THE MATING DYNAMICS AND POPULATION GENETICS OF THE AMERICAN ALLIGATOR (ALLIGATOR MISSISSIPPIENSIS)

By

JOSHUA ZAJDEL

(Under the Direction of Stacey Lance and Benjamin Parrott)

ABSTRACT

Genetic diversity is a key factor in the long-term viability of a population; thus, it is important to understand intrinsic and extrinsic processes that influence genetic diversity within and among populations. We examined parentage in 151 American alligator nests and find that 43% of nests were sired by multiple males and that male reproductive success is strongly influenced by male size. No significant difference was found between the sizes of hatchlings or clutch size from multiply sired clutches and singly sired clutches. However, fertility rates were lower in multiply sired clutches. Our findings suggest that sexual conflict might influence the frequency of multiple paternity. We then examined 192 individuals from 8 populations. We developed DNA sequence capture methods to explore how the populations are connected.

INDEX WORDS: MULTIPLE PATERNITY, AMERICAN ALLIGATOR, MICROSATELLITE, MATING DYNAMICS, PARTIAL MATE FIDELITY, POPULATION GENETICS

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

The maintenance of genetic diversity is a key component of conservation. The loss of genetic variation has been correlated with reduced individual fitness and increased extinction risk (Reed and Frankham, 2003; Reed et al., 2007; Vandewoestijne et al, 2008). The genetic diversity of a wildlife population is influenced by many factors, such as the population's connectedness to other populations, and the species' specific mating dynamics (Juanes et al. 2007; Karl 2008; Saura et al. 2008; Sugg and Chesser, 1994; Valiente et al. 2005).

A species' mating dynamics can influence the genetic diversity within a population (Juanes et al. 2007; Karl 2008; Saura et al. 2008; Sugg and Chesser, 1994; Valiente et al. 2005). This is particularly true for species that have a high amount of multiple paternity (Juanes et al. 2007; Karl 2008; Sugg and Chesser, 1994; Valiente et al. 2005). Not only can high levels of multiple paternity increase a population's effective size, it can increase the genetic diversity within a population and lead to a more viable population (Martinez et al. 2000; Moran and Garcia-Vazquez, 1998; Sugg and Chesser, 1994). However, before the impact of multiple paternity on effective population size can be determined, the overall and average occurrence of multiple paternity within a population must first be described.

Populations that are more connected to other populations often have a higher amount of genetic diversity than isolated populations (Reed, 2004). Populations can become isolated for several reasons including geologic features or anthropogenic change to the environment (Mayr, 1970). However, it may not always be obvious which populations are genetically isolated without conducting genetic studies (Frankham, 1995). Once the degree of connectivity and genetic diversity within a population are known, the information can be used in conservation and management decisions and efforts can be made to preserve and or enhance the genetic diversity within the population (Frankham, 1995).

Multiple Paternity

Multiple paternity is a common and prevalent component of mating systems in a wide array of taxonomic groups (see reviews; Birkhead and Møller, 1998; Boomsma and Ratnieks, 1996; Byrne and Avise, 2012; Griffith et al. 2002; Uller and Olsson 2008). In spite of this prevalence, the evolutionary and ecological drivers of multiple paternity are still not well understood, though several hypotheses have been proposed to explain its occurrence (see review Jennions and Petrie, 2000) as discussed below.

Females may mate with multiple males in order to ensure complete fertilization of her clutch. A single male may not be capable of fertilizing all available eggs and females may require additional matings to ensure complete fertilization (Gibson and Jewell, 1982; Krokene et al., 1998). In addition, females

may mate with multiple males to promote sperm competition (see review Jennions and Petrie, 2000). In this scenario, females are unable to have much precopulatory mate selection but by mating with multiple males and retaining their sperm, the female is able to promote sperm competition and ensures that the higher quality sperm fertilize her gametes (see review Jennions and Petrie, 2000). This strategy has often been referred to as cryptic female choice or the "sexually selected sperm" hypothesis (Bocedi and Reid, 2015). Alternatively, females may be performing genetic bet hedging, wherein a female promotes genetic diversity within her offspring due to uncertain future environmental conditions (Yasui, 1998).

It is possible that females receive direct benefits from mating and multiple paternity is the result of females attempting to increase those benefits. An example of this scenario comes from insects, wherein females often receive nuptial gifts from males prior to mating (Arnqvist and Nilsson, 2000). These nuptial gifts often provide valuable resources to the female and by courting and mating with multiple males, females may be increasing the amount she receives (Arnqvist and Nilsson, 2000).

In all of the above scenarios, females directly benefit from mating multiply. Another possibility is that females mate multiply to avoid the cost of resisting males (Sztatecsny et al. 2006). If male harassment poses a significant cost to the female but the act of mating poses little cost, then females may mate with

multiple males in order to reduce the overall costs to themselves (Sztatecsny et al. 2006). Aligned with this hypothesis is the idea that multiple paternity may simply reflect the encounter rate between males and females. Under this hypothesis, females may not be seeking out direct benefits, rather multiple paternity is a result of a female having access to several males (Martin et al. 2014; Wells et al. 2017). This hypothesis has been referred to as the null model of multiple mating (Kokko et al., 2006).

Population Genetics

Individual populations can have reduced genetic diversity and unique genetic characteristics for several reasons. (Levin, 1970; Mayr, 1942; Mayr 1970; Nei et al., 1975). One such factor is the population's demographic history. Populations that have experienced recent or historical declines may have reduced genetic diversity when compared to other populations of the same species (Nei et al., 1975). This process is known as the bottleneck effect wherein a portion of the genetic diversity in the population was lost due to a population decline (Nei et al., 1975).

A similar effect can be seen if a population has been newly founded. Individuals colonizing a new area often only contain a small portion of the total genetic diversity that can be found in the population that these individuals migrated from. This effect is known as the founder effect and can be observed in many island populations (Mayr, 1942).

Genetic diversity can also be reduced if a population becomes isolated and remains so for a significant period of time (Crow and Kimura, 1970). Under this scenario, genetic diversity is lost through random chance. Individuals with certain alleles may die without being able to pass on those alleles or alleles may not be passed onto the next generation because of the independent assortment of genes. This process occurs in all populations and is known as genetic drift (Crow and Kimura, 1970). However, in isolated populations, insufficient levels of migration occur to counteract the effects of genetic drift resulting in decreasing levels of genetic diversity over time (Frankham, 1995; Levin, 1970).

Populations may be isolated for several reasons including, impenetrable geologic features such as mountain ranges, large rivers, oceans, or by anthropogenic changes such as the development of cities or large highways (Mayr, 1970). Another possibility is that the degree of isolation occurs along a gradient such as distance from large population cores (Levin, 1970; Mayr, 1970; Wright, 1943). This effect can be seen within populations located along the edge of a species' range. These populations often receive fewer migrants and tend to show lower levels of genetic diversity (Levin, 1970).

A similar effect can be seen when differing levels of habitat permeability lead to population isolation. Depending on a species' natural history, different types of landscapes may be more or less difficult for an individual to move through (Sugg et al.,1996). If a population is surrounded by less permeable habitat, the

population may be isolated even if the population is relatively close to other populations (Sugg et al.,1996). No matter the causes of population isolation, the effects to the population's genetics are the same, reduced genetic diversity and distinct allelic characteristics (Levin, 1970; Mayr, 1942; Mayr 1970; Nei et al., 1975; Wright, 1943).

The American Alligator

The American alligator *(Alligator mississippiensis)* is a large predator endemic to the Southeastern United States. It is one of only two species within the genus *Alligator* (Ross, 1989). The species ranges from Eastern Texas to the Atlantic seaboard and from the Southern tip of Florida to North Carolina (Ross, 1989). The American alligator is the largest predator within its range, except where it is sympatric with the American crocodile (Ross, 1989). As such, the species exhibits strong top-down predatory influences on its environment (Jones et al, 1994; Nifong and Silliman, 2013;Williams et al, 2004). Furthermore, the alligator can directly influence its environment with behaviors, such as building and maintaining ponds, leading to its classification as an ecosystem engineer (Jones et al, 1994; Kushland, 1974; Palmer & Mazzotti, 2004).

Historically, American alligator populations have experienced unregulated hunting, which contributed to large population declines during the early twentieth century prompted federal protection under the Endangered Species Act in 1973 (Ross, 1989). The American alligator population has since rebounded and was

removed from the federal endangered species list in 1985. Currently, American alligator populations are actively managed by both federal and state wildlife agencies for recreational hunting and collection of eggs for the alligator ranching industry (Chabreck, 1978; Dutton et al., 2002; Saalfield et al., 2012).

American Alligator Nesting Ecology

Ongoing management efforts at the state and federal level have encouraged numerous studies concerning the life history and ecology of the alligator since its delisting, with nesting ecology being a subject of particular interest. The earliest investigations into the nesting ecology of the American alligator were pioneered by EA McIlhenny (1935) whose studies focused on alligators in Louisiana. Additional research expanded on these early studies to include other areas in the alligator's range. Nesting studies have been conducted in Louisiana, Florida, Georgia, Texas, South Carolina, and North Carolina (Deitz and Hines, 1980; Goodwin and Marion, 1978; Hunt and Ogden 1991; Joanen, 164; Joanen et al., 1977; Joanen and McNease, 1989; Kushlan and Jacobsen, 1990; Kushlan and Kushlan, 1979, 1980; Metzen, 1976; Platt and Brantley, 1995; Saalfield et al., 2012; Wilkinson, 1984; Wilkinson and Rhodes, 1992). Studies have concentrated on characterizing the average clutch size, nest success rates, causes of nest failure, female attendance, and female nest defense. These studies have demonstrated variability in American Alligator nesting ecology, however the averages from these studies closely align. Collectively, these studies have shown that the

American alligator produces an average of 36 eggs per clutch, ranging from 30 to 58 eggs and with an average of 45% of the nests producing at least one successful hatchling. The primary causes of nest failure are flooding and predation by raccoons (*Procyon lotor*) (Deitz and Hines, 1980; Goodwin and Marion, 1978; Hunt and Ogden 1991; Joanen, 164; Joanen et al., 1977; Joanen and McNease, 1989; Kushlan and Jacobsen, 1990; Kushlan and Kushlan, 1979, 1980; Metzen, 1976; Platt and Brantley, 1995; Saalfield et al., 2012; Wilkinson, 1984; Wilkinson and Rhodes, 1992). An interesting exception to these general observations comes from the studies of Metzen (1976) and Hunt and Ogden (1991). These studies found that in the Okefenokee Swamp in southern Georgia from 1972 to 1988, between 90% and 93% of the alligator nests where predated prior to hatching, primarily by American black bear (*Ursus americanus*). Additionally, female attendance was found to vary across the studies. The lowest rate of female attendance was found by Joanen (1964) who found females attending to 9.2% of the nests. While Hunt and Ogden (1991) found a high of 66% of the nests having female attendance.

Alligator nests are typically made in freshwater marshes although alligators have been reported to nest in brackish marshes in South Carolina. Nest building takes place from June to July throughout the alligator's range. Nests are constructed by piling available vegetation into a dome shape. These domes are often constructed with cordgrass (*Spartina spp*.), sawgrass (*Cladium jamaicense)*

and common reed (*Phragmites spp.*) (Deitz and Hines, 1980; Goodwin and Marion, 1978; Hunt and Ogden 1991; Joanen, 164; Joanen et al., 1977; Joanen and McNease, 1989; Kushlan and Jacobsen, 1990; Kushlan and Kushlan, 1979, 1980; Metzen, 1976; Platt and Brantley, 1995; Saalfield et al., 2012; Wilkinson, 1984; Wilkinson and Rhodes, 1992).

American Alligator Mating Behavior

Despite the intensive research into alligator nesting ecology, relatively little research has focused on alligator mate selection and reproduction. Garrick and Lang (1977) describe the mating behaviors of captive American alligators involving highly ritualized courtship behaviors with vocalizations and body posturing. Captive studies have also demonstrated that females seem to accept or reject males based on size and that forced copulations are unlikely (Garrick and Lang 1977, Joanen and McNease 1971). However, *in situ* studies of wild alligator mating behavior are lacking. In the wild, mating behaviors take place in the water, thus definitive copulation between two individuals is difficult to observe. Similarly, because copulation often occurs within groups of indistinguishable individuals, identification of particular mating pairs is difficult (Lang 1987). Some studies have been able to report the minimum size at which wild male alligators have been found to produce sperm. In Louisiana, male alligators as small as 1.83 meters have been reported to be producing sperm during the mating season, while in North Carolina only alligators above 2.2 meters have been found

to be producing sperm during the mating season (Joanen and McNease, 1980; Murphy and Coker 1983).

Advances in genetic techniques have allowed researchers to overcome these obstacles, with microsatellite markers being particularly advantageous (Davis et al 2001, de Oliveira et al 2010, Glenn et al, 1998, Lance et al, 2009). Briefly, microsatellites are short tandem arrays of repeated DNA sequences that are useful in distinguishing individual organisms and populations (Queller et al., 1993). Glenn et al. (1998) developed microsatellite markers for alligators and demonstrated their utility in distinguishing individuals and populations. Facilitated by this advancement, Davis et al. (2001) confirmed that females found guarding nests were indeed the mothers of the nests. Davis et al. (2001) was further able to establish the occurrence and incidence rate of multiple paternity, reporting its occurrence in seven of 22 clutches examined, one of which was fathered by three males. Following Davis et al. (2001), Lance et al. (2009) further examined patterns of multiple paternity in American Alligators over a ten-year period (1995-2005) at the same study location, Rockefeller National Wildlife Refuge, Cameron Parish, Louisiana. This study reported high variability in multiple paternity rates between years, with a range of 32% to 67% of the nests showing multiple paternity in a given year. To date, no other studies have characterized the occurrence of multiple paternity within an American alligator population outside of Louisiana.

