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Multiple Paternity of Caretta Caretta within the Northwestern Atlantic Ocean Population on Wassaw Island, Ga

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MULTIPLE PATERNITY OF *CARETTA CARETTA* WITHIN THE NORTHWESTERN ATLANTIC OCEAN POPULATION ON WASSAW ISLAND, GA

by

JACOB LASALA

(Under the Direction of David C. Rostal)

ABSTRACT

Characterizing the mating system of a species is important for understanding demography and population dynamics and can contribute information to conservation efforts. Mating systems can impact the ecology, evolution, effective population size and genetic variability of a species. Polyandry, resulting in multiple paternity can influence the maintenance of genetic variation within a population. Within Testudines, the frequency of multiple paternity varies extensively among species (0-100% of nests). Previous studies on the loggerhead turtle (*Caretta caretta*) have shown that within the large management unit of peninsular Florida, multiple paternity occurs in approximately 30% of nests. This study examines nests from the smaller and more endangered northern management unit. The primary objectives of this study are to determine if multiple paternity exists in Georgia's smaller nesting population and determine if the percentage of nests with multiple fathers differs significantly from previous studies. Secondary objectives are to compare the incidence of multiple paternity over multiple years, determine if multiple paternity varies over the course of the nesting period. Our final objectives were to determine the relationship between the number of fathers per nest and female size (straight carapace length), as well as hatching success and to determine how many males are actually contributing to this nesting population. Mothers and offspring (up to 20) were initially sampled from more than 90 nests over three nesting seasons on Wassaw Island, GA (2008-2010). We found that multiple fathers contribute to 75% of nests over the three years. There is a difference in number of fathers per nest with relation to the year, but there is no relationship between the number of fathers per nest and the nesting period. There is a positive relationship between female size (SCL) and the number of fathers per nest. There is no relationship between the number of fathers contributing to a clutch and hatching success. Finally I found a total of 195 male genotypes over the course of the study, resulting in a sex ratio of 2.7 males per 1 nesting female. Every male genotype that I discovered only contributed to one nest over the three years samples, indicating there is a large number of males contributing to this nesting population.

INDEX WORDS: *Caretta caretta*, Mating system, Multiple paternity, Georgia, Barrier islands, Genotypes, Straight carapace length, Hatching success, Nest size, Sex ratio

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by

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ABSTRACT

Characterizing the mating system of a species is important for understanding demography and population dynamics and can contribute information to conservation efforts. Mating systems can impact the ecology, evolution, effective population size and genetic variability of a species. Polyandry, the occurrence of a single female mating with multiple males can result in the multiple paternity of progeny arrays. Previous studies on the loggerhead turtle (*Caretta caretta*) have shown that within the large nesting population of peninsular Florida, multiple paternity occurs in approximately 30% of nests. This study tested nests from the smaller and more endangered northern nesting population for the presence of multiple paternal contributions. Mothers and up to 20 offspring were sampled from more than 90 nests over three nesting seasons on Wassaw Island, GA (2008 – 2010). We found that 75% of nests sampled had multiple fathers with an average of 2.65 fathers per nest. We determined that there was a difference in the number of fathers per nest with relation to the year, but not to the period the nest was laid. There was a positive relationship between the number of fathers per nest and female size (SCL), but there was no relationship between number of fathers and hatching success. Finally we found 195 paternal genotypes and determined that each individual only contributed to one nest over all three years.

INTRODUCTION

The study presented here is the first examination of multiple paternity within loggerhead sea turtles *(Caretta caretta*) nesting on Georgia beaches. This is also the first study to look at the frequency of multiple paternity on a small nesting beach and will be the first study to examine the “full” nesting potential of this nesting beach: this research covers three years of nesting data, allowing for an assessment of the whole nesting population on this beach.

