5-2013

Conservation Genetics of Threatened Gopher Tortoises, *Gopherus polyphemus*: An Assessment of Genetic Variation and Parentage in Two Populations in South Mississippi

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CONSERVATION GENETICS OF THREATENED GOPHER TORTOISES, *GOPHERUS POLYPHEMUS*: AN ASSESSMENT OF GENETIC VARIATION AND PARENTAGE IN TWO POPULATIONS IN SOUTH MISSISSIPPI

by

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A Thesis
Submitted to the Graduate School
of The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Master of Science

Approved:

Dean of the Graduate School

May 2013

ABSTRACT

CONSERVATION GENETICS OF THREATENED GOPHER TORTOISES, *GOPHERUS POLYPHEMUS*: AN ASSESSMENT OF GENETIC VARIATION AND PARENTAGE IN TWO POPULATIONS IN SOUTH MISSISSIPPI

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Despite the protection of gopher tortoises, *Gopherus polyphemus*, in the western portion of their range for over twenty years, populations of the De Soto National Forest (DNF) in southern Mississippi experience low recruitment and lower hatching success than populations in the eastern portion of the range, and the causes of this are unknown. Previous work has shown that Mississippi populations of the DNF have lower levels of genetic diversity than eastern populations, which prompted the suggestion that reduced levels of genetic variation may play a part in low hatching success. Small populations can become more susceptible to the effects of inbreeding which can have negative effects on fitness of offspring. Using a microsatellite-based approach, I assessed genetic variation at two sites in south Mississippi that have different levels of recruitment to test for a correlation between genetic variation and survivorship. T44 at Camp Shelby is a low recruitment site, and Hillsdale is a high recruitment site. I found evidence of a heterozygosity fitness correlation among tortoises belonging to different age classes in the Hillsdale population. Multilocus genotypic data was also used to perform parentage assessments to characterize the mating systems and movements of both populations. Both

populations demonstrated unequal reproductive success among adult tortoises, and spatial analyses revealed strong colony fidelity within populations even across several years.

ACKNOWLEDGMENTS

For helping to bring this work to fruition, I would like to thank my committee members, Brian Kreiser, Carl Qualls, and Mark Welch, for their dedication and direction during my graduate studies. I thank Brian and Carl for giving me the opportunity to pursue graduate research in their labs. To Brian, I am deeply grateful for his guidance and commitment to my success. I thank Carl for his enthusiasm for sharing his expertise in herpetology and statistics. I would like to thank Mark for recognizing my potential and giving me my first opportunity to delve into the world of scientific research. I would also like to show my appreciation to Cherilynn Shadding for her faith and encouragement and to all the women in my life who have inspired me to pursue a career in science.

For their financial support and technical assistance, I would like to thank the U.S. Fish and Wildlife Service, the Mississippi Museum of Natural Science, and The Nature Conservancy. I would like to thank my lab mates, especially Daniel Gaillard and Aaron Holbrook. Their guidance and time spent in the field helped make this work possible. I give special thanks to former lab member Josh Ennen for his earlier collection efforts that contributed to this research.

To my fellow graduate students whom I have formed friendships with, you have become my USM family. Thank you for lending an ear, offering helpful advice, and sharing your experiences with me. I am very grateful for my parents, my sisters, and my in-laws who have always been supportive and encouraging. Finally, I would like to thank my husband and best friend, Aaron. I could not have made this journey without his love, support, and positive attitude to see me through it.

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CHAPTER I

INTRODUCTION AND OBJECTIVES

The Gopher Tortoise, Gopherus polyphemus, a Species in Decline

The gopher tortoise, *Gopherus polyphemus*, a species native to the coastal plain of the southeastern United States, has been drastically reduced from its historical range. Auffenberg and Franz (1982) collected data on gopher tortoise densities across the range in the 1970's and estimated that populations had declined by 80% over the past 100 years. The decline of the gopher tortoise is closely coupled to loss of longleaf pine (*Pinus palustris*) habitats due to anthropogenic alterations to land for agriculture, silviculture, and development (Auffenberg and Franz, 1982). This once abundant ecosystem in the eastern and southeastern U.S. has diminished by more than 98% from pre-1880 to 1986 (Noss et al., 1995). The distribution of gopher tortoises is associated with well-drained, sandy soil types for excavating burrows, open canopies for thermoregulation, and persistence of low-lying herbaceous plants for foraging (Auffenberg and Franz, 1982). Periodic wildfires are needed to suppress undesirable hardwoods in longleaf pine stands and clear litter for germination of longleaf pine seeds (Croker and Boyer, 1975). In addition to making the habitat more desirable for gopher tortoises, periodic wildfires can reduce the habitat quality for raccoons (*Procyon lotor*) that frequently prey on gopher tortoise eggs and hatchlings (Jones et al., 2004).

Due to fragmentation of habitat and reduction in gopher tortoise populations, the U.S. Fish and Wildlife Service listed western populations as threatened under the Endangered Species Act of 1973. The federal listing includes populations in Louisiana, Mississippi, and Alabama west of the Tombigbee and Mobile Rivers (Figure 1., U.S. Fish

and Wildlife Service, 1987). The eastern portion of the range includes populations in Alabama east of the Tombigbee and Mobile Rivers, Florida, Georgia, and South Carolina. Although gopher tortoises in the eastern portion of the range are deserving of federal listing, they currently remain federally unprotected due to the need to allocate resources to higher priority listings. Alternatively, these gopher tortoises have been designated a candidate species (U.S. Fish and Wildlife Service, 2011).

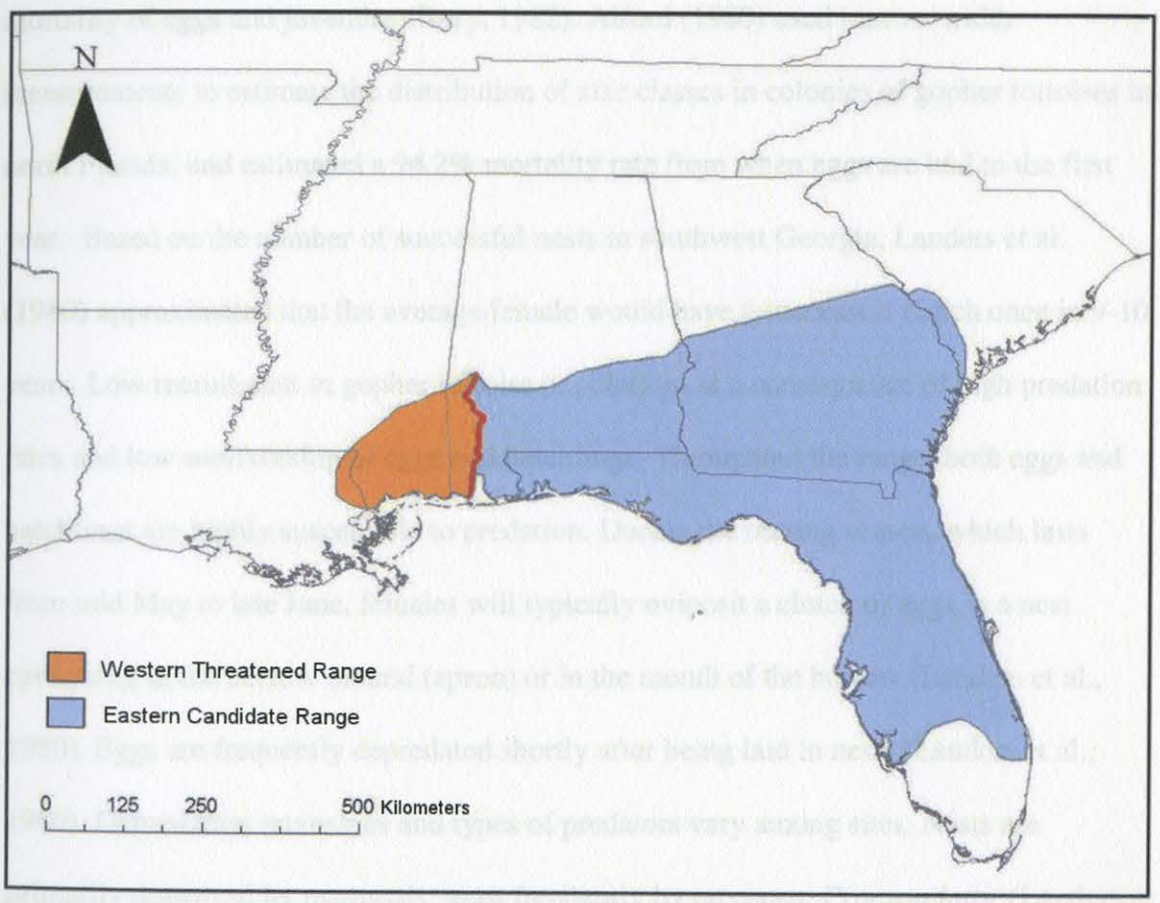

Figure 1. Map of the Approximate Range of the Gopher Tortoise. Populations found west of the Tombigbee and Mobile Rivers in Alabama, Mississippi, and Louisiana are listed as threatened populations. Populations found east of the boundary in Alabama, Georgia, Florida, and South Carolina are listed as candidates for Endangered Species Act listing.

Evidence of Low Recruitment in Gopher Tortoise Populations

Turtles are relatively long-lived and are iteroparous but generally have late maturity and high juvenile mortality. These life history traits heighten their vulnerability to habitat loss and degradation and population declines caused by humans (Ernst et al., 1994). *Gopherus* species require a growth period of 10 or more years before reaching sexual maturity, and populations of tortoises have low rates of recruitment and high mortality of eggs and juveniles (Bury, 1982). Alford (1980) used burrow width measurements to estimate the distribution of size classes in colonies of gopher tortoises in north Florida, and estimated a 94.2% mortality rate from when eggs are laid to the first year. Based on the number of successful nests in southwest Georgia, Landers et al. (1980) approximated that the average female would have a successful clutch once in 9-10 years. Low recruitment in gopher tortoise populations is a consequence of high predation rates and low survivorship of eggs and hatchlings. Throughout the range, both eggs and hatchlings are highly susceptible to predation. During the nesting season, which lasts from mid May to late June, females will typically oviposit a clutch of eggs in a nest cavity dug in the burrow mound (apron) or in the mouth of the burrow (Landers et al., 1980). Eggs are frequently depredated shortly after being laid in nests (Landers et al., 1980). Depredation intensities and types of predators vary among sites. Nests are primarily destroyed by mammals, most frequently by raccoons, *Procyon lotor* (Landers et al., 1980). Hatchlings emerge from the nest following an incubation period which ranges between 97 and 106 days (Landers et al., 1980). Hatchlings are also preyed upon predominantly by mammals (Landers et al., 1980; Butler and Sowell, 1996; Epperson and Heise, 2003), but also by birds, snakes, (Butler and Sowell, 1996) and introduced red fire

ants, *Solenopsis invicta* and *S. saevissima* (Landers et al., 1980; Smith, 1995; Epperson and Heise, 2003). Radio telemetry studies have shown that depredation of hatchlings is highest within the first month after hatching (Epperson and Heise, 2003; Pike and Seigel, 2006), and more than half of tracked individuals were deceased by the end of the first year (Butler and Sowell, 1996; Epperson and Heise, 2003; Pike and Seigel, 2006).

Efforts to protect nests by predator exclusion are typically successful. Throughout much of the range, hatching success of protected, natural nests is generally high; 86% in southwest Georgia (Landers et al., 1980), 67-97% in north-central Florida (Smith, 1995), 80.6% in northeastern Florida (Butler and Hull, 1996), and 78% in eastern Florida (Demuth, 2001). However, nests in Mississippi show a much lower rate of hatching success. Protected, natural nests of the De Soto National Forest (DNF) in south Mississippi yielded 28.8% hatching success over 4 nesting seasons (1997-2000) (Epperson and Heise, 2003), 16.7% in 2003 (Noel, 2006), and 46.6% in 2006 and 2007 combined (Hammond, 2009).