American alligator Population Genetics

The IUCN and the Crocodilian Specialist Group have stated that the preservation of genetic diversity is one of their main conservation objectives (IUCN, 1980; Thorbjarnarson et al., 1992). With that goal in mind, recent studies have focused on understanding the genetic structure of crocodilian populations. Currently, populations of the American crocodile (*Crocodylus acutus*), broad snouted caiman (*Caiman latirostris*), Nile crocodile (*Crocodylus niloticus*), West African crocodile (*Crocodylus suchus*), false gharial (*Tomistoma schlegeii*), Morelet's crocodile (*Crocodylus moreletii*), and the spectacled caiman (*Caiman crocodilus*) have been a part of studies focusing on population genetics. These studies report genetic differentiation among populations of crocodilians and a general trend of low genetic diversity within crocodilian populations (Amavet et al., 2007; Cunningham et al., 2016; de Oliveira et al., 2010; Dever et al., 2002; González-Trujillo et al., 2012; Hekkala et al., 2010; Marques et al., 2016; Mauger et al., 2017; Shafiei-Astani et al., 2015; Serrano-Gómez et al., 2016; Vandewoestijne et al., 2008; Velo-Antón et al., 2014; Versfeld, 2016).

The American alligator (*Alligator mississippinesis*) has also been a part of the increased focus on population genetics. The earliest American alligator genetic studies used allozymes and isozymes to examine the genetic variation. These early studies found low levels of genetic diversity (Adams et al., 1980; Gartside et al., 1977; Menzies et al., 1979). For example, Adams et al. (1980) reported an

average observed heterozygosity of 0.022 and did not find any significant level of genetic differentiation between populations of American alligators in South Carolina, Louisiana, and Florida.

Using microsatellites, Glenn et al. (1998) found a larger amount of genetic variation within and between American alligator populations. They found an average level of heterozygosity of 0.47 and significant differentiation between populations in Louisiana and Florida. This study was expanded upon by Davis et al. (2001), who examined six populations of American alligators: two in Louisianan, one in Alabama, one in Florida, and two in South Carolina. This study found an average observed heterozygosity of 0.637, with the lowest observed heterozygosity (0.52) found in the population at Santee Island, South Carolina. This study also noted significant differences between the Florida, Louisiana, and South Carolina populations. The two South Carolina populations – Santee Island, a barrier island on the coast, and the Savannah River Site, an inland site along the Savannah River – also exhibited significant differentiation from each other.

Davis et al. (2002) then expanded their study to include twelve populations spread across Texas, Louisiana, Alabama, Georgia, Florida, and South Carolina. This study included all sites from Davis et al. (2001) except the Savannah River Site in South Carolina. They found an average level of heterozygosity of 0.64 and found significant differentiation between the western populations (Texas and

Louisiana) and the eastern populations (Georgia, Florida), with the Alabama population showing characteristics of both eastern and western populations. Of particular note are the results from the South Carolina population on Santee Island. This population again had the lowest level of observed heterozygosity (0.547), a complete absence of private alleles, and had allelic distributions unlike any other population in the study. To date Adams et al. (1980), Davis et al. (2002) and Davis et al. (2001) are the only studies to examine the genetic structure of American alligators at the northern extent of their range.

The only fine scale study of alligator population genetics was conducted in East Texas (Ryberg et al. 2002). Specifically, this study examined differences between coastal and inland populations as well as differences between populations inhabiting different river drainages. They found an average observed heterozygosity of 0.565 and significant differentiation between all of the populations regardless of their geographic characteristics. The level of differentiation between populations was best explained by an isolation by distance model. This study indicates the potential for American alligator populations to be differentiated at a fine scale. However, to date, no further studies have examined the fine scale differentiation of other American alligator populations.

Thesis Objective

In order to better understand the ecology the American alligator we will characterize the occurrence of multiple paternity and mate selection dynamics at

one population in South Carolina and examine how the occurrence of multiple paternity within a clutch relates to clutch and hatchling characteristics such as clutch fertility and hatchling size. To do this, our study uses previously collected tissue samples from hatchling and adult alligators from the Tom Yawkey Wildlife Center to genotype these individuals with microsatellites and determine which clutches have been multiply sired. Further, we use adult genotypes to match adults back to any hatchlings they may have sired.

We will also examine the degree of connectivity among American alligator populations. To do this we will collect samples from Alligator populations in South Carolina and North Carolina and incorporate previously collected samples from Florida, Georgia, South Carolina, and North Carolina. We use DNA sequence capture techniques to compare single nucleotide polymorphisms between alligator populations.

My thesis objectives were to 1) characterize the occurrence of multiple paternity at the Tom Yawkey Wildlife Center, 2) examine how alligator morphometrics may influence male alligator reproductive success, 3) examine potential differences in the fitness of offspring from multiply sired and singly sired nests, and 4) examine the degree of connectivity between American alligator populations ranging from North Carolina to Florida.

CHAPTER TWO

MULTIPLE PATERNITY IS ASSOCIATED WITH SEXUAL CONFLICT IN A LONG-LIVED VERTEBRATE

To be submitted to Ecology and Evolution

Abstract

Multiple paternity is relatively common across diverse taxa; however, the drivers and implications related to paternal and maternal fitness are not well understood. Here we investigate mating system dynamics in a historically studied population of the American alligator in South Carolina. We examine parentage in 151 nests across 6 years and find that 43% of nests were sired by multiple males and that male reproductive success is strongly influenced by male size. There was no significant difference in clutch size between singly sired and multiple sired nests. However, fertility rates are lower in multiply sired clutches. No significant difference was found between the sizes of hatchlings from multiply sired clutches and singly sired clutches. Our findings suggest a cost of multiple paternity in regard to female fitness, suggesting that sexual conflict might be the cause of frequency of multiple paternity in wild alligator populations.

Keywords: Multiple Paternity, American alligator, Mate selection, Reproductive success

Introduction

One of the most novel discoveries resulting from modern genetic analysis of mating systems is that multiple paternity, wherein more than one male sires a clutch or litter, is relatively common across vertebrates (Birkhead and Møller, 1998; Coleman and Jones, 2011; Griffith et al, 2002; Uller and Olsson, 2008). However, evolutionary explanations for the occurrence of multiple paternity and whether it is adaptive for females are not always evident (Birkhead and Møller, 1998; Jennions and Petrie 2000; Griffith et al, 2002; Uller and Olsson, 2008). Hypotheses typically include direct or indirect benefits to the female, wherein direct benefits encompass male contribution to parental care, improved genetic quality of offspring, and increased fertilization success (see reviews Birkhead and Møller, 1998; Griffith et al, 2002; Jennions and Petrie, 2000; Uller and Olsson, 2008). Indirect benefits can stem from promoting sperm competition, cryptic female choice or genetic bet-hedging to create a genetically diverse clutch (Eberhard 1996; Jennions and Petrie, 2000; Keller and Reeve, 1995; Yasui, 1997). All of these explanations suggest that multiple mating by females is adaptive. Contrary to this idea, it has also been suggested that multiple paternity might result from sexual conflict and be non-adaptive for females (Andersson, 1994; Arnqvist and Kirkpatrick, 2005; Kokko et al., 2006; Lee and Hays, 2004; Martin et al. 2014; Sztatecsny et al. 2006; Wells et al. 2017). Under this scenario, the

amount of mating by females may increase with mate encounter rate and be limited by the cost of mating to females (Andersson, 1994).

In non-avian reptiles, there is broad support for multiple paternity resulting from female mating frequency being driven by mate encounter rates (Fitze et al. 2005; Garner et al. 2002; Jensen et al. 2006; Laloi et al. 2004; Lee and Hays, 2004; Olsson and Shine, 1997). Non-avian reptiles typically do not provide paternal care and, therefore, offspring would not benefit from increased care from multiple males (Gans, 1996). Furthermore, some studies have failed to find evidence for direct or indirect benefits from multiply paternity (Fitze et al. 2005; Garner et al. 2002; Jensen et al. 2006; Laloi et al. 2004; Lee and Hays, 2004; Olsson and Shine, 1997). However, other studies report correlations between population density and the frequency of multiple paternity (Fitze et al. 2005; Jensen, 2006; Laloi et al., 2004; Lee and Hays, 2004). In olive ridley sea turtles (*Lepidochelys olivacea*), the frequency of multiple paternity varies across nesting sites, with nesting sites having higher densities of turtles also characterized by higher frequencies of multiple paternity (Jensen, 2006). However, given the taxonomic and behavioral diversity in non-avian reptiles, there is still debate as to the drivers of multiple paternity (Amavet et al, 2008; Bryne and Robert, 2000; Budd et al. 2015; Davis et al. 2001; Fitze et al. 2005; Garner et al. 2002; Jensen et al. 2006; Lafferriere et al, 2016; Laloi et al. 2004; Lance et al. 2009; Lee and Hays, 2004; Lewis et al, 2013; Mcvay et al, 2008; Muniz et al. 2011; Ojeda et al,

2017; Oliveira et al, 2014; Olsson and Shine, 1997; Shine, 1988; Wu and Hu, 2010).

Crocodilians, which have widely varying population densities and degrees of male territoriality provide an excellent system to explore the evolutionary and ecological drivers that underlie the observed variation in frequency of multiple paternity (Amavet et al, 2008; Budd et al. 2015; Davis et al, 2001; Lafferriere et al, 2016; Lance et al, 2009; Lewis et al, 2013; Mcvay et al, 2008; Muniz et al. 2011; Ojeda et al, 2017; Oliveira et al, 2014; Wu and Hu, 2010). The frequency of multiple paternity observed across crocodilian taxa ranges from 32% in the Chinese alligator (*Alligator sinensis*) to 100% in black caiman (*Melanosuchus niger*) (Muniz et al. 2011; Wu and Hu 2010). Among crocodilians, it is not clear if the frequency of multiple paternity is driven by population density and/or mate encounter rate (Amavet et al, 2008; Budd et al. 2015; Davis et al, 2001; Lafferriere et al, 2016; Lance et al, 2009; Lewis et al, 2013; McVay et al, 2008; Muniz et al. 2011; Oliveira et al, 2014; Wu and Hu, 2010) though both have been suggested (Budd et al. 2015; Lafferriere et al, 2016).

The most thoroughly studied crocodilian species in terms of multiple paternity and mating behavior is the American alligator (*A. mississippiensis*) (Davis et al, 2001; Garrick and Lang, 1977; Joanen and McNease, 1971; Lance et al, 2009). However, because of the difficulty of observing mating activity in the wild, most research into mate selection dynamics has focused on captive

populations (Garrick and Lang, 1977; Joanen and McNease, 1971). Studies on these animals describe a complex courtship process with larger male alligators holding territories more successfully than smaller males (Garrick and Lang, 1977; Joanen and McNease, 1971). However, whether territorial gains translate into reproductive success remains unknown.

Previous studies investigating mating dynamics of wild alligators using genetic techniques have exclusively examined the population at the Rockefeller National Wildlife Refuge (RNWR) in Louisiana (Davis et al 2001; Lance et al 2009). These studies demonstrated that an average of 46% of nests have multiple sires (Davis et al 2001; Lance et al 2009). The study by Lance et al. (2009) was also the first to demonstrate mate fidelity across years in any crocodilian species. However, in both studies males were identified solely by offspring genotypes and thus, the phenotypic attributes of males that might lead to higher reproductive success could not be inferred. Wild alligators examined in this investigation are part of a long-studied population (Wilkinson et al. 2016) for which data on size, sex, and age of many individuals are available. Here, we examine mating dynamics in the American alligator with respect to the frequency of multiple paternity, the role of male characteristics in male reproductive output, and potential fitness benefits to females with multiply-sired clutches. By examining these questions within the context of the American alligator mating system we seek to better understand whether multiple paternity is driven by evolutionary

fitness advantages across sexes or is the product of population-specific parameters.

Methods

Site Description

This study was conducted on the South Island and Cat Island portions (6,033 ha) of the Thomas A. Yawkey Wildlife Center (YWC), a wildlife management area operated by the South Carolina (SC) Department of Natural Resources. The YWC alligator population is relatively closed, in that it is bordered by saltwater on all sides; the Atlantic Ocean to the east, Winyah Bay to the north, the Intracoastal Waterway to the west, and North Santee Bay to the south. This alligator population is well-characterized due to long-term (1970s to present) mark-recapture efforts resulting in a large database of alligator tissue, nesting, and morphometric data (Bangma et al., 2017; Hale et al., 2017; McCoy et al., 2015; Parrott et al., 2014; Wilkinson et al. 2016). However, information regarding the ongoing mark recapture studies was not available for this study.

Egg and Hatchling Collection

Alligator eggs were collected at YWC from 2011 to 2017. Helicopter surveys were used to locate nests from the air during the peak of the alligator nesting season in SC (early June-early July; Wilkinson, 1984). Nests were visited daily on foot until oviposition was confirmed**.** Fertility rates were determined by observing banding patterns (fertile eggs exhibit an opaque patch or band on the eggshell;

Ferguson, 1982). Eggs were collected within 48 hours of oviposition and transported to the Hollings Marine Laboratory (2011 – 2016) in Charleston, SC or the University of Georgia Savannah River Ecology Laboratory (2017) in Aiken County, SC where they were either necropsied as embryos or reared to hatching. In some cases, entire clutches of eggs were taken while at other nests only a subset (1-8 eggs) was collected (Table 1).

In 2012, 2013, and 2017 twenty-seven full clutches were collected, maintained in damp sphagnum moss and reared until hatching. For all years in which eggs were allowed to hatch, eggs were checked twice daily for the initiation of hatching ("pipping") and once hatchlings had pipped, they were removed from sphagnum and transferred to individual glass jars. Embryos that had not completed the hatching process within 48 hours of pipping were manually assisted in order to limit hatchling loss. Upon hatching, neonates were weighed and snout-vent length (SVL), total length, cloacal tail girth, and both head and snout length and width were measured. Scutes and/or chorioallantoic membrane were collected shortly after hatching. All tissue samples collected from hatchling alligators were immediately stored at -20° C upon collection.

A total of 1657 hatchlings were sampled from 151 nests. For 31 nests, we collected the entire clutch of eggs. For the remaining 120 nests, we collected a subset of the eggs (1-8 eggs).