Loggerheads reach sexual maturity between 20 and 35 years of age (Federal Register, 2011). Every three years, females will travel to their mating sites between late March and early June, males are not restricted and can mate every year (Conant et al., 2009). Mating occurs in offshore wasters near to the nesting beaches several weeks before nesting (Limpus, 1985, Conant et al. 2009, etc). Males will occasionally fight one another for access to females (Schofield et al., 2006) and females can mate with more than one male and then store the sperm for up to a year (Uller and Olsson, 2008). Males return to their foraging grounds after mating, females will remain in the vicinity of the nesting beach (Limpus and Reed, 1985; Dodd, 1988, etc). Between late April and early September, females return to their natal nesting beaches. Females nest on continental beaches and barrier islands close to continental land masses (Dodd, 1985) Females will lay their first clutch and leave the beach, approximately two weeks (14 days) later the females return to nest again (Dodd, 1988; Conant et al., 2009 etc.); this process can be repeated for up to 7 (Dodd, 1988) or 8 (observed) clutches per female.

Determining how mating systems impact population ecology, genetics and evolution has long been of interest to ecology and evolutionary biologists (Wright, 1931;

Bjorndal et al. 1983; etc.). Direct observation of marine turtles mating has been a difficult task to accomplish in the wild, and has had limited success in closed and controlled settings (Moore et al., 2008). Advances in genetic techniques and analytical methods permit allow scientists to infer turtle mating systems that may be relevant to conservation policy.

In 1978 the loggerhead sea turtle was first listed as threatened in its circum-global distribution (Federal Register, 1978). In 2007 a petition circulated to ask that the Western North Atlantic Loggerhead be listed as endangered. In 2008, the "Recovery Plan for the Northwest Atlantic Population of the Loggerhead Sea Turtle (*Caretta caretta*)" highlighted five specific recovery units from Virginia to the Caribbean, including the Northern Gulf of Mexico (NMFS and USFWS, 2008). The primary goal of this recovery plan was to determine if the Northwest Atlantic loggerhead population was declining enough to warrant endangered status.

Currently there are 9 distinct population segments worldwide as defined by the National Marine Fisheries Service and the United States Fish and Wildlife Service (Federal Register, 2011). A distinct population segment is the smallest partition of a species allowed to be protected under the U.S. Endangered Species Act. A distinct population segment is granted to a population if it is both *discrete* and *significant* in relation to the rest of the species (Federal Register, 2011). Five of these 9 distinct population segments are now considered endangered, the remaining 4 distinct population segments are now considered threatened (including the Northwest Atlantic population).

Bowen et al. (2005) examined mitochondrial DNA, typically maternally inherited, suggested that nesting colonies across the southeastern United States had a strong population structure (each beach was genetically distinctive when compared to one another). However, nuclear DNA (microsatellite markers: inherited from both parents) did not show a strong population structure on those same nesting beaches (Bowen et al., 2005). Bowen et al. suggested that the species mixed in the ocean and then as individuals grew, juveniles would group together depending on where they were initially hatched (ie. Western/Eastern North Atlantic etc.). In this model, adults would separate further, such that the loggerheads along the southeastern United States are all one population, but that breeding/nesting populations are distinctive nearer to shore.

Bowen et al.'s data indicates that this species' genetic structure along the Northwest Atlantic coast is driven by natal nesting (Bowen et al., 2005). Natal nesting is the process of nesting females returning to their natal nesting beaches to lay their eggs. In this model, gene flow is attributed to males, as they will move along the coast and not be restricted to the beaches they hatched on (Bowen et al., 2005, Federal Register, 2011). The Northwestern Atlantic population is genetically distinct from populations elsewhere in the Atlantic Ocean (Bowen et al., 2005; NMFS and USFWS, 2008; Federal Register, 2011; Richards et al., 2011). Thus NMFS and USFWS recently determined that a loss of this population would mean a loss of genetic diversity to the species as a whole (Federal Register, 2011).

