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Maternal Effects and Offspring Behavior: Potential Contributors to the Lack of Recruitment in Mississippi Gopher Tortoises (*Gopherus polyphemus*)

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MATERNAL EFFECTS AND OFFSPRING BEHAVIOR:

POTENTIAL CONTRIBUTORS TO THE LACK OF RECRUITMENT

IN MISSISSIPPI GOPHER TORTOISES (*GOPHERUS POLYPHEMUS*)

by

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ABSTRACT

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Federally threatened in Mississippi, gopher tortoise *(Gopherus polyphemus)* populations within the state have strongly variable recruitment and are generally in decline. Hatching success is significantly lower in Mississippi than in any other part of the species' range, and most hatchlings die within the first year. There are few refuges where survival and hatching success is high. Here I compare two populations that differ in recruitment and offspring survival for differences in corticosterone. Corticosterone is a hormone that influences energy availability and is released in elevated levels during stressful events, like living in poor quality habitat. Prolonged corticosterone elevation can impede growth and immune responses and result in early death. To assess adult stress, I utilized leukocyte profiles as they are influenced by prolonged elevation of corticosterone. A viable yolk sampling technique was used to collect yolk samples to determine levels of yolk CORT. Hatchlings were then kept in captivity for two years to observe growth rates and burrowing behavior before being released and monitored via radiotelemetry to monitor dispersal and survival. I found no differences in adult stress or egg yolk CORT between populations. This suggests adults are not excessively stressed. Survival of hatchlings did not differ between sites. Captive-reared hatchlings were larger and may have better survival than similar aged wild hatchlings. Overall, the

poor survival of gopher tortoises may be more strongly linked to environmental variables such as soil structure than physiological ones.

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TABLE OF CONTENTS

LIST OF TABLES

Table

LIST OF ILLUSTRATIONS

Figure

CHAPTER I

GENERAL INTRODUCTION

Before Europeans settled the Southeastern United States, over 90 million acres of longleaf pine (*Pinus palustris*) savanna covered the Southeastern Coastal Plain (Frost 1993). These savannas are characterized by sparse longleaf pine distribution with a diverse herbaceous understory that generally lacks in woody vegetation (Bartram 1791); this fairly open savanna was maintained by frequent, low intensity fires, most often triggered by lightning strikes that promoted longleaf pine establishment while suppressing understory hardwood and shrub growth. Native American tribes also encouraged fire not only as a means to hunt game, but they also used it as a tool to open areas for crops, promote growth of seed and fruit bearing plants, and create habitat for game (Fagan 1991; Fowler and Konopik 2007). As European settlers became established, they may have continued the practice of using fire for preventing woody vegetation encroachment for agricultural and hunting purposes (Pyne 1982). It was not until the 20th century that fire suppression became a common practice, resulting in longleaf pine forests with a dense understory of shrubs and hardwoods. As a result of this fire suppression, coupled with agriculture and development, only three million of the original 90 million acres of longleaf pine savanna remains in the Southeastern Coastal Plain (Frost 1993). Loss of habitat and change in vegetative composition has had broad and severe impacts on the native animal species dependent on longleaf pine habitats such as red-cockaded woodpeckers (*Leuconotopicus borealis*; Conner et al. 2001), gopher frogs (*Lithobates*

capito; Humphries & Sisson 2012), and the gopher tortoise (*Gopherus polyphemus*; Lohoefener and Lohmeier 1981; Auffenburg and Franz 1982).

The gopher tortoise is described as a keystone species known to have over 360 commensal species that rely on them and the burrows they produce for survival. Included among these are multiple species of conservation concern such as the dusky gopher frog (*Lithobates sevosus*; Richter et al. 2001), gopher frog (*Lithobates capito*; Roznik and Johnson 2009), indigo snake (*Drymarchon corais*; Diemer and Speake 1983), Florida mouse (*Podomys floridanus*; Jones and Franz 1990), and the threatened burrowing owl (*Athene cunicularia floridana*; Jackson and Milstrey 1989). There are also several invertebrate species only associated with gopher tortoises such as the gopher tortoise onthophagus beetle (*Onthophagus polyphemi polyphemi*; Guthrie and De Long 1977), a dung beetle found only in and around tortoise burrows and the tortoise shell moth (*Ceratophaga vicinella*; Deyrup et al. 2005), a highly endangered moth whose larval food source is the keratin from dead gopher tortoise shells.

The historic range of the gopher tortoise mirrors much of the historic longleaf pine savanna and is also greatly reduced; the typically open understory of longleaf pine savanna is crucial for thermoregulatory behavior in tortoises, and the herbaceous groundcover is important for foraging (Auffenberg and Franz 1982). Thus, the loss of longleaf pine savanna through fire suppression, agriculture, and urban development has negatively affected tortoise populations by decreasing forage availability, reducing basking opportunities, and displacing individuals from habitats with suitable soils for burrowing (burrows being an important aspect of gopher tortoise ecology). The loss of

habitat and resultant population reductions in gopher tortoises has led to the development of management strategies for this species that have varied in their successes (Berry and Aresco 2014). For the remnant populations in their reduced habitats, the impacts of surviving in marginal habitats on adult physiology, behavior, and reproductive success, and offspring survival and behavior are not fully understood. Understanding more of the mechanisms driving variation in recruitment and survival can lead to more effective management strategies to ensure population growth and stability in the gopher tortoise.

To facilitate research on aspects of physiology and behavior that may impact population stability and recruitment, this study will focus on two gopher tortoise populations in Mississippi that differ in juvenile recruitment; one located at Hillsdale, Mississippi and the other at Camp Shelby Joint Forces Training Center (hereafter Camp Shelby) in Hattiesburg, Mississippi. More specifically, differences in stress hormone physiology, hatching success, and hatchling behavior will be compared between these two areas.

The tortoises at Camp Shelby are located within Training Area 44 (T44), a "gopher tortoise refuge" consisting of a mature longleaf pine stand that is around 750 acres in size. The site is managed by the United States Forest Service (USFS) and is on a two year prescribed burn cycle to maintain an open longleaf pine savannah suitable for gopher tortoises. The refuge is open to the public, and the USFS has only recently begun to restrict vehicle access. The soils are classified as sandy loam to loam (Benndale-Smithdale complex, USDA Web Soil Survey) that due to the clay content can become compacted, leading to limited gas exchange (Brady and Weil 2008) and likely making

burrow excavation more difficult and energetically demanding compared to sandy soils. The tortoise population at T44 is typical of many in Mississippi, consisting of mature individuals with limited recruitment and poor hatching success which ranges from 8 to 43% of eggs hatching (Epperson et al. 2003; Noel et al. 2012) under natural conditions, and a maximum of 58.8% from artificial incubation under ideal conditions (Noel et al. 2012). These hatching success rates are lower than the 67 to 97% hatching success reported in the eastern portion of this species range (e.g., Alabama, Florida, and Georgia; Landers et al., 1980; Smith, 1995; Butler and Hull, 1996).

While the majority of Mississippi gopher tortoise populations are exhibiting recruitment problems, there are a few sites in the state that do exhibit stronger recruitment. The Hillsdale, MS population mostly inhabits a failed subdivision located on well-drained, sandy soil (Troup Sand, USDA Web Soil Survey) with a mix of longleaf pine, sand live oak (*Quercus geminata*), and turkey oak (*Quercus laevis*) that has experienced past fire activity as indicated by fire scarring on many of the trees (A. Holbrook, pers. obs.). The soils limit shrub encroachment, resulting in habitat consisting of an open canopy with sparse herbaceous ground cover and exposed sandy soil. The Hillsdale tortoise population consists of mature, sub-adult, and juvenile tortoises with the latter appearing most abundant based on burrow occurrence (D. Gaillard, A. Holbrook, M. Hinderliter, T. Mann, and T. Radzio pers. obs.); this would suggest Hillsdale, MS has a healthy, viable population. No studies of hatching success in the wild or in captivity have been completed for Hillsdale, MS and it is a relatively unstudied site.

Clearly, one very important difference between Camp Shelby and Hillsdale is the difference in soil structure, but it may not be the only factor impacting differences in these two populations. For example, if soil characteristics alone were driving the recruitment disparity observed, one could predict no differences in hatching success and hatchling characteristics when eggs are collected and incubated in identical conditions, and offspring raised in identical conditions. However, if population, physiological variation, genetics and/or maternal inputs are influencing recruitment and behavior, one would predict higher hatching success, larger hatchlings, and different behaviors from clutches collected from the high recruitment, and potentially higher-quality environment (genetic/physical environment) of Hillsdale, when individuals from both sites are raised in identical conditions. In this work, reproductive and hatchling characteristics will be compared in captive reared individuals from the two sites as a method to assess whether aspects of physiology are a potential factor in the recruitment differences observed in these two areas. From this assessment, outcomes will potentially allow for a greater determination of the many factors impacting recruitment, and provide insight into the aberrant behaviors that have been observed in hatchlings at the T44 site (use of pallet burrows or no burrowing at all by juveniles; M. Hinderliter pers. obs.).

One mechanism whereby variation in physiology could impact reproductive success and recruitment is through alteration in environmental stressors and individual responses to those stressors. Stressors are typically defined as any unpredictable occurrence that moves an individual out of homeostatic norms and can be events such as storms, predator presence, or habitat alterations (Wingfield et al. 1998, Sapolsky et al.

2000). Corticosterone (CORT) is one of the primary hormones responsible for mitigating the response to stress. It does so by preparing the body for a response to stressful situations through the release of energy stores in the form of glycogen, and conserving energy by temporarily suppressing non-vital functions such as digestion, reproduction, and immune responses. CORT also increases awareness and memory to better cope with future stressors or avoid them altogether (Sapolsky et al. 2000, Thaker et al. 2009). Once the stressful event ceases, suppressed functions return to pre-stress levels without measureable negative effects (Wingfield et al. 1998, Romero et al. 2009). The hormone CORT is dual purpose; baseline levels (frequently termed 'non-stressed' levels), are required to regulate energy for everyday function and maintenance (e.g., regulation of glucose levels between foraging events), while elevated levels allow individuals to deal with more serious situations (Wingfield et al. 1998).

Long term, or chronic stress (e.g., destruction of habitat) leads to prolonged elevation of CORT and can result in prolonged suppression of immune responses, reduced reproductive output and altered neurological function (see reviews in Wingfield et al. 1998, Sapolsky et al. 2000, Romero et al. 2009), leading to increased risk of bacterial, viral, and parasitic infections, reproductive failure, decreased memory, and altered behavior (e.g., increased stimuli needed to trigger a future fight or flight response; Meylan & Clobert 2004). The increased energy demand of the body, as energy needs are elevated in an attempt to return to homeostasis, depletes glycogen and fat during prolonged stress. Once fat reserves are depleted, muscle tissue is metabolized for energy, consequently increasing the chance of death through irreversible cardiac muscle damage

(Romero et al. 2009). Additionally, elevated CORT in females can be transferred to developing eggs and embryos which can drive a variety of physiological and behavioral changes in young (Groothuis et al. 2005). For example, prolonged stress responses (e.g., elevated CORT) in adults can have genetic and epigenetic impacts that can alter the hypothalamic-pituitary-adrenal (HPA; set of inter-connected tissues that control CORT production) axis in offspring (Rogers et al. 2013). Altered stress responses in offspring may lead to individuals which are then unable to properly respond to their environment due to a dampened stress response and altered behaviors such as decreased predator response times (*Lacerta vivipara*; Meylan and Clobert 2004) and increased philopatry (*Lacerta vivpara;* de Fraipont et al. 2000). In this work, one potential mechanism for the reduced survival and recruitment of juveniles in marginal habitats may be stress communicated to them from their parents via elevated adult CORT, this may reduce egg hatchability, juvenile size, and body condition (*Lacerta vivipara*; Meylan et al. 2010).

As a method for investigating whether physiological stress is a part of the differences observed in recruitment noted at the two Mississippi sites under study (Hillsdale and T44), CORT levels will be assessed along with differences in egg and hatchling physical characteristics across the two sites. Potential impacts of stress on offspring will be measured via CORT levels in eggs that young hatch from and via immune function in adults (proxy for prolonged elevation of CORT) as a potential mechanism driving poor hatching success and the maladaptive behavior that has been observed in hatchling gopher tortoises (M. Hinderliter, pers. obs.). By conducting this research, it is my intention to better understand the mechanisms driving poor recruitment

in gopher tortoises and provide information that could be used to administer even more effective management decisions. To facilitate the goal of understanding the impacts of stress physiology on recruitment and behavior the following studies were completed and will be detailed in full through this document:

1. Adult tortoises at high and low recruitment sites were captured and had blood samples collected to assess long term CORT elevation (e.g., elevated stress) via leukocyte profiling. Eggs collected from these same sites underwent viable yolk sampling to determine if yolk CORT varied among clutches and sites.
2. Burrowing behavior and survival of captive reared hatchlings was assessed following release of individuals back at their respective sites of origin.

Together these assessments and observations have yielded some insights into the potential for the hormones associated with stress/energy physiology to impact survival and behavior in hatchling tortoises. More work will be needed in the future as the full picture of what is driving variation in recruitment is not completely clear. From this work it appears that environmental quality has a strong impact on survival and recruitment, but the impacts of physiology are not completely irrelevant and should be considered as well.