Adult Alligator Capture and Sampling

Adult alligators were captured using multiple methods including baited-trip snare traps, walk-through traps, snare poles, and snatch hooks (Cherkiss et al. 2004; Murphy et al., 1983; Wilkinson 1994, Wilkinson et al. 2016). Over the course of the study we sampled 204 adult alligators, 120 females and 84 males. Of the 120 females sampled, 76 were captured on or near a nest. Alligator SVL ranged from 63.5 – 176.0 cm (females) and 73.66 – 194.3 cm (males). The preponderance of females in our data set was the result of a research focus on nesting ecology from 2009 to 2017 in which female alligators were captured at their nests (Wilkinson et al. 2016). Following capture, total length, SVL, and tail girth were measured for each animal and scute and blood samples collected. All samples collected in the field were stored on ice until transport to the laboratory where they were stored at -20°C until DNA extraction.

DNA Extraction

Alligator DNA was isolated from a variety of sample types including adult blood and scutes, hatchling chorioallantoic membranes, scutes, and embryos preserved in RNAlater. DNA isolation was performed using the DNeasy blood and tissue kit (Qiagen, Valencia, CA) following the manufacturers' protocols with the following exceptions. Econospin columns (Epoch Life Sciences, Inc., Missouri City, TX) were used during DNA filtration and DNA was eluted with 100 µl of the provided AE buffer. DNA concentrations were determined using a

NanoDrop Spectophotometer ND-1000 (Thermo Scientific, Waltham, MA) and standardized to 20 ng/µl.

Microsatellite Development

We initially screened a subset of samples using the same microsatellite loci used by Lance et al. (2009). However, the YWC samples exhibited insufficient genetic variation for conducting parentage analyses (Lance, unpublished data). Therefore, we developed new microsatellite loci. We extracted DNA from one individual and prepared an Illumina paired-end shotgun library by shearing 1 µg of DNA using a Covaris S220 sonicator and following the standard protocol of the Illumina TruSeq DNA Library Kit using a multiplex identifier adaptor index. Illumina sequencing was conducted on a HiSeq with 100 bp paired-end reads. Five million of the resulting reads were analyzed with the program PAL_FINDER_v0.02.03 (Castoe et al. 2012) to extract those reads that contained di-, tri-, tetra-, penta-, and hexanucleotide microsatellites. Once positive reads were identified, they were batched to a local installation of the program Primer3 (version 2.0.0) for primer design. To avoid issues with copy number of the primer sequence in the genome, loci for which the primer sequences only occurred one or two times in the five million reads were selected. Forty-eight potential loci that met this criterion were chosen. One primer from each pair was modified on the 5' end with an engineered sequence (CAG tag 5'-CAGTCGGGCGTCATCA-3') to enable use of a third primer in the PCR (identical to the CAG tag) that was

fluorescently labeled. The sequence GTTT was added to primers without the universal CAG tag addition.

The 48 potential primer pairs were tested for amplification and polymorphism using DNA obtained from eight individuals. PCR amplifications were performed in a 12.5 μL volume (10 mM Tris pH 8.4, 50 mM KCl, 25.0 μg/ml BSA, 0.4 μM unlabeled primer, 0.04 μM tag labeled primer, 0.36 μM universal dye-labeled primer, 3.0 mM MgCl2, 0.8 mM dNTPs, 0.5 units JumpStart Taq DNA Polymerase [Sigma], and ~20 ng DNA template) using an Applied Biosystems GeneAmp 9700. Touchdown thermal cycling programs (Don et al. 1991) encompassing a 10°C span of annealing temperatures ranging between 65-55°C (TD65) were used for all loci. Touchdown cycling parameters consisted of an initial denaturation step of 5 min at 95°C followed by 20 cycles of 95°C for 30 s, highest annealing temperature (decreased 0.5°C per cycle) for 30 s, and 72°C for 30 s; and 20 cycles of 95°C for 30 s, lowest annealing temperature for 30 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. PCR products were run on an ABI-3130xl sequencer and sized with Naurox size standard prepared as described in DeWoody et al. (2004), except that unlabeled primers started with GTTT. Results were analyzed using GeneMapper version 3.7 (Applied Biosystems).

We further assessed the variability of ten polymorphic loci (Almi 8, Almi 19, Almi 26, Almi 30, Almi 32, Almi 34, Almi 39, Almi 40, Almi 46, and Almi 47)

across all adult individuals using the same conditions described above with a touchdown protocol and highest annealing temperature of 58°C. Allele frequencies for these ten loci were estimated using all adults captured during the course of the study. We estimated the number of alleles per locus (k), observed and expected heterozygosity (H_o and H_e), mean polymorphic information content (PIC), the non-exclusion probability for the first parent (NE-1P), the non-exclusion probability for the second parent (NE-2P), and the non-exclusion probability for the parent pair (NE-PP) with Cervus 3.0.7 (Kalinowski et al. 2007). Tests for deviations from Hardy-Weinberg equilibrium (HWE) and for linkage disequilibrium were conducted using GENEPOP v4.0 (Rousset 2008). Characteristics of the loci are provided in Table 2. After determining that these 10 loci would provide the power needed for parentage analyses, we genotyped 1657 hatchlings across all 10 loci using the same conditions.

Maternal Genotype Comparison and Genotyping Error Rate

Hatchling alligator genotypes were initially screened using the Program Gerud 2.0 to test that each clutch could be explained by a single maternal genotype (Jones 2005). The genotypes of clutches that could not be explained by a single maternal genotype were examined for unexpected alleles. If an unexpected allele occurred at one locus, the allele was considered to be a mutation and the allele calls for that hatchling at the locus were excluded from future analysis. If an individual contained two or more alleles that prevented the clutch

from having a single maternal genotype, the individual was removed from further analysis. Almi 40 consistently produced unreliable alleles and was removed from future analysis.

Following the initial screening process, hatchling genotypes were compared to the genotype of the female caught at the nest to confirm maternity. If the genotype of a female captured at a nest was not consistent with maternity, then the female DNA and hatchling DNA were re-extracted and the female's microsatellite loci were amplified in triplicate and hatchling microsatellite loci were amplified in duplicate. Allele calls from the same individual but different amplifications were compared in order to estimate the genotyping error rate. A total of 457 individuals (28% of the total number of individuals in the study) were re-analyzed to determine the genotyping error rate. Almi 19, Almi 32, Almi 39, and Almi 46 all had genotyping error rates above 10% and were therefore excluded from further analysis. The remaining loci had an average genotyping error rate of 5% with a standard deviation of 2% (Table 2). Table 2 shows the number of alleles per locus (k), observed and expected heterozygosity (Ho and He), mean polymorphic information content (PIC), the non-exclusion probability for the first parent (NE-1P), the non-exclusion probability for the second parent (NE-2P), and the non-exclusion probability for the parent pair (NE-PP) for the remaining loci that were used in parentage assignment and multiple paternity detection.

Parentage Assignment

We used Cervus 3.0.7 to assign parentage (Kalinowski et al. 2007). We ran an initial simulation with 10,000 offspring, the estimated 5% genotyping error rate, and with 90% of all loci having allele calls in Cervus to calculate the confidence of each parental assignment. Confidence intervals were set to 80% relaxed and a strict of 95%. When assigning maternity, if Cervus assigned a single female to the majority of hatchlings from a single nest with a high logarithm of the odds score (LOD), then the genotype of the proposed female was compared to the hatchling genotypes. If the proposed female genotype was consistent with maternity for 90% of the hatchling allele calls, then we assigned the female as the mother of the clutch. Paternity assignments were made based off the LOD scores. If Cervus proposed the same male to have sired multiple individuals within a clutch and those matches fell within the strict 95% confidence interval range, then the male genotype was compared to the clutch genotypes to determine which hatchlings within the clutch were fathered by the proposed male. Less strict criteria were used for paternity assignments in order to allow for the possibility of multiple paternity and multiple males being assigned to a single nest.

Multiple Paternity Detection

Multiple paternity was detected by two separate methods. With clutches where maternity was known, allelic counting was used to determine if multiple paternity occurred. For nests without a known mother, the program Colony was

used to determine intra-clutch relatedness as well as the likely number of sires (Jones and Wang 2010). Colony uses a maximum likelihood full pedigree analysis to assign individuals into either full-sibling or half-sibling categories (Jones and Wang 2010). If a clutch contains individuals who are half-siblings then multiple paternity is determined to have occurred (Jones and Wang 2010, Lafferriere et al. 2016). Colony runs were conducted under the "high precision" likelihood while incorporating the estimated genotyping error rate of 5%.

Our power to detect multiple paternity was tested with Gerudsim 2.0 (Jones 2005). Gerudsim 2.0 uses provided allele frequencies, clutch size, number of males contributing to a clutch, the number of offspring sired by each male, as well as whether or not the maternal genotype is known, to simulate potential clutch genotypes, maternal genotypes, and paternal genotypes. These simulated genotypes are then passed to Gerud 2.0 to test if Gerud 2.0 is able to accurately re-create the correct paternal and maternal genotypes (Jones 2005). We simulated 39 egg clutches sired by three males with one male contributing to 24 eggs, another male contributing to 10 eggs and the final male contributing to 5 eggs. With a known mother, 11 eggs needed to be sampled in order to accurately recreate the paternal genotypes 75% of the time. Without a known mother and 11 eggs sampled, we were able to accurately recreate the paternal genotypes 70% of the time. As a result, our estimates of multiple paternity are likely to be underestimates.

Statistical analysis

All statistical analyses that we preformed were conducted with R statistical software version 3.4.0 (R Development Core 2017). Generalized linear mixed modeling (GLMM) was used to assess models where either nest counts, clutch size or the presence of multiple paternity was a response variable. Models in which clutch size was the response variable were run with a Poisson's error distribution. Models in which the presence of multiple paternity was the response variable were run with a binomial error distribution. Zero inflated GLMM's were used to asses the influence of male morphometric characteristics on the number of nests sired. Zero inflated models were preformed using the function "zerinfl" from the package MuMIN (Barton and Barton, 2018) The effects of male morphometrics on the number of nests sired were compared using Akaike information criterion with a correction for small samples size (AICc) as well as by using Akaike weights (Burnham and Anderson, 2002). AICc values were calculated using the "AICc" function within the package MuMIN (Barton and Barton, 2018). Linear models were used to assess the influence of male size on clutch fertility and the influence of multiple paternity on clutch fertility. The influence of multiple paternity on hatchling mass, the influence of multiple paternity on hatchling SVL, and the influence of multiple paternity on hatchling body condition were examined independently using linear mixed modeling were clutch identity was included as a random effect. These models were run using the

function lmer from the package “lme4” (Bates et al. 2007). P-values were extracted from these models using the function summary from the “lmerTest” R packages (Kuznetsova et al. 2017). Within R, the function “rcorr” within the package Hmisc was used to perform a Pearson’s correlation test on maternal size and paternal size (Harrell and DuPont, 2012; R Development Core 2017). The function “moran.I” within the R package lctools to perform a Global Morans I test was used to determine the degree of spatial autocorrelation between multiply sired and singly sired nests (Kalogirou, 2016; R Development Core, 2017). For nests with multiple paternity, a Wilcoxon ranked sums test was used to compare the contributions from the primary males and secondary males at nests sired by two or three males. All variables were considered significant at P-values of less than 0.05.

Results

Parentage and Clutch Characteristics

Of the 151 nests examined, we assigned a mother to 78 and at least one father to 38. For 28 nests, we assigned both maternity and a paternity. The majority of maternity assignments matched the female that was caught at the nest (81%). However, at 15 nests, the female captured at the nest was determined not to be the maternal female. Three pairs were found to have mated with each other across multiple years (Table 2). No cases of multiple paternity were detected within nests that had been sired by the same pair across years.

Only 12 males contributed to the 38 nests for which paternity was determined, and two males sired 47% of these nests (Figure 1). In order to identify factors that may be causing these males to sire such a large percentage of the nests, we examined the relationship between male snout-vent length, total length, tail girth, the ratio between snout-vent length and tail girth, and the ratio between total length and tail girth and number of nests sired. When modeled separately, snout-vent length, total length, and tail girth were all found to be significantly related to the number of nests sired (snout-vent length: z value= -2.251, p= 0.0244, total length; z value = -2.730, p= 0.00634; tail girth: z value = 2.719, p= 0.00654). Neither the ratio of tail girth to snout vent length nor the ratio of tail girth to total length were significant predictors of the number of nests sired (ratio of tail girth to snout-vent length: z value = 0.713, p= 0.476, ratio of tail girth to total length: z value= 1.292, p= 0.196). Snout-vent length was a factor in the top two models. Table three shows the delta AICc values between all models examined. The top model was Snout-vent length plus the additive effect of tail girth and snout-vent length alone (Table 3). When model averaging was preforming the models receive 56% and 44% of the model weight respectively. Interestingly, tail girth was no longer significant as an additive effect within the top preforming model (tail girth: z value = 0.268, p = 0.7891, Table 3).

Male size was not related to clutch fertilization (t-value= -0.582, p = 0.564, Figure 1) nor clutch size (z-value = 0.935, p = 0.35, Figure 1). We next

tested if larger males were also mating with larger females but detected no significant correlation between paternal and maternal size (Pearson's correlation coefficient = 0.12, p= 0.54, Figure 1). Multiple paternity was confirmed for only three nests for which a known male was identified as the sire of the nest; therefore, the relationship between male size and multiple paternity could not be examined. Together, these data suggest that male size is a determinant of reproductive success.

Multiple Paternity

Based on our simulations we determined that the probability of accurately detecting the number of sires when we collected eight or fewer eggs was less than 70%. Therefore, we excluded nests with eight or fewer eggs from our analyses of multiple paternity. This removed all nests collected in 2011, 2015 and 2016 and a total of 116 nests were excluded from our multiple paternity analysis. We detected multiple paternity at 11 (35%) of the 35 remaining nests, and rates of multiple paternity varied across years with an average of 43.5% per year (Table 1). Within multiply sired nests, we detected up to three males contributing to a clutch. For 80% of multiply sired nests there was a primary male that was responsible for ≥ 50% of the hatchlings in the clutch (Figure 2). We next asked if paternal contribution from a tertiary male detracts from the proportion of eggs sired by either the primary or secondary male. Interestingly, the primary male sired an average of 74.5% of the clutch when there were two sires, but only 57 %

in the presence of a tertiary sire (w = 31, p = 0.04, Figure 2). However, the presence of a tertiary male did not affect the proportion of sired offspring from the secondary male (w = 16, p = 0.8182, Figure 2).

