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The Conservation Genetics of Two Emydid Turtles: *Emydoidea blandingii* and *Malaclemys terrapin*

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The University of Southern Mississippi

THE CONSERVATION GENETICS OF TWO EMYDID TURTLES:

EMYDOIDEA BLANDINGII AND *MALACLEMYS TERRAPIN*

by

Charlotte Lizana Petre

A Thesis

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ABSTRACT

THE CONSERVATION GENETICS OF TWO EMYDID TURTLES:

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Conservation of turtles is complicated by their sensitivity to habitat degradation and overexploitation. I used microsatellites and standard population genetic analyses to explore genetic diversity, population structure, paternity and demographic history in two emydid turtles that are currently experiencing threats to their survival. The Blanding's turtle, *Emydoidea blandingii*, has experienced habitat fragmentation throughout its range, and this study focuses on a population in Massachusetts where hatchlings from one population are being translocated to establish a new population. I found evidence of multiple paternity within clutches and found no significant reduction in genetic diversity when comparing the source population and the offspring being relocated. Genetic structuring of populations of Diamondback terrapins (*Malaclemys terrapin*) has only been detected at the range wide level. However, previous studies failed to obtain abundant samples covering a large spatial scale. I acquired a collection of 556 terrapin tissue samples from across Louisiana where landscape features include two large freshwater rivers expected to act as a barrier to gene flow. I found a highly connected population with no indication of discrete genetic structuring across the major freshwater rivers in Louisiana. Additionally, I found evidence of a stepping stone migration model, isolation by distance and historical bottlenecks.

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CHAPTER I

INTRODUCTION AND OBJECTIVES

Turtles are of considerable conservation concern with over 20% of known extant species estimated to be imperiled or declining (IUCN 2014). Turtles and tortoises have existed on the earth for nearly 300 million years with very little change to their morphology and life history. Turtle survival is reliant upon their cryptic and secretive nature and a durable carapace and plastron. Shell development requires investing a large quantity of energy and resources. Allocating many resources into shell development limits the investment of resources into reproduction. Therefore, the benefit of a strong shell comes at a cost of delaying sexual maturity (Congdon and Van Loben Sels 1993). This delay in sexual maturity exposes turtles to a long interval where the organism could die before reproducing; therefore, high adult survivorship must be maintained to allow populations to persist.

When a species becomes of conservation concern due to population decline, a resource management and/or conservation approach should be implemented. Resource management approaches focus on the individual species of concern, ensuring adequate habitat patches, food sources, the removal of predators and invasive species, and reintroduction of captive-bred organisms or translocation of individuals from other stable populations. Conservation management considers the entire ecosystem and attempts to understand interspecific and intraspecific interactions to manage viable populations (Klemens 2000). Two species of turtle that have benefited from conservation management of ecosystems are the Diamondback Terrapin, *Malaclemys terrapin*, and Blanding's turtle, *Emydoidea blandingii*. For the Diamondback terrapin, the Clean Water

Act has been beneficial throughout the entire range in preserving their estuarine habitat and the prevention of further development within the saltmarsh. The Blanding's Turtle has benefited from land procurement and the establishment of protected preserves similar to the E.S. George Reserve in Michigan (Congdon et al. 1993) and Weaver Dunes in Minnesota (Hamernick 2000).

Habitat destruction can occur in many ways and unequally impact different age classes and sexes of turtles. In places where habitat fragmentation occurs, breeding populations of adults can be isolated from other populations, reducing gene flow. Attempts by adults to traverse the landscape increase an individual's risk of roadside mortality from cars or detection by predators. For many turtle species, juveniles are known to utilize and occupy different habitats than adults (e.g., loggerhead sea turtles Bowen et al. 2005), Diamondback terrapins (Gibbons et al. 2001) and Blanding's turtles (Crockett 2008). Female turtles also need to have access to adequate nesting grounds, which are typically different from breeding grounds. Nesting females have been documented traversing large distances to find a nesting site (Congdon and Loben Sels 1991), which increases the risk of predation or roadside mortality.

Headstarting is another technique employed by conservationists to help increase the survival of hatchling and juvenile turtles. In headstarting, eggs are removed from a nest or from vehicle struck females (Wood and Herlands 1997) and incubated in the lab. This practice has been implemented for a variety of turtle species including ornate box turtles (Bowen et al. 2004), wood turtles (Brewster and Brewster 1991), Diamondback terrapins (Brennessel 2006), Blanding's turtles (Arsenault and Mockford n.d.), and loggerhead sea turtles (Nagelkerken et al. 2003) and Kemp's ridley sea turtles (Dodd and

Seigel 1991). Depending on the research goals and current sex ratios researchers, will either incubate the eggs to preference one sex over the other or a temperature that should achieve a 1:1 sex ratio. Most turtles exhibit temperature dependent sex determination with females produced at warmer temperatures and males at cooler temperatures (Vitt and Caldwell 2009). Hatchlings are then maintained in the laboratory for as long as a few years before being released into the environment. This technique has come under criticism since hatchlings might be missing critical imprinting milestones, disrupting their homing ability to find their natal nesting area or breeding grounds (Heppell et al. 1996). Also, predator avoidance skills may be underdeveloped, making them more susceptible to predation before they reproduce (Heppell et al. 1996). There have been a few documented cases of headstarted turtles experiencing nearly 100% mortality after release (Dodd and Seigel 1991); however, this may not be a reflection of the headstarting practice but normal low survivorship of juvenile turtles. The effectiveness of headstarting is hard to determine and therefore is criticized and a less favorable practice.

More recently, interest and support for the use of genetics in the conservation of imperiled species has developed. Conservation biologists and researchers are interested in understanding current levels of genetic diversity, how they might compare to historical levels (if archived samples are available and not degraded) and how we can improve or avoid loss of observed genetic diversity while implementing other conservation techniques like captive breeding and translocation programs (Alacs et al. 2007). When there is a substantial or persisting reduction in population size, rare alleles are lost, resulting in a reduction of genetic diversity. Further reduction in allelic diversity is seen when individuals are forced to breed with closely related individuals. The smaller a

population becomes the more susceptible it is to the effects of genetic drift and inbreeding. Genetic drift will drive rare alleles to be lost from a population leading to a loss of genetic variation, while mating between closely related individuals can promote the expression of deleterious alleles thus reducing an offspring's fitness. However, if small populations are connected to larger populations the effects of genetic drift or inbreeding will be reduced as migration will introduce new alleles or maintain allele frequencies similar to the larger population.

Although population size can significantly influence the genetic diversity maintained within a population, it is important to consider and understand an organism's ability to disperse away from natal population and their propensity for multiple mating partners. An organism's reluctance or inability to disperse away from relatives increases the possibility of inbreeding in small populations. When there is reduced gene flow due to landscape features there is the potential for population differentiation. These genetically distinct groups can be important for the management of a species, especially with one of conservation concern (e.g., Management Units (MU's)) and Evolutionary Significant Units (ESUs). Traditional management units are often defined by political boundaries or perceived barriers (i.e. mountain ranges and water drainages), which may have little biological relevance. Genetically defined management units are focused on identifying current population structure, where migrants and gene flow is minimal, producing independently reproducing populations (Mills 2007). Management units differ from ESU's in that ESU's correlate phylogeny and ecological data where a population is evolutionarily unique and genetically distinct, affording them conservation protection.

The management of ESU's focuses on long term conservation concerns where potentially adaptive traits exist and may ultimately lead to speciation (Mills 2007).

Genetic research was first introduced into wildlife population management as a tool for researchers to identify species or genetically discrete populations by use of allozymes and restriction digests of mtDNA (Selkoe and Toonen 2006; Alacs et al. 2007). The use of allozymes requires a fresh sample rich in protein which often requires the sacrifice of an organism and is hard to justify when working with protected species (Mills 2007). After allozymes came the use of mitochondrial DNA (mtDNA) which can be harvested from small and degraded samples (Alacs et al. 2007). Mitochondrial DNA is most appropriately used to answer questions about historical population structure or phylogeography as it is only maternally inherited and has a slower mutation rate than microsatellites (Awise et al. 1992). For example, mtDNA has been used to explore the phylogeography of gopher tortoises (Ennen et al. 2012) and nesting natality in loggerhead sea turtles (Bowen et al. 2005). Questions concerning more recent changes in population structure are best evaluated using microsatellites (Awise 1992).

Microsatellites are co-dominant molecular markers found in non-coding regions in genomic DNA and have a higher mutation rate than coding regions of the genome (Selkoe and Toonen 2006). Generation of polymorphisms in microsatellites repeats are from the slippage of the DNA polymerase and the lack of proof reading since this is a non-coding region of the genome (Selkoe and Toonen 2006). Common repeat motifs are dinucleotides, trinucleotides, and tetranucleotides, which describe the number of nucleotides found in the repeat (the tandem unit). Trinucleotides and tetranucleotides are preferable because they are less susceptible to stuttering, which can inflate heterozygosity

scores due to scoring error. Since mutation is relatively common in these non-coding regions (neutral genetic markers) the number of alleles within a locus increases over time and it is the polymorphic nature of the microsatellites and the influences of genetic drift which is used to help answer questions about population structure, connectivity of populations (migration), effective population sizes, and inbreeding (Hartl 2000). Furthermore, with an increase in personal computer computational efficiency, we are able to make inferences about the relatedness of individuals, assign parentage, determine hybridization levels, and detect historical demographic changes.