CHAPTER II

LONG TERM STRESS IN ADULT GOPHER TORTOISES (*GOPHERUS POLYPHEMUS*) AND ITS POTENTIAL COMMUNICATION TO OFFSPRING VIA EGG YOLK CORTICOSTERONE.

Introduction

Stress can be defined as a specific response by the body to a stimulus, such as fear or pain, which disturbs or interferes with the normal physiological equilibrium of an organism (Chrousos 2009). Corticosterone (CORT) is one of the primary hormones responsible for mitigating the stress response and prepares the body for a reaction to stressful situations by releasing energy stores in the form of glucose (both fat and lean muscle mass can be accessed by CORT for glucose production; McEwen and Wingfield 2003). Corticosterone also conserves energy by temporarily suppressing non-vital functions while increasing awareness and memory regarding the stress event (Sapolsky et al. 2000, Thaker et al. 2009). Stressors are typically short term and once the stress event ceases, suppressed functions resume to pre-stress levels without measureable negative effects (Wingfield et al. 1998).

According to the reactive scope model (Romero et al. 2009), long term stressors (e.g., compromised habitat) can lead to a decreased threshold between reactive homeostasis, the range in which a stress event can be mediated without irreversible damage to the organism, and homeostatic overload, levels of stress at which the organism incurs irreparable damage. As a long term stressor continues and the homeostatic overload threshold continues to drop, it will eventually reach a point where even

everyday activities will keep the organism in homeostatic overload (Romero et al. 2009). Once this occurs, there is a rapid decline in health, and homeostatic failure occurs resulting in death. Long term, or chronic stress, can result in prolonged suppression of immune responses; as demonstrated in rats (*Rattus norvegicus*), using restraint and/or shaking as a stressor (Dhabhar and McEwen 1997). Chronic stress can also inhibit reproduction by lowering testosterone in males (e.g., restraint stress in *Urosaurus ornatus*; Moore et al. 1991) and decreasing reproductive success in females through loss of hatchlings (e.g., simulated predator stress in *Sturnus vulgaris*; Cyr and Romero 2007). According to Chrousos (1998), chronic stress resulting in the long term release of corticotropic-releasing hormone (CRH) can trigger the locus coeruleus norepinephrine (LC-NE) system to inhibit digestive function by impeding the secretion of gastric acid and emptying (decreased gastric motility) via vagus nerve suppression, while increasing colonic motility through stimulation of the sacral parasympathetic system. Ultimately, prolonged suppression of the above physiological functions could lead to an increased risk of bacterial, viral, and parasitic infections, reproductive failure, reduced body condition, and endanger future survival. The increased energy demands of the body as it attempts to return to homeostasis depletes glycogen and fat during prolonged stress events; once fat reserves are depleted and muscle is metabolized for energy (Romero et al. 2009). In term of behaviors associated with stress, prolonged stress in adult females (e.g., long term CORT exposure in the common lizard *Lacerta vivipara*) can lead to a need for increased stimuli in order to trigger a future 'fight or flight' response (Meylan and Clobert 2004) which can negatively impact an individual's ability to avoid predators.

In addition to assisting the individual in dealing with the increased energy demands often associated with adverse conditions, CORT has a roll to play in general, daily energy regulation as the animal moves through daily activities and seasonal behavioral/physiological changes (Wingfield et al. 1998). Interestingly, baseline CORT and ability to elevate CORT during stress events interact and often in unexpected ways. There are seasonal fluctuations in baseline CORT levels that are tied to predictable increased energy demands such as seasonal weather changes and reproduction. Within these periods of higher general energy demand, the threshold for triggering a stress response is often reduced and exposure to perceived stressors that increase plasma CORT are more likely to push the organism into a state of chronic stress (Romero et al. 2009). Many environmental and social variables can increase plasma baseline CORT and individual responses to these can vary. For example, (1) poor nutritional resources and decline in body condition (Holberton et al., 1996; Kitayski et al., 1999; Love et al., 2005), (2) poor quality habitat (Marra and Holberton, 1998; Suorsa et al., 2003; Kitayski et al., 2006), (3) exposure to severe weather conditions (Romero et al., 2000; Breuner and Hahn, 2003), (4) increased predation risk (Boonstra et al., 1998; Cockrem and Silverin, 2002; Clinchy et al., 2004), (5) exposure to human disturbance (Fowler, 1999; Creel et al., 2002; Müllner et al., 2004; Walker et al., 2005; Lucas et al., 2006; Pereira et al., 2006) and (6) exposure to socially dominant conspecifics (Creel et al., 1996; DeVries et al., 2003; Goymann and Wingfield, 2004) have all been shown to lead to CORT increases. Suorsa et al. (2003) demonstrated in the Eurasian treecreeper (*Certhia familiaris*) that habitat characteristics, stem density and food availability in this case, can

induce physiological stress and may result in poorer body condition in offspring (lower mass). As for gopher tortoises, the shrubby understory resulting from fire suppression in longleaf savannas not only reduces thermoregulatory opportunities, but inhibits herbaceous plant growth needed for forage and may induce stress responses and/or increased baseline CORT. By existing in sub-optimal conditions adults may be experiencing elevated CORT levels which may have impacts not only on adults but on the offspring they produce, a better understanding of stress physiology in this species can lead to a more complete understanding of the reasons for their decline and help foster their successful recovery.

One concern in gopher tortoises (an egg laying vertebrate) is could elevated levels of CORT in females (e.g., individuals unable to thermoregulate or forage successfully) lead to increased CORT deposition into the yolk of eggs? CORT is a steroid and hence it easily passes cell membranes and collects in the lipid-rich yolk (Ward and Weisz, 1984; Hayward and Wingfield, 2004; Saino et al., 2005; Love and Williams, 2008). Maternal hormones have both organizational and activational effects on offspring behavior and physiology and can communicate maternally-experienced environmental conditions to offspring, which can pre-adapt offspring to maximize fitness (Groothuis et al. 2005). For example, during yolk deposition, maternal CORT accumulates in the yolk and in low levels could indicate good maternal health and low environmental stressors, resulting in offspring that are more likely to disperse, reducing local competition. Elevated maternal CORT can be a sign of declining maternal health, and offspring philopatry would ensure replacement of maternal genes in the local population (see, de Fraipont et al. 2000). High

maternal CORT can have negative physiological effects on offspring (de Fraipont et al. 2000; Hayward and Wingfield 2004; Love and Williams 2008; Saino et al. 2005), including decreased body condition and increased mortality at birth (Cadby et al. 2010), decreased offspring dispersal (Vercken et al. 2007), decreased growth rates (Hayward et al. 2005, 2006), altered anti-predator behavior (Robert et al. 2009) and increased stimuli needed for an escape response by juveniles (Meylan and Clobert 2004). Together these negative, CORT-based maternal effects can lead to lower offspring survival, potentially altered recruitment of young, and if this is occurring in populations of the gopher tortoise it could be an additional factor in its reduced population status in Mississippi.

Taking habitat characteristics of the two sites under study (T44 and Hillsdale) into account, it is possible that there are differences in the stress physiology of adult tortoises in these populations due to variation in necessary environmental variables (i.e., tortoises at T44, where burrowing is potentially more difficult, may have higher CORT to counteract energetic demands of burrow development and maintenance when compared to Hillsdale individuals). This difference in stress could be communicated to offspring, ultimately accounting for some of suspected differences in recruitment at the two sites; elevated CORT in eggs might reduce egg hatchability or alter offspring behavior in some manner. I propose that when compared to the population with greater recruitment at Hillsdale, the sampled adult tortoises from T44 will exhibit higher ratios of heterophils to lymphocytes (H:L; assessment of blood cell types which serves as a proxy measure for prolonged stress responses, see Methods here; Davis et al. 2008), indicating higher CORT in the T44 population. The same technique was used to assess stress in Florida

populations of gopher tortoises infected with upper respiratory tract disease (URTDs) resulting in a mean H:L ratio of 0.52 (McCoy et al. 2005). If, between the two sites, there are significant differences in long term stress among adults as indicated by H:L ratios, I would predict that yolk CORT will follow a similar pattern with higher levels seen in the T44 population. Endogenous egg CORT will be measured in clutches from both sites to determine if levels are higher in either habitat. This increased yolk CORT exposure may then have a negative impact on hatching success.

Materials and Methods

Study Sites. Behavioral observations and assessment of hatchling and juvenile gopher tortoises completed at T44 (Figure 1), a gopher tortoise refuge located within Camp Shelby, have revealed a population with low hatching success (Epperson and Heise 2003; Noel et al. 2012), low offspring dispersal, low growth rates, and poor burrowing behavior (all hatchling characteristics M. Hinderliter, J. Lee, and A. Holbrook pers. obs.) in juveniles. Poor burrowing behavior may increase mortality via exposure to temperature extremes, increased likelihood of predation, and exposure to fire. I propose that the reduced burrowing behavior, low dispersal of juveniles, and the reduced hatching success could be impacted by maternally derived CORT introduced via yolk deposition and that the adults of T44 show evidence of prolonged stress responses and/or elevated baseline CORT. The T44 adult tortoise population characterizes many populations within Mississippi; aging populations that lack juveniles and sub-adults for replacement (M Hinderliter pers. obs.; Epperson et al. unpubl. data), so most of the reproductive effort may be carried out by older individuals potentially stressed by the increased energetic

demand to maintain homeostasis when exposed to poor quality habitat. For refuge restoration and maintenance purposes, T44 is burned every two to three years which has promoted an open understory with diverse herbaceous ground cover (A. Holbrook pers. obs.). However, soils (Benndale-Smithdale complex, USDA Web Soil Survey) at T44 are at best "suitable" (McDearman 2005) for tortoises due to the high clay content, possibly making it more difficult to excavate burrows and potentially reducing gas exchange (Brady and Weil 2008) for buried egg clutches.

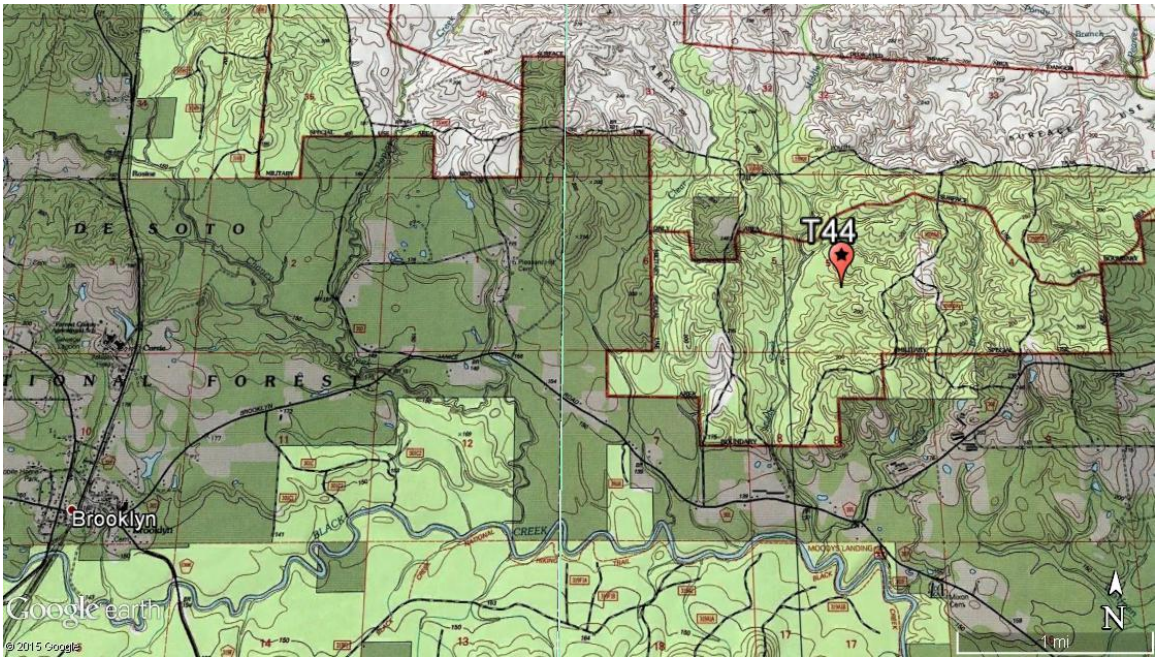

Figure 1. Training area 44 (T44) located within the Camp Shelby Joint Forces Training Center, Hattiesburg, Mississippi.

In contrast, a gopher tortoise population found at Hillsdale, MS (Figure 2) is one of a few sites in the state that exhibits recruitment as hatchling, juvenile, sub-adult, and adults are all present (D. Gaillard, M. Hinderliter, A. Holbrook, and T. Mann pers. obs). Hillsdale has deep, sandy soils (Troup Sand, USDA Web Soil Survey) and is categorized

as “priority” soil for gopher tortoise management purposes (McDearman 2005). The lack of clay requires little effort for burrowing and may provide better drainage and gas exchange (Brady and Weil 2008) for developing clutches of eggs. While burning is infrequent at Hillsdale (A. Holbrook pers. obs.), the soil encourages establishment of drought tolerant plant species that are slow-growing due to the soil being xeric, low in organic material, and low in nutrients (USDA Web Soil Survey). The slow growth may result in slower accumulation of fuels to carry fire, which could also result in a slower rate of woody encroachment.