We next examined how multiple paternity might influence clutch characteristics. In order to ensure that our results were not cofounded with our sampling methods, we examined whether clutch size or clutch viability was correlated with the number of samples taken. We found no significant correlation between clutch viability with the number of samples taken (viability, t=0.903, p=0.374). The occurrence of multiple paternity was not correlated with clutch size (w=70.5, p = 0.1151; Figure 3). However, egg fertilization rates (percentage of fertilized eggs) were significantly greater in nests with only one sire (94%) when compared to those that were multiply sired (86%, w = 179, p = 0.002757, Figure 3). Further, we reasoned that fertility rates and the frequency of multiple paternity might be indirectly linked by maternal traits. However, female size was not correlated to the frequency of multiple paternity or fertilization rates, suggesting that multiple paternity might confer a direct fitness cost to maternal females in terms of reduced fertilization rates (female size and fertility: t = 0.257, p = 0.801; female size and multiple paternity: t =0.528, p = 0.606).

We next asked if the frequency of multiple paternity might be influenced by landscape characteristics and examined the spatial orientation of singly sired and multiply sired nests. We found that multiply sired nests were not clustered

with other multiply sired nests, nor were singly sired nests found to cluster with singly sired nests (Morans I= -0.069, z-resampling = -0.38, z-randomization = -0.36, p-resampling = 0.7, p-randomization = 0.71, Figure 4). However, more detailed analyses are required to determine if landscape characteristics, such as habitat type and quality, associated with nest site might influence the mating dynamics underlying the frequency of multiple paternity.

Implications of Multiple Paternity on Offspring Phenotype

In an effort to further explore the potential benefits and fitness costs associated with multiple paternity, we examined whether multiple paternity influences hatchling phenotypes. We compared the body mass and length (SVL) of hatchlings from 21 complete clutches collected in 2012, 2013 and 2017. No significant difference was found between the hatchling sizes at multiply sired and singly sired nests in terms of mass, length, or body condition (Mass: t value = 1.114, p = 0.279; Length: t value = 1.209, p = 0.241; Body condition: t value = 1.005, p = 0.328).

Discussion

It is well documented that large male alligators are better able to establish and maintain territories when compared to smaller males (Garrick and Lang, 1977; Joanen and McNease, 1971). However, how these territorial advantages influence a male alligator's reproductive output is not known. The present study demonstrates that larger males sire more nests. Interestingly, these larger males do

not sire larger clutches or have higher fertilization rates, suggesting that the advantage of territoriality among larger males is more mating opportunities but perhaps not higher quality mates. In captive studies, female alligators were found to preferentially mate with larger males (Joanen and McNease, 1971). This appears to extend to wild populations as we saw no size-assortative mating but did find that only males > 2.86 m in TL sired offspring. In Louisiana, male alligators as small as 1.83 m in total length produce sperm during the mating season (Joanen and McNease, 1980). It is possible that while these males are physiologically cable of mating, they are excluded from entering the breeding population by the larger males or by female selection (Joanen and McNease, 1971; Garrick and Lang, 1977; Hamlin et al. 2011). Adult males with an SVL of 135 cm or less display seasonal increases in testosterone (T), similar to larger males, until late March. After which, T concentration in smaller males decreases, whereas T concentrations in larger males continues to increase into April (breeding season) and remained much higher through June (Hamlin et al. 2011). This physiological observation is consistent with smaller males being excluded from the breeding population, and is perhaps mediated through social interactions with larger, more dominant males.

Our study represents the first study to describe multiple paternity in the American alligator outside of Louisiana (RNWR). Multiple paternity occurred in 25% to 75% of nests examined from 2012 to 2017 with an average of 43% of

examined nests in a year having multiple paternity. These estimates align closely to the frequency of multiple paternity observed at RNWR (46.6%). Despite this similarity in occurrence of multiple paternity, these sites are characterized by substantial ecological differences. Whereas RNWR is dominated by open marsh, YWC is a series of coastal islands fragmented into diverse habitat types (Joanen, 1969; Obernuefemann, 2013; Wilkinson et al. 2016; Coates et al. 2018), suggesting that habitat characteristics may not be an important determinant of multiple paternity frequency across American alligator populations.

Another possibility is that multiple paternity in American alligators has arisen as a mechanism to reduce infanticide by males (Agrell et al. 1998). American alligators are known to eat hatchling and juvenile alligators and in other species, multiple matings by females are related to reduced infanticide by males (Agrell et al. 1998; Ross. 1989), presumably because males are less certain of their relationships to hatchlings. However, to date no studies have examined whether male alligators preferentially consume unrelated juvenile alligators.

Unfortunately, estimates for population size and density are not available for either YWC or RWNR. Uller and Olsson (2008) suggest that within the non-avian reptiles, the occurrence of multiple paternity may reflect the number of males encountered by a female during her reproductive cycle. This density-driven pattern may be true in other non-avian reptiles. Studies on the Common garter

snake (*Thamnophis sirtalis*) found higher rates of multiple paternity in a population associated with larger communal hibernation and mass-mating behavior (Garner et al., 2002). This pattern may be true for alligators as well. Female alligators increase home range size and movement during the spring mating season and have the potential to contact multiple males (Garrick and Lang, 1977; Goodwin and Marion, 1979; Rootes and Chabreck, 1993). However, without estimates of alligator population density or male-female encounter rates, this hypothesis cannot be directly examined.

This study, as well as those by Lance et al. (2009) and Davis et al. (2001), found no more than three male alligators contributing to a single clutch. All three studies also found that the contribution of the primary male, but not the secondary male, decreases in the presence of a tertiary male. This pattern of paternal contribution might reflect the number of copulation events during ovulation. Because each successive male's contribution to a clutch comes at the expense of the primary male, it is tempting to speculate that paternal contribution of a secondary and tertiary male result from a single mating event. Under this scenario, the primary male maintains a territory to increase the frequency of copulation events and experiences strong evolutionary pressure to prevent other males from contributing to a clutch (Emlen and Oring, 1977). This may lend further support to the idea that the reproductive advantage of larger size in male alligators is the increased ability to hold a territory and exclude other males'

access to females within that territory. An alternative possibility is that the loss of paternal contribution from the primary male reflects the primary male's inability to completely fertilize the clutch. However, multiple paternity would be expected to increase fertilization rates under this scenario, which is the opposite of what we observed. American alligators do have the ability to store sperm within a breeding season, and thus the potential for sperm competition exists (Gist et al. 2008). To date no studies have examined sperm competition in alligators; therefore, the role of male sperm quality in multiple paternity in these animals is unknown.

We found that hatchling alligators from multiply sired clutches were not significantly different in terms of mass, length or body condition when compared to hatchlings from singly sired nests. These findings fail to support the hypothesis that multiple paternity increases fitness through benefits to offspring. However, the implications of hatchling size in alligators in terms of long-term fitness or survival is currently unclear. Other studies have documented increases in fitness-related traits in the offspring of other species resulting from multiply sired clutches (see reviews Griffith et al. 2002; Jennions and Petrie, 2000). Costs or a lack of benefits to hatchlings as a result of multiple paternity is a predicted outcome if multiple paternity is primarily a product of male harassment (Fitze et al. 2005; see review Uller and Olsson, 2008). Studies on the common lizard (*Zootoca vivipara*) have shown that females in male-biased enclosures have decreased reproductive output despite mating with more males as detected

through mating scars (Fitze at al. 2005). Male harassment could explain multiple paternity in American alligators given that we observed decreases in clutch fertility and hatchling size indicating an overall cost to females of mating multiply. Contrary to this idea are other observational studies indicating that female alligators are able to reject male advances and will even kill potential male suitors (Joanen and McNease, 1971; Garrick and Lang, 1977). Though, in these studies the rejected or killed males were smaller than the males we detected within the breeding population at YWC (Garrick and Lang, 1977, Joanen and McNease, 1971). It is possible that once a male reaches a certain size, females are no longer able to avoid mating. The role of male harassment within American alligator mating dynamics remain unclear and require further study.

Our study was able to document three cases in which the same parent pair sired nests across years. These results are similar to the findings of Lance et al. (2009) with the exception that our study found no cases of mate fidelity and multiple paternity within the same clutches. Mate fidelity is often explained with three hypotheses; males assist in parental care in order to increase their own reproductive success, males defend females from rival males to ensure paternity, or females adopt monogamy in order to gain some advantage from the male (Bull, 2000). Male parental care has not been documented in the American alligator, and while males will defend a territory, females will interact with multiple males during a breeding season (Garrick and Lang, 1977, Joanen and McNease, 1971).

At YWC, larger males are better able to maintain territorial advantages and we show they are also able to sire more nests (Garrick and Lang, 1977, Joanen and McNease, 1971). Together, our work and the work of previous researchers suggest that the advantage of size and territory translate into more mating opportunities for male alligators. Further, multiple paternity led to a decrease in clutch fertility, and had no impact on hatchling mass, hatchling length or hatchling body condition. These results are inconsistent with hypotheses in which multiple paternity results in benefits to females or offspring (Arnqvist and Kirkpatrick, 2005; Birkhead and Møller, 1998; Byrne and Robert 2000; Bull, 2000; Eberhard, 1996; Laloi et at. 2004; Lee and Hays, 2004; Olsson and Shine, 1997). However, our findings are consistent with a system in which multiple paternity is the product of sexual conflict (Fitze et al. 2005; Jensen et al. 2006). Thus, this study advances our understanding into the evolutionary and ecological drivers of mating system diversity, particularly in the context of long-lived vertebrates.

CHAPTER THREE

DEVELOPMENT OF CAPTURE PROBES FOR ANALYZING FINE SCALE POPULATION STRUCTURE IN AMERICAN ALLIGATORS (ALLIGATOR MISSISSIPPIENSIS)

To be submitted to Molecular Ecology

Abstract

The amount of genetic diversity within a population has direct impacts on the population's long-term viability. However, the amount of genetic diversity within a population may not be obvious and require examination before effective conservation and wildlife management can take place. The American alligator (*Alligator mississippiensis*) has been a part of several population genetic studies that have found evidence for an East-West phylogeographic split. Yet, few studies have examined the genetic diversity and population connectedness within these groups. Therefore, whether fine scale factors just as habitat influence alligator population genetics is unknown. Using RADseq techniques we examined the population genetics of eight alligator populations representing inland and coastal populations. We were able to uncover 716 potential loci for use in capture probe design and future studies on population genetics.

Keywords: AMERICAN ALLIGATOR, RADSEQ, POPULATION GENETICS

Introduction

The maintenance of genetic diversity is a key component of population viability and conservation. The loss of genetic variation has been correlated with reduced individual fitness and with increased extinction risk (Reed and Frankham, 2003; Reed et al.,2007; Vandewoestijne et al, 2008). For example, studies on the wolf spiders in the genus *Rabidosa* correlated lower genetic diversity within a population to lower fecundity and offspring survivorship to sexual maturity (Reed, 2007; Reed et al. 2007). These effects can be particularly pronounced in recently recovered species, which often have experienced a genetic bottleneck (Frankham, 1995; Nei et al., 1975). The genetic diversity within a population can be influenced by several factors including demographic history and isolation from other populations (Levin, 1970; Mayr, 1942; Mayr, 1970; Nei et al., 1975; Wright, 1943). Populations may become isolated because of geological features, distance from other populations, or because of an impermeable habitat matrix (Mayr, 1970). Identifying the factors influencing population isolation is challenging but imperative for effective conservation (Frankham, 1995).

The American alligator (*Alligator mississippiensis*) has recently recovered from endangered status and is currently actively managed across its range (Ross, 1989). Population genetics studies of the American alligator suggest an East-West split and two populations; one encompassing Georgia, Florida, and South Carolina and one including Alabama, Louisiana, and Texas (Adams et al., 1980;

Davis et al., 2001; Davis et al., 2002; Gartside et al., 1977; Glenn et al. 1998; Menzies et al., 1979; Ryberg et al., 2002). Of these studies, only three have attempted to characterize gene flow within these larger populations (Davis et al., 2001; Davis et al., 2002; Ryberg et al., 2002).

Initially, Davis et al (2001), found evidence for high levels of gene flow among coastal populations and lower levels across inland populations. However, a follow-up study found support for high levels of gene flow among all alligator populations (Davis et al. 2002). Contrary to this, Ryberg et al. (2002) examined gene flow between inland and coastal populations and across river drainages. They found population differentiation that was best explained by isolation by distance not associated with geological features. As a result, the factors influencing gene flow and population connectivity with the American alligator are still unclear.

Thus far, the primary genetic markers used to examine population structure and gene flow in the American alligator have been microsatellites (Davis et al., 2001; Davis et al., 2002; Glenn et al. 1998; Ryberg et al., 2002). While microsatellite markers have been useful in population genetic studies, they are time consuming to develop and difficult to genotype across large sample sizes and therefore may have limited use in detecting fine scale population structure (Baird et al., 2008; Rašić et al., 2014; Vendrami et al., 201). More recently developed techniques, such as RADseq, focusing on single nucleotide polymorphisms

(SNPs), have been found to be useful in detecting fine scale populations structure (Rašić et al., 2014; Vendrami et al., 2017). Individually, SNP loci are less powerful than microsatellite loci in detecting gene flow and population structure but by using large numbers of SNPs it may be possible to uncover population structure that was not detectable with microsatellites (Baird et al., 2008; Rašić et al., 2014; Vendrami et al., 201). Our study aims to use SNPs in order to examine the potential fine scale population genetic differences between coastal and inland populations within the eastern phylogeographic group of American alligators.

Methods

Site Description

We collected samples from eight populations along the eastern edge of the American alligator's range. Populations were located in North Carolina, South Carolina, Georgia and Florida. We sampled two populations per state with one population representing a coastal population and the other population representing the inland population (Figure 6). All coastal populations bordered the Atlantic Ocean while all inland populations were located at least fifty miles away from the Atlantic Ocean.