My research focused on two species of emydid turtles: *Emys blandingii*, Blanding's Turtle, and *Malaclemys terrapin*, Diamondback Terrapin. Both turtles are not federally listed for protection under the Endangered Species Act, but they are managed by some states in their respective ranges. Protection for these turtles range from no protection to being state listed species with regulations that restrict or prohibit the take or disturbance of the species. The Blanding's Turtle experienced a reduction in its population as a result of habitat modification for agricultural use (Congdon et al. 2008). Diamondback terrapin populations began to decline when they were unsustainably harvested for consumption, with some local populations becoming commercially extinct. Ultimately this led to the need to import individuals from other populations to maintain Diamondback terrapin farms (Brennessel 2006). Although both species have been the focus of intense research, many questions still remain where genetic tools can provide some insight.

My study on the Blanding's Turtles examined a population located in Massachusetts in part of its range that is isolated from the larger population and is

currently listed as threatened under the Massachusetts Endangered Species Act (MESA). There are two sites associated with this study: the first site is an established site and the second is where turtles are being reintroduced. At the established population site eggs are collected just after deposition and then incubated in the laboratory by Oxbow and Associates. Hatchlings are released at either the established site where the eggs were collected (source) or at a new site. Data collection on nesting females and hatchlings has occurred over six years and these data were used to look for similarities in levels of genetic diversity and levels of multiple paternity in previously reported populations of Blanding's turtles. An effort to collect tissue and morphometric data on juveniles and males has occurred over the years but representation is limited. With information on both sexes and multiple generations, questions about paternity and levels of multiple paternity and repeat paternity were explored. A general investigation was conducted to determine if the level of genetic diversity in adults at the source site differs from that in the hatchlings being established at the new site. The results of my study will inform biologists managing this population about mating systems and current levels of genetic diversity and may be used to modify their translocation methodologies.

Within my Diamondback terrapin study I analyzed turtles from across the Louisiana coast. Although the Louisiana salt marsh appears to be an expansive and contiguous habitat, there are two major waterscape features within the Diamondback terrapin's range in Louisiana – the Atchafalaya Delta and Mississippi River Delta. These freshwater systems may serve as ecological barriers to movement and gene flow in this salt and brackish marsh-dependent species. To determine if genetic differentiation exists across these freshwater features I analyzed multilocus microsatellite data from sites

across Louisiana (Sabine Pass to the Pearl River). I employed a variety of Bayesian based approaches (e.g., STRUCTURE, TESS, and Geneland) to test for genetic differentiation, and patterns of gene flow were also examined via model testing using Migrate-n. Lastly, I compared geographic trends to demography and genetic diversity.

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CHAPTER II

GENETICS OF A REINTRODUCED POPULATION OF BLANDING'S TURTLES, *EMYDOIDEA BLANDINGII*, IN MASSACHUSETTS

Introduction

Background

The Blanding's turtle (*Emydoidea blandingii* or *Emys blandingii*) is a semi-aquatic turtle whose range is centered around the Great Lakes and extends into eastern Nebraska, eastern Minnesota, eastern Iowa, Nova Scotia and a few small isolated populations in New York, Massachusetts, New Hampshire, and Maine. The Blanding's Turtle is listed as threatened or endangered in much of its range as a result of being extirpated by agriculture (Congdon et al. 2008). Although this turtle is well-studied in terms of its ecology, demographics, and threats to survival, it continues to be of conservation concern.

The Blanding's turtle inhabits freshwater wetlands including swamps, marshes, permanent and temporary ponds including beaver ponds, and slow flowing streams (Congdon et al. 2008). Within these habitats, Blanding's turtles are known to consume mostly crayfish and other crustaceans, and less often consume insects and plant matter (Congdon et al. 2008). Like most other semi-aquatic turtles, the Blanding's turtles can be observed basking during the day and are most active during dawn and dusk for feeding; however, this activity can be influenced by temperature (Rowe and Moll 1991). Blanding's turtles occupy home ranges between 0.6-7.9 ha in smaller wetlands (Ross and Anderson 1990; Rowe and Moll 1991) but will occupy 18.9-74 ha in larger or fragmented habitats. Both male and female Blanding's Turtles have been documented to move in

search of a mate or more abundant resources with a daily average of less than 2 km with occasional movements of >10 km (Rowe and Moll 1991). For females, larger movements were in search of adequate nesting grounds whereas males moved as a result of mating competition (Schuler and Thiel 2008). The increased movements of these turtles leaves them more vulnerable to roadside mortality events (Gibbs and Shriver 2002).

Blanding's Turtles are known to be one of the longest lived freshwater turtles in North America (Congdon et al. 1993) with individuals documented living >60 years while still remaining reproductively active. Once females reach sexual maturity, clutches of 3-19 eggs are produced (Congdon and Loben Sels 1991). Clutch size will increase with body size, demonstrating that the initial reproductive bout is not equivalent to maximum reproductive output for an individual (Congdon et al. 2001). Blanding's turtles rarely will nest more than once in a season, and most adults do not nest (Congdon and Loben Sels 1991). This is not uncommon in turtles, as it is costly, and if food resources are limited it may not be feasible to produce a clutch every season (Congdon et al. 1993; Anthonyamy et al. 2013). Egg size, clutch size, and nesting frequency generally increase with age until maximum adult size is reached and then they remain constant (Congdon et al. 2001).

Blanding's turtles do not mature until 14-20 years of age with a mean age of maturity at 17 and a cohort generation time of 37 years (Congdon et al. 1993). An advantage of delayed sexual maturity is that females are able to invest more energy into each offspring she produces, increasing their chances of survival (Klemens 2000; Refsnider 2009). The drawback in delaying sexual maturity is that an individual runs the risk of dying before reproducing. This strategy is selected for when there are relatively

high levels of juvenile survivorship. Congdon et al. (1993) constructed a life table based off a long term study at the E. S. George Reserve in Michigan and determined that in order to maintain a stable population of Blanding's turtles, the following annual survivorship is necessary for each life stage: nest survivorship of 26%, juvenile survivorship (hatching to 13 years) of 76%, and adult survivorship (>13 years) of 96%. Small deviations in survivorship did not destabilize the population.

Today's threats to survival of Blanding's Turtles are centered on habitat degradation, reduction and fragmentation through residential development, and the construction of roadways (Congdon et al. 2008). Road side mortality continues to be a major contributor to many reptilian and amphibian declines (Gibbs and Shriver 2002). Adult turtles are most susceptible to road side mortality when searching for nest grounds, new suitable habitat, or mates. As a result of high roadside mortality, roadside fences and culverts have been installed and proven effective in reducing road mortality (MWPARC 2010). Nest predation by foxes, raccoons, skunks, and opossums also impact survivorship of the Blanding's turtle, especially since the collapse of the local fur industry which has allowed for population expansion of these known nest predators (Congdon et al. 1993). Collection for the pet trade has also been linked to the decline of the Blanding's Turtle (Levell 2000).

A considerable amount of effort has been applied to the conservation of Blanding's turtle throughout its range, such as nest protection, headstarting, wetland management, habitat restoration, invasive species removal, establishment of reserves and wildlife refuges, relocation, predator removal, genetic studies, turtle fences, and movement corridors (MWPARC 2010). Nest protection is common in turtle conservation

and includes predator exclusion cages, which prevent nest predators from excavating the nest. More invasive forms of nest protection require relocating nests into more ideal habitats or to a laboratory for a controlled incubation environment. This controlled environment also allows researchers to manipulate the sex ratio of a developing clutch because turtle sex determination is driven by temperature and not sex chromosomes. When eggs are incubated in the laboratory hatchlings can be directly released into existing habitats or into new sites. Headstarted hatchlings are delayed in their release in order to rear them to a more advanced stage of development which should offset hatchling mortality rates (Heppell et al. 1996). Headstarting has become a contested issue on its effectiveness as turtles are adapted to having high hatchling mortality. Through the construction of a life table and survivorship models, Congdon et al. (1993) found small fluctuations in juvenile and adult survivorship can have large impacts on a population's persistence. Implementation of multiple efforts which affect the broadest range of age classes will have the greatest long term effect on these long lived turtles (Congdon et al. 1993).

Headstarted individuals have also been used in translocation and reintroduction programs. In translocation programs, individuals are moved from one population, usually large and well established, to a place where a population may be in decline or have experienced a catastrophic event. Within reintroduction programs the purpose is to reestablish a population in a location where a population was extirpated (Tracy et al. 2011). Within both situations considerations should be made to ensure the restocking of the second site is conducted with the most appropriate individuals (genetically similar). Additionally, it is important to avert the founder effect by moving an adequate number of

individuals to capture the genetic diversity which is representative of the source population to avoid a reduction in alleles (Hartl 2000; Mills 2007; Tracy et al. 2011).