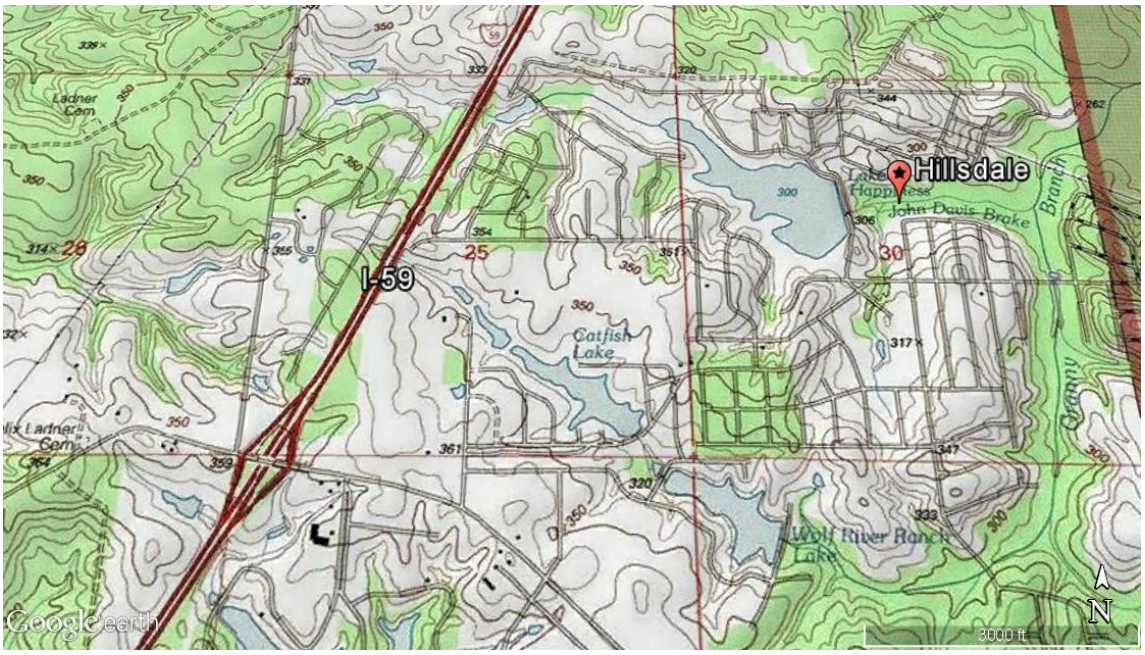

Figure 2. The Hillsdale, Mississippi research area, the majority of which is an abandoned housing development from the 1970’s.

Sampling techniques. From 15 May -10 June 2013, adult tortoises were trapped for blood sampling at T44 and Hillsdale using trapping and handling techniques approved by the University of Southern Mississippi’s Institutional Animal Care and Use Committee (protocol #09051403, Mississippi Department of Wildlife Fisheries and Parks

permits 2010-2013). Modified Tomahawk® live traps were placed in front of the burrow entrance and covered with vegetation to provide shade for captured tortoises. Traps were opened and closed daily and while they were open, were checked every two hours. Blood smears for H:L ratios were made from blood collected from a brachial vein with a 25 gauge needle and syringe, smears were allowed to air dry prior to staining.

Leukocyte profiles. A typical method of assessing long term stress in tortoises is to set traps and obtain a blood sample within 5 minutes of the tortoise being removed from the trap to determine baseline CORT (Ott et al. 2000). However, under normal trapping regimes for tortoises, traps must be left undisturbed allowing the animal to come out of its burrow and tortoises are typically in the trap for more than 30 minutes (two hours between trap checks; Ott et al. 2000) before a blood sample is drawn. Gopher tortoises can elicit a stress response in less than 30 minutes, making trapping animals for accurate baselines very difficult. Other potential confounding factors influencing CORT are the perceived barrier the trap creates in front of the burrow and previous trap experience. To remove the potential influence of trap stress affecting CORT results (e.g., ability to get true baselines), we opted to use leukocyte profiling to assess long term stress (as in Davis et al. 2008). When reptiles are exposed to a stressor, or when glucocorticoids are administered experimentally, there is a long-term decrease in lymphocytes and an increase in heterophils (Davis et al. 2008; Dhabhar et al. 1996). The resulting heterophil to lymphocyte (H:L) ratios can then be used to quantify long-term stress, with higher values indicating greater long-term stress (Davis et al. 2008; Dhabhar et al. 1996). Leukocyte profiles do not begin to change for hours or even days after

exposure to the stressor with endotherms being the quickest to show variation (birds over one hour; Davis 2005; mammals over two hours; Burguez et al. 1983) and ectotherms varying based on body temperature and species (alligator over 4 hours with no mention of temperature, Lance and Elsey 1999). I therefore use H:L measures as a proxy for the indication that an individual has been under prolonged elevated stress. This will permit comparison between the two populations assessed here to determine if stress profiles differ.

Collected blood smears were subjected to a staining process using a Sigma-Aldrich® periodic acid-Schiff (PAS) staining system (#395B-1KT). This system includes four solutions used in a series of steps and consists of a formalin-ethanol fixative, a periodic acid solution that stains cytoplasm and oxidizes carbohydrates in tissues, this allows for binding of the third solution, Schiff's reagent, which binds to aldehydes. The fourth solution is hematoxylin solution which stains cell nuclei. Following kit instructions, slides were stained and allowed to air dry before being assessed with oil immersion. Under oil immersion at 1000x and using a manual cell counter, cell counts and leukocyte profiles were determined by members of Dr. Jennifer Owen's Avian Health and Disease Ecology Lab at Michigan State University.

Yolk corticosterone. From 15 May to 30 June 2010 daily nest searches for newly laid clutches were completed at Hillsdale and T44 to ensure eggs were collected within 24 hours of oviposition; searches consisted of excavation of adult burrow aprons. During excavation and prior to transport, the top of exposed eggs were marked with graphite pencil so that they could be kept in the same orientation in which they were found; this

guards against embryo damage following attachment of extraembryonic membranes onto the inside of egg shells (Limpus et al. 1979). Once marked, eggs were removed and kept in individual containers with moistened vermiculite, covered with plastic wrap, and placed in a foam padded cooler for protection from heat and to reduce vibration during travel. Eggs were then transported to the University of Southern Mississippi where egg mass as well as minimum and maximum diameters were recorded. Next, viable yolk sampling was performed on all collected eggs, drawing from two techniques to better suit gopher tortoise eggs and incubation (Bowden et al. 2001; Lipar and Ketterson 2000). To remove any possibility of disturbing the developing embryo, the site of needle insertion was positioned 90 degrees from the mark added during egg collection, indicating the top of the egg as positioned in the nest and where embryonic development occurs. Before needle insertion, the withdrawal site was thoroughly cleaned with a 10% Betadine® solution (Purdue Frederick, Stamford. CT). Next, a 25-gauge winged infusion set (Terumo Medical Corporation, Somerset, NJ) was attached to a 1ml tuberculin syringe (Becton Dickinson, Franklin Lakes, NJ), and a mark was placed on the tubing 13mm above the needle, which equates to 10 µl of yolk. To penetrate the eggshell, the needle was rotated back and forth while applying gentle pressure until the needle pushed through the egg shell. The needle was then pushed towards the center of the egg and the depth of the needle was kept consistent between subsequent yolk draws. Once the needle was in position, yolk was then drawn to the 13mm mark by applying gentle suction with the syringe. Once the sample was collected the needle was gently removed and the hole covered with New Skin® liquid bandage (Prestige Brands, Tarrytown, NY). Yolk

samples were placed into a 1.5ml Eppendorf tube, syringes were then cleared of remaining yolk by rinsing them with 0.5ml of distilled water which was added to the Eppendorf tube containing the main yolk sample for each egg. Finally, two 3mm glass beads (Thermo Fisher Scientific, Waltham, MA) were added to the Eppendorf tube, and tubes vortexed until yolk was homogenized, and then frozen until assays. After yolk collection, eggs were then placed in individual containers containing vermiculite that was sterilized and dried before being rehydrated with distilled water at a rate of 0.7 g water per 1.0 g of vermiculite (Packard et al. 1987). Since the sex of the gopher tortoise is determined by incubation temperature, eggs were incubated at 29.3°C, the pivotal temperature for a 50:50 sex ratio (Demuth 2001).

The yolk samples were analyzed using a competitive binding radioimmunoassay developed by Wingfield and Farner (1975) and modified by Schwabl (1993). Approximately 2,000 cpm of H^3-CORT (PerkinElmer) were added to each yolk sample so that recovery percentages could be calculated after extraction and chromatography had been performed. The tritiated yolk samples were then extracted with a (30:70) solution of petroleum and diethyl ether and treated with 95% ethanol to precipitate excess proteins and lipids. Extracts were then evaporated and re-dissolved in 10% ethyl acetate in isooctane before being added into the chromatography columns containing Celite, ethylene glycol, and propylene glycol in the upper portion and a mixture of Celite and water in the lower portion of the column. Corticosterone was then eluted by adding 45% ethyl acetate in isooctane to the columns and the resulting solution was collected for the

assay. Egg samples were randomly assigned to one of three assays to quantify yolk CORT.

The concentration of yolk CORT was measured using a competitive binding radioimmunoassay with tritiated CORT and anti-CORT antibody (Fitzgerald Industries International) and compared to a standard curve that was constructed from a serially diluted standard CORT solution. Intra-assay variation was determined from multiple, randomly placed standards of known CORT concentration throughout the assays. Intra-assay variation (variation among standards within a single assay that occurs due to uncontrollable issues such as variation in antibody binding mechanics) was 11%, only one assay was performed and there is no inter-assay variation to report.

Unfortunately, after the first assay, we were forced to use a different supplier for antibody and the preferred Celite became unavailable allowing us to only complete one assay successfully. The changes in assay components resulted in decreased recovery rates and undetectable levels of CORT for all samples in the remaining two assays, and only results from the first assay will be reported on.

Statistical analyses. To determine the effects of population and sex on adult tortoise H:L ratios, a two factor, factorial ANOVA was used with population and sex as factors. A nested ANOVA, with clutches of eggs as nested blocks, was used to test for population differences in yolk CORT. This nested design was used to avoid pseudoreplication due to non-independence of multiple eggs from an individual clutch, and so that variation among clutches could be quantified and accounted for. Eggs are typically yolked simultaneously in turtles (Congdon and Tinkle 1982) and this may lead

to similar yolk CORT levels among the eggs within single clutches. Data was analyzed using SAS JMP 11 (SAS, Cary, North Carolina).

Results

Leukocyte profiles as an indicator of long term stress. From 15 May through June 10, 2013 blood samples were collected from a total of 30 adult tortoises at Hillsdale (n = 13) and T44 (n = 17). The H:L ratios for Hillsdale (both sexes: $\bar{x}$ = 1.15, SD = 0.87; Figure 3. Females: $\bar{x}$ = 1.50, SD = 1.10, n = 7; males: $\bar{x}$ = 0.73, SD = 0.31, n = 6; Figure 4) and T44 (both sexes: $\bar{x}$ = 2.018, SD = 1.309; Figure 3. Females: $\bar{x}$ = 1.703, SD = 0.98, n = 8; males: $\bar{x}$ = 2.33, SD = 1.57, n = 8; Figure 4). Population consisted of two levels (Hillsdale and T44) and sex included two levels as well (male and female). None of the factors had a significant effect on H:L ratios and there were no interactions (Table 1). One female outlier from T44 was not included in the analyses as her H:L ratio of 13.07 may indicate illness or error.

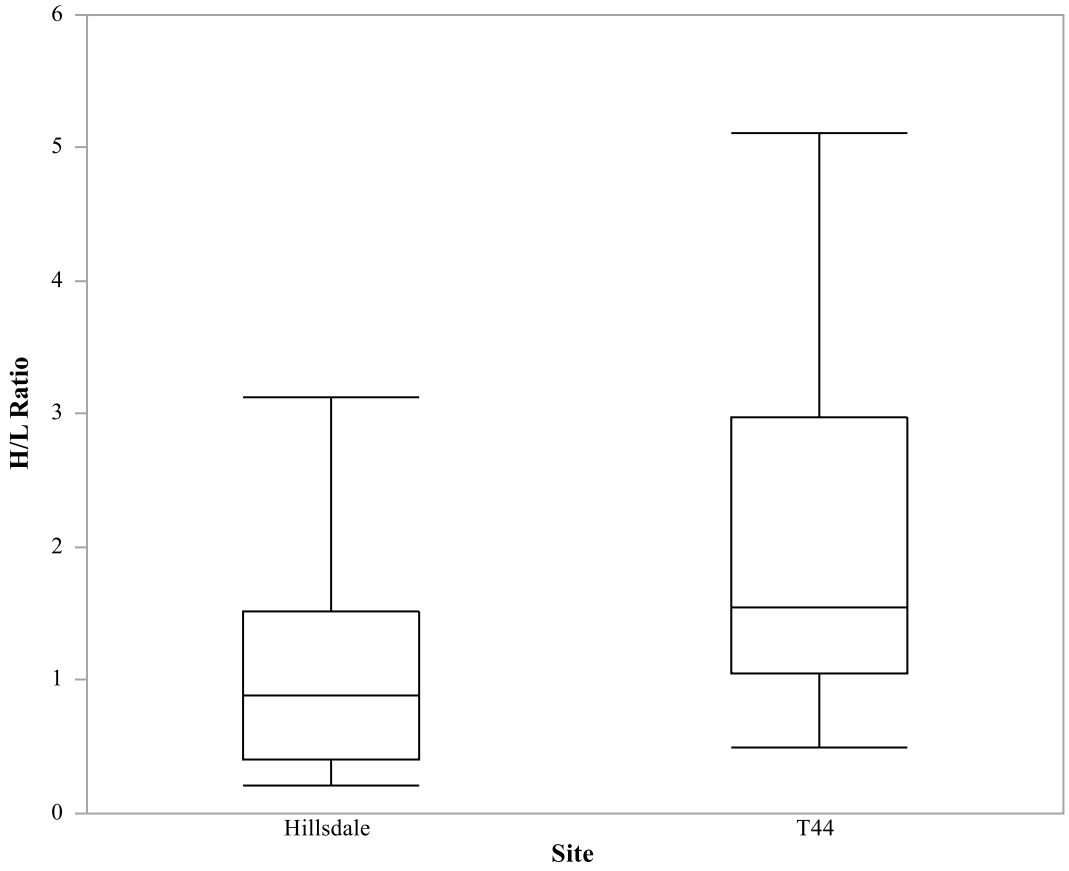

Figure 3. A boxplot comparison of heterophil/lymphocyte ratios between Hillsdale and T44 gopher tortoises (sexes combined). Whiskers represent minimum and maximum values, top and bottom of the box first and third quartiles, and the middle line represents the median.