North Carolina

We included two populations from North Carolina: Albemarle Sound and in Lake Waccamaw. Albemarle Sound is a large estuary along the coast of North Carolina and represents one of the most northern populations of alligators. The

estuary is spread across numerous North Carolina counties but we only collected samples from Hyde and Dare county. Lake Waccamaw is a large freshwater Carolina bay located in south central North Carolina within Columbus County. The lake is a part of the Lake Waccamaw State park and is managed by the North Carolina Division of Parks and Recreation.

South Carolina

We included two populations in South Carolina: the Thomas A. Yawkey Wildlife Center and from Lakes Marion and Moultrie. The Thomas A. Yawkey Wildlife Center is located in Georgetown County, South Carolina and is a series of three barrier islands located along the coast of South Carolina. The islands are bordered by the Atlantic Ocean to the east, Winyah Bay to the north, the Intracoastal Waterway to the west, and North Santee Bay to the south. Lake Marion and Lake Moultrie are two man made reservoirs located in central South Carolina. While these lakes span multiple counties in South Carolina, we only collected samples from Clarendon County and Berkeley County. Lake Marion flows into Lake Moultrie and the two lakes are only separated by a distance of three miles. Therefore, for this study alligators from Lake Marion and alligators from Lake Moultrie were considered a part of the same population.

Georgia

The two populations sampled in Georgia are Sapelo Island and at the Joseph E. Jones Ecological Research Center at Ichauway. Sapelo Island is a barrier island

located along of the coast of Georgia located entirely within McIntosh County. The island is currently managed by the Georgia Department of Natural Resources. The Joseph E. Jones Ecological Research Center at Ichauway is an ecological research center located in Baker County, Georgia. The Joseph E. Jones Ecological Research Center has been the site of long-term ecological studies since 1991. This population is unique to our study in that this population lies within the Gulf of Mexico watershed.

Florida

The sampled populations from Florida include Guana Lake and Silver Lake. Guana Lake is located on the Atlantic coast of northern Florida. It is a man-made saltwater lake and is a part of the larger Guana Tolomato Matanzas National Estuarine Research Reserve. Silver lake is located in central Florida within Lake County. Silver lake is a naturally occurring freshwater lake.

Sample Collection

We captured alligators using multiple methods including baited-trip snare traps, walk-through traps, snare poles, and snatch hooks (Cherkiss et al. 2004; Murphy et al., 1983; Wilkinson 1994, Wilkinson et al. 2016). Table 6 described the locations and years in which we collected samples. Samples from the Joseph E. Jones Ecological Research Center were collected from 2009 until 2010 as a part of other studies examining the movement and ecology in inland alligator populations (Subalusky et al., 2009). Samples from Sapelo Island, Guana Lake,

and Silver Lake were collected between 2011 and 2014 as a part of studies examining alligator ecology and the role alligators in food web dynamics (Nifong and Silliman, 2013; Nifong et al., 2015). Samples from the Thomas A. Yawkey Wildlife Center were collected between 2011 and 2016 as a part of studies examining maternal mating behavior (Bangma et al., 2017; Hale et al., 2017; McCoy et al., 2015; Parrott et al., 2014; Wilkinson et al. 2016). Samples from the Albemarle Sound were collected between 2011 and 2015 as a part of ongoing capture mark recapture studies (Dunham et al., 2014). Samples from Waccamaw, Lake Marion and Lake Moultrie were collected in 2017 specifically for this study. All samples collected in the field were stored on ice until transport to the laboratory where they were stored at -20°C until DNA extraction.

DNA Extraction

Alligator DNA was isolated from a variety of sample types including adult blood and scutes, hatchling chorioallantoic membranes and scutes, and embryos preserved in RNAlater. DNA isolation was performed using the DNeasy blood and tissue kit (Qiagen, Valencia, CA) following the manufacturers' protocols with the following exceptions. Econospin columns (Epoch Life Sciences, Inc., Missouri City, TX) were used during DNA filtration and DNA was eluted with 100 µl of the provided AE buffer. DNA concentrations were determined using a Qubit 2.0 Fluorometer (Thermo Scientific, Waltham, MA) and standardized to 20 ng/µl.

DNA Digestion and Library Preparation

Our protocols for generating reduced representation genomic libraries closely followed those outlined in Glenn et al. (2017). In order to reduce the complexity of the Alligator genome prior to sequencing, DNA was digested using the enzymes BAM-HI and Cla-I. The enzyme Msp-I was used to prevent the formation of primer dimers. All enzymes and enzyme buffers were supplied by New England Biolabs (NEB; Beverly,MA, USA). All itru-5 and itru-7 adapters were supplied by the Glenn Lab at the University of Georgia's Department of Environmental Health Science (Glenn et al., 2016). Sequences for internal and external tags can be found in table 7.

Digestions were performed in a 15 μL volume (1.5 μL 10X Cut Smart Buffer, 10,000 units MSP-I,10,000 units BAM-HI, 10,000 units Cla-I, 5 μM read one adapter, 5 μM read two adapter, 100 ng of DNA, 4.5 μL of water) and incubated for one hour at 37° C. Immediately following the digestions, products were ligated with the addition of 15 μM ATP, 0.5 μL 10X ligase buffer, 100 units of ligase, and 2.75 μL of water and incubated in an Applied Biosystems GeneAmp 9700 Thermo Cycler using two cycle of ligation with an initialization at 22°C for 20 minutes and ligation at 37°C for 10 minutes. After those two cycles enzymes were heat killed at 80°C for 20 minutes.

Following digestion and ligation, samples were pooled and external itru-5 and itru-7 tags were added. Itru-5 external tags were added using primers that would

add eight unknown bases to the end of DNA strands. This was done in order to detect PCR duplicates. Itru-5 external tags were added in a 50 µL volume (10 µL 5X kapa Hifi Buffer, 15 µM dNTP's, 25 µM itru-5 8N primer, 300 ng of pooled DNA, 17.5 µL water, 1 unit of Kapa Hifi Polymerase). Kapa Hifi buffer and polymerase were provided by Roche Holding AG (Basel, Switzerland). Itru-5 external tags were added using an Applied Biosystems GeneAmp 9700 Thermo Cycler to preform one cycle of denaturation at 98°C for one minute, annealing at 60°C for 30 seconds, and elongation at 72°C for six minutes. Itru-7 external tags were immediately added after the addition of the itru-5 tags. The addition of the itru-7 was performed in at 50µl volume (10 µL 5X kapa Hifi Buffer, 15 µM dNTP's, 25 µM P5 primer, 25 µM itru-7 primer, 200 ng of pooled DNA, 20 µL water, 1 unit of Kapa Hifi Polymerase). Itru-7 external tags were added using an Applied Biosystems GeneAmp 9700 Thermo Cycler to perform six cycles of PCR consisting of an initial denaturation at 98°C for two minutes, followed by six cycles of denaturation at 98°C for 20 seconds, annealing at 60°C for 15 seconds, and elongation at 72°C for 30 seconds, followed by a final elongation at 72°C for five minutes.

We prepared two libraries of 96 individuals, used a BluePippin (Sage Science, Beverly, MA, USA) to select fragments between 100 – 600 bp, and sequenced them on an Illumina NextSeq device using a 150 Cycles PE75 Mid Output flow cell at The Georgia Genomics and Bioinformatics Core.

Bioinformatic Processing

Initial bionformatic processing was done using Stacks 2.2 (Catchen et at., 2013). Libraries were separated using the external itru-7 sequences and the command “process_radtags”. Library sequences were demultiplexed for each individual using the internal tags as well as the Bam-HI and Cla-I cut cites. The command “process_radtags” was also used during this step except the flags -c, -q were used in order to remove sequences that contained uncalled bases or low quality reads. PCR clones were removed using the command “clone filter”. All sequences were trimmed to 140 bp. Following initial processing, sequences were aligned to the alligator genome using BWA 7.17 (Li and Durbin, 2009). Our study used the alligator genome assembled by Dovetail Genomics and submitted to the National Center for Biotechnology Information (NCBI) on January 26, 2016. The resulting files were converted from SAM format to BAM format using SAMtools 1.6 (Li et al., 2009). Loci were derived and processed using Stacks 2.2 (Catchen et al., 2013). The command “ref_map” was used to derive loci from reference aligned sequences and the command “populations” was used to filter derived loci and calculate population summary statistics including F_{st} comparisons between all populations. Only loci that could be found in 75% of the sample locations and 50% of the individuals within a location were used for our analysis. In order to ensure that loci were independent, only one SNP was retained per locus.

Sequence Capture Design

Our methods closely follow those outlined in Hoffberg et al. (2016). Twenty-eight samples from four locations were processed using the aforementioned RADseq methods in order to identify potential loci for sequence capture. An important exception to our processing is that we allowed each locus to contain five SNPs instead of one SNP allowed for the analysis of our RADseq data. Eight samples were collected from Rockefeller National Wildlife Refuge, Louisiana; St. John's River, Florida; Thomas A Yawkey Wildlife Center, South Carolina; Everglades National Park, Florida. After potential loci were identified, a subset was selected for bait design. Loci were chosen if they did not contain repeated sequences and were not linked to any of the other found loci. Selected loci were sent to Arbor Bioscience (Ann Arbor, Michigan) for oligo synthesis of our targeted sequence capture probes. Two capture probes were designed for each selected locus.

Results

3RAD

Twenty-four alligators were sampled at each of the eight locations for a total of 192 individual samples. Sampled alligators' snout-vent lengths ranged from 16.8 cm to 194.3 cm. Sequencing produced 252,461,458 reads for library one and 349,271,550 reads for library two. After processing, an average of 1,044,896 reads remained per individual. 252 loci were found using the the "ref_map" and

"populations" commands within stacks. Harvester indicated that our data were best explained by two clusters. Samples were clustered based on which library they had been sequenced in. In order to determine how library biases may be influencing our results, the commands "ref_map" and "populations" were re-run with the libraries being analyzes separately rather than together. Our analyses resulted in 17819 loci from library one and 30944 loci from library two. Harvester indicated that five clusters are the most likely number of clusters from library two and four clusters are the most likely number of clusters for library one. No clear pattern of cluster is evident for the populations in either library.

RADcap

After processing, an average of 1,370,274r reads emained per individual. 3,267 loci were found using the the "ref_map" and "populations" commands within stacks. After removing loci that contained repeated sequences or were linked to other loci, 716 loci remained. A total of 1432 capture probes were designed and produced by Arbor Biosciences.

Discussion

It is important to note that our study found a strong library bias with the program Structure clustering samples according to the library in which they were sequenced. Library biases has been reported from another study examining the effectiveness of ddRAD techniques to population genetic analysis (DaCosta and Sorenson, 2014). One potential explanation for this bias in our data could be that

the libraries were sequenced independently on different dates. Library two was prepared in September 2018 while library one was prepared in December 2018 and library two was sequences in November 2018 while library one was sequenced in March 2019. DaCosta and Sorenson (2014) found inconsistent recovery of loci across different sequencing runs. While the explanations for this inconsistency vary, the end result would explain why when both libraries were analyzed together, the samples clustering according to the library in which they were sequenced.

In order to help alleviate some of these issues, we are currently in the process of using sequence capture methods such as RADcap (Hoffberg et al., 2016). RADcap allows to the consistent recovery of the same loci across sequencing runs and between individuals. RADcap also allows for the selective sequencing of the most informative loci. We were able to find 3,267 potential loci share across populations in Louisiana, South Carolina, and Florida. Of these only 716 were found to be unlinked and without repetitive sequences. Our future studies focusing on the population genetics of the American alligator will be able to use these loci and baits in order to ensure consistent recovery of the same loci across sequencing runs and between individuals.

CHAPTER FOUR

CONCLUSION

The amount of genetic diversity within a population has direct impacts on the population's long-term viability (Reed and Frankham, 2003; Reed et al.,2007; Vandewoestijne et al, 2008). This is especially true of recently recovered species (Frankham, 1995). The level of genetic diversity within a population is impacted both by the species' connectedness to other populations and by the natural history of the species (Sugg and Chesser, 1994). Both of these factors need to be understood before effective conservation and wildlife management can take place (Frankham, 1995).

A key component of a species' natural history are the species' specific mating dynamics. A recent discovery regarding mating dynamics has been that multiple paternity is a common and widespread occurrence (Birkhead and Møller, 1998; Coleman and Jones, 2011; Griffith et al, 2002; Uller and Olsson, 2008). However, the drivers and implications of multiple paternity are still debated (Birkhead and Møller, 1998; Jennions and Petrie 2000; Griffith et al, 2002; Uller and Olsson, 2008). One set of hypotheses explains multiple paternity through direct and indirect benefits to females and offspring (see reviews Birkhead and Møller, 1998; Eberhard 1996; Griffith et al, 2002; Jennions and Petrie, 2000; Keller and Reeve, 1995; Yasui, 1997). Yet, another set of hypotheses explains multiple paternity through random chance and male harassment (Andersson, 1994;

Arnqvist and Kirkpatrick, 2005; Kokko et al., 2006; Lee and Hays, 2004; Martin et al. 2014; Sztatecsny et al. 2006; Wells et al. 2017). Therefore, we sought to examine these hypotheses in the context of the American alligator, a long lived recently recovered species with a previously established occurrence of multiple paternity (Davis et al., 2001; Lance et al., 2009).

In order to examine these hypotheses, we used previously collected eggs, hatchlings, and adult tissue samples from a population of American alligators at the Thomas A. Yawkey Wildlife Center (YWC). The population at YWC has been extensively studied, especially in regard to nesting habitat, and provides a unique opportunity to examine the hypotheses surrounding multiple paternity as well as to examine alligator specific mating dynamics. Our goals for this part of the study were to 1) characterize the occurrence of multiple paternity at the Thomas A. Yawkey Wildlife Center, 2) examine how alligator morphometrics may influence male alligator reproductive success, 3) and to examine potential differences in the fitness of offspring from multiply sired and singly sired nests.