Recent studies (Mockford et al. 2005; Sethuraman et al. 2014) on the Blanding's turtles have looked at range wide genetic structure and genetic structure within a site. Populations within the United States have been examined for range wide structure where it was determined (n=5 microsatellite loci) the Appalachian Mountains and Hudson river present geographic barriers to gene flow dividing this part of the range into three Evolutionary Significant Units (ESU) (Moritz 1994). A study within the ESU to the west of the Appalachian Mountains found 4-5 unique genetic populations of Blanding's turtles which did not conform to isolation by distance but were proposed to be the product of Pleistocene glaciers (Sethuraman et al. 2014). The presence of genetic population structure is important to maintain as turtles may be locally adapted to their geographic region of the range and therefore any translocation efforts should consider these divisions in their management plans.

The use of genetics has also become useful in better understanding the mating systems present in the Blanding's turtle. Both male and female Blanding's turtles will vary in their home range and also display variation in the distance an individual is willing to travel for mating or nesting purposes (Hamernick 2000; Crockett 2008; Schuler and Thiel 2008; McGuire et al. 2013). Movement of breeding Blanding's turtles in and out of their home ranges promotes gene flow amongst neighboring fragmented patches (McGuire et al. 2013). These movements also increase the potential for multiple mating events between populations, leading to multiple paternity within a single clutch. Within the Blanding's turtles multiple paternity has been documented with as many as three sires

in a clutch (Refsnider 2009). However, multiple sires do not necessarily mean that multiple mating events took place within a single season. The capacity for sperm storage is observed in many turtle species and therefore multiple paternity may be the result of using stored and fresh sperm. Even though multiple mating systems are not well understood, it may have evolved as a mechanism to maintain adequate genetic diversity within populations, especially with organisms with long generation times and low mutation rates (Congdon and Van Loben Sels 1993; Refsnider 2009).

Purpose

The focus of my research was a population of Blanding's Turtles from Massachusetts that is part of a headstarting and reintroduction program being managed by Oxbow and Associates. Due to confidentiality agreements, it is not known how well established or isolated our source and reintroduced populations are from each other or other populations of Blanding's turtles. My first goal was to compare the level of genetic diversity in adults to the hatchlings being headstarted and sent to the reintroduction site. I anticipated that if an appropriate sample was collected for reintroduction then we would not see a reduction in measures of genetic diversity between adults and hatchlings or among years of hatchlings. My second goal was to use genetic data and parentage programs to assess levels of multiple paternity and repeat paternity. With this study having spanned multiple years, another goal was to determine if a few males were disproportionately contributing to the mating events.

Materials and Methods

Sample Collection and Molecular Techniques

Technicians from Oxbow and Associates are responsible for data and tissue collection of the Blanding's Turtles in this study. We provided Oxbow and Associates with sample collection tubes containing SED buffer for whole blood samples and 100% ethanol for scute clips. Whole blood was collected from encountered adult Blanding's turtles. Hatchlings had scute clippings taken prior to release. Females were collected for identification after nesting and released while males were collected opportunistically. Clutches were collected and incubated and headstarted by Oxbow and Associates. Half of each clutch was returned to the same site as the mother and the other half to a reintroduction site.

Blood or scute clippings were digested and extracted for genomic DNA using Qiagen DNeasy extraction kits (Qiagen Inc., Valencia, California, USA). Each individual was amplified at 6 or more microsatellite loci. Microsatellites used in this research included *Eb17*, *Eb19*, *Eb09*, and *Eb11* developed for the Blanding's Turtle (Osentoski et al. 2002), *Gmu28*, *Gmu70*, *GmuD87*, *GmuD90*, *GmuD93*, and *GmuD121* loci developed for *Glyptemys muhlenbergi*, Bog turtle (King and Julian 2004), and a locus developed for *Carretta carretta*, Loggerhead sea turtle (*Cc7*) by FitzSimmons et al. (1995). Loci were chosen based on an initial estimate of polymorphic representation within a subset group of total samples and ability to multiplex with other loci. Polymerase Chain Reactions (PCR) were performed in 12.5 ul reactions containing 100-200 ng of DNA, 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 μ M of M13 labeled primer (LI-COR), and water to the final volume. PCR cycling conditions were performed as follows: initial denaturing step at 94°C for 2 minutes followed by 35 cycles of denaturing for 30 seconds at 94°C, primer annealing for 30 seconds at 56-60°C, and elongation for 1 minute at 72°C, with a final 10 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.

Genetic analyses

Tests of Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were conducted in GENEPOP version 3.4 (Raymond and Rousset 1995). ML-Null (Kalinowski and Taper 2006) was used to determine if null alleles were present. I used GenAlex 6.5 (Peakall and Smouse 2006) to calculate the number of alleles per locus (N_a), expected heterozygosity (H_E) and observed heterozygosity (H_O) for each group, and allelic richness (A_R) was calculated to account for differences in sampling with FSTAT version 2.9.3.1 (Goudet 2001). For comparisons between adults and hatchlings, I used only one hatchling randomly selected from each clutch. This avoided a potential bias in the measures of genetic diversity due to relatedness among member in a clutch. I also compared clutches among years using the same representative individuals. For the comparison between adults and hatchlings, I used a pooled two-tailed t test when assumptions of a normal distribution and equal variances were met or a non-parametric Wilcoxon rank sums test when they were not. When I compared clutches among years I used an analysis of variance since assumptions for parametric tests were met. All statistical tests were performed with JMP (SAS Institute Inc. 2007).

For parentage assignments I used COLONY (Jones and Wang 2010) and CERVUS (Marshall et al. 1998; Kalinowski et al. 2007). CERVUS uses either strict exclusion or categorical allocation to assign parentage and is not designed to use loci with null alleles. CERVUS will assign one parent or parent-pairs with a likelihood of detection score (LOD). COLONY uses a maximum likelihood method and that jointly considers sibship and parentage. COLONY considers more *a priori* information about mating systems, mutation rates, typing errors, and can handle allelic dropout (null alleles). COLONY also assigns assignment scores to parent(s). If a parent was not included in the analysis then COLONY will indicate that the parent was not sampled.

For COLONY, I assumed polygamy for both the male and female mating systems and provided the allele frequencies calculated in GenAIEx. Since the female producing a clutch was known, I increased the probability of having the mother in my maternal genotypes to 0.9. Many of the other parameters were left at the default setting as I felt they met the recommendations defined by the authors. Run length was set to *medium* 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.

The use of two parentage programs allowed me to look for congruence between their assignments to ensure the most appropriate possible parent was assigned and if multiple paternity can be detected within a clutch. When COLONY and CERVUS could not assign a sire to a clutch I used the same method as McGuire et al. (2013) to look for evidence of multiple paternity. A clutch was deemed to be the result of multiple paternity

when a minimum of three paternal alleles were detected in offspring from the clutch in a minimum of two loci or confirmed by the same alleles in more than one offspring.

Results

A total of 338 hatchlings and 226 adults (males = 19, females = 207) were genotyped representing 5 nesting years (2008 n=115, 2009=21, 2010=42, 2011=133, 2012=27). For purposes of estimating genetic diversity in the hatchling population, one hatchling was randomly selected to represent each clutch (2008 n=16, 2009 n=8, 2010 n=9, 2011 n=51, 2012 n=26). The 6 microsatellite loci (*Eb17*, *Eb19*, *Gmu121*, *Gmu88*, *Gmu87* and *Cc7*) were used to answer questions about genetic diversity loci. Loci *Eb09* and *Eb11* were eliminated because they could not be consistently scored. After a sequential Bonferroni correction (Rice 1989), no loci deviated significantly from Hardy Weinberg equilibrium nor was there evidence of linkage disequilibrium.

Genetic diversity was estimated for all adults, all hatchlings, and then hatchlings by year. Measures of genetic diversity included calculating the following population parameters: number of alleles (N_a), allelic richness (A_R), observed heterozygosity (H_o), and expected heterozygosity (H_E) (Table 1). The average number of alleles per locus was greater in adults than hatchlings (5.17 and 4.0, respectively); however, in both age classes the fewest number of alleles observed in a locus was 2. Both observed and expected heterozygosity were higher in hatchlings. Comparisons between adults and hatchlings released at the new site yielded no significant differences among the number of alleles, allelic richness, or observed and expected heterozygosity (Table 2). When hatchlings were compared across years using a single representative for each clutch there were no significant changes in these same measures of genetic diversity (Table 3).

Table 1

Measures of genetic diversity for Blanding's turtle adult and hatchlings.