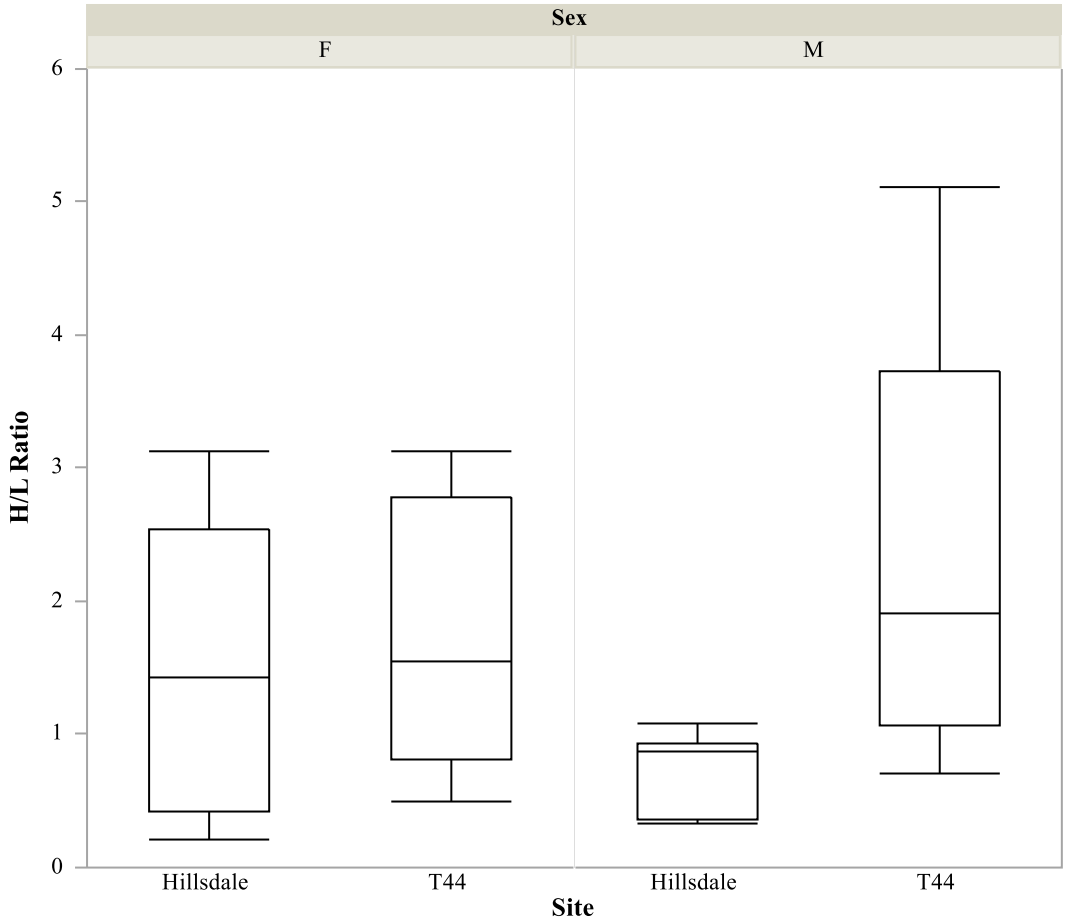

Figure 4. Gopher tortoise heterophil/lymphocyte ratios at Hillsdale and T44 separated by sex. Whiskers represent minimum and maximum values, top and bottom of the box first and third quartiles, and the middle line represents the median.

Table 1

Effects of population and sex on H:L ratios using a two factor, factorial ANOVA with population and sex as factors.

ANOVA	F-ratio	P-value
Population	F1,26=3.06	0.092
Sex	F1,26=0.640	0.431
Population * Sex	F1,26=0.006	0.938

Yolk corticosterone. Yolk samples were collected from a total of 92 eggs from 18 clutches in 2010, however changes in assay component availability only allowed one assay to be completed (see Methods above). The yolk CORT assay had samples from 31 eggs; 18 eggs from six clutches that came from Hillsdale and 13 eggs from seven clutches collected from T44. Mean yolk CORT for all of the eggs in the assay was 97.35 ng/ml (range 39.84 – 179.66 ng/ml), with a T44 mean of 96.57 ng/ml (range 60.55 – 179.66) and Hillsdale 97.91 ng/ml (range 39.84 – 166.86). Taking clutch into account, I found no differences in yolk CORT between T44 and Hillsdale (Table 2).

Table 2

Yolk CORT comparison between T44 and Hillsdale using a nested ANOVA with site as the fixed factor and clutch as a nested block.

ANOVA	F-ratio	P-value
Site	F1,12.51=0.085	0.775
Clutch[nested]	F11,18=1.82	0.125

Discussion

Using H:L ratios as a means to quantify long term stress in adults, I found no significant differences between the Hillsdale and T44 populations or among the sexes when the populations were pooled. Additionally, assessment of egg yolk CORT also found no significant differences between the populations in this parameter. These nonsignificant results could be an artifact of small sample sizes and high individual variation. With larger sample sizes, assessing aspects of behavior, physiology, reproduction, or condition in relation to individual H:L ratios and egg yolk CORT may yield interesting results in both adults and the offspring they produce. While no significant differences between the sites were found there are still some interesting outcomes to be noted.

Female H:L ratios. Female tortoises at the two sites exhibited less variation in their H:L ratios than the males (see Figure 4), and they were very similar to each other. Inhibition of the stress response in females during reproduction has been documented in

marine turtles (*Carretta carretta*, *Chelonia mydas*, and *Eretmochelys imbricata*; Jessop et al. 2001, 2004). This inhibition may be a strategy to maximize reproductive output where reproductive opportunities are limited due to competition for nesting areas or temporal constraints as is seen in arctic birds, which have a very limited temporal window for reproduction (Wingfield et al. 1998). The suppression of the stress response ensures that maximum energy is being diverted to reproductive effort. Future studies of female hypothalamic-pituitary-adrenal (HPA) axis feedback function and assessment of H:L ratios at times outside of egg production would be of great interest and may help clarify why females between the two sites are so similar while males sampled at the same time exhibit greater variability.

Egg Yolk CORT. There were no differences in yolk CORT found between the two populations and this could be linked to the evidence that females are dampening their stress responses during egg production. While the lack of differences in egg yolk CORT between T44 and Hillsdale suggest that egg CORT concentration may not be a factor in the different recruitment suggested between sites, there are a number of other potential levels where the CORT found in egg yolks could impact individual physiology and behavior of developing tortoises. Genetics of the developing animal (HPA axis activity is heritable, Hayward et al. 2005; impacts of genetic sex on CORT organization effects, Hayward et al. 2006), glucocorticoid receptor number in target tissues, their density or activity (Shahbazi et al. 2011), concentration of yolk CORT compared to yolk volume (injection studies in birds, Saino et al. 2005, Rubolini et al. 2005, Hayward et al. 2006), and the speed with which the yolk is consumed (egg incubation rates vary between 70-

120 days, faster developing embryos could consume yolk at a higher rate than slower developing individuals; Rostal and Wibbels 2014) could all have impacts on the full development of individual hatchling behavior or physiology. While eggs within a clutch from a single female were similar in CORT levels, they were not 100% identical and these variations could also have impact. How and if CORT impacts behavioral development in this species still remains an open question; at this point, I can say that there does not appear to be any significant difference between sites in yolk CORT levels or in measures of experienced stress in adult females.

Male H:L Ratios. While this investigation was focused on the potential for maternal effects on offspring behavioral development, the leukocyte profiles of males showed more variability when compared to females and warrant some discussion. Given the differences observed between the populations in described soil structure, distribution of burrows, and individuals, it could be predicted that males at T44 might be more stressed; soils appear more difficult to burrow in and burrows are clumped in small groupings throughout the landscape at T44, making individual interactions more likely to occur. The lack of significant differences may be due to the strong individual variability in H:L ratios observed in T44 males and the overlap in H:L ratios between sites (Figure 4). Additional sampling at a variety of time points coupled with behavioral observations and perhaps additional physiological measures may help resolve this confusing outcome regarding stress in male gopher tortoises.

Determining whether males are more or less stressed in one population is needed as stress in males can have important impacts on the offspring they produce. Paternal

influence was once thought to be restricted to DNA/genes passed in sperm. Clearly this does have an effect on offspring, but epigenetic effects passed from males may also have a strong influence on offspring development and behavior. Recent research in mice (Rogers et al. 2013) demonstrated that stressed males fathered offspring who ultimately had dampened stress responses. It was discovered that the stressed adult males (fathers) had higher amounts of microRNA in their sperm, suggesting that epigenetic reprogramming could have a direct effect on the HPA axis. Offspring born with a dampened stress response can have difficulty making appropriate responses in a changing environment and altered psychological and behavioral responses to stressors. While the current findings do not support a direct impact of adult or yolk CORT on gopher tortoises, stress as experienced by adults and passed on to offspring as epigenetic changes impacting offspring stress response management or behavior, cannot be ruled out and deserve further investigation in this and other species.

In summary, while not significantly different in stress related H:L profiles, the adults at T44 and Hillsdale do present intriguing outcomes regarding this measure of prolonged CORT elevation. Evidence is suggestive of females mitigating their stress responses and this is reflected in the similar H:L ratios and egg yolk CORT levels between the two sites. Males at the two sites show no evidence of differentially elevated H:L ratios, although males at T44 show a wider range of H:L ratios. Within each population, variation in individual H:L ratios and egg yolk CORT levels does exist. Future population studies comparing H:L values to specific measures of physiology and/or behavior in adults, as well as individual yolk CORT levels to later offspring

physiology and/or behavior may provide greater insight into the impacts of stress on gopher tortoise populations in Mississippi.

In summary, while not significantly different in stress related H:L profiles, the adults at T44 and Hillsdale do present intriguing outcomes regarding this measure of prolonged CORT elevation. Evidence is suggestive of females mitigating, possibly by dampening, their stress responses and this is reflected in the similar egg yolk CORT levels between the two sites. Males at the two sites show evidence of differentially elevated CORT levels, males at T44 have higher H:L ratios and this could be potentially linked to a variety of social/environmental variation that future studies may be able to tease apart. Within each population, variation in individual H:L ratios and egg yolk CORT levels does exist. Future population studies comparing H:L values to specific measures of physiology and/or behavior in adults, as well as individual yolk CORT levels to later offspring physiology and/or behavior may provide even greater insight into the impacts of stress on gopher tortoise populations in Mississippi.

CHAPTER III

BURROWING BEHAVIOR, DISPERSAL, AND SURVIVAL IN RELEASED GOPHER TORTOISES (*GOPHERUS POLYPHEMUS*)

Introduction

The gopher tortoise is federally threatened in its range west of the Mobile River, which includes Mississippi and Louisiana and is under consideration for federal listing throughout its entire range in the Southeastern United States. Mississippi populations have been severely reduced or even extirpated from some portions of their range, and recruitment of new reproductive individuals is low (Epperson and Heise 2003). Possible factors contributing to the low recruitment rates in the western range are habitat loss from development and fire suppression, lack of genetic diversity (Ennen et al. 2010), low natural hatching success (Epperson and Heise 2003; Noel et al. 2012), and increased risk of predation by domestic dogs (*Canis familiaris*), coyotes (*Canis latrans*), nine-banded armadillos (*Dasypus novemcinctus*), and red imported fire ants (*Solenopsis invicta*). At the Camp Shelby gopher tortoise refuge (T44), the reported 8 to 43% hatching success in Gopher Tortoises (Epperson and Heise 2003; Noel 2012) is much lower than the 67 to 97% hatching success reported in the eastern portion of the species range (Landers et al. 1980; Smith 1995; Butler and Hull 1996). This low hatching success, combined with the range-wide estimated 94% chance of being predated before one year of age (Alford 1980; Landers et al. 1980), can result in continued local population declines and extirpation unless there are strategies devised to increase hatchling survival.