A total of 151 nests were sampled with 31 nests being completely sampled. We assigned a mother to 78 nests and at least one father to 38 nests. We found a significant correlation between male SVL and number of nests sired but did not find a significant correlation between male SVL and clutch fertility or clutch size. These results suggest that the advantages of size in male alligators translates into more nests sired but not better quality mates or offspring. Multiple paternity

occurred in 25% to 75% of nests examined from 2012 to 2017, with an average of 43% of examined nests in a year having multiple paternity. This average closely aligns with the average occurrence of multiple paternity at the Rockefeller National Wildlife Refuge (Davis et al., 2001; Lance et al., 2009). Clutch fertility was lower, on average, in multiply sired nests than in singly sired nest but there was no difference in average clutch size, hatchlings length, hatchlings mass, or hatchling body condition. Given the lack of observable differences between multiply sired offspring and singly sired offspring, and the decrease in average clutch fertility, potential explanations for these results include that multiple paternity in alligators may be driven by a population parameter such as density or mate encounter rate. Unfortunately, population estimates for YWC or RNWR are unavailable. However, both populations occur in vastly different habitats suggesting that habitat characteristics may not be an important determinant in the occurrence of multiple paternity within the American alligator.

These effects of low genetic diversity can be particularly pronounced in recently recovered species, which often have experienced a genetic bottleneck (Frankham, 1995; Nei et al., 1975). The genetic diversity within a population can be influenced by several factors including demographic history and isolation from other populations. Previous studies on the populations genetics of the American alligator have demonstrated an East West phylogeographic split within the American alligator range (Davis et al., 2001; Davis et al., 2002; Glenn et al. 1998;

Ryberg et al., 2002). However, the influence of habitat on the fine scale population structure of the American alligator remains unknown. Therefore, we sought to examine the amount of gene flow and population connectivity between inland and coastal populations within the Eastern phylogeographic group.

In order to examine these effects, we gathered samples from eight American alligator populations. Two populations were sampled from each state bordering the Atlantic coast within the American alligator's range. These states included North Carolina, South Carolina, Georgia, and Florida. We used recently developed RADseq techniques to find SNPs and infer population differentiation. Using a method of RADseq known as 3RAD resulted in inconsistent results. However, we have designed sequence capture probes in order to ensure the consistent recovery of loci across sequencing runs.

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Table 1. The number of clutches and hatchlings sampled during each year of the study and the results regarding multiple paternity.

Year	Total Clutches Sampled	Full Clutches	Hatchlings Collected	Multiply Sired Clutches (%)
2011	10	0	66	-
2012	11	8	267	2 (25%)
2013	20	9	305	4 (44%)
2014	19	4	110	3 (75%)
2015	19	0	135	-
2016	44	0	319	-
2017	28	10	455	3 (30%)
Total	151	31	1657	12

Table 2. The forward and reverse primer sequence used for each microsatellite loci used in the analysis as well as the repeated sequence motif for each loci. Each sequence includes the CAG forward primer and the pigtailed reverse primer.

Loci	**Primer Sequence 5' -> 3'**	**Motif**
Almi 8	**F**:CAGTCGGGCGTCATCACCTTAATTATGAATTATCCGGAGGG **R:** GTTTAATCCTCCCTGACATTTCCC	ATCT (56)
Almi 19	**F:**CAGTCGGGCGTCATCAGCAGACTCTAGAGCATTTAGAATAGTCC **R:** GTTTCAAGTCAGGTTCACTTGTATCTAAACTAGC	ATCT (52)
Almi 26	**F:**CAGTCGGGCGTCATCAGAACCAGTAAGTGCCCTCCC **R:** GTTTCGAAACAGAAGTCACACATCCC	ATCT (68)
Almi 30	**F:**CAGTCGGGCGTCATCATTAGACCCTGTTGCCCATCC **R:** GTTTGCCCTCTTCTTCATCATGCC	ATCT (60)
Almi 32	**F:**CAGTCGGGCGTCATCATGTCTGGCCTGGAAAGATCC **R:** GTTTGGGAGTACCTGCCTGTTCCC	ATCT (56)
Almi 34	**F:** CAGTCGGGCGTCATCAGGAGTGCAGATGTCCAGG **R:** GTTTGTTCGGACCAGCAGCACC	ATCT (48)
Almi 39	**F:**CAGTCGGGCGTCATCAAGTCTCCCTACACACAGGG **R:** GTTTGCAGTCAGGGACAGACTACC	ATCT (52)
Almi 40	**F:**CAGTCGGGCGTCATCAGGCTCTGTGCATCTTGCTCC **R:** GTTTGGTATGGGATGCTAAGCCC	ATCT (64)
Almi 46	**F:**CAGTCGGGCGTCATCATTTGTTTCCTATCTTTCCTCCC **R:** GTTTGAGAACACTTCAACGTTTCC	ATCT (56)

Almi 47	**F:**CAGTCGGGCGTCATCAGGAGTTCTCTGATGATCCTATCCC **R:** GTTTATTGGAGGATGTCATTGGG	ATCT (64)

Table 3. Details on the loci used for parentage analysis and multiple paternity detection. H_o is the observed heterozygosity, H_e is the expected heterozygosity, PIC is the mean polymorphic information content, NE-1P is the non-exclusion probability for the first parent, NE-2P is the non-exclusion probability for the second parent, and NE-PP is the non-exclusion probability for the parent pair.

Loci	k	H_o	H_e	PIC	NE-1P	NE-2P	NE-PP	Error Rate
Almi 8	12	0.81	0.814	0.791	0.530	0.355	0.169	0.04
Almi 26	11	0.797	0.815	0.789	0.539	0.364	0.183	0.02
Almi 30	20	0.839	0.841	0.822	0.476	0.31	0.134	0.07
Almi 34	15	0.813	0.851	0.833	0.458	0.296	0.125	0.08
Almi 47	9	0.667	0.67	0.627	0.732	0.557	0.362	0.06
Total	-	-	-	-	0.046	0.0066	0.00188	0.05

Table 4. The nests in which mate fidelity occurred. (5), 1264-1278.*Molecular*

Year	Nest ID	Female	Male
2016	13_2016	CF-35	CG-18
2017	35_2017	CF-35	CG-18
2013	7_2013	BE-02	BF-05
2015	3_2015	BE-02	BF-05
2012	3_2012	CF-40	CI-28
2016	1_2016	CF-40	CI-28
2017	24_2017	CF-40	CI-28

Table 5. An AICc table of the AICc scores, Delta AICc and model weight or each model used to examine the effect of male morphometrics on the number of nests each male sired.

Formula	AICc	ΔAICc	Weight
Nest Sired~SVL+Tail Girth	72.8818	0	0.56
Nest Sired~SVL	73.38822	0.50642	0.44
Nest Sired~Total Length+Tail Girth	87.31301	14.43121	0
Nest Sired~Tail Girth	89.35854	16.47674	0
Nest Sired~Total Length	94.09322	21.21142	0
Nest Sired~ Ratio of SVL to Tail Girth	125.482	52.6002	0
Nest Sired~ Ratio of Total Length to Tail Girth	125.8006	52.9188	0

Table 6. The location and years in which samples were collected

Population	State	Type	Years Collected
Albemarle Sound	NC	Coastal	2011-2015
Lake Waccamaw	NC	Inland	2017
Thomas A. Yawkey Wildlife Center	SC	Coastal	2011-2017
Lake Marion/ Lake Moultrie	SC	Inland	2017
Sapelo Island	GA	Coastal	2011-2014
Joseph E. Jones Ecological Research Center	GA	Inland	2009-2010
Guana Lake	FL	Coastal	2011-2014
Silver Lake	FL	Inland	2011-2014

Table 7. Sequences of each internal and external tag used per sample.

Sample ID	Library	Itru-5 Internal Tag 5' -> 3'	Itru-7 Internal Tag 5' -> 3'	External Itru-7 Tag 5' -> 3'
SAM153	1	CCGAATAT	CTAACGC	TGTTCGAG
SAM240	1	TTAGGCAAT	CTAACGC	TGTTCGAG
SLV120	1	AACTCGTCAT	CTAACGC	TGTTCGAG
SLV141	1	GGTCTACGTAT	CTAACGC	TGTTCGAG
WA3716	1	GATACCAT	CTAACGC	TGTTCGAG
WA3810	1	AGCGTTGAT	CTAACGC	TGTTCGAG
MUSC101	1	CTGCAACTAT	CTAACGC	TGTTCGAG
CG59	1	TCATGGTCAAT	CTAACGC	TGTTCGAG
SAM179	1	CCGAATAT	TCGGTACC	TGTTCGAG
SAM247	1	TTAGGCAAT	TCGGTACC	TGTTCGAG
SLV121	1	AACTCGTCAT	TCGGTACC	TGTTCGAG
SLV157	1	GGTCTACGTAT	TCGGTACC	TGTTCGAG
WA3718	1	GATACCAT	TCGGTACC	TGTTCGAG
WA3812	1	AGCGTTGAT	TCGGTACC	TGTTCGAG
CF29	1	CTGCAACTAT	TCGGTACC	TGTTCGAG
CG61	1	TCATGGTCAAT	TCGGTACC	TGTTCGAG
SAM180	1	CCGAATAT	GATCGTTGC	TGTTCGAG
SAM249	1	TTAGGCAAT	GATCGTTGC	TGTTCGAG

SLV122	1	AACTCGTCAT	GATCGTTGC	TGTTCGAG
SLV158	1	GGTCTACGTAT	GATCGTTGC	TGTTCGAG
WA3723	1	GATACCAT	GATCGTTGC	TGTTCGAG
WA3841	1	AGCGTTGAT	GATCGTTGC	TGTTCGAG
MUSC209	1	CTGCAACTAT	GATCGTTGC	TGTTCGAG
YK45	1	TCATGGTCAAT	GATCGTTGC	TGTTCGAG
SAM182	1	CCGAATAT	AGCTACACTC	TGTTCGAG
SAM250	1	TTAGGCAAT	AGCTACACTC	TGTTCGAG
SLV123	1	AACTCGTCAT	AGCTACACTC	TGTTCGAG
SLV160	1	GGTCTACGTAT	AGCTACACTC	TGTTCGAG
WA3744	1	GATACCAT	AGCTACACTC	TGTTCGAG
WA3845	1	AGCGTTGAT	AGCTACACTC	TGTTCGAG
MUSC93	1	CTGCAACTAT	AGCTACACTC	TGTTCGAG
YK49	1	TCATGGTCAAT	AGCTACACTC	TGTTCGAG
SAM191	1	CCGAATAT	ACGCATC	TGTTCGAG
SAM255	1	TTAGGCAAT	ACGCATC	TGTTCGAG
SLV124	1	AACTCGTCAT	ACGCATC	TGTTCGAG
SLV161	1	GGTCTACGTAT	ACGCATC	TGTTCGAG
WA3755	1	GATACCAT	ACGCATC	TGTTCGAG
WA8258	1	AGCGTTGAT	ACGCATC	TGTTCGAG

MUSC94	1	CTGCAACTAT	ACGCATC	TGTTCGAG
YK54	1	TCATGGTCAAT	ACGCATC	TGTTCGAG
SAM222	1	CCGAATAT	GTATGCAC	TGTTCGAG
SAM290	1	TTAGGCAAT	GTATGCAC	TGTTCGAG
SLV126	1	AACTCGTCAT	GTATGCAC	TGTTCGAG
SLV162	1	GGTCTACGTAT	GTATGCAC	TGTTCGAG
WA3757	1	GATACCAT	GTATGCAC	TGTTCGAG
WA8265	1	AGCGTTGAT	GTATGCAC	TGTTCGAG
MUSC95	1	CTGCAACTAT	GTATGCAC	TGTTCGAG
CG86	1	TCATGGTCAAT	GTATGCAC	TGTTCGAG
SAM223	1	CCGAATAT	CACATGTCC	TGTTCGAG
SAM292	1	TTAGGCAAT	CACATGTCC	TGTTCGAG
SLV127	1	AACTCGTCAT	CACATGTCC	TGTTCGAG
SLV163	1	GGTCTACGTAT	CACATGTCC	TGTTCGAG
WA3764	1	GATACCAT	CACATGTCC	TGTTCGAG
WA8267	1	AGCGTTGAT	CACATGTCC	TGTTCGAG
MUSC218	1	CTGCAACTAT	CACATGTCC	TGTTCGAG
CG92	1	TCATGGTCAAT	CACATGTCC	TGTTCGAG
SAM225	1	CCGAATAT	TGTGCACGAC	TGTTCGAG
SAM299	1	TTAGGCAAT	TGTGCACGAC	TGTTCGAG

SLV128	1	AACTCGTCAT	TGTGCACGAC	TGTTCGAG
SLV165	1	GGTCTACGTAT	TGTGCACGAC	TGTTCGAG
WA3765	1	GATACCAT	TGTGCACGAC	TGTTCGAG
WA9800	1	AGCGTTGAT	TGTGCACGAC	TGTTCGAG
MUSC216	1	CTGCAACTAT	TGTGCACGAC	TGTTCGAG
CI27	1	TCATGGTCAAT	TGTGCACGAC	TGTTCGAG
SAM227	1	CCGAATAT	GCATCAC	TGTTCGAG
SAM320	1	TTAGGCAAT	GCATCAC	TGTTCGAG
SLV129	1	AACTCGTCAT	GCATCAC	TGTTCGAG
SLV166	1	GGTCTACGTAT	GCATCAC	TGTTCGAG
WA3772	1	GATACCAT	GCATCAC	TGTTCGAG
BF7	1	AGCGTTGAT	GCATCAC	TGTTCGAG
CG34	1	CTGCAACTAT	GCATCAC	TGTTCGAG
YK53	1	TCATGGTCAAT	GCATCAC	TGTTCGAG
SAM228	1	CCGAATAT	ATGCTGTC	TGTTCGAG
SAM323	1	TTAGGCAAT	ATGCTGTC	TGTTCGAG
SLV134	1	AACTCGTCAT	ATGCTGTC	TGTTCGAG
SLV167	1	GGTCTACGTAT	ATGCTGTC	TGTTCGAG
WA3777	1	GATACCAT	ATGCTGTC	TGTTCGAG
MUSC191	1	AGCGTTGAT	ATGCTGTC	TGTTCGAG