Group	Number of Alleles Na	Allelic Richness A _R	Observed Heterozygosity H _O	Expected Heterozygosity H _E
Adult	5.17 ±3.06	4.50±2.41	0.47±0.19	0.51±0.19
Hatch	4.0±1.60	4.17±2.56	0.56±0.22	0.54±0.18
Hatch				
by year:				
2008	4.17±1.60	3.22±1.20	0.50±0.209	0.55±0.160
2009	3.83±1.47	3.36±1.64	0.63±0.23	0.60±0.16
2010	3.83±1.47	3.33±1.49	0.58±0.27	0.56±0.20
2011	4.50±2.07	3.14±1.15	0.58±0.17	0.56±0.18
2012	3.67±1.51	2.84±1.09	0.49±0.26	0.45±0.23

Note: table reports means and standard deviations

Table 2

Results of a pooled two tailed t-test or Wilcoxon rank sums comparing adults to all hatchlings

Number of Alleles Na	Allelic Richness A _R	Observed Heterozygosity H _O	Expected Heterozygosity H _E
$X^2 = 0.3831$ Df = 1 $p = 0.5359$	$t = -0.2389$ Df = 1 $p = 0.8160$	$X^2 = 0.3831$ Df = 1 $p = 0.4445$	$X^2 = 0.3831$ Df = 1 $p = 0.5523$

Table 3

Results of ANOVAs comparing clutches among years

Number of Alleles Na	Allelic Richness A _R	Observed Heterozygosity H _O	Expected Heterozygosity H _E
$F_{(4,25)} = 0.2336$ $p = 0.9168$	$F_{(4,25)} = 0.1456$ $p = 0.9633$	$F_{(4,25)} = 0.4534$ $p = 0.7690$	$F_{(4,25)} = 0.4885$ $p = 0.7441$

A total of 31 clutches from 24 females were analyzed for parentage with clutch sizes ranging from 3-13 individuals. The clutches sampled had 4 or more individuals and were collected from 2008-2011. Neither COLONY nor CERVUS produced reliable estimates of parentage. Some females had identical genotypes. We attribute the failure of these analyses to the limited number of alleles found for most loci (3 of 6 loci only had 2 or 3 alleles). I was still able to visually inspect the genotype data for evidence of multiple paternity. Of the 31 clutches analyzed 9 showed indications of multiple paternity (29%). Since the results for parentage analyses were inconclusive we were unable to determine the contributions of individual males to reproduction at this site over the course of the study. However, one female did nest every year of the study and an examination of her offspring made it clear that the male who sired the 2008 and 2009 clutches was different than the male who sired the 2010 and 2011 clutches.

Discussion

Overall levels of heterozygosity were low (this study 6 loci, adults H_O=0.474 H_E=0.507 and hatchlings H_O=0.556 H_E=0.543), but similar to other studies (Refsnider 2009, 4 loci H_E=0.64-0.78 H_O=0.61-0.78; McGuire et al. 2013, 8 loci H_E=0.54-0.85; Davy et al. 2014, 12 loci H_E=0.41-.85 H_O=0.41-.86). Davy et al. (2014) used many of the same loci as I did (*Gmu87*, *Gmu88*, *Eb19*, and *Eb17*) and found a consistently higher

number of alleles in their study sites than observed in ours. A study comparing several small populations of Blanding's turtles near densely populated cities to larger populations in more ideal habitats found a loss in genetic diversity between the two habitat sites (Rubin et al. 2001). Overall, the level of genetic diversity is in line with the expectations of a species that is thought to be in decline and found within isolated populations.

It is important that founder individuals be representative of the adult population so that genetic diversity is not lost. A loss in genetic diversity can translate to a loss in adaptive potential and can have a negative impact on the population's long-term persistence (Frankham 2005; Tracy et al. 2013). Reduction in genetic diversity was not observed between the adults and hatchlings located at the new site. Furthermore, there was no significant difference in the amount of genetic diversity seen in the hatchlings across years. Even though the initial amount of diversity seen in the hatchlings is not different from the adults in the source population, there is still the potential for a loss of variation to take place before the hatchlings mature. Within a population located on a refuge, hatchling and juvenile, ages 1-13 years, annual survivorship is 0.78 in contrast to adults, 0.96 (Congdon et al. 1993), and imposes a risk of mortality and loss of alleles before reproductive maturity is reached. Therefore, both survivorship and the population growth rate should be considered when trying to determine an adequate number of individuals to reintroduce or translocate (Tracy et al. 2011). Thrimawithana et al. (2013) suggest proximity to other populations should be another consideration. Headstarting the hatchlings before release does provide them with a size advantage; however, they may have poorly developed defensive or hunting behaviors (Klemens 2000). It is unlikely the effort invested into headstarting will translate into substantial gains in juvenile

survivorship and more adults. More successful translocations have been conducted using a variety of age classes, especially when the focus is on translocating reproductive or nearly reproductive individuals (Dodd and Seigel 1991). However, translocating adults is not always an option so headstarting is sometimes the best available strategy.

There was evidence of multiple paternity among the clutches sampled. However, COLONY and CERVUS were unable to assign paternity to any clutch from the males sampled. Several of the loci used in this study were uninformative for paternity studies due to low number of alleles. Low levels of observed heterozygosity within the population also did not aid in the success of paternity assignments. Inability to assign paternity prevented me from being able to answer my questions about repeat paternity or if a single male was disproportionately siring clutches. Clutching behavior was consistent with other studies where females nested every other year (Congdon et al. 1993; McGuire et al. 2013). However, one individual did nest every year, although it appears sperm storage may have played a role in her ability to clutch every year. In previous paternity studies the reported incidence of multiple paternity per year ranged from 81% (Refsnider 2009) to 47.6% (McGuire et al. 2013). Our study found an average of 29% multiple paternity (annual range from 0-46%). Due to low levels of genetic diversity and few informative loci, my multiple paternity estimates may be underestimated.

Currently, many management strategies have been implemented to help protect remaining populations of Blanding's turtles. Although, this study does not provide insight on the level of connectivity among other populations it does identify the level of genetic diversity and occurrence of multiple paternity. With low levels of genetic diversity seen in the adult population, it is even more important to ensure enough individuals are

translocated and persist to ensure the founding population does not experience a reduction in genetic diversity by the time individuals reach reproductive age. Long-term research should be conducted to determine the actual level of survivorship and levels of genetic diversity once these individuals reach reproductive age.

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CHAPTER III
THE POPULATION GENETICS OF THE DIAMONDBACK TERRAPIN,
MALACLEMYS TERRAPIN, IN LOUISIANA

Introduction

Background

Diamondback terrapins (*Malaclemys terrapin*) are the only emydid turtle that is endemic to coastal areas inundated with brackish water where salinity ranges from 0-34 parts per thousand (ppt), averaging around 12-17 ppt, and tidal ranges from 1-8 feet (Ernst and Lovich 2009). These coastal habitats include brackish and saline marshes, estuarine bays, lagoons, tidal creeks, and mangrove forests. Diamondback terrapins function as one of the keystone predators of invertebrates in their coastal habitat. Common prey items for Diamondback terrapins include periwinkle snails (*Littorina littorea*), mud snails (*Ilyanassa obsoleta*), fiddler crabs (*Uca spp.*), marsh mud invertebrates (Gibbons et al. 2001; Brennessel 2006). The predation pressure exerted by Diamondback terrapins on the periwinkle snail is essential for the health of the salt marsh. Excessive grazing on epiphytic microorganisms on the dominant salt marsh grass (*Spartina alteriflora*) when periwinkle snail populations are large harms the plants and has been linked to salt marsh decline and die off (Levesque 2000). Therefore, an adequate population of Diamondback terrapins is essential to maintain the health of this important part of the salt marsh.

The distribution of Diamondback terrapins ranges from Cape Cod, Massachusetts to Corpus Christi, Texas (Figure 1), as well as an isolated population on the island of Bermuda. Diamondback terrapins have historically been divided into seven subspecies

based on morphological differences: *M.t. terrapin*, *M.t. centrata*, *M.t. tequesta*, *M.t. rhizophoratum*, *M.t. macrospilota*, *M.t. pileata*, *M. t. littoralis* (Figure 1). However, genetic studies using mitochondrial DNA and microsatellite loci have failed to find support for all subspecies designations although there is population structure across its range (Lamb and Avise 1992; Hauswaldt and Glenn 2005; Hart et al. 2014).

Morphological characteristics attributed to one subspecies can be seen in distant parts of the range (Will Selman per. comm), which suggests that these morphological characteristics are not good taxonomic characters, but rather they reflect traits that are variable across much of the range.

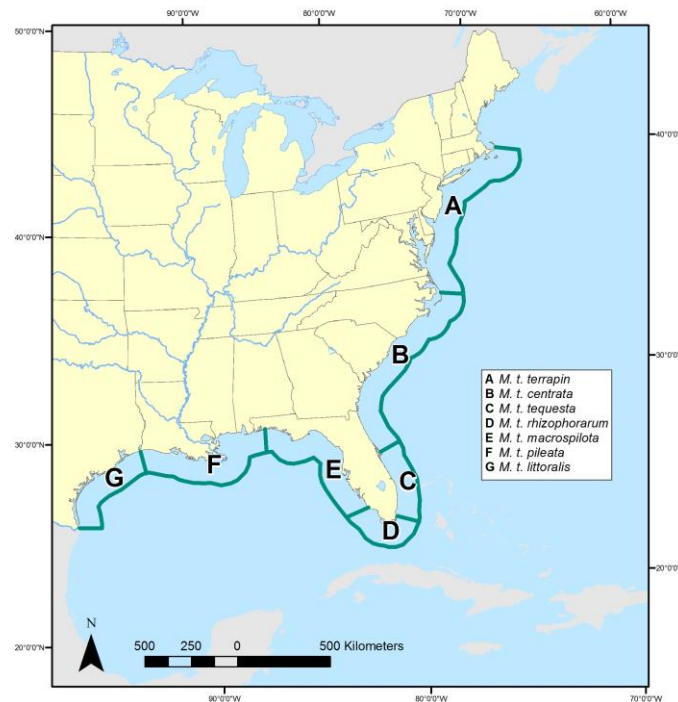


Figure 1. Range map for Diamondback terrapins. This map identifies the approximate divisions between the seven accepted subspecies of the Diamondback terrapin.