Potential Causes for Low Levels of Hatching Success in Mississippi Populations

Causes for the discrepancy in hatching success between gopher tortoise populations in the DNF of Mississippi and eastern populations remain unknown. It is difficult to determine whether developmental mortality in natural nests is due to environmental factors, maternal effects, genetic factors, or a combination of these. Environmental factors, that may affect the survivorship and resulting phenotype of developing embryos, include nest characteristics such as embryo incubation temperature, moisture, and gas exchange. In desert tortoises, *G. agassizii*, a close relative of the gopher tortoise, both temperature and substrate moisture affected hatching success of laboratory

incubated eggs. Eggs incubated at low (26.0° C) and high (35.3° C) temperatures had lower hatching success than intermediate temperatures, and substrate of high moisture content (−5 kPa) lowered hatching success at both temperatures that were tested (Spotila et al., 1994). In an attempt to quantify the affects of nest environmental factors on gopher tortoises, Noel (2006) split clutches from the DNF between protected natural nests and laboratory incubations in which temperature and hydric conditions were kept constant. Hatching success was significantly lower among naturally incubated eggs (16.7%) than among artificially incubated eggs (58.8%). Even when incubated under controlled conditions in the laboratory, clutches from three sites in Mississippi still had lower hatchling success compared to naturally incubated nests of eastern populations, 58.8% to 67-97%, respectively. In gopher tortoises, laboratory incubation at a constant 34° C was lethal to eggs, but natural nests did not seem adversely affected by periodic exposure to temperatures at or above 34° C (Demuth, 2001). However, Noel (2006) found that successful clutches had significantly fewer hours per day at or above 34° C than did clutches with no hatching success. While nest environmental factors such as nest temperature, soil clay content, slope of terrain, and percent shrub cover did seem to play a role in success rates, Noel (2006) estimated that approximately 40% of the eggs were impeded by one or more unknown intrinsic factors. In a later study of nests within the DNF, nest locations were compared to soil classification data to assess the effect of soil type on hatching success (Hammond, 2009). The three soil classifications defined by the USFWS are "priority," "suitable," and "marginal" soil (McDearman, 2005, p. 7). Hammond (2009) found hatching success to be 53.4% on priority soil, 45.2% on suitable soil, and 13.6% on marginal soil. However, only the difference between hatching success

on priority soil and that of marginal soil was significant, demonstrating that hatching success does not seem to be greatly limited by particular soil types (Hammond, 2009).

In addition to a low level of hatching success in Mississippi populations, studies spanning multiple years have reported an increased frequency in late stage embryo mortalities compared to reports in the eastern portion of the range. Most often when eggs fail to hatch, no discernible embryo is found when eggs are dissected (Landers et al., 1980; Hurley, 1993; Butler and Hull, 1996; Noel, 2006). However, embryo mortality is elevated in clutches of the DNF, and many embryos are almost fully developed and near hatching (Figure 2., Noel, 2006; Hammond, 2009). Noel (2006) found 22.6% of field incubated eggs (14 of 62) and 13.9% of laboratory incubated eggs (5 of 36) to contain dead embryos, most of which were late stage embryos. Similarly, Hammond (2009) found 27.8% of field incubated eggs (90 of 324) to suffer late stage embryo mortality, but the frequency of late stage embryos did not differ significantly between nests of different soil types.

Factors influencing offspring fitness are not limited to those of the nest environment, but also include factors influenced by the mother. Maternal effects can influence the nest environment by means of nest site selection, but can also affect egg viability via mate choice, maternal nutritional condition, and energy allocation. Very little is known about the impact maternal effects may have on hatchling fitness. Female gopher tortoises do not exhibit parental care, but there is some evidence that they may exhibit nest site selection. In southern Mississippi, burrow aprons that contained nests had more bare soil and lower clay content than random burrow aprons without nests (Lamb et al., in press). It is unclear to what degree mate choice is exhibited by female gopher tortoises.

Size may be important for male social rank. Larger males often dominate aggressive male-male interactions (McRae, 1981), and paternity studies have shown higher reproductive success in larger males (Moon et al., 2006, Tuberville et al., 2011). However, it is not known if females prefer to mate with larger males or if larger males dominate more territory, and thus gain access to more females. Diet, age, and overall health of the mother can influence maternal nutritional condition and gamete quality, which can impact egg viability and embryo/offspring fitness. An ongoing study is investigating the affects of the stress-induced hormone, corticosterone, in eggs (A. Holbrook, pers. comm.). Elevated levels of corticosterone in bird eggs have been shown to produce low quality offspring (Saino et al., 2005).

Figure 2. A Dead, Late Stage Embryo, After Being Dissected from an Unhatched Egg. Photograph by Jennifer Y. Lamb.

Low Genetic Diversity in Mississippi Populations

Lack of evidence pointing to any one critical nest environmental factor contributing to low hatching success or late stage embryo mortalities suggests that the

problem is multifaceted and is likely to be a combination of environmental factors, maternal effects, and/or genetic factors. Reduced genetic variation within the gopher tortoise populations of Mississippi may be a genetic factor affecting hatching success, thus affecting recruitment. A microsatellite study of gopher tortoise populations revealed that Mississippi populations of the DNF have lower genetic diversity than their eastern conspecifics of Florida and Georgia. DNF populations had significantly fewer alleles per locus, reduced heterozygosity, and fewer polymorphic loci than eastern populations included in the analysis (Ennen et al., 2010).

Gopher tortoise populations have historically been declining across the range since the late 1800's (Auffenberg and Franz, 1982), but more recently, declines have been more precipitous. Hammond (2009) documented that on specific sites within the DNF the number of active and inactive gopher tortoise burrows had decreased by approximately 35.7% between 1995 and 2007. Steep population declines, such as that observed in the DNF, may account for a loss of genetic variation. A period of small population size, called a population bottleneck, results in a decrease in the effective population size and is expected to reduce population heterozygosity and average number of alleles per locus (Wright, 1969; Nei and Chakraborty, 1975). While Ennen et al. (2010) did not find genetic evidence of a historic population bottleneck, the demographic evidence certainly suggests that one is currently in progress.

Population declines can increase the probability of individuals sharing alleles that are identical by descent due to inbreeding. Inbreeding decreases heterozygosity throughout the genome and can have negative effects on fitness in cases of overdominance (heterozygote superiority) and/or when deleterious alleles are expressed

in the homozygous state (Charlesworth and Charlesworth, 1987). Pedigrees can be used to calculate the inbreeding coefficient, the probability that two alleles at a locus are identical by decent (Wright, 1969). This estimate of inbreeding can then be compared to a fitness phenotype to characterize the relationship between degree of inbreeding and individual fitness. This reduction in fitness, termed inbreeding depression, may become evident during different life stages and is often perceived as a reduction in growth rate, fertility, fecundity, and offspring viability (Wright, 1977). A variety of studies have demonstrated that inbreeding between closely related individuals can result in a decline in the fitness of their offspring. Studies involving populations of wild bird species frequently report reductions in hatching rate, offspring survivorship, or recruitment (reviewed in Keller and Waller, 2002). Using a pedigree analysis to calculate inbreeding coefficients, Daniels and Walters (2000) found a reduction in hatching rates, recruitment of females, and fledgling survivorship of offspring produced from closely related pairs in a population of red-cockaded woodpeckers, *Picoides borealis*. Inbreeding depression in song sparrows, *Melospiza melodia*, was characterized by a reduction in egg survival to breeding age, adult survival, and reproductive success of females mostly due to lower hatching success of their eggs (Keller, 1998). In the great tit, *Parus major*, hatching rate was reduced in inbred offspring and in offspring of inbred females (Van Noordwijk and Scharloo, 1981). A population of Mexican jays, *Aphelocoma ultramarina*, exhibited smaller broods among related parents than unrelated parents, and inbred offspring had low survivorship to the following year and no recruitment to the breeding population (Brown and Brown, 1998).

Pedigree analyses have been the preferred means for estimating inbreeding, but accurate long-term data necessary for pedigree analyses are often not available or not feasible to obtain for wild populations. As a substitute for detailed pedigrees, researchers may utilize molecular markers to estimate inbreeding indirectly (reviewed in Hansson and Westerberg, 2002). Inbreeding increases homozygosity due to allelic co-ancestry, and can cause correlations between loci, identity disequilibrium, throughout the genome (Weir and Cockerham, 1973). The inbreeding coefficient is then calculated by comparing the observed heterozygosity to the expected heterozygosity from multi-locus genotypes (Wright, 1969). Relationships between the level of genetic diversity and one or more fitness traits are known as heterozygosity-fitness correlations (HFCs). Studies of vertebrate species have demonstrated correlations between multilocus heterozygosity and fitness. For example, in a study of house sparrows, *Passer domesticus*, there was a strong negative relationship between heterozygosity of microsatellite loci and pedigree-based estimates of the inbreeding coefficient (Jensen et al., 2007), and recruitment of fledglings decreased as level of inbreeding increased.

There are some caveats to inferring inbreeding depression using HFCs because homozygous individuals can also arise through outcrossing, and multilocus heterozygosity is an indirect estimate of the level of inbreeding. There are different theoretical explanations for HFCs detected using selectively neutral microsatellite loci. Under the local effect hypothesis, HFCs are considered to be the result of linkage disequilibrium between selectively neutral marker loci and loci affecting fitness. According to the general effect hypothesis, HFCs are detectable through selectively neutral marker loci because of homozygosity of unlinked fitness loci throughout the

genome (reviewed in Hansson and Westerberg, 2002), and only under the general effect hypothesis can HFCs be used to infer inbreeding depression (Slate et al., 2004).

Objective and Description of Thesis Chapters

As gopher tortoise populations become smaller and more isolated, it becomes increasingly important to understand the genetic variability of populations from a conservation perspective. Low hatching success, increased number of late stage embryo mortalities, and low recruitment observed in gopher tortoise populations in Mississippi can obstruct a recovery of these populations and ultimately lead to their extirpation. These factors and low genetic diversity indicate that these populations might be experiencing inbreeding depression. The objective of this thesis research was to use molecular genetic data to study the genetic variability of two populations of gopher tortoises in Mississippi. The second chapter of this thesis tests for a correlation between genetic variation and fitness using survivorship as a fitness parameter. I investigated genetic variation among gopher tortoises from two sites that have different recruitment success, and I compared the genetic variation between tortoises of different age classes at both sites to look for any disparity in heterozygosity. In the third chapter, I present the results of parentage analyses for populations from two study sites and patterns in the reproductive contributions of adults from temporal and spatial perspectives. I report the patterns in morphological characteristics of reproducing and non-reproducing adults from one of the study sites.

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CHAPTER II

GENETIC VARIATION AND RECRUITMENT IN TWO POPULATIONS OF GOPHER TORTOISES, *GOPHERUS POLYPHEMUS*, IN SOUTH MISSISSIPPI

Introduction

The longleaf pine, *Pinus palustris*, ecosystem was once the dominant forest ecosystem of the southeast US and covered approximately 38 million ha (Frost, 1993). With the advent of anthropogenic habitat alteration for agriculture, silviculture, urbanization, and wildfire suppression, the longleaf pine ecosystem has been reduced by more than 98% from pre-1880 to 1986 (Noss et al., 1995). The decline of the gopher tortoise, *Gopherus polyphemus*, is closely associated with that of the longleaf pine ecosystem (Auffenberg and Franz, 1982). Due to habitat fragmentation and population declines in the western portion of the species' range, gopher tortoises in Louisiana, Mississippi, and west of the Tombigbee and Mobile Rivers in Alabama are listed as federally threatened (U.S. Fish and Wildlife Service, 1987). Because of range-wide declines, populations found in the eastern portion of the range, in Alabama east of the Tombigbee and Mobile Rivers, Florida, Georgia, and South Carolina, are also of conservation concern and are protected on the state level.

The De Soto National Forest (DNF) contains the largest remnants of gopher tortoise populations in Mississippi and consequently, it has been the focus of most of the gopher tortoise research and conservation efforts in the western portion of the species' range. Although these populations are protected, they continue to decline. On specific sites within the DNF, number of active and inactive gopher tortoise burrows had decreased by approximately 35.7% between 1995 and 2007 (Hammond, 2009). Multiple

studies have suggested that these gopher tortoise populations suffer from problems that do not affect the eastern populations so dramatically. For example, recruitment in gopher tortoise populations across the range is generally low; however, recruitment in Mississippi populations may be further hindered by unusually low hatching success rates. Multiple year studies have shown that protected nests of the DNF in south Mississippi yield lower hatching success rates than protected nests in the eastern portion of the range (Epperson and Heise, 2003; Noel, 2006; Hammond, 2009). Additionally, frequency of embryo mortality is elevated in clutches of the DNF. Embryos discovered in eggs that failed to hatch were almost fully developed and near hatching (Noel, 2006; Hammond, 2009). Populations of the DNF also have lower genetic diversity than their eastern conspecifics. A microsatellite study of gopher tortoise populations revealed that Mississippi populations of the DNF had significantly lower number of alleles per locus, lower heterozygosity, and lower percent of polymorphic loci than eastern populations of Florida and Georgia included in the analysis (Ennen et al., 2010). Steep population declines, such as that observed in the DNF, may account for a loss of genetic variation. Low hatching success, increased number of late stage embryo mortalities, and low recruitment observed in gopher tortoise populations in Mississippi are alarming because these factors will obstruct a recovery of these populations and could ultimately lead to their extirpation. These factors and low genetic diversity indicate that these populations might be experiencing inbreeding and its unfavorable consequences.

