretta caretta*, while Wood and Ebanks (1984) found that heterophils accounted for only 6% of the circulating leukocytes prior to the breeding season (April) in adult Green Sea Turtles (*Chelonia mydas*) from a turtle farm on the Grand Cayman Island of the British West Indies. In addition, there were no significant correlations of WBCs with corticosterone, body condition, or hemolysis in fall 2013. Allender (2012) suggested that complete blood cell counts could possibly be indicative of *Ranavirus*. For example, Allender (2012) found that *T. c. carolina* infected with *Ranavirus* had fewer lymphocytes than uninfected turtles. Infected box turtles actually had no lymphocytes, and it is suggested that it is possible that all of the lymphocytes were in transit to the location of the virus (Allender 2012). No other WBCs differed in infected and uninfected individuals (Allender 2012). Lymphocytes were not measured for fall 2013 in the current study, but the single turtle (LPW) that tested positive for *Ranavirus* did seem to have greater than average number of circulating heterophils (94.65/10,000 RBCs). Additional research is needed to determine the validity of this finding.

Lipids, including triglycerides, are used as energy reserves, and Derickson (1976) proposed four patterns of lipid cycling: 1) no cycling, 2) lipids stored and

used for hibernation, 3) lipids stored and used for reproduction, and 4) lipids stored and used for both hibernation and reproduction. Derickson (1976) also stated that lipid storage and cycling is completely dependent on food availability, which is likely to cause seasonal fluctuation in lipid levels depending on abundance and accessibility of food. A study on the Stinkpot Turtle (*Sternotherus odoratus*) in Alabama found that female turtles had higher lipid levels than male turtles in all months except December and February (McPherson and Marion 1982). Female turtles had the highest lipid levels in fall (September: approximately 700 mg/dl) which was most likely linked to reproductive requirements (McPherson and Marion 1982). The mean lipid level for males was 175 mg/dl and was highest during January/February at about 275 mg/dl (McPherson and Marion 1982). In the female Painted Turtle (*Chrysemys picta*) triglyceride levels were highest in early spring (March to April: 211.6 mg/dl) during pre-ovulation and peaked again in late fall just before the redevelopment of the ovaries (November: 176.7 mg/dl), indicating a strong connection of triglyceride levels with reproduction and vitellogenesis (Duggan et al. 2001). Ballinger et al. (1992) found that the Red-Chinned Lizard (*Sceloporus undulatus erythrocheilus*) in Colorado has the highest percentage of triglycerides in fat bodies in May during the early reproductive season (88.29%) and the lowest in August after egg deposition (66.32%). It seems certain that seasonal variability in fat cycling does occur in many reptile species and is usually associated with reproduction and/or winter dormancy. This study at Nickajack found triglyceride

levels to be highest in females across both years, but there was no significant seasonal variation. However, although not significant, levels were highest in spring in males and females across both years, which is indicative of possible seasonal cycling, and can probably be attributed to mobilization of lipid stores following emergence from hibernacula in preparation for mating. The higher levels in females across both years can most likely be accredited to reproductive demands of mating and nesting. Because of the opportunistic mating nature of *T. c. carolina*, it is possible that there is no seasonal variability because there is no set reproductive period.

Uric acid, the major nitrogenous waste product in terrestrial turtles, constitutes 60% of excreted nitrogenous waste and can be used to assess nutritional status (Moyle 1949). Few studies have reported mean uric acid levels in *T. c. carolina*, but those that do found the average to be 1.75 and 2 mg/dl, respectively (Hutton and Goodnight 1957, Dessauer 1970). My study found means of 1.5 and 1.6 for 2013 and 2014, respectively, which is somewhat lower than what other studies have reported. Kimble and Williams (2012) reported seasonal uric acid levels in *T. c. carolina* in Indiana with medians of 0.75 for spring (shortly after emergence), 0.55 for summer (mid-summer), and 0.50 for fall (just before hibernation). These values are somewhat lower than what my study found, but the seasonal measuring times were very similar, and there was no significant seasonal difference (Kimble and Williams 2012). Rangel-Mendoza et al. (2009) found that captive Central American River Turtles (*Dermatemys mawii*)

in Mexico at a “turtle farm” had significantly higher plasma uric acid levels during the dry season (April-May) than the wet season (September-October) with median levels of 6.2 mg/dl and 1.5 mg/dl, respectively. The higher uric acid level could be due to a change in the condition of the water between dry and rainy seasons or because of improper nutrition or disease in the farmed turtle population (Rangel-Mendoza et al. 2009). It seems reasonable that uric acid levels vary seasonally in some turtle species, but many co-occurring conditions such as nutritional status and environment may cause variation as well.\

Potentially confounding variables

Time that blood was drawn, time it took to collect blood, and hematocrit did not affect any variables in this study based on supplemental ANCOVAs (not reported). Blood was drawn between 8:20 AM and 2:30 PM in this study in order to control for daily changes in hormone levels. A major concern of this study was the possible influence of time to bleed on stress hormone levels and of hematocrit on physiological measures such as triglycerides, uric acid, and hemolysis. For example, a very low hematocrit could be caused by hemodilution; this could result in artificially low uric acid levels. Ott et al. (2000) found that there was no effect of time it took to collect blood on corticosterone levels in *Gopherus polyphemus*, with the longest blood draw lasting 24 minutes. Romero and Reed (2005) found that corticosterone levels in 6 species of birds can be considered baseline if collected in 2 minutes, and at 3 minutes is still considered close, if not

equal, to baseline. Cash et al. (1997) found that there was no considerable change in corticosterone levels drawn within the first ten minutes of capturing Red-Eared Slider Turtles (*Trachemys scripta elegans*) in Mississippi, but after ten minutes there was a noticeable rise in corticosterone levels. The mean time to collect blood for this study was 4 minutes and 49 seconds in 2013 and 3 minutes and 10 seconds in 2014. The longer time to collect blood in 2013 may somewhat account for the higher corticosterone levels in 2013, but I do not believe it is completely responsible for the higher levels. The safe cut-off time for drawing blood can be variable for different taxa, but I believe time to collect blood was not a confounding factor in corticosterone levels in this study based on supplemental ANCOVA results.

Allender (2012) found packed cell volume, or hematocrit, to be 23% and 27% in 2010 and 2011, respectively, in *T. c. carolina*. Kimble and Williams (2012) found levels closer to this study with a spring median of 22%, a summer median of 16%, and a fall median of 14%, and a significant decrease in levels across seasons. The mean percent hematocrit for this study was 17.79% in 2013 and 13.50% in 2014 which is probably somewhat low due to lymph dilution, but close to the levels of *T. c. carolina* in Kimble and Williams' (2012) study. However, I found no seasonal differences, which is also what Kimble and Williams (2012) found. Hematocrit was correlated with hemolysis and uric acid and may have influenced the values of these two physiological tests. However, it seems unlikely that it greatly affected the results because it is believed that dilution was

somewhat equal across the seasons and years and would cancel out any negative effects on the overall results. It may have simply lowered values. Perfection of extracting blood with no lymph dilution would be beneficial.

The demographic data seem to indicate a healthy, growing population of turtles at Nickajack. The one *Ranavirus* positive turtle at Nickajack makes it imperative that continual monitoring occur in the population. It is also important to continue to monitor physiological conditions, including hormone levels of the turtles at Nickajack. In conclusion, the basic demographic characteristics, information on infection status, and physiological measures reported herein can be used to monitor the health of the Nickajack population, and can be used as baseline levels for monitoring other box turtle populations as well.

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