Knowledge of a species' dispersal ability and sex ratio is important for developing appropriate management practices. However, previous studies demonstrate that dispersal

abilities and sex ratios are inconsistent across the range. Two non-gravid females in New Jersey were tagged, recaptured >8km away, and found to be gravid; this long distance movement was inferred to be in response to a search for an appropriate nesting habitat (Sheridan et al. 2010). However, most females can be found in or close to the same tidal creeks as in previous years (Lovich and Gibbons 1990; Sheridan et al. 2010; Butler and Heinrich 2013). Male Diamondback terrapins will travel to breeding aggregations (Seigel 1980; Butler 2002), but during the non-breeding season they will exhibit high site fidelity with small home ranges (Roosenburg et al. 1999; Gibbons et al. 2001; Sheridan et al. 2010). The dispersal ability of hatchling and juvenile Diamondback terrapins is not well understood. Due to their small size and cryptic behavior (burrowing in the mud) the detectability of younger Diamondback terrapins is challenging. Sex ratios also vary across the range and equal sex ratios are rarely seen. In South Carolina a male biased population was observed (Lovich and Gibbons 1990; Gibbons et al. 2001), while a well-studied population in Maryland was female biased (Roosenburg 1990), as was a population in New Jersey (Sheridan et al. 2010). Reported sex ratios should be interpreted with caution as there may be a collection bias due to size (females are easier to detect due to a larger size) or season (movement due to breeding or search for appropriate nesting locations). Adequate understanding of dispersal ability and sex ratios can influence the interpretation of the results from genetic analyses.

A growing number of threats are putting populations of Diamondback terrapins at risk. Nests are depredated by a variety of human-subsidized organisms including raccoons, armadillos, and foxes, while shore birds and ghost crabs will consume hatchlings (Roosenburg 1990; Gibbons et al. 2001). Additional threats to Diamondback

terrapins include the invasive fire ants (*Solenopsis invicta*) which may overtake a nest or hatchling, roadside mortality (Wood and Herlands 1997), habitat degradation or fragmentation (Wood and Herlands 1997), crab pots - especially abandoned “ghost” crab pots (Roosenburg et al. 1997), and collection for pet trade or legal/illegal harvesting. Roadside mortality may be responsible for creating male skewed populations by disproportionately killing females looking for higher ground nesting habitats. Alternately, crab traps may be biased towards killing male and juvenile Diamondback terrapins due to females being too large to enter the openings. However, the most influential threat to Diamondback terrapin persistence may be the intense harvesting pressures applied by humans (Roosenburg 1990; Roosenburg et al. 1999).

Historically, Diamondback terrapins were harvested for their meat, starting with reports as early as the 1700’s to feed the continental army (Hart and Lee 2006). During the height of Diamondback terrapin consumption, Maryland was the center for harvesting, farming (including captive breeding), and distributing Diamondback terrapin. As early as 1902, the United States Federal Bureau of Fisheries established “artificial propagation” regimes for restocking, commercial exploitation and experimental cross breeding (Hay 1917; Coker 1920; Hart and Lee 2006). During this time, Diamondback terrapins from Louisiana and North Carolina were harvested and exported to Maryland. The price of Diamondback terrapins from Louisiana was second only to the ones from Chesapeake Bay. Over time this industry collapsed as Diamondback terrapins popularity declined, a trend that was also influenced by the Great Depression. Currently, only 3 states (Massachusetts, Rhode Island, and Georgia) have outlawed the possession or taking of Diamondback terrapins, while others have a variety of statues that regulate

commercial/recreational taking. For example, within Louisiana, there are regulations for both personal and commercial fishing for Diamondback terrapins which prohibit collection during the nesting season, April 15-June 15, and require a minimum carapace length of 6 inches and no traps can be used (Louisiana Department of Wildlife and Fisheries 2014).

The coastal ecosystem is always changing as a result of ocean currents and large-scale disturbances (e.g., hurricanes), natural erosion, and subsidence processes. However, more recently changes are being brought about by anthropogenic sources of disturbance such as pollution, salt marsh ditching and draining for mosquito control and pasturing, marsh flooding for rice, and filling in the marsh for causeways structures (Brennessel 2006). The marshes in Louisiana have been heavily altered by dredging of oil canals, channelization, levee and dyke construction. These structures have been installed to improve access to inland areas, prevent saltwater intrusion, and were once used to assist with agriculture. While the impact of these practices may not be initially apparent over time they often lead to reduction in habitat quality or quantity (erosion). Since 1932, Louisiana has experienced increased marsh erosion predominantly in the eastern part of the range (Couvillion et al. 2011). Sea level rise, which is largely attributed to the action of humans, is submerging historic salt marsh beds which is further reducing available habitats for Diamondback terrapins (Gibbons et al. 2001).

Purpose

The main focus of this study is to determine the extent of genetic connectivity among Diamondback terrapin populations within the salt marshes of Louisiana. Louisiana has approximately 653,000 hectares of brackish or saline marshes that are

suitable habitat for Diamondback terrapins (Sasser et al. 2008). This is not necessarily a homogenous habitat, as the western and eastern portions of Louisiana's brackish and saline marshes are separated by the expansive freshwater marsh at the base of the Atchafalaya River. Major freshwater inputs like the Atchafalaya Delta and Mississippi River Delta may isolate populations by creating a barrier to gene flow, thus ultimately leading to the formation of genetically distinct groups. Further, most of the human population within Louisiana has occurred east of the Atchafalaya River. Proximity of large cities (i.e., New Orleans) or highly populated areas has also likely influenced historical harvest pressures and population genetic patterns. Roads extending in the marshes and bayous will fragment the habitat and increase road mortality (Seabrook 2012). With the increase in accessibility from a highly populated area and lack of wildlife refuges, fishing activity will be high, which increases the risk of mortality, particularly from crab pots.

Previous genetic studies of Diamondback terrapins have been conducted on small spatial scales in various parts of their range. Drabeck et al. (2014) included 31 Diamondback terrapins from the marshes east of the Atchafalaya River in Louisiana and found no evidence of genetic structure amongst their sites. Similarly, both Alabama (Coleman 2011) and Galveston Bay (Glenos 2013) demonstrated no evidence of genetic structure within their respective ranges of the gulf coast. On the Atlantic coast, Hauswaldt and Glenn (2005) included a small spatial scale study within the Charleston Harbor, a large port and river, and also found no genetic structure within the harbor. The limitations of previous studies conducted within Louisiana Diamondback terrapins have restricted their focus in range, spatial coverage, and sample size. These limitations may

provide incomplete information on the demographic history of these populations. Determining the extent to which populations are genetically connected is important for making well supported recommendations for conservation and management efforts. With this in mind, the goals of my study were to determine the influence of landscape features across Louisiana coastline (e.g., large freshwater inputs) on population structure and patterns of gene flow. I also wanted to examine the demographic history of this region in terms of genetic bottlenecks and effective population size. These questions are all centered on better understanding the Diamondback terrapin demography and the influences of landscape features on gene flow and how this knowledge can be applied to wildlife management practices.

Materials and Methods

Sample Collection and Molecular Techniques

Will Selman from Rockefeller Wildlife Refuge (RWR) and his collaborators provided Diamondback terrapin samples from 26 locales. Samples were distributed from the Sabine River in the west to Lake Eugenie in the east. Diamondback terrapins were collected either by hand or with fyke nets set into salt marsh tidal creeks (Selman and Baccigalopi 2012). The sex and spatial coordinates for each sample were recorded. Tissue samples were collected (tail tips) and preserved in 95% ethanol. Genomic DNA was extracted from the tissue samples using Qiagen DNeasy extraction kit reagents (Qiagen Inc., Valencia, California, USA) and Econospin spin columns (Epoch Life Science, Inc., Fort Bend County, Texas). Each individual was genotyped at 13 microsatellite loci. Microsatellites used in this research were developed for *Malaclemys terrapin* (*TerpSH1*, *TerpSH2*, *TerpSH7*) by Hauswaldt and Glenn (2003); loci developed

for *Glyptemys muhlenbergi*, Bog turtle (*GmuB08*, *GmuD87*, *GmuD90*, *GmuD93*, *GmuD121*, *GmuD51*, *GmuD28*, *GmuD62*, *GmuD21*) by King and Julian (2004), and a locus developed for *Carretta carretta*, Loggerhead sea turtle (*Cc7*) by FitzSimmons et al. (1995). Loci were chosen based on initial estimate of polymorphic representation within a subset group of total samples and their ability to multiplex with other loci. Polymerase Chain Reactions (PCR) were performed in 12.5 μ L reactions containing 100-200 ng of DNA, 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 μ M of M13 tailed forward primer, 0.3 μ M reverse primer, 0.1 μ M of M13 labeled primer (LI-COR), and water to the final volume. PCR cycling conditions for primers developed for *Glyptemys muhlenbergi* were performed as follows: initial denaturing step at 94°C for 2 minutes followed by 35 cycles of denaturing for 45 seconds at 94°C, primer annealing for 45 seconds at 56-60°C, and elongation for 2 minutes at 72°C, with a final 10 minute elongation step at 72°C. PCR cycling conditions for primers developed for *Carretta carretta* or *Malaclemys terrapin* were performed using touchdown PCR and, follows: initial denaturing step at 94°C for 2 minutes followed by 15 cycles of denaturing for 30 seconds at 94°C, primer annealing for 30 seconds at 65°C decreasing by 0.5°C every cycle, and elongation for 1 minute at 72°C, followed by 15 cycles of denaturing for 30 seconds at 94°C, primer annealing for 30 seconds at 56°C, and elongation for 1 minute at 72°C, with a final 10 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.