One strategy for increasing hatchling survival is to raise juveniles in captivity (Pedrono and Sarovy 2000). Tuberville et al. (2015) found that in 6-9 month old hatchlings, head-starting increased juvenile survival rates, body size and performance similar to those of non-head-started tortoises that are 2–3 years older. Releasing larger head-started tortoises may reduce predation by eliminating those predators that feed on the smaller, softer shelled hatchlings. Larger tortoises are also stronger and capable of digging deeper burrows, which is critical in marginal soil types. This not only decreases the risk of predation, but deeper burrows also offer a wider range of thermoregulatory options and increased protection from fire. Here I describe a successful housing protocol for *G. polyphemus* (and potentially other burrowing tortoise species) that arose from research attempting to understand the decline of this species in Mississippi and report on the fate of captive reared hatchlings following their release from captivity.

In 2010, gopher tortoise eggs were collected from a site with low recruitment rates (Camp Shelby) and one of the state's few sites with high recruitment rates (Hillsdale, MS). One aim of this movement of eggs/hatchlings into captivity was to collect yolk samples to quantify maternally-deposited corticosterone (Chapter 2), a hormone that mitigates the stress response and regulates metabolism, and where elevated levels have been shown to decrease hatching success and hatchling in other species (Eriksen et al. 2003; Love et al. 2005). An important aspect of this work was the housing and care of captive tortoises prior to and during these assessments and the investigation of whether captive reared hatchlings can survive in the wild. Here I describe the husbandry protocol that I developed for raising hatchlings in captivity and provide

comparative data from a previous study at Camp Shelby which looked at survival in tortoises released directly after hatching in an effort to show that this is a beneficial care protocol that could be adapted for other research and head-starting programs. I also report on the survival and activities of captive reared individuals in a common garden and back in their home populations of origin. While head-starting is not the sole answer for maintaining tortoise populations in perpetuity, it is a useful tool if the protected and faster growing head-started juveniles have significantly higher survival than wild juveniles and have a reduced age to sexual maturity (Heppell et al. 1996). If successful, head-starting can at the very least slow down population decline and loss of genetic diversity as effective management strategies are being devised and implemented.

Materials and Methods

Gopherus polyphemus burrows were checked daily from 15 May to 30 June 2010 to ensure that eggs were collected within 24 hours of oviposition. Eggs were collected from Camp Shelby, and Hillsdale. During excavation and prior to transport, the tops of exposed eggs were marked with a graphite pencil so that they could be kept in the same orientation in which they were found; this guards against damage to the embryo following its attachment to the inside of the egg shell (Limpus et al. 1979). Eggs were placed in individual plastic containers containing vermiculite that was sterilized and dried before being rehydrated with distilled water at a ratio of 0.7 g water per 1.0 g of vermiculite (Packard et al. 1987), and then transported in a foam-lined cooler to reduce vibrations and maintain a constant temperature. Upon arrival at the University of

Southern Mississippi, the eggs were weighed, measured, and then incubated at 29.3°C, a recommended temperature for producing a 50:50 sex ratio in this species (Demuth 2001).

Body mass, carapace (CL), and plastron (PL) lengths were recorded within 24 hours of hatching, and individual numbers were assigned to each animal using a unique pattern of notches cut from the marginal scutes with a pair of dissecting scissors, similar to those described by Cagle (1939). Each tortoise was housed individually in a plastic tub (Rubbermaid®, Wooster, Ohio, USA) measuring 67.8 x 40.1 x 17.5 cm [L x W x H] to monitor food intake and eliminate stress from resource competition. Each tub was labeled with the occupant's number to assure that tortoises were returned to the proper tubs after measurements and soaking. All tubs contained 8 cm of sandy soil that was collected from the Hillsdale field site (Troup sand), a PVC pipe "burrow" (10 cm wide by 13 cm long) with one end plugged by an ABS test cap (to prevent the tortoises from burrowing through the back and pushing the burrow around the enclosure), and one separated petri dish (FisherScientific®, Waltham, Massachusetts, USA; 60 x 15 mm) (Figure 5). The petri dish lid and base were recessed into the soil and used for food and water, respectively. After one year, the water dishes were switched to larger and more durable glass culture dishes (63 x 28 mm), but plastic petri dishes continued to be used for food. Basking lamps with 125 watt Sylvania® (Osram Sylvania, Danvers, Massachusetts, USA) brooder bulbs were placed above and in between tubs so that each lamp would heat two tubs simultaneously. This resulted in a basking zone temperature around 34°C, burrow temperatures around 21°C, and a temperature gradient between the basking and burrow areas. Fluorescent light fixtures measuring 1.2 m long with 36 watt ReptiSun®

10.0 UVB fluorescent bulbs (Zoo Med Laboratories, San Luis Obispo, California, USA) were mounted approximately 20 cm above the enclosures so that two bulbs could light five tubs simultaneously (Figure 6). Fluorescent lamps were maintained on a 12:12 h (light:dark) cycle, while the basking lamps were maintained on a 10:14 h cycle, coming on an hour after and then shutting off an hour before the fluorescent lights.

Figure 5. Tortoise container with food, water, and PVC burrow. Burrows were placed in the coolest part of the tub, opposite of the basking lamp.

Figure 6. Lighting and container arrangement in the lab.

Between 0800 h and 1100 h each day, feces and uneaten food were removed from the tubs, and petri dishes were washed with Dawn® (Proctor & Gamble, Cincinnati, Ohio, USA) dish detergent and rinsed thoroughly before being replaced. Tortoises were provided with tap water daily (aged for one day so chlorine could evaporate), and a commercial tortoise diet (Natural Grassland Tortoise Food; Zoo Med Laboratories, San Luis Obispo, California, USA) was reconstituted with distilled water and provided *ad libitum*, totaling around 15 g, or one tablespoon of food daily. Initial encouragement was needed to stimulate consumption of the commercial diet; therefore chopped turnip, mustard, or collard greens were mixed in with the reconstituted food for the first month. After one month, when the tortoises were readily consuming the commercial diet, turnip or collard greens were offered weekly and dusted with calcium (Calcium with Vitamin

$D_{3;}$ Rep-Cal, Los Gatos, California, USA) and vitamin supplements (Herptivite™ with Beta Carotene; Rep-Cal, Los Gatos, California, USA). To promote natural foraging behavior and add variety to the diet, rye grass (*Lolium sp*.) was planted in the back of the tub away from the basking lamp (Figure 7). Old rye grass was removed, and tubs were replanted every two weeks. Tubs were misted weekly to maintain the rye grass, stimulate feeding, and reduce dust. As the tubs were being watered, burrows were refilled with soil to encourage continued burrowing behavior. To ensure hydration, tortoises were soaked weekly in groups of up to six individuals for 10 min in a tub of warm tap water deep enough to cover the lower half of the smallest individual. Body mass, carapace (CL) and plastron (PL) lengths were recorded monthly when the tortoises were soaked.

Figure 7. Rye grass (Lolium sp.) planted to supplement the commercial diet and encourage natural foraging behavior.

After two years in captivity, hatchlings were transferred to individual 1.8 x 1.8 m outdoor enclosures (Figure 8) from 7 June to 28 July 2012 to observe natural burrowing behavior and assess their ability to acclimate to outdoor conditions prior to their release into the wild. Each enclosure contained a shallow dish to collect water and a large section of tree bark to provide shelter and shade until the tortoises excavated burrows of their own. An irrigation timer was set to water the enclosures every two days to keep the water dishes filled and to facilitate the growth of grasses and forbes.

Figure 8. Outdoor enclosures used for acclimation and observation of burrowing behavior at the University of Southern Mississippi's Lake Thoreau Environmental center.

To provide adequate time for the tortoises to acclimate and dig suitable burrows, I released them before cold weather arrived on 1 August 2012. Prior to their release, the

tortoises were excavated from their burrows, CL, PL, and weights were recorded, and burrow depth and length were measured. Ten tortoises from each site were then randomly selected to receive custom radio transmitters (BD-2; 2g Holohil®, Carp, Ontario, Canada) with the antenna rotated 90 degrees to protrude from the middle of the transmitter. The traditional design required the antenna to be bent to point towards the posterior of the tortoise, which could negatively impact transmitter performance. The pulse rate was also reduced to extend the transmitter lifespan an additional four weeks from the original 10–20 week lifespan. Transmitters were affixed to the fifth vertebral scute with a two part epoxy (Protective Coating Company's PC-7®, Allentown, Pennsylvania, USA) (Figure 9) and held in place with masking tape overnight while the epoxy cured. The epoxy is strong enough to prevent transmitter loss through burrowing activity, but can easily be removed without any damage to the scutes. Unlike many other epoxies, PC-7® is not exothermic and will not burn the scutes while curing.

Figure 9. A hatchling tortoise with transmitter from earlier Nature Conservancy research. The modified transmitters for this project had an antenna protruding from the middle of the transmitter so it would not have to be bent to face posteriorly. Pulse rates were reduced as well in order to extend the lifespan of the transmitter. Photo courtesy of Matt Hinderliter

Prior to their official release, tortoises were placed in temporary enclosures at their original site of egg collection. This technique, known as a "soft release," is meant to encourage site fidelity and prevent roaming into undesirable areas. The enclosures consisted of a 0.91 m high, 2.4 m diameter circle of hardware cloth held in place with 1.2 m long metal U-posts and secured to the ground with sod staples to prevent tortoises from pushing under the wire. Burlap was draped over the top of the entire enclosure for shading and to prevent bird and snake predation. Camp Shelby and Hillsdale each had

two enclosures installed and each received eight tortoises except for one of the enclosures at Camp Shelby, which only received seven. Tortoises were then held in the pens for five days before it was disassembled and they were allowed to roam freely. Tortoises with transmitters (N = 20) were then tracked three times per week for two months using an ICOM® (Kirkland, Washington, USA) IC-R20 receiver and a Wildlife Materials Incorporated® (Murphysboro, Illinois, USA) three element folding antenna. After two months, The Nature Conservancy at Camp Shelby took over telemetry and continued throughout the remaining lifespan of the transmitters.

Results

A total of 93 eggs were collected in 2010 from Hillsdale (N = 47) and Camp Shelby (N = 46). Average clutch size at Hillsdale was 5.88 ± 1.81 SD (Range: 4–9) and 4.6 ± 1.07 SD (Range: 3–7) at Camp Shelby. Average incubation time for all eggs (N = 36) averaged 81.00 ± 3.36 days (Range: 76–89), with a Hillsdale hatching success of 46.8% (22/47) and 30.4% (14/46) for Camp Shelby. While a total of 36 tortoises hatched, three died within a day of hatchling, another perished in April 2011 after flipping over under its basking lamp, and one tortoise never developed full function of its rear legs, leaving 31 individuals that were eventually released. While I found no significant differences in growth rates between captive-reared tortoises from the high and low recruitment sites, captive tortoises (both sites combined) grew at faster rates than wild tortoises of the same age that were released directly after hatching and monitored via telemetry (Table 3).

Table 3

Comparison of two-year-old captive and two-year-old wild Camp Shelby tortoises.

	Captive-reared			Wild*			t-test		
	N	Mean	SD	N	Mean	SD	t-value	df	p
Final Mass (g)	31	203.87	45.8	20	53.32	6.58	14.57	49	<.0001
Final CL (mm)	31	97.18	8.37	20	62.25	3.59	17.6	49	<.0001
Final PL (mm)	31	95.39	8.31	20	60.19	3.87	17.7	49	<.0001
Mass per day (g)	31	0.242	0.06	20	0.021	0.006	15.45	49	<.0001

Wild*= Tortoises that hatched in captivity, were outfitted with radio transmitters, released afield shortly after hatching and tracked via radio telemetry for 2 years (at which time measurements reported above were taken). CL = Carapace length, PL = Plastron length

Although *G. polyphemus* are most likely capable of sperm storage, as documented in *G. agassizii* (Palmer et al. 1998; Pearse and Avise 2001), I assumed single paternity for each clutch and accounted for clutch effects using a blocked ANOVA. Even under this level of analysis, all variables of growth in the captive tortoises remained significantly greater than the wild tortoises: final CL ($F_{1,17.3} = 176.141$, $p < .0001$), final PL ($F_{1,17.1} = 178.271$, $p < .0001$), final mass ($F_{1,18.5} = 133.471$, $p < .0001$), and mass gain per day ($F_{1,18.7} = 151.054$, $p < .0001$). The wild tortoise data, collected by M. Hinderliter and J. Lee (unpubl. data), also shows that captive-reared tortoises at two years of age were greater in size than two of the only known wild hatchlings still alive seven years after release (CL = 77.48 and 89.61 mm; PL= 70.96and 82.59 mm; mass = 129.57 and 160.67g). From outdoor enclosure observations, the time required for two-year-old *G. polyphemus* (N = 30) to construct burrows deep enough to cover the entire body ranged from 1 to 21 days with an average of 7.1 days (SD = 5.86). One two-year-old Camp

Shelby tortoise remained in a pallet burrow (a shallow burrow or depression where the tortoise is still exposed) during the entire 51 day trial. These burrows were dug much quicker than those dug by a group of 2011 hatchlings (N = 26) that were placed directly into the enclosures where burrowing times ranged from 5 to 71 days, with an average of 23.2 days (SD = 17.4). I found a strong correlation between burrow depth and burrow length (Spearman rank correlation: $r_s = 0.47$, $p < 0.0001$, N = 30; Figure 10), burrow depth and mass ($r_s = 0.32$, $p = 0.001$, N = 30; Figure 11) and burrow depth and carapace length ($r_s = 0.25$, $p = 0.005$, N = 30; Figure 12). At Camp Shelby, one year after release and long after transmitters ceased functioning, The Nature Conservancy staff found that 90% (9/10) of the Camp Shelby captive hatchlings that had transmitters were still alive and all but one individual had stayed within 40 m of the release site. One of the Camp Shelby hatchlings went missing for several weeks and was assumed to have been lost due to predation or transmitter failure. After changing out receivers the tortoise was rediscovered, having travelled over 250 m from the release site before digging a burrow. At Hillsdale, five tortoises with transmitters were lost within a day of release due to depredation by what appeared to be a domestic dog (based on the size of the canine tracks left near the excavated burrows). After three months at Hillsdale, only two of the remaining five transmittered tortoises could be accounted for; two were lost to depredation by a small mammal and the third possibly due to transmitter failure.