Inbreeding depression is a particular concern in populations that have undergone population declines which increase the likelihood that close relatives will breed. Negative effects on fitness traits of inbred offspring have been observed in captive and wild

vertebrate populations. Studies involving populations of wild bird species frequently report reductions in hatching rate, offspring survivorship, or recruitment (reviewed in Keller and Waller, 2002). Lower heterozygosity throughout the genome, a result of inbreeding, may be detectable using neutral microsatellite loci. Heterozygosity-fitness correlations (HFCs), statistical relationships between marker heterozygosity and one or more fitness traits, have been documented in wild populations.

Recently, a gopher tortoise population was discovered just outside of the western most portion of the DNF in Hillsdale, Mississippi (Tom Mann, pers. comm.). The population is located on privately owned land that is predominantly an undeveloped subdivision. The Hillsdale population is atypical for the listed portion of the range in that it has an unusually high proportion of juvenile and subadult burrows. Approximately half of all active burrows belong to tortoises of the juvenile and subadult age classes (pers. obs.). The high proportion of burrows of multiple age classes suggests that there is higher recruitment compared to populations of the DNF. The genetics of this population have not been previously studied. The discovery of this high recruitment site in Mississippi presented an opportunity to assess the genetic variability of this population and compare it to a low recruitment site and test for positive relationships between genetic diversity and fitness.

In this study, I used molecular genetic data based on 13 microsatellite loci to test for a correlation between genetic diversity and fitness using survivorship as a fitness parameter. Measures of genetic diversity used for analyses included observed number of alleles adjusted for sample size (adjusted allelic richness), observed heterozygosity, expected heterozygosity (as predicted from Hardy-Weinberg expectations), and

multilocus heterozygosity (proportion of heterozygous loci per individual). I compared these measures at three levels to test for positive associations between genetic diversity and survivorship. On the site level, I compared genetic diversity between gopher tortoise populations from two sites that have different recruitment success. On the age class level, I compared the genetic variation between hatchlings, juvenile/subadults, and adults at both sites to look for any disparity in genetic variability between age classes. Lastly, I compared genetic diversity between successful hatchlings and late stage embryo mortalities from both sites in south Mississippi.

Materials and Methods

Low Recruitment Study Site

The Camp Shelby Joint Forces Training Center, a Mississippi Army National Guard training site, is located within the DNF in Forrest and Perry Counties, Mississippi. Camp Shelby currently supports approximately 2000 tortoises throughout the installation (Matt Hinderliter, pers. comm.). The topsoil at Camp Shelby is a sandy loam with a moderate clay content and is designated a suitable soil site for gopher tortoises as defined by the USFWS (McDearman, 2005). This study focuses on gopher tortoises located on Training Area 44 West Road and Training Area 44 East Road (henceforth referred to collectively as T44) at Camp Shelby. At T44 there are approximately 100-150 tortoises (Matt Hinderliter, pers. comm.). Forested areas at T44 are dominated by longleaf pine (*Pinus palustris*) in the overstory, winged sumac (*Rhus copallina*), blueberry (*Vaccinium* spp.), and oaks (*Quercus* spp.) in the midstory, and bluestem grasses (*Andropogon* spp. and *Schizachyrium* spp.) in the understory. Ruderal areas are dominated by the same

grasses, as well as goat's rue (*Tephrosia virginiana*) and sensitive briar (*Schrankia microphylla*) (habitat description from Noel, 2006).

High Recruitment Study Site

The Hillsdale population is a relatively small population of gopher tortoises located on private land in Pearl River County, Mississippi. The population has not been previously studied and is not currently managed. Hillsdale is designated a priority soil site for its deep sandy soil (McDearman, 2005). Hillsdale is a longleaf pine (*Pinus palustris*) and turkey oak (*Quercus laevis*) sandhill with a midstory of yaupon (*Ilex vomitoria*), blueberry (*Vaccinium* spp.), and other oaks (*Quercus* spp.). The understory is dominated by bluestem grasses (*Andropogon* spp. and *Schizachyrium* spp.), reindeer moss (*Cladonia* spp.), prickly pear cactus (*Opuntia humifusa*), and gopher apple (*Licania michauxii*) (pers. obs.).

Sample Collection

Gopher tortoise eggs were collected during the nesting seasons of 2010 and 2011 from T44 at Camp Shelby and Hillsdale. To find nests, active burrow aprons were probed by hand daily during the nesting season. Once nests where identified, all eggs were excavated and incubated in the laboratory. After emergence from eggs, each hatchling was assigned a unique number and marked by clipping the marginal scutes accordingly (Cagle, 1939). Scute clippings were stored in 95% ethanol until DNA extraction. Any unhatched eggs were dissected and tissue samples from any failed late stage embryos were collected and stored similarly to scute clips. In 2006, gopher tortoises were trapped from all active burrows at T44, and blood samples were collected for a genetic study comparing diversity between Mississippi gopher tortoises and those in the eastern portion

of the range (Ennen et al., 2010). Because adult mortality is low and no subadults were found in 2006, we expect these sixty-three adults or a reduced subset thereof to be the same individuals producing offspring in 2010 and 2011, and we will use them as the adult age class from T44 for our analyses. Adult tortoises from Hillsdale were captured in July and September of 2011. To trap adult tortoises, Tomahawk Model 18 Live Traps (81.28 × 25.4 × 30.48 cm) were placed over the mouth of active burrows and were checked each day that the traps were set. Juveniles and subadults from Hillsdale were sampled in June and August of 2010 using bucket traps. Buckets (3.47 L) were buried in front of burrows so that the top of the bucket was even with ground level. A piece of newsprint was placed over the opening concealing it but allowing the tortoise to fall into the bucket upon exiting the burrow. A 23-gauge needle and 1 mL syringe were used to draw a blood sample from the brachial or femoral vein of captured tortoises. Approximately 0.5-1.0 mL of blood was drawn and stored in 0.5 mL tissue preservation buffer (Seutin et al., 1991). Sex of mature adults was determined by examining the degree of concavity of the plastron and length of gular projections (McRae et al., 1981).

Molecular Techniques

Total genomic DNA was extracted from scute clips, late stage embryo tissue, or whole blood using Qiagen DNeasy extraction kits (Qiagen Inc., Valencia, California, USA). Dilutions of DNA samples were performed when necessary to achieve successful amplifications. Each individual was genotyped at 13 microsatellite loci: *GopoA009*, *GopoB103*, *GopoC001*, *GopoD004*, *GopoD006*, *GopoD007*, *GopoD011*, *GopoD107*, *GopoD128* (Kreiser et al., 2013), *Gopo-01*, *Gopo-02*, *Gopo-05*, and *Gopo-12* (Tuberville et al., 2011). Loci were chosen based on variability in a range wide study (Gaillard,

unpubl. data) or ability to be multiplexed with highly variable loci. Polymerase chain reactions (PCRs) were performed in 12.5 µl reactions consisting of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.0 mM MgCl2, 0.6 mM dNTPs, 0.1875 units of Taq DNA polymerase (New England BioLabs), 0.3 µM of M13 tailed forward primer, 0.3 µM reverse primer, 0.1 µM of M13 labeled primer (LI-COR), 10-100 ng of template DNA, and water to the final volume. PCR cycling conditions consist of an initial denaturing step at 94°C for 2 minutes followed by 35 cycles of denaturing for 30 s at 94°C, primer annealing for 1 minute at 56-60°C, and elongation for 1 minute at 72°C, with a final 7 minute elongation step at 72°C. Microsatellite alleles were visualized on acrylamide gels using a LI-COR 4300 DNA Analysis system, and gel images were scored using Gene ImagIR v. 3.55 (LI-COR Biosciences, Lincoln, Nebraska, USA) or scored visually. In cases of ambiguous allele scores, samples were rerun for verification.

Analyses

I used GENEPOP v. 4.0.10 (Raymond and Rousset, 1995) to calculate number of alleles per locus, expected heterozygosity, and observed heterozygosity. GENEPOP was used to test for deviations from Hardy-Weinberg equilibrium (HWE) at each locus using a Fisher's exact test with a Markov chain method to estimate the *p* value (Guo and Thompson, 1992). Tests for linkage disequilibrium between loci were also performed in GENEPOP. MICRO-CHECKER v. 2.2.3 was used to detect typing errors and null alleles. It uses observed alleles to create randomized genotypes and compares those to the observed genotypes. Null alleles are characterized by a significant excess in homozygotes evenly distributed across all allele size classes (van Oosterhout et. al, 2004). Prior to any statistical tests, allelic richness (A_R) was adjusted using a rarefaction method in HP-

RARE 1.0 (Kalinowski, 2005) to correct for differences in sample size of groups being compared. All univariate statistical analyses were completed in JMP v. 7.0.1 (SAS Institute, 2007), and a significance level of 0.05 was used for all tests. I used a two factor, factorial analysis of variance (ANOVA) for each measure of genetic diversity with site and age class as fixed factors to compare genetic diversity between adults and hatchlings and to compare genetic diversity between sites in a single analysis. When parametric assumptions were met, I used *t*-tests (with pooled or unpooled variances) to compare genetic diversity between adults and hatchings at a single site. When assumptions for parametric tests were not met, I used the nonparametric equivalent, Wilcoxon Rank Sum or Kruskal-Wallis tests with Chi Square approximation.

Additionally, I used an information-theoretic approach to determine which factors are the best predictors of genetic diversity. The Akaike Information Criterion (AIC) was used to determine which a priori models of observed variables best explained the observed pattern. It is not a statistical test, and therefore, does not use an arbitrary significance level. Rather, AIC chooses variables that form the most parsimonious model to explain a response variable and then ranks the models (Anderson et al., 2000). Akaike weights (w_i) are used to compare the fit of one model relative to other models. To correct for small sample size, I used the modified criterion (AIC_c) as suggested by Anderson et al. (2000). For each AIC_c, the response variable was one of the three measures of genetic diversity: adjusted allelic richness, observed heterozygosity, and expected heterozygosity. All AIC analyses were performed in R v. 2.15.0 (R Development Core Team, 2012).

Results

A total of 222 individuals from three age classes were collected from the two sites. A total of 28 adults, 47 hatchlings, and 22 juveniles/subadults from Hillsdale were genotyped, as were 63 adults and 62 hatchlings from T44. No juveniles or subadults were collected from T44 because this population lacked individuals in these age classes. A large proportion of the eggs that were collected and incubated in the laboratory failed to hatch. Most failed eggs revealed that development had arrested in the earliest stages. Across both years, late stage embryo mortalities occurred in 5.1% (4 of 78) of eggs collected from Hillsdale and 12.1 % (11 of 91) of eggs collected from T44. Three hatchlings that died within 3 to 6 days post hatching were also genotyped. Two of the three belonged to the same clutch, and the third had an abnormally soft shell (A. Holbrook, pers. comm.). Because these individuals did hatch out of the egg, these were categorized as successful hatchlings for analyses.

Across all samples genotyped, the 13 microsatellite loci had 2 – 13 alleles per locus with observed heterozygosity ranging from 0.39 – 0.80 (mean = 0.59, SE ± 0.02) and expected heterozygosity ranging from 0.41 – 0.81 (mean = 0.60, SE ± 0.02). Linkage disequilibrium was detected between loci *B103* and *C001* among T44 adults after a sequential Bonferroni correction (Rice, 1989), but not among any loci in Hillsdale adults. Locus *D004* and locus *Gopo01* deviated significantly from Hardy Weinberg expectations, and MICRO-CHECKER revealed that the presence of null alleles might be responsible for departures from Hardy Weinberg expectations at these two loci in T44 adults. Due to an excess of missing data at locus *D007* among Hillsdale juveniles and subadults, locus *D007*was omitted from the analysis comparing Hillsdale adults, juveniles/subadults, and

hatchlings. Two late stage embryo mortalities were excluded because of unsuccessful PCR amplifications which were likely due to poor DNA quality.