Genetic analysis

Traditional population genetic analyses require the *a priori* delineation of individuals into some set of groups. Rather than treat each site as distinct we pooled them into eight groups (Figure 2) that are geographically explicit relative to distinct features of the landscape (e.g., rivers and bays). For some analyses, we grouped sites as they were defined by breaks associated with the major freshwater inputs of the Atchafalaya and Mississippi rivers.

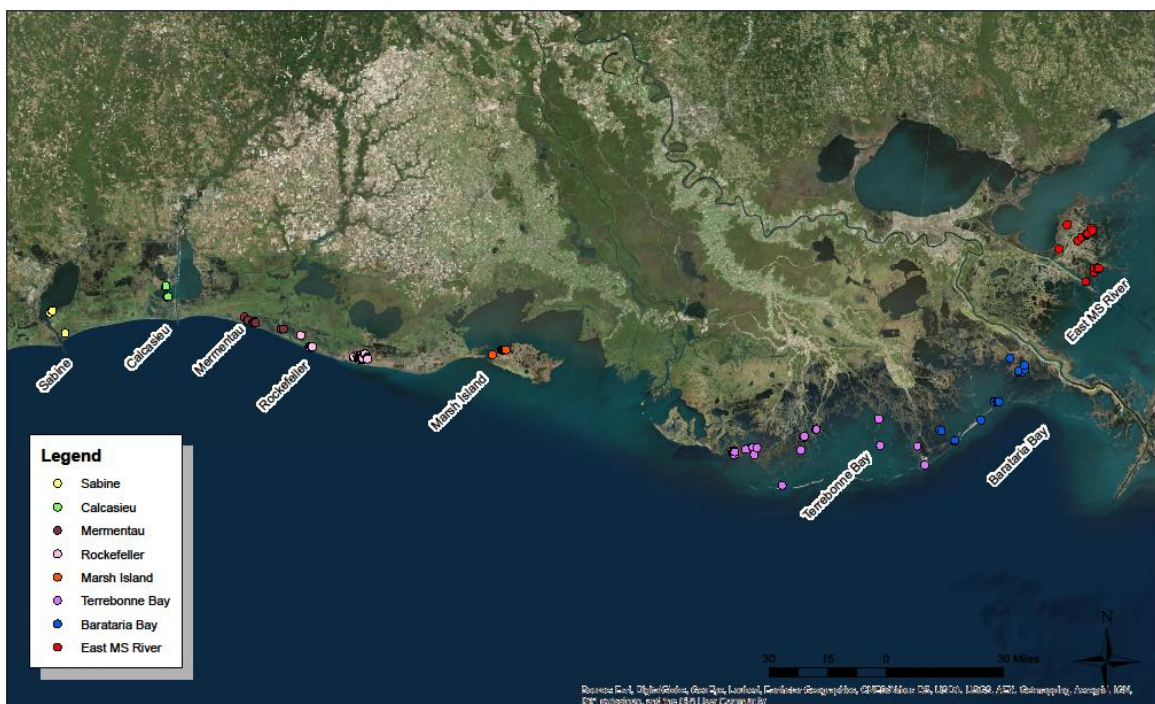


Figure 2. *A priori* groups of Diamondback terrapin. Dots represent locality of collected Diamondback terrapins. Colors represent *A priori* groups which are required for traditional genetic analyses. These groups were determined based on drainages and distance to other individuals.

Tests of Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were conducted in GENEPOP version 3.4 (Raymond and Rousset 1995). ML-Null (Kalinowski and Taper 2006) was used to determine if null alleles were present. I used

GenAlex 6.5 (Peakall and Smouse 2006) to calculate the number of alleles per locus (N_a), expected heterozygosity (H_E), and observed heterozygosity (H_O) for each group, and allelic richness (A_R) was calculated to account for differences in sampling with FSTAT version 2.9.3.1 (Goudet 2001) and was also used to calculate and test the significance of θ , Weir and Cockerham's (Weir and Cockerham 1984) unbiased estimator of F_{ST} . I performed an Analysis of Molecular Variance (AMOVA; Excoffier et al. 1992) using ARLEQUIN 3.5 (Excoffier et al. 2010) at two different grouping levels: the 8 groups defined by distance and drainages and 3 larger groups defined by the Atchafalaya and Mississippi river (west, central, and east). Measures of genetic diversity were also compared among groups using an ANOVA when assumptions of a normal distribution and equal variances were met. Otherwise, I used the non-parametric Kruskal-Wallis ranked sums test. All statistical tests were performed with JMP (SAS Institute Inc., 2007).

The number of genetically distinct groups was estimated using the Bayesian approach implemented by STRUCTURE v. 2.3.4 (Pritchard et al. 2000; Hubisz et al. 2009). Values of K were tested from 1-12 using the admixture model with correlated allele frequencies among groups, and population location was used as a prior. Twenty replicates for each value of K were performed with a burn-in of 5,000,000 generations followed by a subsequent 500,000 generations. The best estimate of K was determined by first examining the probability scores for each value of K and comparing this with the method of Evanno et al. (2005; ΔK) as calculated by Structure Harvester v 6.92 (Earl and von Holdt 2012).

The combination of genetic marker data, geospatial data, and statistical methods has developed into the field of landscape genetics (Manel et al. 2003; Storfer et al. 2006). Spatially explicit programs like TESS(Chen et al. 2007) and GENELAND(Guillot et al. 2005) were specifically designed to simultaneously consider genotypic data and geospatial coordinates to evaluate the role landscape features play in shaping population structure. Both Geneland and TESS use a Bayesian clustering method to find populations structure by simultaneously considering geographic location and multilocus genotype data. TESS operates using a Bayesian approach similar to STRUCTURE. GENELAND calculates the assignment of an individual to a population using posterior probabilities. The differing approaches these two programs take to assign membership and define genetic boundaries or gradients between populations permits an evaluation of congruence between the outputs to ensure confidence in the assignments. For TESS, values of K were tested from 2-12 using the admixture model. Twenty replicates for each value of K were performed with a burn-in of 100,000 generations followed by a subsequent 50,000 generations with admixture. The best estimate of K was determined by first examining the probability scores for each value of K and viewing the hard clustering analysis tessellation. Geneland was run under the advanced model to accommodate a sample size greater than 300 individuals. Values of K were tested from 1-5 with allele frequencies both correlated and uncorrelated. Each simulation was run with 100,000 iterations with 1,000 thinnings. The thinnings are utilized for the post processing. Runs were performed with and without uncertainty (0.05) in the coordinates. Including uncertainty within the coordinate is appropriate for organisms in which they are expected to disperse from the

site of capture. Post processing was completed using 100 points by 150 points with a burn-in of 25.

Isolation by distance was assessed using an Isolation by Distance Web Service version 3.23 (Jensen et al. 2005). Geographic distance was calculated using distances between two centroids between the 8 groups and the genetic distance used pairwise F_{ST} values. A mantel test for a matrix correlation between the two distance matrices was conducted to determine if a reduction in genetic similarity could be related to an increase in geographic distance.

Five possible models of gene flow (Table 4) were tested using Migrate-n (version 3.6.4); (Beerli and Felsenstein 1999). The marginal likelihood of the model was estimated followed by a ranking of the Bayes factor of each one (Beerli and Palczewski 2010). The models tested were based on the known dispersal ability of Diamondback terrapins while others focused on restricting gene flow across a suspected barrier (Atchafalaya River). For each analysis we used the Brownian mutation model, which is appropriate for microsatellite loci. The starting genealogy was taken from a UPGMA tree and initial theta and M values were derived from the F_{ST} calculation. Priors for theta were kept as uniform with minimum, maximum, and delta values set to 0.01, 100.0, and 9.99, respectively. Static heating was applied to four independent chains using temperature settings of 1.0, 1.5, 3.0 and 1,000,000.0. A total of 500,000 steps were run, recorded every 100 generations, of which 10,000 were discarded as the burn-in. Stationarity was assessed by examining the effective sample size (ESS) and distribution of each parameter, where values >1000 and a unimodal distribution were taken to indicate that convergence was reached.