The recovery plan of 2008 highlights two recovery units along the coast of the Northwestern Atlantic Ocean: The Northern (Virginia – North Florida) and the Penninsular Florida Recovery Unit (NRU and PRFU respectively) (NMFS and USFWS, 2008). From 1989-2008, annual nest totals of the NRU averaged 5,125 nests and from 1989-2007, annual nests totals of the PFRU average 64,513. Loggerhead nests have decreased 1.3% annually since 1983 in the NRU; overall the unit has experienced a "long-term decline" (NMFS and USFWS, 2011). PFRU nesting has decreased as well by 1.6% annually in the last 20 years. However, NMFS and USFWS suggest that recent abnormally high nesting years (2008-2010) represent a positive trend for the NRU (Federal Register, 2011). The nesting data suggests that the Northwestern Atlantic population is declining, but there could be signs for recent recruitment.

NMFS and USFWS determined that the Northwestern Atlantic discrete population segment should be considered threatened as opposed to endangered in 2011 (Federal Register, 2011). The factors cited were population abundance and trend: they suggested that the adult population (over 60,000 by conservative estimate) exceeds those of other endangered species in the Atlantic. Furthermore, while acknowledging that the population is experiencing decline, NMFS and USFWS stated that recent nesting events appear to be positive and could stabilize the region. NMFS and USFWS estimate that the nests in Florida comprise approximately 87% of all nesting effort in the Northwest Atlantic population. Subsequently, the majority of the research concerning this discrete population segment has occurred in Florida regarding nesting counts and female characteristics (Bjorndal et al., 1983; Bollmer et al., 1999; Bowen, 2004; Caldwell, 1962; LeBuff and Beatty, 1971; Dodd et al., 2003; Hanson et al., 1998; Moore and Ball, 2002; Steinitz et al., 1998 etc.).

Our understanding of this discrete population segment is incomplete: there is little information available about the mating males as we cannot obtain a reliable sex ratio because males rarely leave the ocean. Without knowledge of male contribution, the effectiveness with which we manage designated populations will always be limited

(Wibbels et al., 1991;Hanson et al., 1998, Casale et al., 2005, Casale et al., 2011 etc.)

Examining paternity of hatchlings we can evaluate the number of contributing males indirectly (ie. without physically counting each individual). Using genetic techniques we can determine how many males sired a nest, even if we are not able to observe mating behavior. Exclusion paternity analysis can estimate the potential genotypes of contributing fathers, allowing an estimate of how many individuals contribute to nesting sites to be calculated. NMFS and USFWS estimate that there are 30,000 nesting females in the Northwest Atlantic discrete population segment and in 2011 Richards et al. Used models to conservatively estimate that there was just over 38,000 females contributing to the discrete population segment from 2001 - 2010. The current approximation of adult sex ratios are 1:1 suggesting there are 60,000 – 75,000+ adult loggerheads in this discrete population segment over the course of this last decade (Federal Register, 2011; Richards et al., 2011). The problem with this approximation is that it assumes that all males are reproducing and does not assess the number of males that are actually contributing to this discrete population segment. By looking at the number of genotypes through paternity, we can approach the sex ratio from a different angle and perhaps determine a more robust population estimate.

Previous studies of multiple paternity in sea turtles are summarized in Table 1. These studies indicate that multiple paternity occurs in sea turtle nests (Moore & Ball, 2002; Zbinden et al., 2007; Fitzsimmons, 1998; Crim et al., 2002; etc.) but varies across populations within a species (Jensen et al., 2006, Zbinden et al. 2007; etc.). It is interesting to note the difference between nesting sites regarding *Lepidochelys olivacea:* in a regular rookery, only 30% of nests have multiple paternal contributions, but within an arribada nesting site (mass mating) the frequency rises significantly to 92% (Jensen et al., 2006). Jensen et al. attributed the difference in sites due to the abundance of individuals in the mating system, suggesting that as the number of nesting females rise, so does the chance of multiple mating. Following this assessment, larger nesting beaches (more nesting females) should have a higher percentage of nests with multiple paternity. Do smaller nesting beaches (ie. less nesting females) follow this model?

This study examined the occurrence of multiple paternity over the full nesting interval (three years) on Wassaw Island, GA. Loggerheads from the Northwestern Atlantic distinct population segment, return once every three years (Conant et al., 2009), by analyzing 92 non-repeating mothers and 72 nests we are essentially examining a snapshot of the nesting population on Wassaw Island, GA. During the three years, we sampled 19.5% of the nests laid in that time period.