CG36	1	CTGCAACTAT	ATGCTGTC	TGTTCGAG
MUSC143	1	TCATGGTCAAT	ATGCTGTC	TGTTCGAG
SAM233	1	CCGAATAT	CATGACCTC	TGTTCGAG
SAM326	1	TTAGGCAAT	CATGACCTC	TGTTCGAG
SLV135	1	AACTCGTCAT	CATGACCTC	TGTTCGAG
SLV172	1	GGTCTACGTAT	CATGACCTC	TGTTCGAG
WA3780	1	GATACCAT	CATGACCTC	TGTTCGAG
MUSC96	1	AGCGTTGAT	CATGACCTC	TGTTCGAG
CG4	1	CTGCAACTAT	CATGACCTC	TGTTCGAG
GTM124	1	TCATGGTCAAT	CATGACCTC	TGTTCGAG
SAM239	1	CCGAATAT	TGCAGTGAGC	TGTTCGAG
SLV119	1	TTAGGCAAT	TGCAGTGAGC	TGTTCGAG
SLV137	1	AACTCGTCAT	TGCAGTGAGC	TGTTCGAG
SLV173	1	GGTCTACGTAT	TGCAGTGAGC	TGTTCGAG
WA3791	1	GATACCAT	TGCAGTGAGC	TGTTCGAG
MUSC97	1	AGCGTTGAT	TGCAGTGAGC	TGTTCGAG
CG50	1	CTGCAACTAT	TGCAGTGAGC	TGTTCGAG
GTM122	1	TCATGGTCAAT	TGCAGTGAGC	TGTTCGAG
AP10	2	CCGAATAT	CTAACGC	CCAAGCAA
AP33	2	TTAGGCAAT	CTAACGC	CCAAGCAA

AP9	2	AACTCGTCAT	CTAACGC	CCAAGCAA
GTM174	2	GGTCTACGTAT	CTAACGC	CCAAGCAA
IC304	2	GATACCAT	CTAACGC	CCAAGCAA
IC408	2	AGCGTTGAT	CTAACGC	CCAAGCAA
SAB10	2	CTGCAACTAT	CTAACGC	CCAAGCAA
SAD3	2	TCATGGTCAAT	CTAACGC	CCAAGCAA
AP12	2	CCGAATAT	TCGGTACC	CCAAGCAA
AP34	2	TTAGGCAAT	TCGGTACC	CCAAGCAA
GTM132	2	AACTCGTCAT	TCGGTACC	CCAAGCAA
GTM177	2	GGTCTACGTAT	TCGGTACC	CCAAGCAA
IC306	2	GATACCAT	TCGGTACC	CCAAGCAA
IC411	2	AGCGTTGAT	TCGGTACC	CCAAGCAA
SAB11	2	CTGCAACTAT	TCGGTACC	CCAAGCAA
SAD4	2	TCATGGTCAAT	TCGGTACC	CCAAGCAA
AP13	2	CCGAATAT	GATCGTTGC	CCAAGCAA
AP36	2	TTAGGCAAT	GATCGTTGC	CCAAGCAA
GTM138	2	AACTCGTCAT	GATCGTTGC	CCAAGCAA
GTM178	2	GGTCTACGTAT	GATCGTTGC	CCAAGCAA
IC312	2	GATACCAT	GATCGTTGC	CCAAGCAA
IC413	2	AGCGTTGAT	GATCGTTGC	CCAAGCAA

SAB2	2	CTGCAACTAT	GATCGTTGC	CCAAGCAA
SAD5	2	TCATGGTCAAT	GATCGTTGC	CCAAGCAA
AP17	2	CCGAATAT	AGCTACACTC	CCAAGCAA
AP47	2	TTAGGCAAT	AGCTACACTC	CCAAGCAA
GTM140	2	AACTCGTCAT	AGCTACACTC	CCAAGCAA
GTM188	2	GGTCTACGTAT	AGCTACACTC	CCAAGCAA
IC313	2	GATACCAT	AGCTACACTC	CCAAGCAA
IC414	2	AGCGTTGAT	AGCTACACTC	CCAAGCAA
SAB3	2	CTGCAACTAT	AGCTACACTC	CCAAGCAA
SAD6	2	TCATGGTCAAT	AGCTACACTC	CCAAGCAA
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AP48	2	TTAGGCAAT	ACGCATC	CCAAGCAA
GTM142	2	AACTCGTCAT	ACGCATC	CCAAGCAA
GTM190	2	GGTCTACGTAT	ACGCATC	CCAAGCAA
IC328	2	GATACCAT	ACGCATC	CCAAGCAA
IC415	2	AGCGTTGAT	ACGCATC	CCAAGCAA
SAB4	2	CTGCAACTAT	ACGCATC	CCAAGCAA
SAD7	2	TCATGGTCAAT	ACGCATC	CCAAGCAA
AP19	2	CCGAATAT	GTATGCAC	CCAAGCAA
AP49	2	TTAGGCAAT	GTATGCAC	CCAAGCAA

GTM143	2	AACTCGTCAT	GTATGCAC	CCAAGCAA
GTM193	2	GGTCTACGTAT	GTATGCAC	CCAAGCAA
IC330	2	GATACCAT	GTATGCAC	CCAAGCAA
IC416	2	AGCGTTGAT	GTATGCAC	CCAAGCAA
SAB5	2	CTGCAACTAT	GTATGCAC	CCAAGCAA
SAD8	2	TCATGGTCAAT	GTATGCAC	CCAAGCAA
AP23	2	CCGAATAT	CACATGTCC	CCAAGCAA
AP50	2	TTAGGCAAT	CACATGTCC	CCAAGCAA
GTM148	2	AACTCGTCAT	CACATGTCC	CCAAGCAA
GTM195	2	GGTCTACGTAT	CACATGTCC	CCAAGCAA
IC37	2	GATACCAT	CACATGTCC	CCAAGCAA
IC418	2	AGCGTTGAT	CACATGTCC	CCAAGCAA
SAB6	2	CTGCAACTAT	CACATGTCC	CCAAGCAA
SAD9	2	TCATGGTCAAT	CACATGTCC	CCAAGCAA
AP24	2	CCGAATAT	TGTGCACGAC	CCAAGCAA
AP51	2	TTAGGCAAT	TGTGCACGAC	CCAAGCAA
GTM156	2	AACTCGTCAT	TGTGCACGAC	CCAAGCAA
GTM205	2	GGTCTACGTAT	TGTGCACGAC	CCAAGCAA
IC402	2	GATACCAT	TGTGCACGAC	CCAAGCAA
IC44	2	AGCGTTGAT	TGTGCACGAC	CCAAGCAA

SAB7	2	CTGCAACTAT	TGTGCACGAC	CCAAGCAA
SAD10	2	TCATGGTCAAT	TGTGCACGAC	CCAAGCAA
AP25	2	CCGAATAT	GCATCAC	CCAAGCAA
AP52	2	TTAGGCAAT	GCATCAC	CCAAGCAA
GTM160	2	AACTCGTCAT	GCATCAC	CCAAGCAA
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IC404	2	GATACCAT	GCATCAC	CCAAGCAA
IC441	2	AGCGTTGAT	GCATCAC	CCAAGCAA
SAB8	2	CTGCAACTAT	GCATCAC	CCAAGCAA
SAD11	2	TCATGGTCAAT	GCATCAC	CCAAGCAA
AP26	2	CCGAATAT	ATGCTGTC	CCAAGCAA
AP54	2	TTAGGCAAT	ATGCTGTC	CCAAGCAA
GTM167	2	AACTCGTCAT	ATGCTGTC	CCAAGCAA
GTM241	2	GGTCTACGTAT	ATGCTGTC	CCAAGCAA
IC405	2	GATACCAT	ATGCTGTC	CCAAGCAA
IC443	2	AGCGTTGAT	ATGCTGTC	CCAAGCAA
SAB9	2	CTGCAACTAT	ATGCTGTC	CCAAGCAA
SAD12	2	TCATGGTCAAT	ATGCTGTC	CCAAGCAA
AP28	2	CCGAATAT	CATGACCTC	CCAAGCAA
AP7	2	TTAGGCAAT	CATGACCTC	CCAAGCAA

GTM170	2	AACTCGTCAT	CATGACCTC	CCAAGCAA
IC26	2	GGTCTACGTAT	CATGACCTC	CCAAGCAA
IC406	2	GATACCAT	CATGACCTC	CCAAGCAA
SAB0	2	AGCGTTGAT	CATGACCTC	CCAAGCAA
SAD10	2	CTGCAACTAT	CATGACCTC	CCAAGCAA
SAM000	2	TCATGGTCAAT	CATGACCTC	CCAAGCAA
AP32	2	CCGAATAT	TGCAGTGAGC	CCAAGCAA
AP8	2	TTAGGCAAT	TGCAGTGAGC	CCAAGCAA
GTM173	2	AACTCGTCAT	TGCAGTGAGC	CCAAGCAA
IC301	2	GGTCTACGTAT	TGCAGTGAGC	CCAAGCAA
IC407	2	GATACCAT	TGCAGTGAGC	CCAAGCAA
SAB1	2	AGCGTTGAT	TGCAGTGAGC	CCAAGCAA
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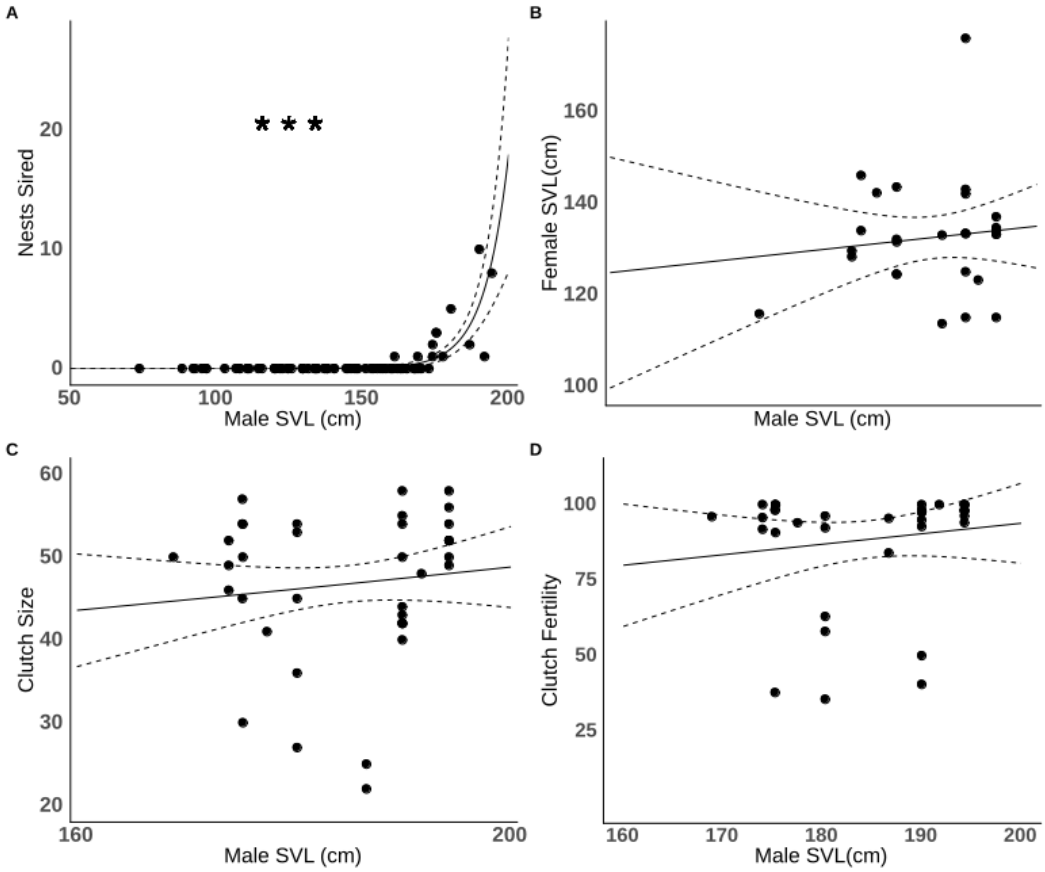

Figure 1. Relationships between male snout-vent length and (A) the number of nests sired, (B) size of female mate, (C) clutch size and (D) clutch fertility. Asterisks indicate significant results.

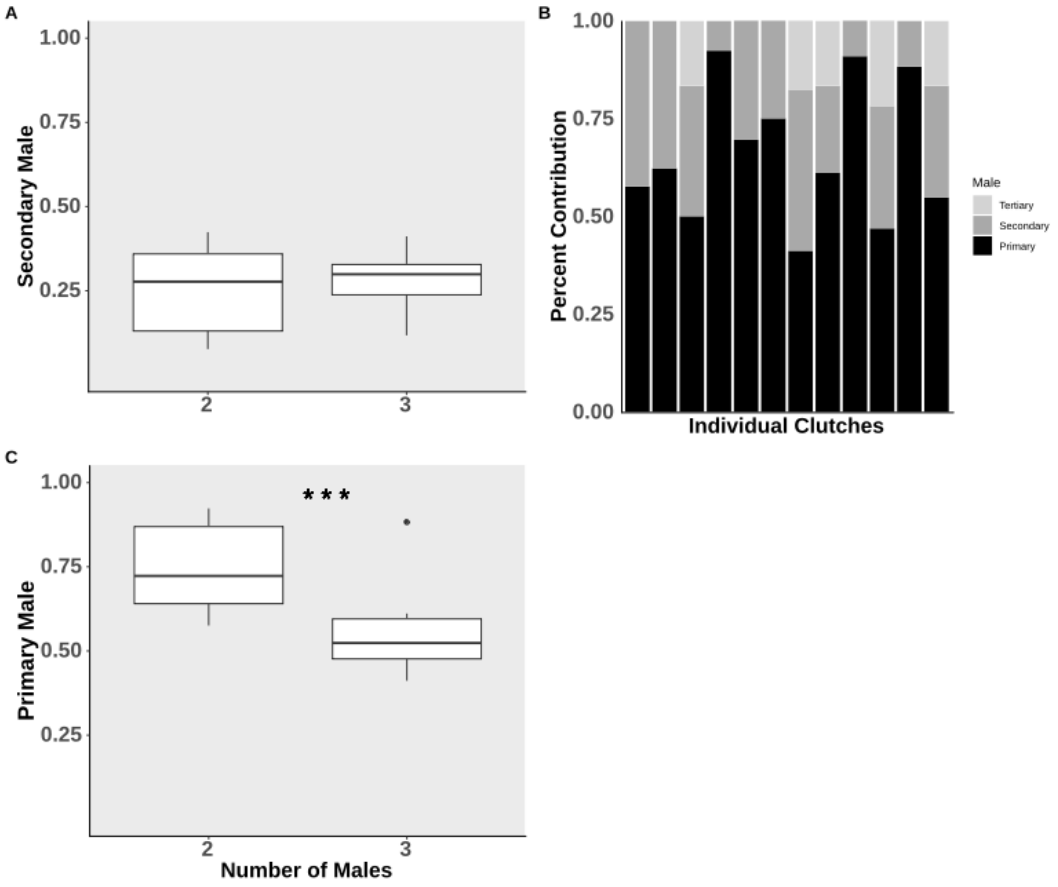

Figure 2. Examination of male contributions to nests with (A) the distribution of contributions across secondary males, (B) the distributions of contributions across primary, secondary and tertiary males, and (C) the distribution of contributions across primary males. N= 35. Asterisks indicate a significant difference.