Table 4

Description of migration models performed in Migrate-n

Model	Description of gene flow
Stepping Stone	from one site to any adjacent site
Full	from one site to any other site – null hypothesis
Bisected stepping Stone	From one site to any adjacent site but not across the Atchafalaya River
Bisected Full	From one site to any other site but not across the Atchafalaya River
Panmixia	One single population – null hypothesis

I characterized the demographic history of these groups by calculating effective population size (N_e) and testing for genetic bottlenecks. I estimated N_e for each site using NeEstimator (Peel et al. 2004). During a genetic bottleneck, allelic diversity is lost faster than population heterozygosity, which produces an excess of heterozygosity relative to the observed number of alleles (Cornuet and Luikart 1996). BOTTLENECK (Cornuet et al. 1999) was used to detect a significant excess of heterozygosity under the two-phase mutation model in each of the eight populations. The two-phase mutation model is an improvement upon the stepwise mutation model in that it allows for larger jumps in mutation size. Both models are appropriate for microsatellite data.

Results

A total of 573 individuals from across the Louisiana coastline were collected from 26 sites. These sites were clustered into 8 groupings from west to east: Sabine (n=8), Calcasieu (n=46), Mermentau (n=89), Rockefeller (n=141), Marsh Island (n=13), Terrebonne Bay (n=108), Barataria Bay (n=229), and East of Mississippi River (n=75). Eight individuals were eliminated prior to analysis due to missing data at four or more loci. Individuals in our collections were primarily adults with a relatively equal sex ratio.

Null alleles were detected in *GmuD21* and therefore, this locus was excluded from further analysis. The remaining 12 microsatellite loci had 2-19 alleles per locus with observed heterozygosity ranging from 0.308-1.00 (mean=0.730, SE±0.015) and expected heterozygosity ranging from 0.320-0.909 (mean=0.746, SE±0.012) per locus. After a sequential Bonferroni correction (Rice 1989), no loci deviated significantly from Hardy Weinberg equilibrium nor was there evidence of linkage disequilibrium.

Within measures of genetic diversity (Table) neither H_O and H_E showed significant differences across groups (Table 6). However, the number of alleles was significantly different. This appeared to be driven by the lower diversity at two sites with small sample sizes (Sabine and Marsh Island). After excluding these sites, allelic richness was not significantly different among groups. Measures of genetic diversity were also quite similar among the three regions (West, Mid, and East of the MS River).

Table 5

Measures of genetic diversity for Diamondback terrapin groups and regions across Louisiana

Group	Number of Alleles N_a	Allelic Richness A_R	Observed Heterozygosity H_o	Expected Heterozygosity H_E
Sabine	5.769	N/A	0.760±0.125	0.743±0.204
Calcasieu	9.615	9.951 ±3.390	0.772±0.103	0.779±0.215
Mermentau	9.615	8.942 ±3.143	0.740±0.103	0.772±0.216
Rockefeller	10.385	9.184 ±3.602	0.749±0.116	0.778±0.216
Marsh Island	6.308	N/A	0.765±0.172	0.749±0.207

Table 5 (continued).

Group	Number of Alleles Na	Allelic Richness A _R	Observed Heterozygosity H _o	Expected Heterozygosity H _E
Terrebonne Bay	9.615	9.076 ±3.502	0.762±0.131	0.781±0.216
Barataria Bay	9.077	8.693 ±3.734	0.750±0.111	0.763±0.212
East MS River	9.000	8.581 ±3.723	0.760±0.142	0.765±0.210

Table 6

Results of ANOVA for different measures of genetic diversity & Kruskal-Wallis

Number of Alleles Na	Allelic Richness A _R	Observed Heterozygosity H _o	Expected Heterozygosity H _E
F _(7,88) 2.857	F _(5,71) 0.230	χ ² 1.518	F _(7,88) =0.205
p=0.0098**	P=0.948	Df=7, p=0.982	P=0.9836

Note. Number of alleles is significant however, it contains Marsh Island and Sabine when these sites are removed and rarefied allelic richness is not significantly different among sites. No significant differences in measures of genetic diversity.

Pairwise F_{ST} values were relatively small (Table 3), with values ranging from 0.0174-0.0000, and exhibited little significant differentiation across sites. However, 9 of the 28 pairwise F_{ST} values were significantly different from zero. Analysis of molecular variance was calculated using ARELQUIN (Table 4) and in both model groupings the significant majority of the variation in the data was explained by variation within individuals. Within the three group AMOVA, sites separated by major rivers, a small yet significant amount of the variation was explained.

Table 7

Pairwise F_{ST} comparisons among the eight groups and p-values

Group	Sabine	Calcasieu	Mermentau	Rockefeller	Marsh Island	Terrebonne Bay	Barataria Bay	East MS River
Sabine		0.57321	0.43036	0.92321	0.79643	0.88214	0.94107	0.61429
Calcasieu	-0.0045		0.00179	0.00179	0.11250	0.00179	0.00179	0.00179
Mermentau	-0.0065	0.0047		0.00179	0.32679	0.00179	0.00179	0.00179
Rockefeller	-0.0073	0.0067	0.0045		0.25893	0.18571	0.00357	0.12500
Marsh Island	-0.0174	0.0036	-0.0026	0.0030		0.51071	0.05357	0.26607
Terrebonne Bay	-0.0101	0.0105	0.0087	0.0012	0.0002		0.00714	0.03393
Barataria Bay	-0.0120	0.0083	0.0081	0.0026	0.0066	0.0027		0.38393
East MS River	-0.0128	0.0066	0.0046	0.0015	-0.0002	0.0013	-0.0002	

Note: F_{ST} comparisons (below the diagonal) and associated sequential Bonferroni corrected p-values (above the diagonal) for twelve microsatellite loci. P-values in bold are significant

Table 8

Results of the AMOVA from for groups and regions

Subdivision of groups	Amount of Variation explained among populations	Amount of Variation explained among individuals within populations	Amount of variation explained within individuals
3 regions	0.16 (<i>p</i> =0.02639)	1.19 (<i>p</i> =0.39687)	98.65 (<i>p</i> =0.03324)
8 groups	0.35 (<i>p</i> =0.06354)	0.99 (<i>p</i> =0.64712)	98.66 (<i>p</i> =0.02542)

Note: The 3 region subdivision level clusters the samples divided by the two major freshwater rivers within the Louisiana landscape while the 8 groups level allows for smaller clusters that are defined by smaller rivers and proximity to other groups.

The highest likelihood score from the STRUCTURE run was for a K of 1 (average $\ln L = -25187.1$; $SD=0.2$; Figure 4). Similarly, both TESS and GENELAND failed to detect any evidence of population structure across the range of our samples. However, despite the lack of distinct population structure, Louisiana terrapins are not one panmictic group. The Mantel test of geographic and genetic distances demonstrated a significant, although weak, positive correlation ($p=0.0090$, $r=0.243$; Figure 3). These results were congruent with the outcome of the model testing using Migrate-n. The stepping stone pattern had the highest probability ($p = 1.0$) of any of the five models with a marginal likelihood of -66076.06. The next best model was the full migration model ($mL = -70057.13$), while the panmixia model ($mL = -337314.51$) and models that restricted movement across the Atchafalaya River (bisected full model ($mL = -415679.42$) and bisected stepping stone ($mL = -222828$) had by far the lowest marginal likelihoods.

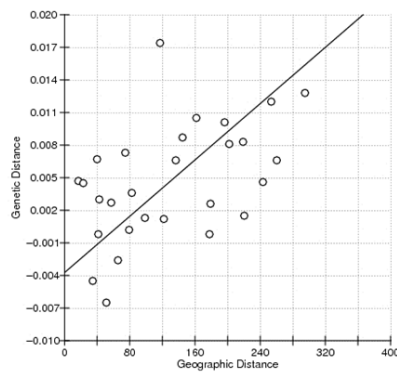


Figure 3. Isolation by distance graph for Diamondback terrapins. Correlation of genetic distance (pairwise F_{ST} values) and geographic distance (distance between centroids of sites).

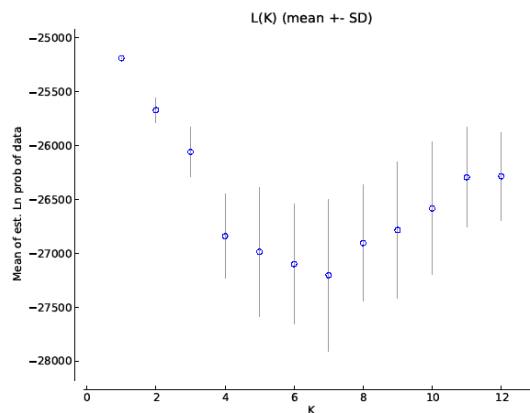


Figure 4. $-\ln K$ plot from STRUCTURE. Plotted mean estimated Ln probability scores with standard deviations for each value of K tested. The least negative value is the number of genetic structures with the highest likelihood.

BOTTLENECK detected significant excesses of heterozygotes in two diamondback terrapin groups: Barataria Bay ($p=0.0017$) and east of the Mississippi River ($p=0.0031$). The two sites with the smallest sample sizes (Sabine and Marsh Island) also corresponded to the lowest N_e estimates. Other sites tended to have values in the range of several hundred. Terrebonne Bay did have a much larger estimate of (226,099), although the lower end of the confidence interval fell within the range of values for most of the

other sites. In general, larger effective population sizes were found in the eastern portion of Louisiana.