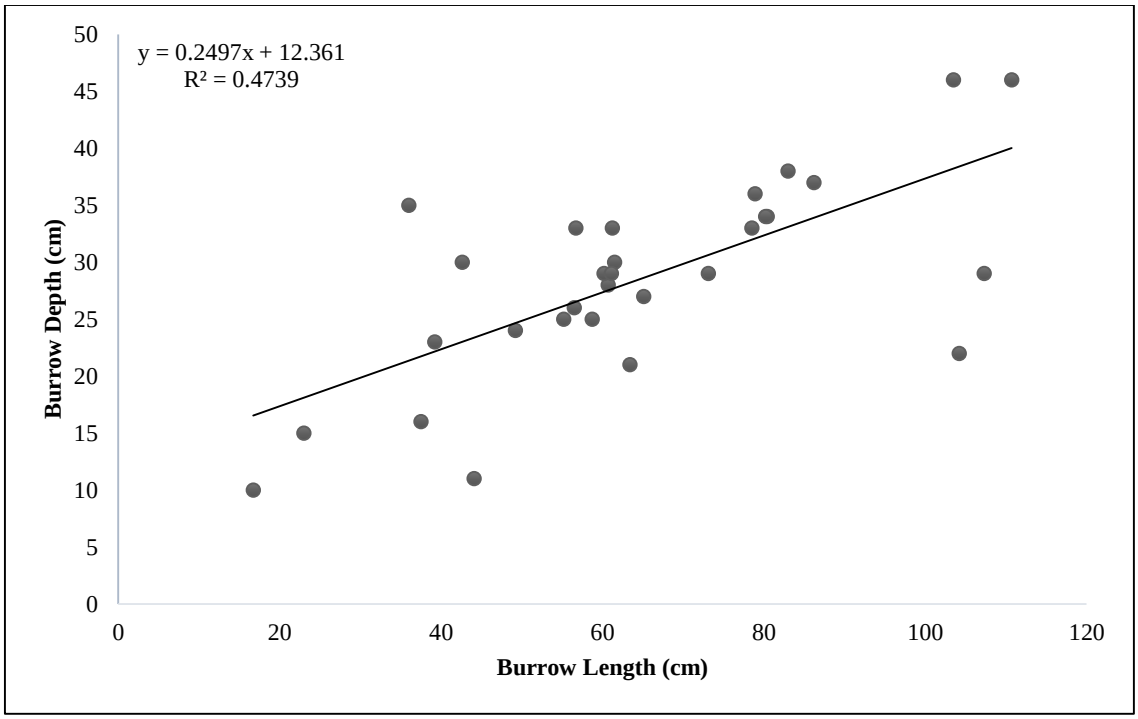

Figure 10. Burrow depth and length after 51 days in an outdoor enclosure before release.

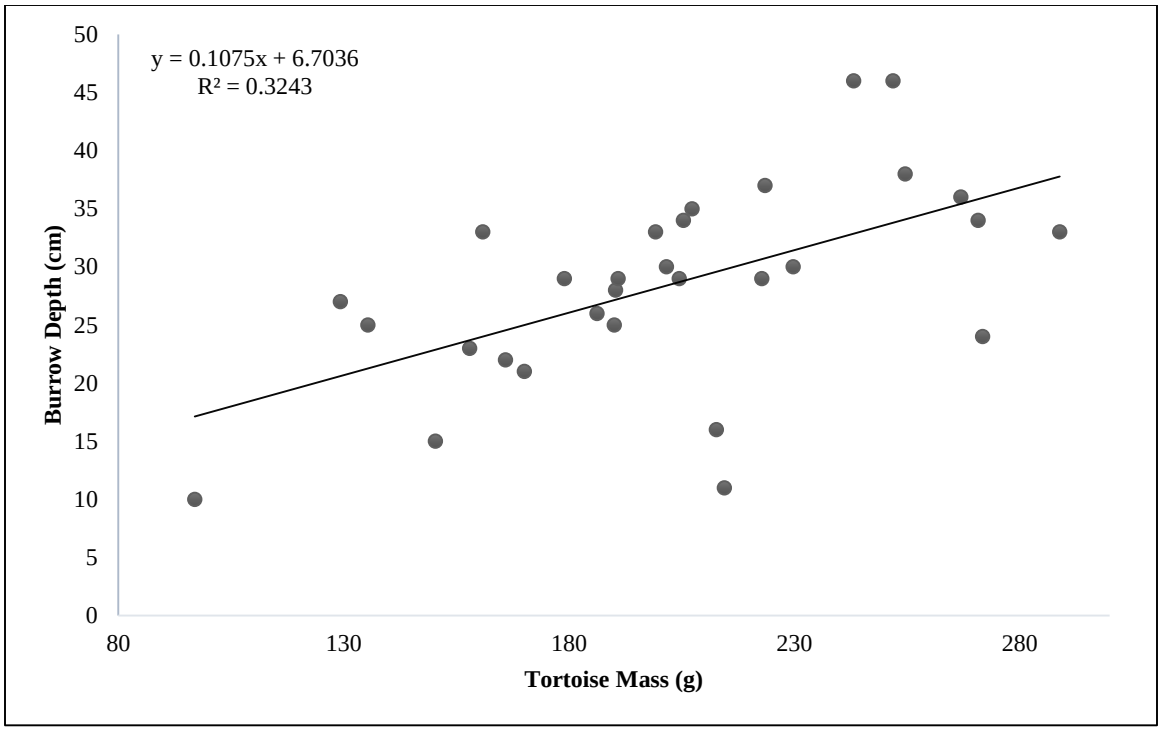

Figure 11. Tortoise mass and burrow depth after 51 days in and outdoor enclosure before release.

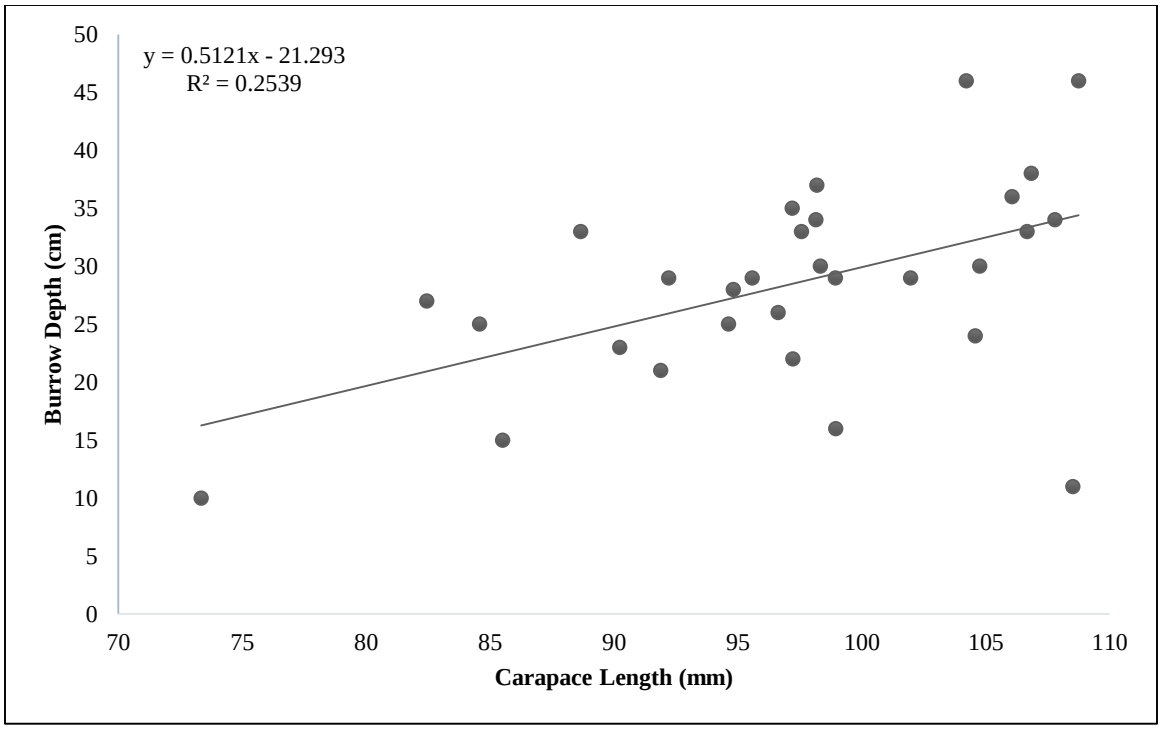

Figure 12. Tortoise carapace and burrow depth after 51 days in an outdoor enclosure before release.

Discussion

This husbandry protocol was initially designed to facilitate controlled investigations on the effects of stress on behavior and growth rates in hatchlings as part of a different study. In terms of producing tortoises for captive research and/or release with a very high survival rate, this protocol requires minimal materials for set up and is fairly low effort in terms of husbandry-related tasks. However, there are some caveats that may differ for others using this protocol. I chose a 29.3°C incubation temperature to ensure an equal sex ratio in our captive population so the effects of stress could be assessed in both sexes while eliminating the potential effects of temperature on stress response, growth rates, incubation period, and possibly behavior. However, if this

protocol is to be used for head-starting programs, temperature optimums for either male (28°C) or female (31°C) *G. polyphemus* should be used since wild clutches of turtles are most often unisex (Vogt and Bull 1984; Limpus et al. 1993; Janzen 1994) and incubation at pivotal temperatures can result in intersexed hatchlings, as demonstrated in European pond turtles (*Emys orbicularis*; Pieau et al. 1998). When compared to typical males and females, little is known about the fecundity of intersexed individuals and this could negatively impact head-starting programs. Incubation temperatures in head-starting programs should also be considered when an equal number of males and females is desired or when producing more of one sex would be advantageous for the program.

This technique for rearing *G. polyphemus* hatchlings in an indoor setting is simple, utilizes affordable and readily available equipment, and requires minimal human contact with tortoises which can reduce stress and possibly encourage natural behavior. Although a single visit per day is all that is required for daily feeding, dishwashing, and wellness checks, one problem encountered was when the tortoises would occasionally flip onto their backs when attempting to climb the side of the tub. In most instances the tortoise would right itself, but sometimes tortoises would have to be righted during morning feedings. The only mortality encountered in two years of captivity occurred when a tortoise flipped over underneath its basking lamp and overheated. To reduce basking lamp mortality, an additional wellness check may be necessary in the afternoon.

How captive-reared hatchlings are released into the wild may require careful consideration with respect to the habitat individuals are released into. When releasing translocated adult tortoises, soft releases where tortoises are penned for up to 12 months,

can increase site fidelity and survival by discouraging long distance roaming (Tuberville et al. 2005) and decreasing the likelihood of predation and road mortality. With head-started tortoises, this technique may be unnecessary as their small size limits them from roaming long distances. Also, in the case of two year old tortoises, most established burrows within seven days of being transferred to acclimation pens and less than three days at the release sites. A drawback of soft releases, which was experienced at the Hillsdale site, is that they concentrate individuals in a small area, making them more vulnerable to mass predation. Despite all of the juveniles having dug burrows, half of the juveniles at Hillsdale were predated by a single domestic dog within a day of the enclosures being removed. An alternative to the soft release is a hard release, where juveniles are directly released at the burrows from which they were collected as eggs. This technique would eliminate concentrating tortoises in a few enclosures, instead scattering smaller clutch groups back to their respective burrows of egg origin. This should reduce the likelihood of a mass predation event by a single predator, and there would not be a fenced structure to draw the unwanted attention of potential predators and humans.

Additional behavior and growth research required the tortoises to be kept in captivity for two years. With the sizes reached after just one year in captivity, it may not be necessary to keep them any longer than one year. If the objective of the project is to overcome the 90% juvenile/hatchling mortality rate found in first year juveniles and/or head-starting a larger quantity of individuals, then retaining juveniles for one year may be sufficient. By captive rearing indoors, tortoises can attain a large size in a relatively short

period of time, as they gain at least an extra five months (November-March) of foraging and growth over wild *G. polyphemus* and individuals head-started in outdoor enclosures. The increased size reduces the chance of predation by smaller predators and can decrease the time required to reach sexual maturity since sexual maturity depends on size rather than age (Landers et al. 1980; Iverson 1980). At the time of release, the two year old captive tortoises in the present study were equal in size to 5-7 year old wild juveniles at Camp Shelby (Hinderliter and Lee, unpubl. data), which suggests the time required to reach sexual maturity could be reduced by as much as five years. Additionally, while outdoor enclosures may offer protection from many predators, pesticides have to be applied to control fire ants and some predators still manage to get into the enclosures (e.g. a juvenile *Crotalus adamanteus* found in an outdoor enclosure at Camp Shelby). The Camp Shelby enclosures also required yearly prescribed burning of the vegetation to maintain suitable groundcover for foraging and for sustaining an open understory. The risks of burning the enclosure vegetation not only increased risks for wildfires outside the enclosure and enclosure damage but also increased the risk of tortoise fire mortality. Exposure to excessive heat, cold, and drought can also increase mortality in outdoor head-start enclosures.