The following measures of genetic diversity were calculated for each group: allelic richness (A_R), observed heterozygosity (H_o), expected heterozygosity (H_e), and multilocus heterozygosity (MLH) are reported with standard deviations (Table 1). For ease of reporting results, mean number of alleles (A) is reported in Table 1 instead of A_R because a separate rarefaction was run for each comparison between groups. When testing the effects of site and age on genetic diversity measures, parametric test assumptions were met when allelic richness, observed heterozygosity, and expected heterozygosity were the dependent variables. For each of the three ANOVAs, the interaction term was not significant and neither of the two factors, site nor age, was significantly different between Hillsdale and T44 (Table 2). To test the effect of site and age class on MLH, nonparametric methods were necessary. Two Wilcoxon Rank Sum tests were used. MLH was not significantly different between adults and hatchlings ($\chi^2 = 2.371$, $P = 0.124$), but MLH was significantly higher among tortoises of T44 than those of Hillsdale ($\chi^2 = 17.906$, $P < 0.0001$).

Table 1

Mean Number of Alleles (A), Observed Heterozygosity (H_o), Expected Heterozygosity (H_e), and Multilocus Heterozygosity (MLH)

	Hillsdale Hatchlings (N = 40)	Hillsdale Juveniles/sub -adults (N = 22)	Hillsdale Adults (N = 28)	T44 Hatchings (N = 51)	T44 Adults (N = 63)
A	3.85 ± 1.14	4.33 ± 2.02	5.15 ± 2.19	5.15 ± 2.61	6.00 ± 2.94
H_o	0.514 ± 0.146	0.578 ± 0.172	0.590 ± 0.162	0.627 ± 0.166	0.618 ± 0.161
H_e	0.519 ± 0.124	0.602 ± 0.132	0.620 ± 0.156	0.610 ± 0.147	0.629 ± 0.148
MLH	0.516 ± 0.132	0.579 ± 0.136	0.589 ± 0.103	0.628 ± 0.117	0.618 ± 0.117

Note. Reported values with standard deviations arranged by site and age class.

Table 2

ANOVA or Wilcoxon Rank Sum Test Results for Both Hillsdale and T44

	A_R	H_o	H_e	MLH
Site	$F_{1,48} = 1.531$; $P = 0.222$	$F_{1,48} = 2.588$; $P = 0.114$	$F_{1,48} = 1.519$; $P = 0.224$	$\chi^2 = 17.906$; $P < 0.0001$*
Age class	$F_{1,48} = 2.357$; $P = 0.131$	$F_{1,48} = 0.591$; $P = 0.446$	$F_{1,48} = 2.247$; $P = 0.140$	$\chi^2 = 2.371$; $P = 0.124$
Interaction	$F_{1,48} = 0.774$; $P = 0.383$	$F_{1,48} = 0.940$; $P = 0.337$	$F_{1,48} = 1.031$; $P = 0.315$	

Comparing the level of genetic diversity in adults and hatchlings from each site revealed differences between sites. For the Hillsdale population, observed heterozygosity and expected heterozygosity of adults was not significantly different than that of hatchlings. However, both allelic richness ($t = -2.166$; $P = 0.044$) and MLH ($t = -2.489$; $P = 0.015$) among Hillsdale adults was significantly higher than that of hatchlings. For the T44 population, none of the measures of genetic diversity tested were significantly different among adults and hatchlings (Table 3).

Table 3

The Results of t-test Comparisons or Wilcoxon Rank Sum Tests between Adults and Hatchlings at Each Site

	A_R	H_o	H_e	MLH
Hillsdale	$t = -2.166$; $P = 0.044$*	$t = -1.265$; $P = 0.218$	$t = -1.821$; $P = 0.081$	$t = -2.489$; $P = 0.015$*
T44	$t = -0.616$; $P = 0.544$	$t = 0.138$; $P = 0.891$	$t = -0.334$; $P = 0.741$	$\chi^2 = 0.293$; $P = 0.589$

Because juveniles and subadults were captured from the Hillsdale population in addition to adults, a Kruskal Wallis test was used to determine whether differences in genetic diversity among the three age classes at that site were significant. Only one Kruskal Wallis test yielded a significant result: MLH was significantly different among age classes ($\chi^2 = 6.705$; $P = 0.035$). A post-hoc comparison of all pairs of age classes using the Wilcoxon method revealed that MLH of adults was significantly higher than that of hatchlings ($P = 0.015$), but MLH of juveniles/subadults was not significantly different from that of adults ($P = 0.760$) or hatchlings ($P = 0.088$). There were no

significant differences in allelic richness, observed heterozygosity, or expected heterozygosity among the three age classes from Hillsdale (Table 4).

Table 4

Kruskal Wallis Test Results for Comparisons Between Hatchlings, Subadults, and Adults at Hillsdale

	A_R	H_o	H_e	MLH
Hillsdale	$\chi^2 = 2.367$; $P = 0.306$	$\chi^2 = 1.156$; $P = 0.561$	$\chi^2 = 1.484$; $P = 0.476$	$\chi^2 = 6.705$; $P = 0.035$*

Note. Locus *D007* was omitted from these comparisons due to missing data for the juvenile/subadult age class. If a significant difference was detected from a Kruskal Wallis test, I performed multiple comparisons for each pair using the Wilcoxon method. For MLH there was a significant difference between adults and hatchlings ($P = 0.0147$), but no significant difference between hatchlings and juveniles/subadults ($P = 0.088$) or juveniles/subadults and adults ($P = 0.760$).

To assess differences in genetic diversity between successful hatchlings and late stage embryos mortalities, I pooled hatchlings from both sites and both years due to small sample size of late stage embryo mortalities. Allelic richness, observed heterozygosity, and expected heterozygosity did not differ significantly among successful hatchlings and late stage embryo mortalities (Table 5). However, MLH of late stage embryo mortalities was significantly higher than that of successful hatchlings ($\chi^2 = 7.118$, $P = 0.008$).

Table 5

The Results of t-test Comparisons or Wilcoxon Rank Sum Tests Comparing Successful Hatchlings to Late Stage Embryo Mortalities from Both Sites

	A_R	H_o	H_e	MLH
Hillsdale and T44 combined	$t = -0.182$; $P = 0.857$	$t = -1.814$; $P = 0.082$	$t = 0.038$; $P = 0.970$	$\chi^2 = 7.118$; $P = 0.008$*

I performed four AIC_cs to determine whether age, site, or a combination of the two formed the best model for explaining each measure of genetic diversity in our groups. For each AIC_c, I assessed 5 models: null with no variables, age, site, age and site, and age and site with an interaction effect. In both the AIC_c for allelic richness and the AIC_c for expected heterozygosity, none of the models with variables had a better fit than the null model (Table 6). The AIC_c for observed heterozygosity resulted in the site model having the best fit ($\Delta AIC_c = 0.0$, $wi = 0.4056$). In the AIC_c for MLH, the age and site interaction model was the best fit ($\Delta AIC_c = 0.0$, $wi = 0.668$), followed by the site model ($\Delta AIC_c = 2.5$, $wi = 0.192$) and the age and site model ($\Delta AIC_c = 3.1$, $wi = 0.140$). Because the models with site were generally better than those with age, there appeared to be strong site (population) differences, weak age differences and a strong interaction between the two.

Table 6

Results of AIC_c Analysis Including Their ΔAIC_c Scores and Weights

Response variable	Model	ΔAIC_c	w_i	Evidence ratio
	Null	0.0	0.2667	
	Age	0.0	0.2642	1.009
A_R	Site	0.6	0.1948	1.369
	Age, Site	0.7	0.1908	1.398
	Age * Site	2.3	0.0836	3.190
	Site	0.0	0.4056	
	Null	0.9	0.2636	1.539
H_o	Age, Site	2.0	0.1481	2.739
	Age	2.8	0.1000	4.056
	Age * Site	3.2	0.0828	4.899
	Null	0.0	0.2984	
	Age	0.2	0.2761	1.081
H_e	Site	1.0	0.1834	1.627
	Age, Site	1.2	0.1661	1.797
	Age * Site	2.7	0.0759	3.931
	Age * Site	0.0	0.668	
	Site	2.5	0.192	3.479
MLH	Age, Site	3.1	0.140	4.771
	Age	17.1	<0.001	>668
	Null	18.2	<0.001	>668

Note. The AIC_c is scaled so that the minimum AIC_c is 0 ($\Delta_i = AIC_{ci} - \min AIC_c$). The Akaike weights ($w_i$) for each model and evidence ratio (w_j / w_i) in which the weight of the best model (w_j) is compared to the weight of any particular model (w_i).

Discussion

Despite lower recruitment within the T44 population, genetic diversity was higher among gopher tortoises of T44 than those of Hillsdale. This unexpected result may be due to inherent differences between the two sites. Possible explanations for differences in recruitment include differences in population demographics and environmental factors.

However, within Hillsdale, some differences in genetic variability do seem to be associated with age class.

Variation in Population Demographics between Sites

One aspect that differs between the two study sites is adult population size. At Hillsdale we were able to thoroughly trap most, if not all, of the adults present in areas where we conducted nest searches. Thus, the 28 adults probably represent the bulk of the population at Hillsdale. This is substantially lower than the estimated 100-150 tortoises at T44. Population size, and more importantly effective population size (N_e), can strongly influence population genetic diversity. When populations are small, random genetic drift will drive alleles to loss or fixation at faster rates than larger populations. The loss or fixation of alleles results in loss of heterozygosity and lower population genetic diversity (Crow and Kimura, 1970).

Potential differences in the level of gene flow with neighboring populations could be another factor generating higher genetic diversity at T44 than at Hillsdale. The gopher tortoise population at T44 is in close proximity to other known gopher tortoise populations within Camp Shelby. Camp Shelby encompasses nearly 546 km^2 (211 mi^2) and has approximately 2000 tortoises throughout the installation (Matt Hinderliter, pers. comm.). In a study of 34 gopher tortoise populations (colonies) of Camp Shelby, very little genetic differentiation was identified between the colonies, indicating that perhaps some genetic connectivity exists (or recently existed) between nearby colonies (Richter et al., 2011). The seemingly isolated nature of the Hillsdale gopher tortoise population may be limiting gene flow, contributing to high rates of genetic drift, and lowering population genetic diversity. Admittedly, the degree of isolation of this population may be an

overestimation as surveys of the surrounding area are not extensive. Given the difficulty in accessing private lands, surveys focused on lands adjacent to the known population and areas with priority soil and open canopy identified using soil maps and available aerial imagery. No areas neighboring the Hillsdale population that harbor any significant number of tortoises have been recently identified.

Variation in Habitat Quality between Sites

Apparent differences in recruitment levels between sites could be driven by differences in environmental factors affecting gopher tortoise habitat quality. Soil type, vegetation, and sunlight at ground level have all been recognized as key components limiting gopher tortoise distribution. gopher tortoise densities are usually higher in xeric habitats which have well-drained, sandy soils (Auffenberg and Franz, 1982). The well-drained, sandy soils are thought to be better suited for the excavation of burrows for refugia than other more compacted soil types. However, when other habitat characteristics are favorable, they can occur in areas with higher clay content soils, such as those more common in Mississippi (Auffenberg and Franz, 1982). Perhaps, soil type has had a more profound impact on recruitment and should be given more consideration. Shortly after emergence from the nest, hatchlings must dig their own burrows, and hatchlings are especially vulnerable during this period. Priority soils at Hillsdale may explain why more hatchlings are able to persist, while more clay rich soils at T44 may keep hatchlings exposed to the environment and defenseless against predators. Future research is needed to determine hatchlings' ability to dig in sandy versus clay rich soils.

Types of predators and predation intensity vary throughout the range and also vary between sites. Gopher tortoise hatchlings and eggs have low survivorship, mainly

due to increased vulnerability to predators during these early life stages. Mammals have most often been identified as predators of hatchlings throughout the range (Landers et al., 1980; Butler and Sowell, 1996; Epperson and Heise, 2003), but fire ants also contribute considerably to hatchling mortality (Landers et al., 1980; Smith, 1995; Epperson and Heise, 2003). Causes of hatchling mortality have been studied at Camp Shelby using radio telemetry. Across sites at Camp Shelby, 26.8% of hatchlings (over four years) appeared to have died from red imported fire ants, *Solenopsis invicta* (Epperson and Heise, 2003). In a two-year study of north-central Florida hatchlings, 12% of hatchlings died from ants but most frequently from a native fire ant species, *Solenopsis geminata* (Smith, 1995). During a survey of baited gopher tortoise burrows in southeast Florida, *Solenopsis invicta* was the most abundant ant species present; they recruited more individuals to baits, and occurred more often at "edge burrows" than "interior burrows" (Wetterer and Moore, 2005, p. 352). If fire ants are less pervasive at Hillsdale, as they seem to be (Tom Mann, pers. com.), then the threat that fire ants pose to hatchlings could be substantially lower. However, causes of hatchling mortality and predation intensity at Hillsdale have not yet been studied.