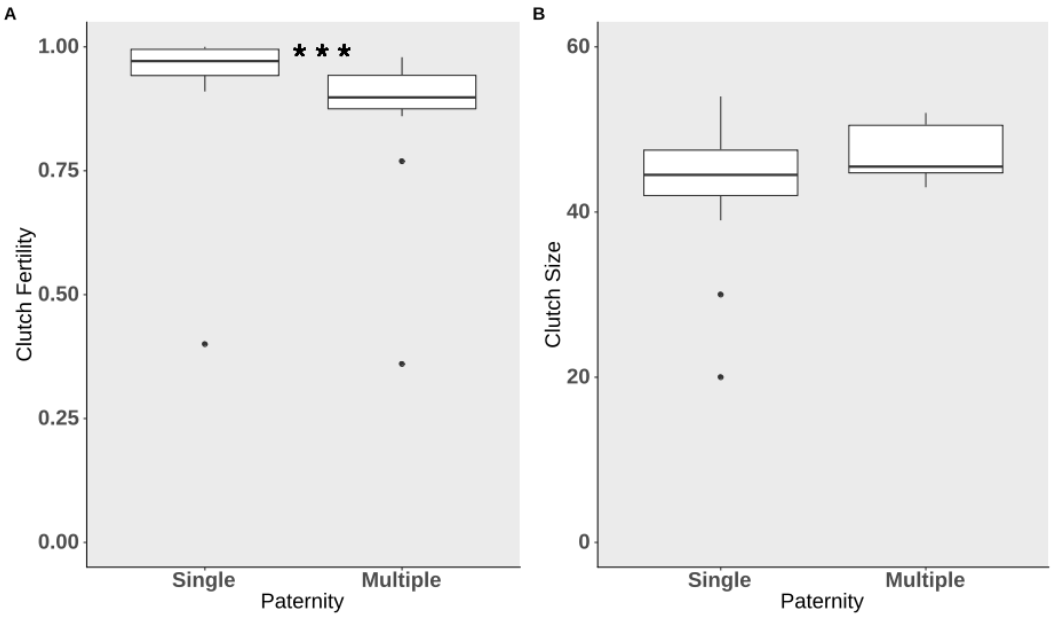

Figure 3. Relationships between fitness related traits and multiple paternity including (A) clutch fertility and (B) clutch size across singly sired and multiply sired nests. N= 35. Asterisks indicate significant results.

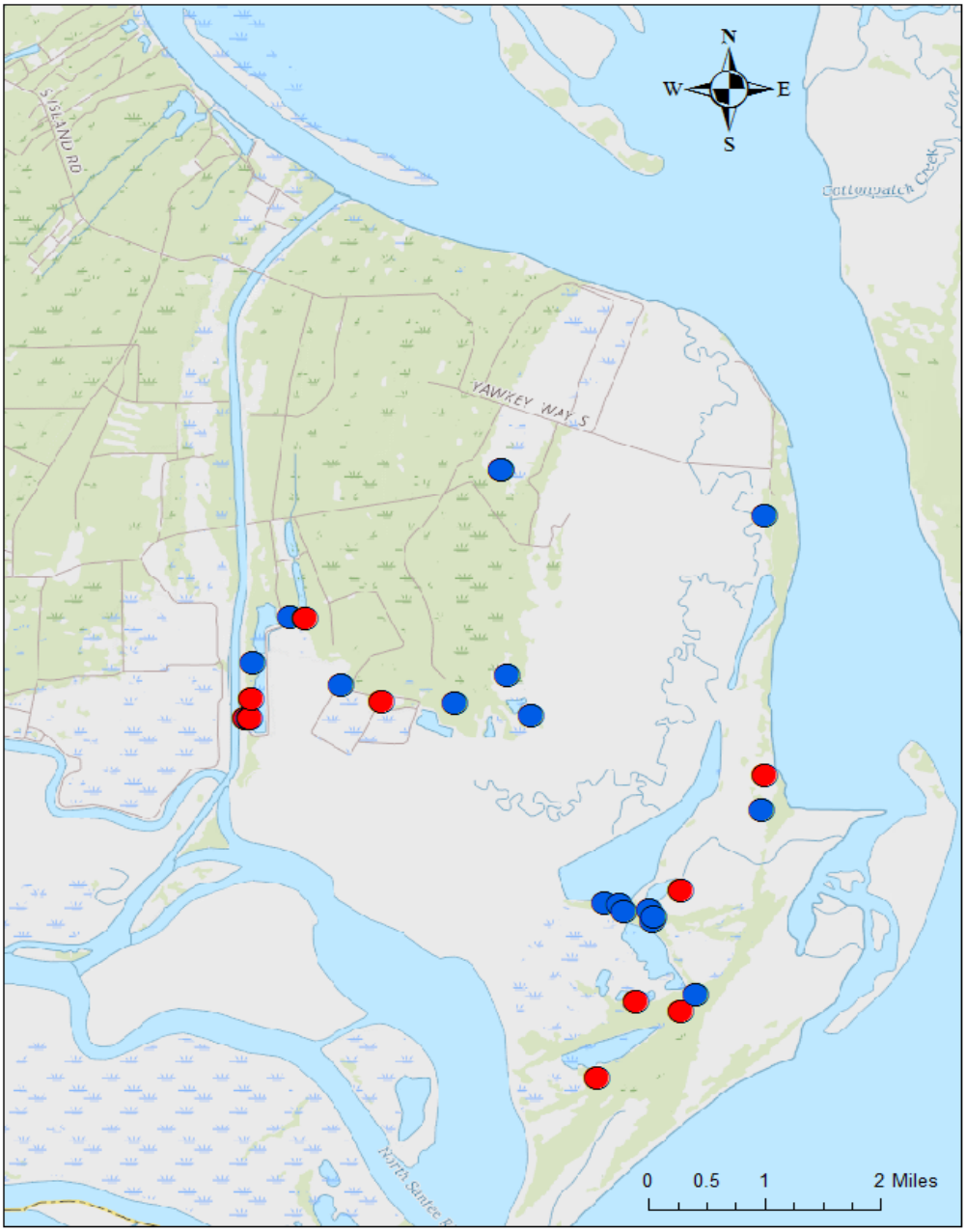

Figure 4. Map of YWC with points indicating nests for which the entire clutch was sampled (N= 35) Blue points represent nests that were singly sired and red points represent nests that were multiply sired.

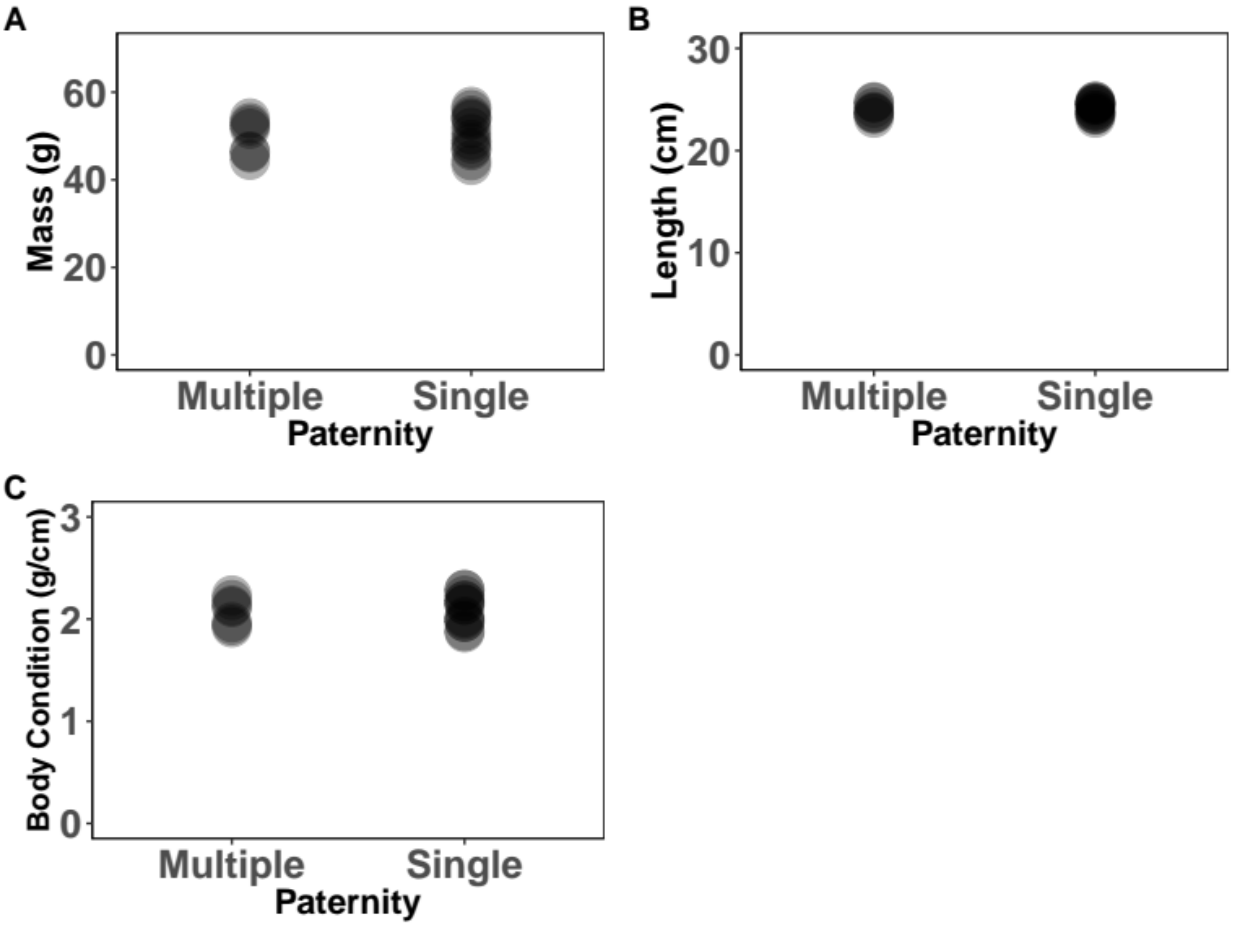

Figure 5. Relationships between hatchling phenotypes and patterns of paternity with (A) hatchling mass (B) hatchling length and (C) body condition across singly sired and multiply sire nests. Each point represents one clutch. N=35. Asterisks indicate significant differences.

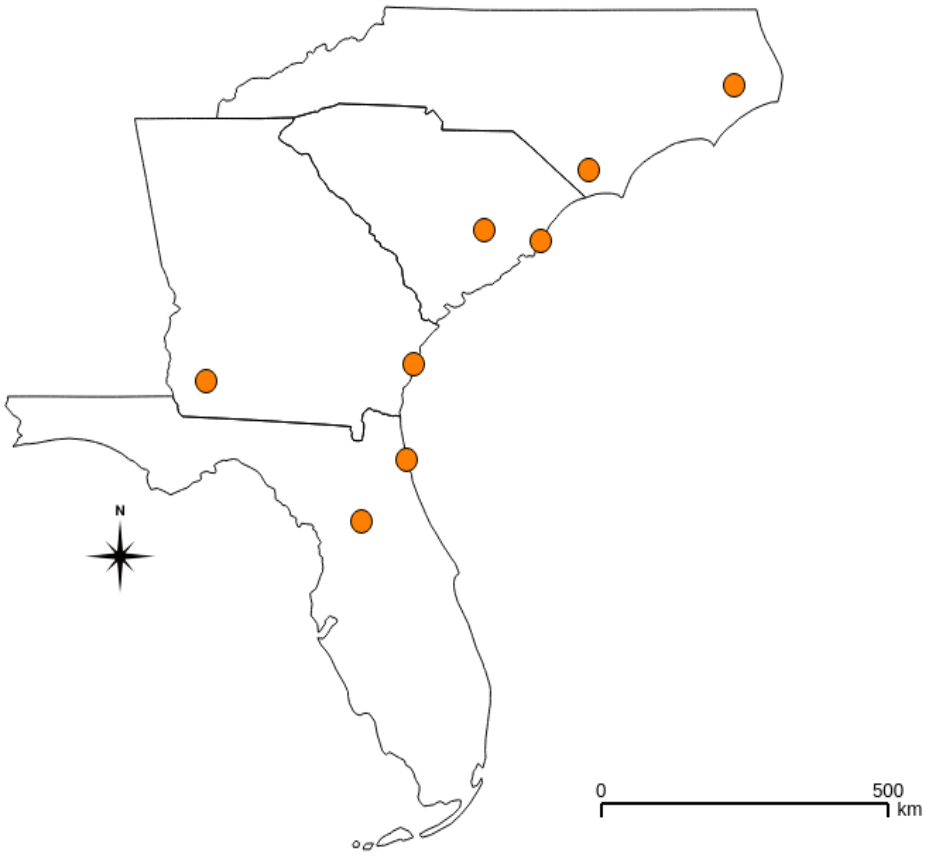

Figure 6. Map of sampled alligator locations. Orange dots represent one population that was included in the analysis of population connectivity.