In conclusion, this captive rearing protocol was able to produce larger juveniles with the ability to build sufficient burrows and promoted juvenile survival in the wild. Around Camp Shelby, wild hatchlings were often observed seeking shelter under leaves, logs, or in shallow pallet burrows that offered limited protection. All of the released captive-reared two-year-old tortoises were able to dig burrows within two days at the

Camp Shelby site. This suggests that larger captive tortoises may be more adept at digging burrows deep enough to protect themselves from extreme temperatures, predators, and wildfires, in contrast to recent hatchlings which may be unable to dig quality burrows; especially in soils with a high clay content as observed at Camp Shelby. Wild hatchling burrows are often found beneath clumps of grass, logs and woody debris, which offer little protection from extreme temperatures, fires and predators. However, if feral dogs and coyotes are present, only adult burrows appear to be deep enough to escape predation.

APPENDIX A

SCIENTIFIC COLLECTING PERMIT, 2010

MISSISSIPPI
DEPARTMENT OF WILDLIFE, FISHERIES, AND PARKS

Sam Polles, Ph.D.
Executive Director

14 June 2010

MODIFIED ADMINISTRATIVE SCIENTIFIC COLLECTION PERMIT NUMBER 0428101

TO WHOM IT MAY CONCERN:

Permission is granted to:

Carl Qualls
Biological Sciences
University of Southern Mississippi
118 College Drive, Box 5018
Hattiesburg, MS 39406-5018,

assisted by Aaron Holbrook and Daniel Gaillard, to collect and incubate in the laboratory at the University of Southern Mississippi 20 clutches of gopher tortoise eggs for CORT assay. Collections will be taken on DeSoto National Forest, as well as Forrest, and Lamar counties. For the CORT assays, 5 µl yolk samples from the 20 clutches of eggs will be taken. Twenty-four hours after hatching 160 µl subcarapacial blood samples will be taken, also 2ml of blood from 20 adult tortoises will be collected in September and October 2010 using a 25 gauge needle and syringe to draw from the brachial vein. Hatchlings will be held in captivity for follow-up CORT assays, as well as monitoring other aspects of fitness and simple physiological and behavioral assessments. Gopher tortoise hatchlings will be released at the conclusion of the study.

Adult and subadult gopher tortoises may be captured for tissue collection (blood samples) from private and state lands (with the appropriate authorization) in the counties of Jefferson-Davis, Covington, Jackson and Pearl River. A maximum of 70 samples may be taken. All captured tortoises must be released at their home burrow after tissue collection and measurements are recorded.
All hatchlings will be individually marked by clipping marginal scutes, and the scute clippings will be retained for DNA analysis.

This permit is valid from 15 May 2010 to 14 May 2011.

SPECIFIC CONDITIONS AND RESTRICTIONS

1) Hand searching will be used for egg location.

2) Adult gopher tortoises will be captured using modified bucket traps that signal when a tortoise has been captured. Sub-adult tortoises will be collected using smaller buckets. The buckets will have a wireless motion sensor that will sound an alarm at a receiver when an animal falls in the bucket. Tortoises may also be trapped using Tomahawk traps. All traps will be covered with burlap or shade cloth to avoid overheating of trapped tortoises, and will be checked at least twice each day (more frequently during hot, sunny weather).

3) All captured tortoises will be released. No specimens will be taken.

4) Collection gear left unattended in the field must be properly identified.

5) With respect to handling of this federally threatened species, the permittees are to be regarded as agents of the Mississippi Department of Wildlife, Fisheries, and Parks (MDWFP), pursuant to the Endangered and Threatened Fish and Wildlife Cooperative Agreement between the U.S. Dept. of the Interior, Fish and Wildlife Service, and the MDWFP, Section 6(c) of the Endangered Species Act of 1973, as amended.

GENERAL CONDITIONS AND RESTRICTIONS:

1) Specimens retained after collection must be placed in a public museum or collection where they will be available for examination by the scientific community. The Mississippi Museum of Natural Science (MMNS), 2148 Riverside Drive, Jackson, MS 39202-1353, ph: (601) 354-7303, is the principal repository of terrestrial and freshwater vertebrates, freshwater mollusks, and crayfish collected in Mississippi, and welcomes additional specimens. **Unless alternative arrangements are made with the MMNS Collections manager (Scott Peyton, 601-354-7303) or curatorial staff at the MMNS, all collections of federally listed and state listed species will be deposited at the Mississippi Museum of Natural Science.**

2) **This permit does not authorize the taking of any federally threatened or endangered species or any state endangered species (list attached), unless otherwise specified in this permit.**

3) All wildlife, including fish and invertebrates, collected under the permit are considered to be a natural resource of the State of Mississippi. Collected specimens should be handled humanely, and live, uninjured specimens not needed for permanent collections should be returned to appropriate habitat at the capture locality when no longer required. Specimens that die incidental to collection activities or which are intentionally preserved must be maintained in a scientifically acceptable fashion in a study/research collection where they will be available for examination by the general scientific community, or should be offered to a museum. The intent of the scientific collecting permit is to encourage meaningful study and to discourage the loss of specimens and information.

4) The issuance of a permit does not authorize trespass by the permittee. Permit is also void if permittee has not obtained other necessary permissions/permits for collection activities on public lands.
5) Collection of migratory birds, their nests, or eggs, collection of federally listed endangered species, and collection of federally listed threatened species (when the collector is not an agent of the State of Mississippi) requires a federal permit in addition to a state permit.

6) Copies of publications, survey reports, and other printed materials produced as a result of this collection should be sent to the Mississippi Museum of Natural Science (Attn: Scientific Collection Permit Review Committee.) 2148 Riverside Dr., Jackson, MS 39202.

REQUIRED COLLECTING PERMIT REPORTS

1) **A collecting permit report using format described below must be filed within 15 days of the expiration of the permit. A new permit will not be issued until the report has been received. Collection reports should list taxa collected, number of individuals of each, exact collection locality and date of collection. Locality information must include the county of collection, and it is preferred that precise locality information be provided in latitude/longitude (GPS) or in the township, range, and section (TRS) system. If the TRS system is used, precise location within a section should be indicated (e.g.: NW4 of SE4 of Sec 11), if possible. If GPS or TRS information is not provided, include instead a clear and precise description of the location of the collection site relative to the nearest named or numbered road, town, intersection, and/or other feature(s) likely to be mapped on a USGS quad map. For aquatic species, provide the name of the stream in which collections were made.**

Instructions for completing Scientific Collections Report

Below is a list of information that should be included in scientific collecting reports, if it applies to the activities covered by the collecting permit. Because of the broad spectrum of activities covered by collecting permits, individual reports may require an altered format or other information not described below. If possible, reports should be submitted electronically in a spreadsheet format (preferably in Excel or Access). A blank spreadsheet with the requested fields can be provided to you by Email. Please include the following fields in the spreadsheet, if they apply to the work conducted under the permit. If you cannot provide an electronic version of the collections report, a blank hard copy of a collections report form can be provided to you. If you have any questions, please contact Scott Peyton at 601-354-7303 or collections.manager@mmns.state.ms.us.

A. SPECIES - species name (scientific name), or lowest taxonomic description possible, for each collected taxon.

B. SACRIFICED - If specimens were killed for vouchers or other scientific purposes, indicate the number taken.
C. NUMBER – total number of each species collected or handled. Include both the number taken and the number released in this total.
D. DATE – specific date of each collection.
E. COUNTY – county where each collection occurred.
F. COORDINATES (X) - latitude/longitude, UTM coordinates
G. COORDINATES (Y) - latitude/longitude, UTM coordinates
H. UTM ZONE – UTM coordinates only
I. TRS - Township, Range and Section (optional, but please include if possible)
J. LOCALITY - brief description of locality, e.g. Chickasawhay River 100m upstream from HWY 84 bridge.
K. COLLECTOR(S) – person or persons who made the collection.
L. TISSUE - Indicate the number of specimens from which tissue samples were taken for genetic analysis or other purposes. If no tissue samples were taken, this column can be omitted.
M. DISPOSITION - For sacrificed specimens or tissue samples, list institution(s) where specimens/samples were deposited. For specimens released, indicate where the specimens were released.
N. TEMP EXP or TEMP PROP - If specimens are held in captivity temporarily for experimental purposes or for propagation and later released, a field should be included to capture this information.
O. TAGGED - If specimens are marked or tagged and released, a field should be included to capture this information.

2) **Those collecting federally listed species specified in this permit must submit an additional report to the state, due the first week of October, detailing collections of listed species made between 1 October of the previous year and 30 September of the current year.**

Libby Hartfield, Director
Mississippi Museum of Natural Science
Mississippi Department of Wildlife, Fisheries, & Parks

LH:ly, conservation biology section
cc: Randall Miller, Assistant Chief, Field Operations, MDWFP
Museum

Enclosure

APPENDIX B

SCIENTIFIC COLLECTING PERMIT, 2011

MISSISSIPPI
DEPARTMENT OF WILDLIFE, FISHERIES, AND PARKS

Sam Polles, Ph.D.
Executive Director

12 June 2011

ADMINISTRATIVE SCIENTIFIC COLLECTION PERMIT NUMBER 0519112

TO WHOM IT MAY CONCERN:

Permission is granted to:

Carl Qualls
Biological Sciences
University of Southern Mississippi
118 College Drive, Box 5018
Hattiesburg, MS 39406-5018,

assisted by Aaron Holbrook, Angie Getz, Brian Kreiser, and Daniel Gaillard, to capture and collect blood samples from gopher tortoises for DNA analysis. DNA samples will be taken in Pearl River, Marion, Covington, Perry, Forrest, and Lamar counties. A hypodermic needle and syringe will be used to collect a 0.5ml blood sample from the brachial or femoral vein of each captured tortoise. All captured tortoises will be released into the burrow from which they were captured following collection of blood samples.
A total of 20 clutches of gopher tortoise eggs will be collected in Camp Shelby, and Hillsdale, MS., and incubated in the laboratory at the University of Southern Mississippi for CORT assay. Twenty-four hours after hatching 160 μl subcarapacial blood samples will be taken, also 2 separate 1ml of blood from 20 adult tortoises will be collected in August and October 2011 using a 25 gauge needle and syringe to draw from the brachial vein. Hatchlings will be held in captivity for follow-up CORT assays, as well as monitoring other aspects of fitness and simple physiological and behavioral assessments. Gopher tortoise hatchlings will be released at the conclusion of the study.

All hatchlings will be individually marked by clipping marginal scutes, and the scute clippings will be retained for DNA analysis.

This permit is valid from 12 June 2011 to 31 October 2011.

SPECIFIC CONDITIONS AND RESTRICTIONS

1) Hand searching will be used for egg location.

2) Adult gopher tortoises will be captured using modified bucket traps that signal when a tortoise has been captured. Sub-adult tortoises will be collected using smaller buckets. The

buckets will have a wireless motion sensor that will sound an alarm at a receiver when an animal falls in the bucket. Tortoises may also be trapped using Tomahawk traps. All traps will be covered with burlap or shade cloth to avoid overheating of trapped tortoises, and will be checked at least twice each day (more frequently during hot, sunny weather).

3) All captured tortoises will be released. No specimens will be taken.

4) Collection gear left unattended in the field must be properly identified.

5) With respect to handling of this federally threatened species, the permittees are to be regarded as agents of the Mississippi Department of Wildlife, Fisheries, and Parks (MDWFP), pursuant to the Endangered and Threatened Fish and Wildlife Cooperative Agreement between the U.S. Dept. of the Interior, Fish and Wildlife Service, and the MDWFP, Section 6(c) of the Endangered Species Act of 1973, as amended.

GENERAL CONDITIONS AND RESTRICTIONS:

1) Specimens retained after collection must be placed in a public museum or collection where they will be available for examination by the scientific community. The Mississippi Museum of Natural Science (MMNS), 2148 Riverside Drive, Jackson, MS 39202-1353, ph: (601) 354-7303, is the principal repository of terrestrial and freshwater vertebrates, freshwater mollusks, and crayfish collected in Mississippi, and welcomes additional specimens. **Unless alternative arrangements are made with the MMNS Collections manager (Scott Peyton, 601-354-7303) or curatorial staff at the MMNS, all collections of federally listed and state listed species will be deposited at the Mississippi Museum of Natural Science.**

2) **This permit does not authorize the taking of any federally threatened or endangered species or any state endangered species (list attached), unless otherwise specified in this permit.**

3) All wildlife, including fish and invertebrates, collected under the permit are considered to be a natural resource of the State of Mississippi. Collected specimens should be handled humanely, and live, uninjured specimens not needed for permanent collections should be returned to appropriate habitat at the capture locality when no longer required. Specimens that die incidental to collection activities or which are intentionally preserved must be maintained in a scientifically acceptable fashion in a study/research collection where they will be available for examination by the general scientific community, or should be offered to a museum. The intent of the scientific collecting permit is to encourage meaningful study and to discourage the loss of specimens and information.

4) The issuance of a permit does not authorize trespass by the permittee. Permit is also void if permittee has not obtained other necessary permissions/permits for collection activities on public lands.
5) Collection of migratory birds, their nests, or eggs, collection of federally listed endangered species, and collection of federally listed threatened species (when the

collector is not an agent of the State of Mississippi) requires a federal permit in addition to a state permit.