Correlation of Genetic Variation and Survivorship to Next Age Class

Our analyses did find some disparity in genetic variation between age classes of gopher tortoises. Within the Hillsdale population, allelic richness and multilocus heterozygosity of adults was greater than that of hatchlings. Juveniles/subadults had intermediate levels of genetic variation, although no measures were significantly different from that of the adult or hatchling age classes. While only allelic richness and multilocus heterozygosity were significantly higher among adults, all other measures of genetic

variation also followed this trend. This suggests that genetic diversity and survivorship were correlated: older individuals in the Hillsdale population had higher levels of genetic variation than younger individuals. Surprisingly, this correlation was not consistent across both sites. No significant differences in genetic variation were observed between adult and hatchling age classes of gopher tortoises from T44, and there was no directionality in the level of genetic variation toward either age class observed in this population.

A greater proportion of heterozygotes in the adult age class may be indicative of a heterozygosity-fitness correlation (HFC) in the Hillsdale population. HFCs have been detected in natural and captive populations and are frequently used to indirectly study the effect of inbreeding on fitness. Although the utility of marker heterozygosity as a proxy for inbreeding coefficients is hotly debated, HFCs have been reported in microsatellite studies of natural populations of vertebrates. In wild wolves, *Canis lupus*, multilocus heterozygosity and breeding success were positively correlated (Bensch et. al, 2006). A study that genotyped sibling dyads of great reed warblers, *Acrocephalus arundinaceus*, showed that siblings recruited into the population had significantly higher multilocus heterozygosity than non-surviving siblings (Hansson et. al, 2004). In house sparrows, *Passer domesticus*, a significant positive relationship was detected between recruitment of fledglings and multilocus heterozygosity (Jensen et al., 2007). Inbreeding increases homozygosity due to allelic co-ancestry, and can cause correlations between functional loci and selectively neutral marker loci throughout the genome (i.e., identity disequilibrium) (Weir and Cockerham, 1973). Although, there was not a statistically significant correlation between genetic diversity and survivorship among gopher tortoises

at T44, it is premature to dismiss that inbreeding could be occurring in the population. Lack of evidence of an HFC can be influenced by historic population size and historical population genetic structure. Small populations such as Hillsdale may be more likely to exhibit evidence of an HFC because it may be more prone to the effects of random genetic drift on mutational load (Kimura et al., 1963).

Successful Hatchlings vs. Late Stage Embryo Mortalities

Despite expectations, late stage embryo mortalities had a significantly higher MLH than successful hatchlings. However, we should treat this result with caution for a variety of reasons. Due to small sample sizes of late stage embryo mortalities, I combined individuals from both sites. Absence of a HFC at T44 could have negated the signal at Hillsdale. It is also important to note these hatchlings which I consider "successful" were hatched under ideal laboratory conditions and may not have hatched in the wild. Additionally, selection may have already acted upon highly inbred individuals by preventing embryo formation altogether and purging those individuals prior to hatching.

Conclusions

Among gopher tortoise populations in Mississippi, Hillsdale is a rarity in that it has a higher level of recruitment than many other populations, even those managed for years on federal land. After examination of the genetic variability in all age classes, my data show evidence of potential inbreeding effects in this population. When compared to a larger population within the DNF, Hillsdale had lower genetic diversity and its age classes demonstrated a HFC. Evidence of a HFC can be used as an indicator that genetic variability is declining in a population (Szulkin et al., 2010). Decline of genetic variability can increase the likelihood of extirpation of a population because it lacks the

ability to adapt to environmental stochasticity (Allendorf and Leary, 1986). Survival of this species in the state and in the western portion of the species range depends on the protection and management of populations on federal and private lands. Although much attention is paid to improving habitat for adults, long-term viability depends on propagation and recruitment of younger individuals who are genetically variable.

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CHAPTER III

PARENTAGE ASSESSMENTS OF TWO POPULATIONS OF THREATENED GOPHER TORTOISES, *GOPHERUS POLYPHEMUS*

Introduction

Conservation efforts of threatened and endangered species mainly focus on acquiring and managing habitat and preserving or increasing population sizes for population viability. However, from an evolutionary perspective there is also a need to maintain genetic variation in populations. Dramatic reductions in population size can lower genetic diversity. Small populations can become more susceptible to the effects of genetic drift and inbreeding. Genetic drift can lead to a loss of genetic variation, while mating between closely related individuals can have negative effects on the fitness of offspring.

In addition to the size of a population, particular life history traits such as reproductive strategy and the ability or propensity to disperse can influence the amount of genetic variation maintained within a population, and thus have important conservation implications. Type of mating system can impact genetics and viability of threatened and endangered species. Mating systems have important implications for conservation because they reveal information about effective population size.

Gopher tortoises, *Gopherus polyphemus*, are semi-fossorial land tortoises that are well adapted to xeric habitats with sandy soil types for excavating burrows, open canopies for thermoregulation, and persistence of low herbaceous plants for foraging (Auffenberg and Franz, 1982). *Gopherus*, like other turtle species, have late maturity and high juvenile mortality which heightens their vulnerability to habitat loss, degradation,

and population declines caused by humans (Ernst et al., 1994). Gopher tortoise populations have historically been declining across the range since the late 1800's (Auffenberg and Franz, 1982), and significant population declines and habitat fragmentation in portions of the range have led to western populations being listed as federally endangered (U.S. Fish and Wildlife Service, 1987).

Success of gopher tortoise populations depends not only on the protection and persistence of adult tortoises, but also on the ability of those adults to produce successful offspring. Hatching success of predator-excluded nests from the De Soto National Forest (DNF) in south Mississippi ranges from 16.7 - 46.6% (Epperson and Heise, 2003; Noel, 2006; Hammond, 2009). In the eastern portion of the range, hatching success of predator-excluded nests is generally higher and ranges from 67-97%; (Landers et al., 1980; Smith, 1995; Butler and Hull, 1996; Demuth, 2001). Low hatching success and low recruitment of Mississippi populations may be rooted in environmental factors, maternal effects, genetic factors, or any combination of these. One factor that may be reducing egg viability is genetic variability. When genetic diversity in populations of the DNF was compared to eastern populations of Florida and Georgia, there were lower levels of genetic diversity in the Mississippi populations than those in the east (Ennen et al., 2010).

Much attention is paid to census population sizes, but from a genetics perspective effective population size (N_e) reveals more about the state of a population. A variety of factors influence N_e, one of which is the variance in reproductive success among individuals in the population. The mating system of a species (e.g., monogamy vs. multiple mating; sperm storage; male-male competition) plays a role in determining the variance in reproductive success. Within populations, reproductive success may not be

distributed equally among all individuals and a large amount of variance might exist. Therefore, the passing of genetic content from one generation to the next largely depends on the particular mating system of a population. In gopher tortoises, both sexes mate with multiple partners (Boglioli et al., 2003; Johnson et al., 2007). Early behavioral studies indicated that female defense polygyny might be the mating system used by gopher tortoises. Occasions of aggressive male-male interactions and dominant behavior of larger males over smaller males supported the thought that males defended access to an aggregation of females (Douglass, 1976). Studies involving radio telemetry suggest that the mating system may be more similar to scramble competition polygyny where females are not in defendable aggregations, and success in males has more to do with encounters with receptive females (Boglioli et al., 2003, Johnson et al., 2009). Only two paternity studies have been conducted on gopher tortoises, and far less is known about the mating systems of populations within the federally listed portion of the species' range. *Gopherus* is among many genera of turtles in which females are capable of long-term sperm storage and are able to store sperm from multiple mating events (Palmer et al., 1998). A mixed-paternity clutch can therefore result from multiple matings in the same season or use of stored sperm from a previous season or a combination of the two. This kind of temporal polyandry, polyandry in the stricter sense, and polygyny among gopher tortoises increases the effective size of a population. In paternity studies of gopher tortoises, Moon et al. (2006) detected multiple paternity in 28.6% of clutches (2 of 7) and Tuberville et al. (2011) detected it in 57.1% of clutches (8 of 14) of a translocated population. Multiple paternity is important in assessing the potential for higher reproductive contributions from a variety of males as opposed to strict monogamy, but it is just one aspect of what

can be inferred from the parentage analysis. This type of analysis can reveal which individuals are mating, how often they are reproducing, and how successful are they when they reproduce. Moon et al. (2006) found that males assigned as sires had significantly larger carapace length than males who did not sire clutches which could be evidence that larger males (and/or older males) have a reproductive advantage over smaller males. Tuberville et al. (2011) found a similar trend, and also found that previously established males were more reproductively successful than males recently translocated into the population.

I conducted microsatellite-based parentage analyses to investigate patterns of reproductive success of gopher tortoises at two sites that vary in many aspects such as size and habitat quality within the protected portion of the species' range. Using assignments from parentage analyses, I examined individual reproductive success and frequency of reproduction in adults over two years. In two populations, how many individuals are contributing offspring and are they doing so both years? Secondly, I looked for patterns in the morphological characteristics of reproductively successful individuals compared to non-reproducing individuals. For example, do larger males contribute disproportionately to the reproductive efforts within a year? Lastly, I examined the spatial dynamics between the burrows of mating individuals and nest sites at both sites. Radio telemetry studies have established home range sizes for many populations of gopher tortoises, but without genetic testing we are unable to discern which movements are for mating purposes and which are for other purposes. Spatial dynamics of small isolated populations also could be vastly different than that of larger, more contiguous populations.

Materials and Methods

Study Sites

I conducted a parentage assessment to investigate the mating system of populations of gopher tortoises from two sites in south Mississippi. One of the sites, Training Area 44 (T44) at the Camp Shelby Joint Forces Training Center, is located in Forrest and Perry Counties and is a part of the Desoto National Forest (DNF). The second study site, Hillsdale, is approximately 33 km southwest of T44 and lies just outside of the DNF in Pearl River County. The population of gopher tortoises at Hillsdale is on privately owned land, which unlike T44, is not managed for gopher tortoises. Although both populations occur in the protected portion of the species range, both differ in several characteristics including habitat quality, population size, recruitment, and genetic variation (See Chapter II).

Sample Collection

During the 2010 and 2011 nesting seasons, which last from mid-May to late-June, clutches of eggs were collected from both sites. The aprons of active adult burrows were probed by hand daily. When a freshly laid nest was discovered, the burrow location was recorded, and the entire clutch was excavated and carefully transported to the lab where they were artificially incubated until hatching. Within a few days of hatching, tissue samples were taken by clipping the marginal scutes of each hatchling, and samples were stored in 95% ethanol. This also served as a way to uniquely mark each hatchling (Cagle, 1939). Any eggs that grew fungus, became discolored during incubation, and did not hatch were preserved in 95% ethanol for later dissection or were dissected after all other eggs had hatched. If a failed late stage embryo was discovered upon dissection, a tissue

sample was collected and stored in 95% ethanol. Adult tortoises at T44 were sampled in 2006 as a part of a genetic study by Ennen et al. (2010). Adults were trapped by placing Tomahawk Model 18 Live Traps (81.28 × 25.4 × 30.48 cm) over the mouth of active burrows. Approximately 0.5-1.0 mL of blood was collected from the brachial or femoral vein of adult tortoises using a 23-gauge needle with a 1 mL syringe. Blood was stored in 0.5 mL tissue preservation buffer (Seutin et al., 1991) and held at –20°C. Sex of mature adults was determined by examining the degree of concavity of the plastron and length of gular projections (McRae et al., 1981a). Burrow of capture was recorded and associated with known GPS coordinates for previously mapped burrows. Adult tortoises from Hillsdale were collected as exhaustively as possible during July and September of 2011. Trapping methods of tortoises at Hillsdale were the same as those used by Ennen et al. (2010) at T44. At the time of capture, GPS coordinates were taken at the burrow and standard morphological measurements were recorded including mass, carapace length, plastron length, total length, width, thickness, anal notch, and anal width (McRae et al., 1981a).

Molecular Techniques

Qiagen DNeasy extraction kits (Qiagen Inc., Valencia, California, USA) were used to extract total genomic DNA from tissue samples or blood samples. I used 13 polymorphic microsatellite loci to genotype all individuals including nine from Kreiser et al. (2013; *GopoA009*, *GopoB103*, *GopoC001*, *GopoD004*, *GopoD006*, *GopoD007*, *GopoD011*, *GopoD107*, *GopoD128*) and four from Tuberville et al. (2011; *Gopo-01*, *Gopo-02*, *Gopo-05*, and *Gopo-12*). For polymerase chain reaction conditions refer to Chapter II. Microsatellite alleles were visualized on acrylamide gels using a LI-COR

4300 DNA Analysis system. Gel images were scored using Gene ImagIR v. 3.55 (LI-COR Biosciences, Lincoln, Nebraska, USA) or scored visually. In any cases of uncertainty of allele scores, samples were rerun for verification. Samples containing rare alleles were also rerun for confirmation.