Table 9

Effective population size for groups and regions

Sites	95% Confidence		Region	95% Confidence	
	Ne	Interval		Ne	Interval
Sabine	26	(16, 63)			
Calcasieu	521	(229, ∞)			
Mermentau	302	(214, 529)			
Rockefeller	830	(506, 2,131)			
Marsh Island	31	(21, 53)	West	983	(734, 1,459)
Terrebonne Bay	226,099	(902, ∞)			
Barataria Bay	488	(284, 1516)	Mid	1707	(858, 30,322)
East MS River	5762	(568,∞)	East	5762	(568,∞)

Note: Values were calculated by NeEstimator. Values in bold were significant for a population bottleneck detected by BOTTLENECK, $p < 0.05$.

Discussion

Population Structure

Significant population structure was not detected across the Louisiana coastline, even with sites that were up to 100km apart across an area suspected to be unsuitable habitat for diamondback terrapins (Marsh Island to Terrebonne Bay – across the Atchafalaya river). Similar to other small-scale studies, low F_{ST} values (for my study

average $F_{ST} = 0.0004$) were observed with no meaningful differentiation among groups (Drabeck et al. 2014; Sheradin et al. 2010, NJ average pairwise F_{ST} 0.0001; Hauswaldt and Glenn 2005, Charleston Harbor $F_{ST} = -0.0003$ to -0.011). This often led the authors to group small spatial scale clusters together when comparing to range wide data (Drabeck et al. 2014, Coleman 2011; Hauswaldt and Glenn 2005). Low F_{ST} values indicate a high degree of genetic connectivity between sites. Consistent with Glencos (2013; Galveston Bay and associated shipping channel, TX) and Drabeck et al. (Mississippi River, 2014), we found large freshwater rivers (Atchafalaya and Mississippi River) do not act as barriers to gene flow for diamondback terrapins. Previous approaches to assessing genetic structure across landscape features had been done using traditional genetic approaches (e.g., STRUCUTRE, *F-statistics*). In this study I also employed spatially explicit programs (TESS and GENELAND) and included much more intensive spatial coverage in sampling. Hence, I have considerable evidence to suggest that the genetic connectivity of diamondback terrapins is not impacted by large bodies of freshwater.

Isolation by Distance and Connectivity

Despite lack of genetic structuring I do not suggest there is a single panmictic population in Louisiana, but rather highly connected populations, which demonstrate isolation by distance. Both the Mantel test and Migrate-n results support this observation. There was a significant correlation between genetic distance and geographic distance. Likewise, the stepping stone model had the highest probability in the Migrate-n analysis. Even the model that allowed for stepping stone migration amongst

populations but not over the Atchafalaya River did not out-compete the stepping stone model. This is further support that the large rivers may not act as a barrier to gene flow.

It is unclear how the connectivity of Diamondback terrapins is maintained across major freshwater rivers as seen in this and other studies (Galveston, Atchafalaya and Mississippi). The gap produced by the Atchafalaya river delta (approximately 100km) is larger than the largest recorded movement of a Diamondback terrapin (Sheradin et al 2010, 8508m). Diamondback terrapins (age >3) maintain high site fidelity during the non-breeding season (Roosenberg 1999; Gibbons et al. 2001; Sheradin et al. 2010), which would presumably lead to population differentiation. However, this may be offset by male diamondback terrapins moving to meet in dense breeding aggregations (Seigel 1920). Since it is unknown how far males will travel to these breeding aggregations it is assumed that they facilitate gene flow, however, the magnitude is not clear. The inability of mark and recapture studies (Lovich and Gibbons 1990; Gibbons 2001; Sheridan et al. 2010) to demonstrate a Diamondback terrapin's natural ability or propensity to make large or regular movements as supported by molecular studies also suggests the possibility of high juvenile dispersal or stochastic events like hurricanes and storms to translocate individuals.

Historic Harvesting and Bottlenecks

Demographic influences of historical bottlenecks (TPM) within the Barataria Bay and East of the Mississippi River support historical documentation of diamondback terrapins being harvested and nearly extirpated from these regions. The sites are the same general location from which Hart et al. (2014) sampled and also detected a population bottleneck. An article in the Louisiana Conservationist magazine documents a

diamondback terrapin trapper who recalls his experiences in the Barataria bay and bayous (Davis 1973). The fisherman identifies other successful trappers and elaborates on the preference for female diamondback terrapins, how they would trap them on nesting shoals or in mud flats and then sell the diamondback terrapins at local markets (Davis 1973). These eastern populations also have effective population sizes with confidence intervals that can more realistically be interpreted as large populations that have either recovered or are still in the process of recovering from a demographic bottleneck. To the west of the Atchafalaya there is no indication of bottlenecks, and the confidence interval for the effective population size estimates is less broad, ensuring greater confidence in their estimates. Within the western part of Louisiana is Rockefeller Wildlife Refuge (RWR) owned and operated by the Louisiana Department of Wildlife and Fisheries. The refuge was donated to the Louisiana department of Wildlife and Fisheries from the Rockefeller Foundation in 1920. Within the deed of donation it made it a criminal offense to “destroy, kill, or pursue game, fish, birds, fur bearing animals or terrapins.” The wildlife refuge contains approximately 30,000 hectares maintained as saline, brackish, or freshwater marshes. This expansive refuge may be the key to maintaining genetic diversity and a presumably healthy population. It is the western part of the range where we have the RWR and Marsh Island Wildlife refuge that we find no indication of genetic bottlenecks. This part of the range is also less populated than the eastern part, so access to the salt marsh may have protected the Diamondback terrapins from overharvesting which likely occurred on the eastern part of the range.

Genetic diversity observed in this study is comparable to measures of diversity seen in east coast populations which were intensely studied using similar loci (this study,

n=566, 12 loci, average $H_O=0.74-0.77$, $H_E=0.74-0.78$; Sheradin et al. 2010, NJ, n=1558, 6 loci, $H_O=0.82$, $H_E=0.81$; Hauswaldt and Glenn 2005, Charleston Harbor, SC, n=130, 6 loci, $H_O=0.84$, $H_E=0.85$; Hart et al. 2014, NC, n=120, 12 loci, $H_O=0.66$, $H_E=0.68$).

Among genetic studies in other gulf coast states, lower levels of genetic diversity have been documented (Coleman 2011, AL, n=53, 12 loci, $H_O=0.51$, $H_E=0.51$ and Glencos 2013, Galveston Bay, TX, n=61, 12 loci, $H_O=0.43$). Lack of reduction in genetic diversity found within Louisiana could be a reflection of a more moderated bottleneck where it did not persist for many generations. However, the lower levels of genetic diversity in the AL and TX studies may be the result of using markers with low levels of polymorphism and small sample sizes. Also consistent with the ideas of small or short-lived bottlenecks is the detection of large effective population sizes in the eastern portion of Louisiana.

Conclusions

The results of this study suggest that further research should be done to effectively detect movement or use across the Atchafalaya delta especially during the mating season (March and April) when diamondback terrapins are most likely to be moving throughout the estuaries. Finding individuals that utilize the Atchafalaya river delta as corridor between Marsh Island and Terrebonne Bay would support a highly connected population as my genetic data suggest. If no resident populations or transient individuals are found then the focus should be applied to how stochastic environmental influences may be redistributing a random few number of individuals. Long term mark and recapture studies should be conducted before and after these large environmental stochastic events (e.g., hurricanes and tropical storms). It is not clear how effective these large chaotic effects are at moving individuals to adjacent populations or across longer

distances. Lastly, effort needs to be applied toward better understanding the dispersal ability of juvenile hatchlings (<3 years) and how far in a lifetime they might move from natal beaches.

Management implications of this study suggest the east and west “populations” (as defined by the Atchafalaya River) might be managed differently as demographic histories and current geophysical threats are different. In the west there are multiple wildlife refuges that protect diamondback terrapins and their ecosystem. The amount of development to these areas is limited, and the use by humans for fishing is somewhat restricted. East of the Atchafalaya is where habitat fragmentation and salt marsh erosion (Couvillion et al. 2011) is most severe. The instability in the habitat and presence of historical bottlenecks creates uncertainty in the persistence of diamondback terrapin populations east of the Atchafalaya River. Although there was no discrete genetic structuring across Louisiana, it is clear there are historical and present day concerns which may dictate the use of different management strategies when preserving the Diamondback terrapins that reside within Louisiana.

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APPENDIX



The University of
Southern Mississippi

*Institutional Animal Care
and Use Committee*

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

TO: Brian Kreiser, Ph.D.
118 College Drive #5018
Hattiesburg, MS 39406-0001

FROM: Jodie M. Jawor, Ph.D.
IACUC Chair

PROTOCOL NUMBER: 11092206
PROJECT TITLE: Population Genetics & Systematics of Freshwater Fishes

Enclosed is The University of Southern Mississippi Institutional Animal Care and Use Committee Notice of Committee Action taken on the above referenced project proposal. If I can be of further assistance, contact me at (601) 266-4748, or you may e-mail me at Jodie.Jawor@usm.edu. Good luck with your research.



The University of
Southern Mississippi

Institutional Animal Care
and Use Committee

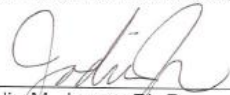
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**INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
NOTICE OF COMMITTEE ACTION**

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



DATE