6) Copies of publications, survey reports, and other printed materials produced as a result of this collection should be sent to the Mississippi Museum of Natural Science (Attn: Scientific Collection Permit Review Committee.) 2148 Riverside Dr., Jackson, MS 39202.

REQUIRED COLLECTING PERMIT REPORTS

1) **A collecting permit report using format described below must be filed within 15 days of the expiration of the permit. A new permit will not be issued until the report has been received. Collection reports should list taxa collected, number of individuals of each, exact collection locality and date of collection. Locality information must include the county of collection, and it is preferred that precise locality information be provided in latitude/longitude (GPS) or in the township, range, and section (TRS) system. If the TRS system is used, precise location within a section should be indicated (e.g.: NW4 of SE4 of Sec 11), if possible. If GPS or TRS information is not provided, include instead a clear and precise description of the location of the collection site relative to the nearest named or numbered road, town, intersection, and/or other feature(s) likely to be mapped on a USGS quad map. For aquatic species, provide the name of the stream in which collections were made.**

Instructions for completing Scientific Collections Report

Below is a list of information that should be included in scientific collecting reports, if it applies to the activities covered by the collecting permit. Because of the broad spectrum of activities covered by collecting permits, individual reports may require an altered format or other information not described below. If possible, reports should be submitted electronically in a spreadsheet format (preferably in Excel or Access). A blank spreadsheet with the requested fields can be provided to you by Email. Please include the following fields in the spreadsheet, if they apply to the work conducted under the permit. If you cannot provide an electronic version of the collections report, a blank hard copy of a collections report form can be provided to you. If you have any questions, please contact Scott Peyton at 601-354-7303 or collections.manager@mmns.state.ms.us.

A. SPECIES - species name (scientific name), or lowest taxonomic description possible, for each collected taxon.
B. SACRIFICED - If specimens were killed for vouchers or other scientific purposes, indicate the number taken.
C. NUMBER – total number of each species collected or handled. Include both the number taken and the number released in this total.
D. DATE – specific date of each collection.
E. COUNTY – county where each collection occurred.

F. COORDINATES (X) - latitude/longitude, UTM coordinates
G. COORDINATES (Y) - latitude/longitude, UTM coordinates
H. UTM ZONE – UTM coordinates only
I. TRS - Township, Range and Section (optional, but please include if possible)
J. LOCALITY - brief description of locality, e.g. Chickasawhay River 100m upstream from HWY 84 bridge.
K. COLLECTOR(S) – person or persons who made the collection.
L. TISSUE - Indicate the number of specimens from which tissue samples were taken for genetic analysis or other purposes. If no tissue samples were taken, this column can be omitted.
M. DISPOSITION - For sacrificed specimens or tissue samples, list institution(s) where specimens/samples were deposited. For specimens released, indicate where the specimens were released.
N. TEMP EXP or TEMP PROP - If specimens are held in captivity temporarily for experimental purposes or for propagation and later released, a field should be included to capture this information.
O. TAGGED - If specimens are marked or tagged and released, a field should be included to capture this information.

2) **Those collecting federally listed species specified in this permit must submit an additional report to the state, due the first week of October, detailing collections of listed species made between 1 October of the previous year and 30 September of the current year.**

Libby Hartfield

Libby Hartfield, Director
Mississippi Museum of Natural Science
Mississippi Department of Wildlife, Fisheries, & Parks

LH:ss, conservation biology section

Enclosure

APPENDIX C

SCIENTIFIC COLLECTING PERMIT, 2012

MISSISSIPPI
DEPARTMENT OF WILDLIFE, FISHERIES, AND PARKS

Sam Polles, Ph.D.
Executive Director

22 June 2012

ADMINISTRATIVE SCIENTIFIC COLLECTION PERMIT NUMBER 0622124

TO WHOM IT MAY CONCERN:

Permission is granted to:

Carl Qualls
Biological Sciences
University of Southern Mississippi
118 College Drive, Box 5018
Hattiesburg, MS 39406-5018,

assisted by Aaron Holbrook, Angie Getz, Brian Kreiser, and Daniel Gaillard, to capture and collect blood samples from gopher tortoises for DNA analysis. DNA samples will be taken in Pearl River, Marion, Covington, Perry, Forrest, and Lamar counties. A hypodermic needle and syringe will be used to collect a 0.5ml blood sample from the brachial or femoral vein of each captured tortoise. All captured tortoises will be released into the burrow from which they were captured following collection of blood samples. Additionally, blood samples of up to 1.5 ml may be taken from up to 40 adult Gopher Tortoises from Hillsdale (Pearl River County) and Camp Shelby, Mississippi to assess long-term stress.
Permittee may also continue to hold juvenile gopher tortoises to monitor growth and fitness of juveniles. Experiments may be conducted assessing digestive efficiency in juveniles held in captivity. Additionally, lab-reared juvenile gopher tortoises may be used in burrowing efficiency/soil composition experiments before release. These experiments will take place near Hillsdale, Pearl River County, and at Camp Shelby, Mississippi. Gopher all tortoise juveniles will be released at the conclusion of the study.

This permit is valid from 22 June 2012 to 21 June 2013.

SPECIFIC CONDITIONS AND RESTRICTIONS

1) Tortoises will be trapped using Tomahawk traps. All traps will be covered with burlap or shade cloth to avoid overheating of trapped tortoises, and will be checked at least twice each day (more frequently during hot, sunny weather).

2) Standard aseptic techniques must be followed when taking blood samples. In addition, permitttess must wear surgical gloves when handling tortoises. Also, all traps must be sterilized with a mild bleach solution between captures.

3) All trapped tortoises will be released. No specimens will be taken.

4) Any mortality of captured tortoises should be reported to MDWFP within 48 hours.

5) Collection gear left unattended in the field must be properly identified.

6) After completion of experiments, all captive-reared tortoises must be released at the burrow from which eggs were obtained.

7) With respect to handling of this federally threatened species, the permittees are to be regarded as agents of the Mississippi Department of Wildlife, Fisheries, and Parks (MDWFP), pursuant to the Endangered and Threatened Fish and Wildlife Cooperative Agreement between the U.S. Dept. of the Interior, Fish and Wildlife Service, and the MDWFP, Section 6(c) of the Endangered Species Act of 1973, as amended.

GENERAL CONDITIONS AND RESTRICTIONS:

1) Specimens retained after collection must be placed in a public museum or collection where they will be available for examination by the scientific community. The Mississippi Museum of Natural Science (MMNS), 2148 Riverside Drive, Jackson, MS 39202-1353, ph: (601) 354-7303, is the principal repository of terrestrial and freshwater vertebrates, freshwater mollusks, and crayfish collected in Mississippi, and welcomes additional specimens. **Unless alternative arrangements are made with the MMNS Collections manager (Scott Peyton, 601-354-7303) or curatorial staff at the MMNS, all collections of federally listed and state listed species will be deposited at the Mississippi Museum of Natural Science.**

2) **This permit does not authorize the taking of any federally threatened or endangered species or any state endangered species (list attached), unless otherwise specified in this permit.**

3) All wildlife, including fish and invertebrates, collected under the permit are considered to be a natural resource of the State of Mississippi. Collected specimens should be handled humanely, and live, uninjured specimens not needed for permanent collections should be returned to appropriate habitat at the capture locality when no longer required. Specimens that die incidental to collection activities or which are intentionally preserved must be maintained in a scientifically acceptable fashion in a study/research collection where they will be available for examination by the general scientific community, or should be offered to a museum. The intent of the scientific collecting permit is to encourage meaningful study and to discourage the loss of specimens and information.

4) The issuance of a permit does not authorize trespass by the permittee. Permit is also void if permittee has not obtained other necessary permissions/permits for collection activities on public lands.

5) Collection of migratory birds, their nests, or eggs, collection of federally listed endangered species, and collection of federally listed threatened species (when the collector is not an agent of the State of Mississippi) requires a federal permit in addition to a state permit.

6) Copies of publications, survey reports, and other printed materials produced as a result of this collection should be sent to the Mississippi Museum of Natural Science (Attn: Scientific Collection Permit Review Committee.) 2148 Riverside Dr., Jackson, MS 39202.

REQUIRED COLLECTING PERMIT REPORTS

1) **A collecting permit report using format described below must be filed within 15 days of the expiration of the permit. A new permit will not be issued until the report has been received. Collection reports should list taxa collected, number of individuals of each, exact collection locality and date of collection. Locality information must include the county of collection, and it is preferred that precise locality information be provided in latitude/longitude (GPS) or in the township, range, and section (TRS) system. If the TRS system is used, precise location within a section should be indicated (e.g.: NW4 of SE4 of Sec 11), if possible. If GPS or TRS information is not provided, include instead a clear and precise description of the location of the collection site relative to the nearest named or numbered road, town, intersection, and/or other feature(s) likely to be mapped on a USGS quad map. For aquatic species, provide the name of the stream in which collections were made.**

Instructions for completing Scientific Collections Report

Below is a list of information that should be included in scientific collecting reports, if it applies to the activities covered by the collecting permit. Because of the broad spectrum of activities covered by collecting permits, individual reports may require an altered format or other information not described below. If possible, reports should be submitted electronically in a spreadsheet format (preferably in Excel or Access). A blank spreadsheet with the requested fields can be provided to you by Email. Please include the following fields in the spreadsheet, if they apply to the work conducted under the permit. If you cannot provide an electronic version of the collections report, a blank hard copy of a collections report form can be provided to you. If you have any questions, please contact Scott Peyton at 601-354-7303 or collections.manager@mmns.state.ms.us.

A. SPECIES - species name (scientific name), or lowest taxonomic description possible, for each collected taxon.

B. SACRIFICED - If specimens were killed for vouchers or other scientific purposes, indicate the number taken.
C. NUMBER – total number of each species collected or handled. Include both the number taken and the number released in this total.
D. DATE – specific date of each collection.
E. COUNTY – county where each collection occurred.
F. COORDINATES (X) - latitude/longitude, UTM coordinates
G. COORDINATES (Y) - latitude/longitude, UTM coordinates
H. UTM ZONE – UTM coordinates only
I. TRS - Township, Range and Section (optional, but please include if possible)
J. LOCALITY - brief description of locality, e.g. Chickasawhay River 100m upstream from HWY 84 bridge.
K. COLLECTOR(S) – person or persons who made the collection.
L. TISSUE - Indicate the number of specimens from which tissue samples were taken for genetic analysis or other purposes. If no tissue samples were taken, this column can be omitted.
M. DISPOSITION - For sacrificed specimens or tissue samples, list institution(s) where specimens/samples were deposited. For specimens released, indicate where the specimens were released.
N. TEMP EXP or TEMP PROP - If specimens are held in captivity temporarily for experimental purposes or for propagation and later released, a field should be included to capture this information.
O. TAGGED - If specimens are marked or tagged and released, a field should be included to capture this information.

2) **Those collecting federally listed species specified in this permit must submit an additional report to the state, due the first week of October, detailing collections of listed species made between 1 October of the previous year and 30 September of the current year.**

Libby Hartfield, Director
Mississippi Museum of Natural Science
Mississippi Department of Wildlife, Fisheries, & Parks

LH:cc, conservation biology section

Enclosure

APPENDIX D

INSTUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL, 2009-2011

THE UNIVERSITY OF SOUTHERN MISSISSIPPI

Institutional Animal Care and Use Committee

118 College Drive #5147
Hattiesburg, MS 39406-0001
Phone: 601.266.4063
Fax: 601.266.4377

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the three year approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes (see attached) should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER:	**09051403**
PROJECT TITLE:	**Maternal Effects and Behavior in Gopher Tortoises, the Influence of Stress**
PROPOSED PROJECT DATES:	**May 2009 – September 2011**
PROJECT TYPE:	**New**
PRINCIPAL INVESTIGATOR(S):	**Jodie Jawor**
DEPARTMENT:	**Biological Science**
FUNDING AGENCY/SPONSOR:	
IACUC COMMITTEE ACTION:	**Full Committee Review Approval**
PROTOCOL EXPIRATON DATE:	**September 30, 2011**

Gordon C. Cannon, Ph.D.
Vice President for Research

4/29/13
Date

*This letter is a replacement for the original which has been misplaced.

APPENDIX E

INSTUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL, 2011-2014

The University of Southern Mississippi
Institutional Animal Care and Use Committee

118 College Drive #5147
Hattiesburg, MS 39406-0001
Tel: 601.266.6820
Fax: 601.266.5509
www.usm.edu/spa/policies/animals

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the three year approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes (see attached) should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER: **11092201**
PROJECT TITLE: **Maternal Effects and Behavior in Gopher Tortoises, The Influence of Stress**
PROPOSED PROJECT DATES: **10/01/2011 to 09/30/2014**
PROJECT TYPE: **Renewal/Continuation of a Previously Approved Project**
PRINCIPAL INVESTIGATOR(S): **Jodie Jawor, Ph.D.,**
COLLEGE/DIVISION: **College of Science & Technology**
DEPARTMENT: **Biological Sciences**
FUNDING AGENCY/SPONSOR: **N/A**
IACUC COMMITTEE ACTION: **Full Committee Review Approval**
PROTOCOL EXPIRATION DATE: **09/30/2014**

Gordon C. Cannon, Ph.D.
Associate Vice President for Research

9/27/2011
Date

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