Parentage Analyses

To assign parentage to hatchlings, I compared the results from two programs that take different approaches. The program CERVUS v. 3.0 (Marshall, 1998; Kalinowski et. al, 2007) uses a categorical likelihood based approach to assign parentage based on the methods developed by Thompson (1975, 1976) and Meagher (1986). A log-likelihood ratio (LOD) between offspring and a potential parent is calculated and the parent with the highest LOD score is assigned parentage. When neither parent is known, a breeding likelihood is calculated. This is the likelihood of a parental pair producing a specific offspring's multilocus genotype. Assignments are given certain levels of statistical confidence based on the difference in LOD scores (Δ) between the most likely parent or parent pair and the second most likely parent or parent pair. A larger Δ therefore suggests higher statistical confidence in the assignment (Kalinowski et al., 2007). Likelihood methods are useful in parentage assessments because genotyping error, whether it is introduced by scoring error, null alleles, or mutation, can lead to apparent genotypic mismatches between true offspring and parent pairs, causing the false exclusion of an actual parent. Because members of clutches were known in this study, I also used a parental reconstruction method to analyze parentage. COLONY, version 2.0.1.7 (Jones and Wang, 2010) simultaneously considers the genotypes of all members of a progeny

array whether they are full or half siblings when determining parentage. Program parameters can also be adjusted to account for genotyping error and allelic dropout rate.

For the analyses, I separated individuals by site and year, and for each data set I performed 10 replicate runs in COLONY and averaged the likelihood probability for assignments across runs. For each replicate I assumed polygamy for both the male and female mating systems based on previous reports of multiple paternity in gopher tortoises (Moon et al., 2006; Tuberville et al., 2011) and observed courtship behavior (Boglioli et al., 2003). Many of the default parameters were used as suggested by program authors. If replicate runs at *medium* length converged on the same answer, then longer runs were not necessary. Each run was of *medium* run length and used the full-likelihood method, *medium* likelihood precision, and a different random number seed to begin the simulated annealing algorithm. I assumed an error rate of allelic dropout of 0.01 and a genotyping error rate of 0.02.

Special attention was paid to assignments when Cervus or COLONY assigned two or more sires to a clutch. In cases where one program assigned a second sire to a clutch and the other assigned different sires or assigned only one sire, the multilocus genotypes of the putative fathers, putative mother, and offspring were examined by hand. The presence of more than four alleles per clutch for any locus would corroborate the need for a second sire to produce the offsprings' genotypes. When four or fewer alleles were present, but two males were assigned to a clutch I examined whether or not one of the sires could solely explain all of the offsprings' multilocus genotypes. A final assignment was based on determining the minimum number of males necessary to sire a clutch. Although these could represent actual multiple paternity clutches, it is more likely

that the program's assignment of two or more males to a clutch is probably due to those males having similar multilocus genotypes. In cases where a female was assigned to two clutches in the same year, precedence was given to the clutch with the higher probability and the other clutch was left unassigned. When the programs disagreed and there was no clear consensus, the clutch was designated as *unassigned.* This mainly occurred when both programs returned assignments with low probabilities and/or low confidences.

Analysis of Morphological Characteristics

I performed a Principle Components Analysis (PCA) using seven morphological characteristics to determine if any patterns among morphological characteristics existed between reproducing and non-reproducing individuals. Morphological characteristics used in the analysis were total length (TL), plastron length (PL), anal width (AW), anal notch (AN), thickness (TH), width, and mass. I performed separate PCAs for both sexes. Analyses were performed in R v. 2.15.0 (R Development Core Team, 2012).

Analysis of Spatial Dynamics

Using Google Earth v. 6.1.0.5001 (Google, Inc., 2011), I measured the straight line distances between GPS locations of burrows where nests were found and burrows where putative parents were captured. I measured the nearest distance between a putative parent and the location of the nest where the assigned clutch was found. If either parent was captured more than once, I also measured the farthest distance between a putative parent's location and their nest. For each parent pair that was assigned to a clutch, I also measured the nearest distance between mothers and fathers. I used JMP v. 7.0.1 (SAS Institute, 2007) to calculate mean distances for spatial relationships and standard deviations.

Results

Over two nesting seasons, a total of 78 eggs from 12 clutches were collected from the Hillsdale population, and 91 eggs from 20 clutches were collected from the T44 population. Low hatching success was evident in clutches from both populations even though eggs were incubated under ideal laboratory conditions. When failed eggs were dissected, most showed little or no signs of development. However, late stage embryos were found in 15 unhatched eggs: 4 from Hillsdale and 11 from T44. This yielded a total of 47 offspring from Hillsdale (23 eggs from 6 clutches in 2010 and 22 eggs from 6 clutches in 2011) and 62 offspring from T44 (20 eggs from 8 clutches in 2010 and 42 eggs from 10 clutches in 2011). Of the estimated 100-150 tortoises present at T44 (Matt Hinderliter, pers. comm.), 63 adult tortoises were captured in 2006. Of the 63 adults, 31 were males and 32 were females. Extensive trapping efforts at Hillsdale in 2011 led to the capture of 28 adults: 15 males, 13 females, and 3 adults of undetermined sex. All adult, hatchling, and late stage embryo gopher tortoises that were sampled were genotyped at 13 microsatellite loci. Across all samples genotyped, the 13 microsatellite loci had 2 – 13 alleles per locus with observed heterozygosity ranging from 0.39 – 0.80 (mean = 0.59, SE ± 0.02) and expected heterozygosity ranging from 0.41 – 0.81 (mean = 0.60, SE ± 0.02). Because null alleles were suspect in locus *D004* and locus *Gopo01* (See Chapter II), genotypes from those loci were omitted and parentage analyses were performed using genotypes from the 11 remaining loci.

After careful comparison of the results from the two parentage assignment methods, I arrived at a final assignment for each clutch (Table 7 and Table 8). Of the clutches analyzed from Hillsdale, no multiple paternity was detected. Two females from

Hillsdale reproduced in consecutive years, and for one of the females (381A), both clutches were sired by the same male (372A). At the time of capture, the sex of three adult tortoises was not able to be determined using secondary sex characteristics, but the parentage analysis revealed that two of the adults were female. All Hillsdale clutches were single paternity clutches. Two males sired 58.3% of the clutches, 372A sired two clutches both years, and 368A sired one clutch in 2010 and two clutches in 2011. Four cases of multiple paternity were observed in the T44 population in 2011. In clutches 101 and 103, visual examination of multilocus genotypes confirmed five alleles were present among offspring at locus *Gopo-05*. For clutch 105, no more than four alleles were present at any locus, but neither male could have produced all of the offspring genotypes of the clutch. Two females (GPFT18 and GPFT12) from T44 produced clutches in 2010 and 2011. Both of GPFT18's clutches assigned to GPMT48, and both of GPFT12's clutches assigned to the same unsampled putative male. Two males (GPMT26 and GPMT9) were assigned at least partial paternity to more than one clutch in a single year.

Table 7

Maternity and Paternity Assignments for Clutches from Hillsdale

Year	Clutch ID	Mother ID	Father ID	Number of genotyped offspring assigned to Father
2010	86	Unassigned	Unassigned	2 of 2
	88	370A	372A	7 of 7
	95	381A	372A	5 of 5
	96	374A	368A	5 of 5
	97	#1	376A	2 of 2
	98	#1	369A	2 of 2

Table 7 (continued).

Year	Clutch ID	Mother ID	Father ID	Number of genotyped offspring assigned to Father
2011	104	367A	368A	3 of 3
	107	#2	372A	5 of 5
	108	370A	368A	7 of 7
	110	381A	372A	6 of 6
	109	389A	382A	1 of 1
	116	365A	366A	1 of 1

Table 8

Maternity and Paternity Assignments for Clutches from T44

Year	Clutch ID	Mother ID	Father ID	Number of genotyped offspring assigned to Father
2010	89	Unassigned	Unassigned	1 of 1
	90	#1	*1	3 of 3
	91	Unassigned	Unassigned	1 of 1
	92	Unassigned	Unassigned	1 of 1
	93	Unassigned	Unassigned	3 of 3
	94	GPFT18	GPMT48	4 of 4
	99	GPFT12	*2	6 of 6
	100	Unassigned	Unassigned	1 of 1
2011	101	#3	GPMT32	4 of 5
			GPMT44	1 of 5
	102	GPFT41_F	Unassigned	4 of 4
	103	GPFT10_F	GPMT26	3 of 4
			GPMT53	1 of 4
	105	GPFT49_F	GPMT40	3 of 5
			GPMT50	2 of 5
	106	#2	GPMT6	3 of 4
			GPMT9	1 of 4
	111	GPFT18_F	GPMT48	4 of 4

Table 8 (continued).

Year	Clutch ID	Mother ID	Father ID	Number of genotyped offspring assigned to Father
2011	112	GPFT37_F	GPMT36	3 of 3
	113	GPFT12_F	*2	6 of 6
	114	GPFT43_F	GPMT26	1 of 4
			Unassigned	3 of 4
	115	GPFT3_F	GPMT9	3 of 3

The first two components produced from the PCA for characteristics of reproducing and non-reproducing females explained respectively, 75.9% and 13.5% of the total variance in morphological measurements (Figure 3). Axis 1 was highly correlated with morphological characters that were associated with size (Table 9). There was no visibly apparent separation between reproducing and non-reproducing groups. Axis 2 was correlated with anal width and anal notch and the two variables were inversely correlated. Anal width was wider among most reproducing females compared to non-reproducing females. For the PCA among reproducing and non-reproducing males, the first two components explained 73% and 17.2% of the total variance (Figure 4). Axis 1, again was highly correlated with morphological characters that were associated with size and there was no pattern between reproducing and non-reproducing males (Table 10). Similar to the PCA for females, anal notch and anal width were inversely correlated along axis 2. However, among males there was no pattern of separation among reproducing and non-reproducing males.

Mean distances between nests and putative parents, minimum distances, and maximum distances are reported in Table 11 and frequency of occurrences at different ranges is illustrated in Figure 5 and Figure 6. For Hillsdale, a female tortoise and her

assigned nest were collected at the same burrow in 2 of the 8 assigned clutches. A nest and its putative sire were found at the same burrow in 4 of the 12 assigned clutches, and a female and the sire of her clutch were captured at the same burrow, but at different times in 2 of the 8 assigned clutches. Of the 10 assigned clutches from T44, one female and one male were captured at the same burrow where their assigned nest was found.

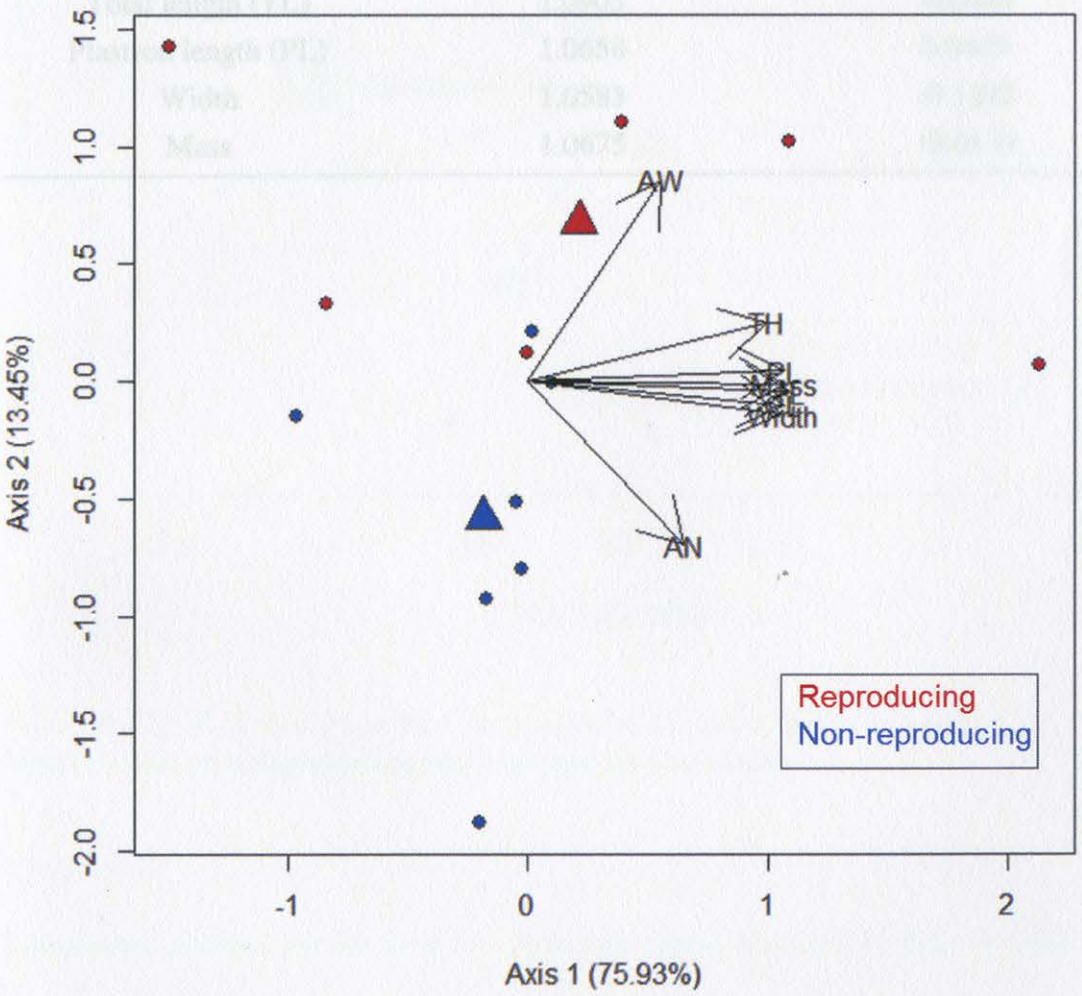

Figure 3. The First Two Principle Components of Morphological Characteristics Measured between Reproducing and Non-reproducing Females.

Table 9

Component Loadings for the First Two Principle Components for the PCA between Reproducing and Non-reproducing Females

Variable	PC1	PC2
Anal width (AW)	0.5572	0.8566
Anal notch (AN)	0.6516	-0.6973
Thickness (TH)	0.9942	0.2519
Total length (TL)	1.0903	-0.0449
Plastron length (PL)	1.0658	0.0449
Width	1.0583	-0.1422
Mass	1.0675	-0.0134

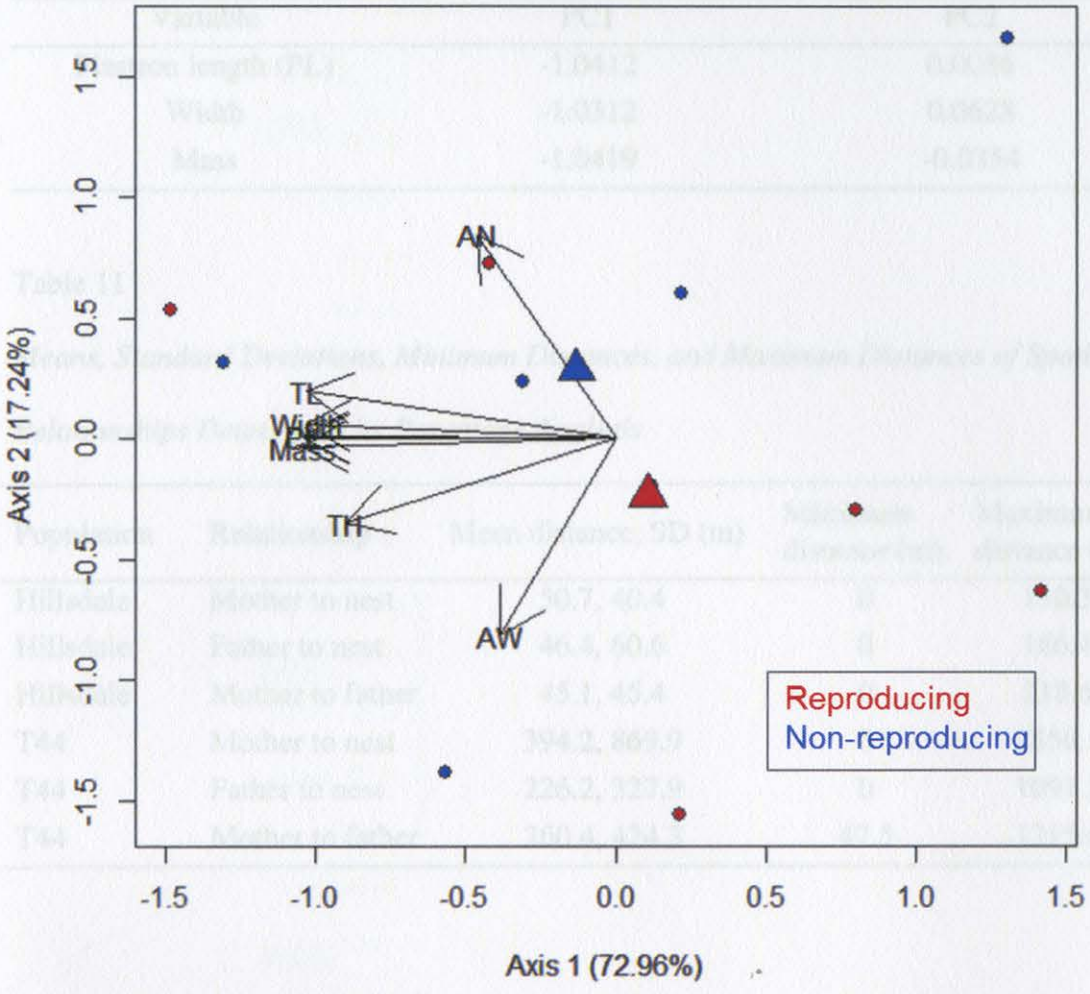

Figure 4. The First Two Principle Components of Morphological Characteristics Measured between Reproducing and Non-reproducing Males.

Table 10

Component Loadings for the First Two Principle Components for the PCA between Reproducing and Non-reproducing Males

Variable	PC1	PC2
Anal width (AW)	-0.3837	-0.8166
Anal notch (AN)	-0.4583	0.8419
Thickness (TH)	-0.8976	-0.3499
Total length (TL)	-1.0277	0.1973

Table 10 (continued).

Variable	PC1	PC2
Plastron length (PL)	-1.0412	0.0086
Width	-1.0312	0.0628
Mass	-1.0419	-0.0354

Table 11

Means, Standard Deviations, Minimum Distances, and Maximum Distances of Spatial Relationships Determined by Parentage Analysis

Population	Relationship	Mean distance, SD (m)	Minimum distance (m)	Maximum distance (m)
Hillsdale	Mother to nest	50.7, 40.4	0	130.3
Hillsdale	Father to nest	46.4, 60.6	0	186.4
Hillsdale	Mother to father	45.1, 45.4	0	218.6
T44	Mother to nest	394.2, 869.9	0	2856.1
T44	Father to nest	226.2, 327.9	0	1091.2
T44	Mother to father	260.4, 424.3	47.5	1215.6

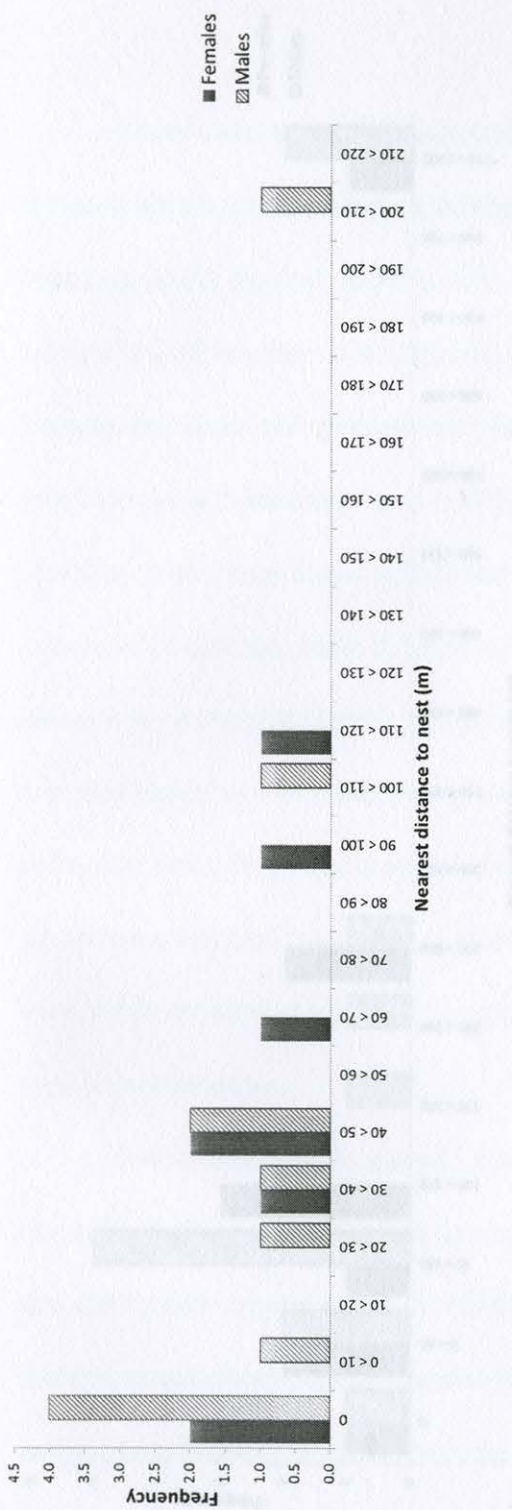

Figure 5. Frequency Distribution of Nearest Distance in Meters from Adults to Assigned Nests of the Hillsdale Population.

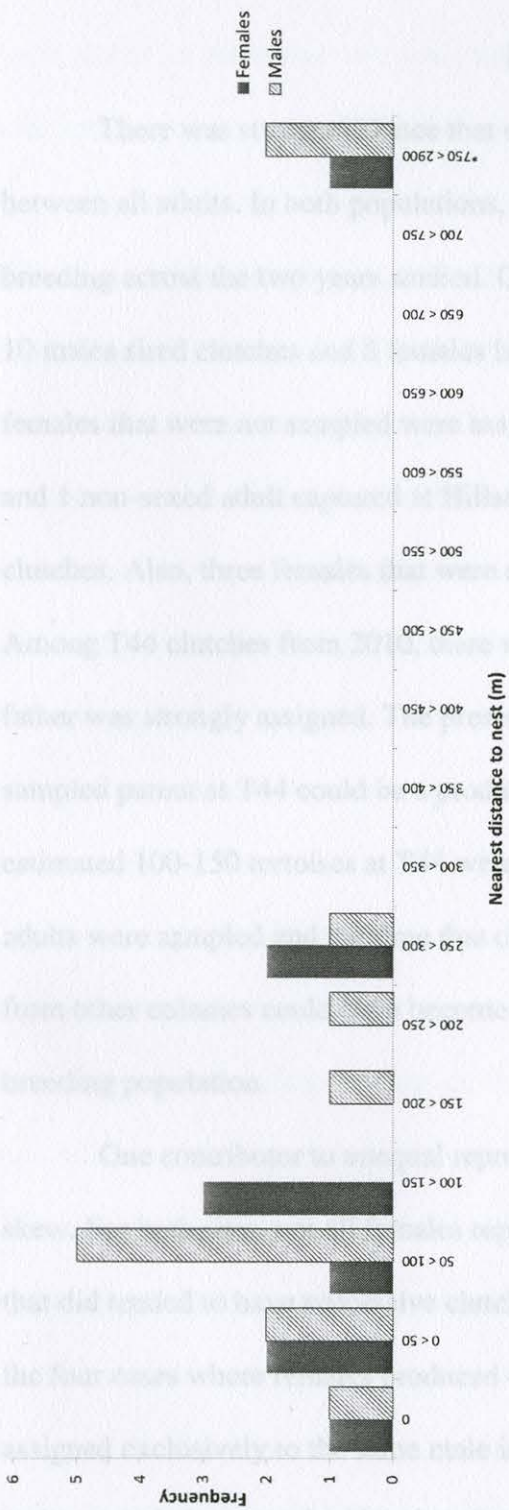

Figure 6. Frequency Distribution of Nearest Distance in Meters from Adults to Assigned Nests of the T44 Population. *Three events were at distances greater than 750 m. Distances of 766.4 m, 1091.2 m, and 2856.1 m were grouped together in one distance category (750 < 2900).

Discussion

There was strong evidence that reproductive success is not equally distributed between all adults. In both populations, there were very few adults contributing to breeding across the two years studied. Of the 31 males and 32 females captured at T44, 10 males sired clutches and 8 females laid clutches. Additionally, two males and three females that were not sampled were assigned to clutches. Of the 15 males, 15 females, and 1 non-sexed adult captured at Hillsdale, 6 males sired clutches and 6 females laid clutches. Also, three females that were not sampled were assigned to clutches as well. Among T44 clutches from 2010, there were five clutches in which neither a mother nor father was strongly assigned. The presence of clutches lacking assignment to any sampled parent at T44 could be a product of the larger population size. Only 63 of the estimated 100-150 tortoises at T44 were sampled. Furthermore, between 2006 when adults were sampled and the time that clutches were collected in 2010 and 2011, migrants from other colonies could have become established at T44 and become part of the breeding population.

One contributor to unequal reproductive success within populations was maternity skew. For both sites, not all females reproduced in consecutive years. However, those that did tended to have successive clutches that were sired by the same male. In three of the four cases where females produced clutches in both 2010 and 2011, their clutches assigned exclusively to the same male in both years. This could indicate that sperm from males were being stored within females and used across both years. Isolation treatments of desert tortoises, *G.agassizii*, a close relative of the gopher tortoise, has shown that

sperm stored for more than two years still produced viable offspring (Palmer et al., 1998). Additionally, females may be receptive to the same males year after year.

Reproductively successful males and reproductively unsuccessful males did not differ in any of the morphological characteristics measured. This was surprising given the evidence produced in past studies that reproductively successful males tended to be larger (Moon et al., 2006; Tuberville et al., 2011). In this study, the size of males did not seem to determine reproductive success. For example, male 372A sired 50% of the eggs produced in clutches across both years, but was below average for carapace length (234 mm) compared to all other sampled males (245 mm). Frequent burrow sharing between males suggests that aggressive male-male interactions are probably infrequent, and uniform distribution of female burrows does not support a female defense polygyny mating system (Johnson et al., 2009). Therefore, relative size of males would not play a large role in reproductive success in a scramble competition polygyny mating system. Reproductively successful females tended to have wider anal widths than non-reproducing females. One explanation for this is residual widening of the pelvic girdle from recent egg laying or widening over multiple years of egg laying. There were no other morphological characteristics that differed between reproducing and non-reproducing females; however, among reproducing females, the females that produced clutches in consecutive years were the two largest females (CL = 317 mm and 294 mm). This suggests that it may be favorable for smaller females to alternate years or reproduce even less frequently. Yolking eggs each year may be too energetically costly for smaller females to produce a clutch every year.

The geographic patterns of parentage in this study support the idea that gopher tortoises tend to form rather spatially restricted colonies. Although gopher tortoises have been known to traverse distances of a few thousand meters, the majority of movements related to social interactions are less than 30 m (McRae et al., 1981b). The spatial data from Hillsdale showed that within two years mean distances between mating individuals and their nests were mostly between 0 and 50 m. Individuals and clutches sampled from Hillsdale were collected from one of three groups of burrows (Figure 7). The highest density of burrows was in the central group of burrows (pers. obs.), and the majority of nests and adults were found in this area. The group of burrows that made up the northernmost part of the population was approximate 340 m away from the central group. The group of burrows that made up the southernmost part of the population was approximately 410 m south of the central group. Parentage analyses revealed that no individuals in one group were assigned parentage in another group. Therefore, these groups of aggregated burrows may be seen as distinct "colonies." One possible reason that adult gopher tortoises did not move between the central colony and the northern colony is that there is a 200 m strip of habitat with dense, woody understory that separates these two groups. However, there are no obvious obstacles that would impede movement from the central colony to the southern colony. There is evidence of colony substructure within the T44 population as well, but on a larger spatial scale (Figure 8). Longer distances between parental pairs and between nests at T44 were to be expected because the time between the capture of adults and collection of clutches spanned a much longer time scale than at Hillsdale. Longer time scales made long range dispersal movements more likely. The T44 site is made up of two major groups of burrows. One

group of burrows is found along T44 West Road to the west, and another is found along T44 East Road to the east. T44 West and T44 East are approximately 2.74 km apart. Despite the time span, distances for the majority of assigned parents were less than 300 m. Only one adult from one colony assigned to a clutch from the other colony. Female GPFT43, captured from T44 East in 2006, assigned to clutch 114 from T44 West approximately 2.86 km away in 2011. This demonstrates a striking amount of colony fidelity across five years.

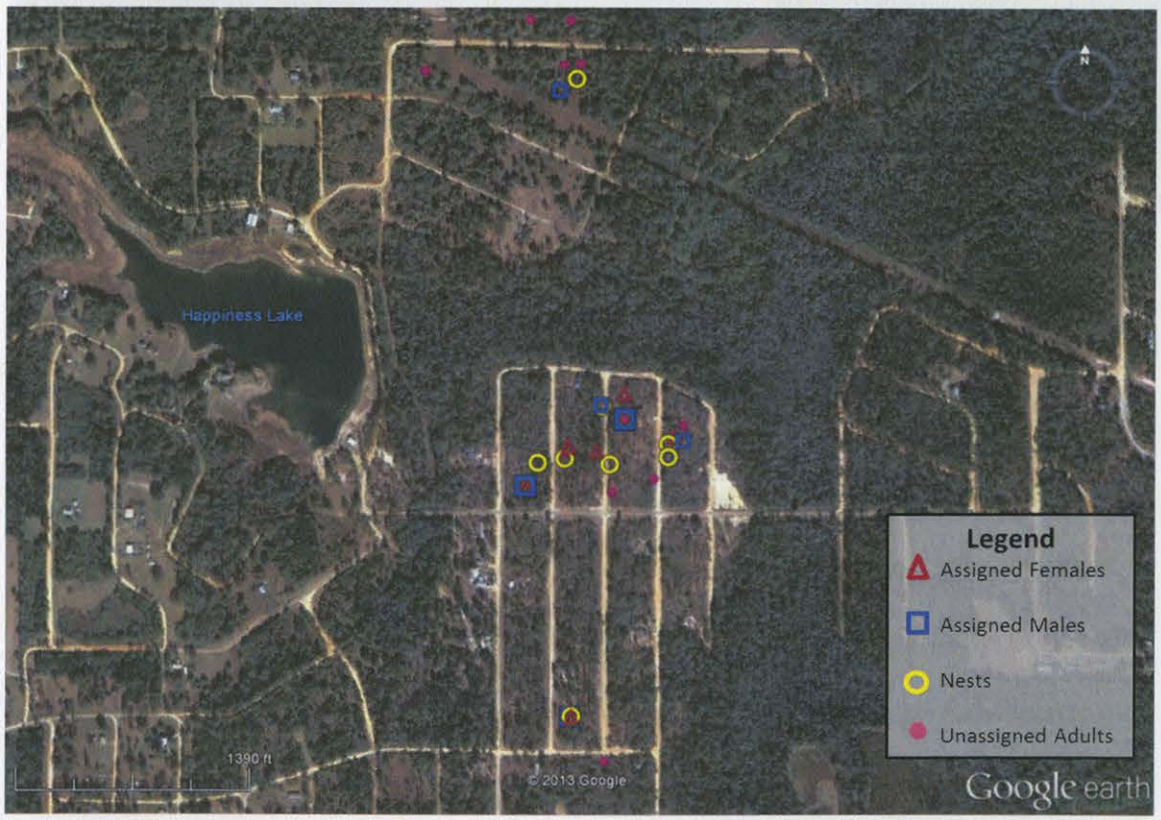

Figure 7. Aerial Imagery of Hillsdale with Nest Locations for Both Years and Capture Locations of Adult Gopher Tortoises. Three distinct groups of burrows were identified within the population: a northern colony, central colony, and southern colony. No parents from one colony were assigned to clutches found in a different colony.

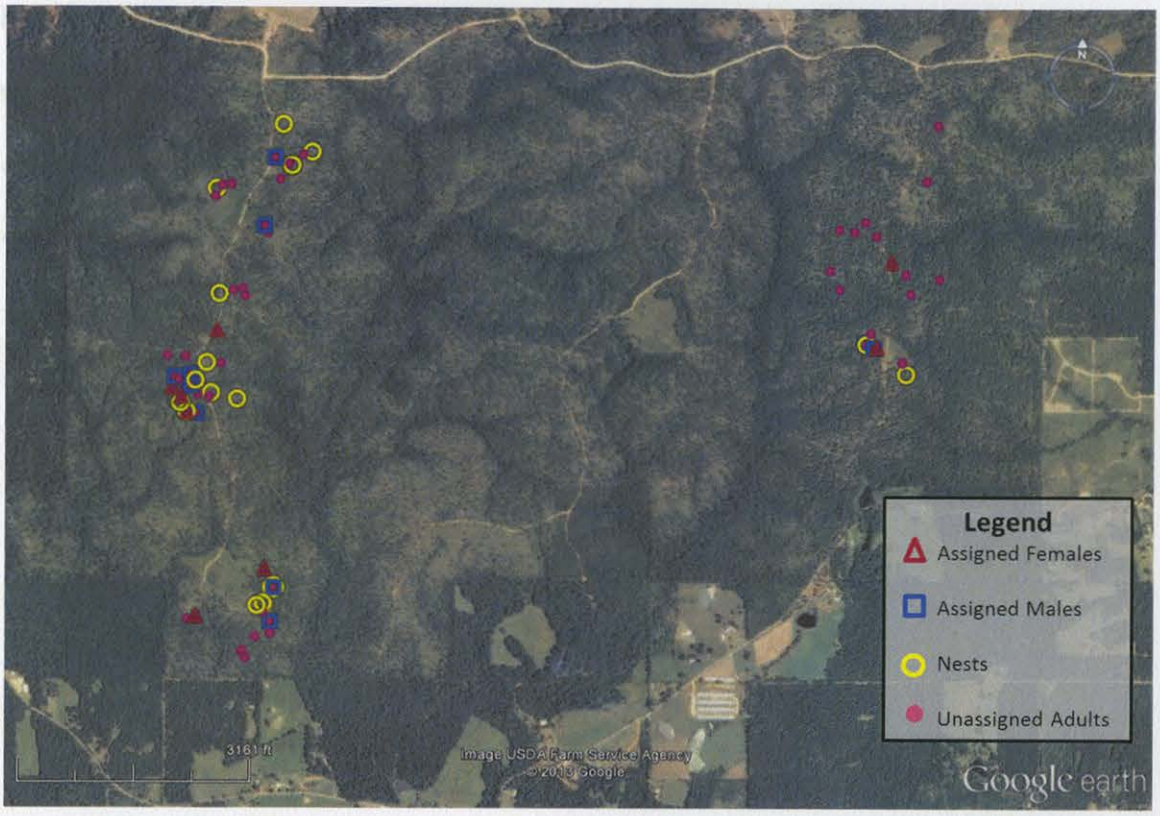

Figure 8. Aerial Imagery of T44 with Nest Locations for Both Years and Capture Locations of Adult Gopher Tortoises. The T44 site is made up of two major groups of burrows. One group of burrows is found along T44 West Road to the west, and another is found along T44 East Road to the east.

I used a combination of parentage analyses to assess the extent of individual reproductive contributions and the spatial scale across which these matings took place. This work is unique in that it represents the only study of the mating systems of gopher tortoises in the western portion of the species range. Similar to other gopher tortoise populations studied, unequal reproductive success among individuals was observed at both sites. However, unlike other studies, the larger males did not seem to be over represented in the reproductive class. The parentage analysis also supported previous work suggesting that individuals tend to be found in spatially restricted colonies and most movements are within the confines of that colony. Interestingly, this tendency seemed to

hold up even across a relatively long time scale at the T44 site. Since unequal reproductive success and a restricted tendency for dispersal both act to lower N_e, this will only serve to exacerbate the problems of fragmentation and small population sizes in the western portion of the species range.

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APPENDIX

IACUC APPROVAL

The University of
Southern Mississippi

Institutional Animal Care and Use Committee

118 College Drive #5147
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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: **11092206**
PROJECT TITLE: **Population Genetics & Systematics of Freshwater Fishes**
PROPOSED PROJECT DATES: **10/01/2011 to 09/30/2014**
PROJECT TYPE: **Renewal/Continuation of a Previously Approved Project**
PRINCIPAL INVESTIGATOR(S): **Brian Kreiser, Ph.D.**
COLLEGE/DIVISION: **College of Science & Technology**
DEPARTMENT: **Biological Sciences**
FUNDING AGENCY/SPONSOR: **Departmental**
IACUC COMMITTEE ACTION: **Full Committee Review Approval**
PROTOCOL EXPIRATION DATE: **09/30/2014**

Jodie M. Jawor, Ph.D.
IACUC Chair

9/28/2011
DATE