I will quantify the occurrence of multiple paternity within this nesting population and how it relates to what we know about the whole discrete population segment (specifically with respects to Florida findings). Using genetic data I will estimate how many males are contributing to this nesting beach. Finally I will assess the relationship between the number of fathers per nest and female size, nest hatching success and the timing of the nesting event.

MATERIALS AND METHODS

Field Methods

Samples were obtained on the Wassaw National Wildlife Refuge (WASI, 31°54’1.08” N/ 80°58’55.92”) for three summer nesting seasons (2008-2010). Female loggerheads nest on this 11.3 km beach from May until early August and nests hatch from late June until early October.

During nightly patrols with the Caretta Research Project adult nesting females were identified from their crawls up the beach (sea turtles all have distinctive crawls, see Figure X). Female sampling was divided into three periods of the nesting season in correspondence to reproductive hormone levels (Drake, 2001): early nests from May 15 – June 10; middle nests from June 11 – July 8 and late nests from July 9 – August 4.

Drake (2001) determined that nesting female testosterone levels peaked at the beginning of the nesting season and would drop (step wise) with each subsequent nesting event. Using this step down pattern as a model, we can estimate when nesting events will occur according to our calendar. The first two nesting events roughly from April 30- June 9 (Early Nesting Period), the next two nesting events occur from June 10- July 9 (Middle Nesting Period) and the final nesting events occur from July 10 – August 18 or until the end of the nesting season (Late Nesting Period). There is some overlap between nesting events and this time scale, but if we assume nesting events occur randomly throughout the nesting season, then independent females could be collected in sampling were distributed throughout each nesting period.

Ten individual females from each nesting period (resulting in 90 females) were tagged and measured according to U.S. Fish and Wildlife protocols (Barnard and Keinath, 1999; Williams and Frick, 2000; Drake, 2001). Blood samples were taken from the cervical sinus using a 21Gx1-1/2" needle and retained in a 5ml Sodium Heparin vacutainer. No individual was sampled more than once.

Nests of these females were promptly caged to prevent predation as well as the escape of hatchlings. A GPS reading was taken to mark the location of each nest. Stakes were also added at the dune line to approximate location of nest from the water line, to document the date the nest was laid and to also designate what kind of turtle laid the nest: *Caretta caretta* (CC); *Chelonia mydas* (CM); *Dermochelys coriacea* (DC) were the three typical species. At hatching, 20 random hatchlings were removed from each loggerhead nest, weighed, measured and sacrificed. Residual yolk sacs were removed from these hatchlings, weighed and placed in Whirl-Paks® and stored at -20°C.

Molecular Techniques

Maternal DNA was extracted by adding 2 µL of blood to a lysis buffer (10mM Tris pH 8.3, 50mM KCl and 0.5% Tween 20) and the mixture was incubated with proteinase K (20 µL, >600 mAU/ml) at 65^oC for one hour followed by 100^oC for 15 minutes. Hatchling DNA was extracted from residual yolk sacs using the DNeasy blood and tissue kit (QIAGEN) following manufacturer protocol. Polymerase chain reaction (PCR) amplification was carried out using primers for five microsatellite loci (CcP7E05, CcP2F11, CcP7D04, CcP7C06, CcP8D06) designed for *Caretta caretta* (Shamblin et al., 2009). PCR reactions were carried out as a multiplex reaction in 25 µL volumes, consisting of 2 µL of extracted DNA, 10 µL of Apex Taq Master Mix (150 mM Tris-HCl pH 8.5, 40 mM $(NH_4)_2SO_4$, 3.0mM $MgCl_2$, 0.2% Tween 20; 0.4 mM dNTPs; 0.05 units/µL Apex Taq DNA polymerase & stabilizer), 3µL H_2O, 2 2 µL P7E Forward and Reverse, 1 µL P2F F&R, 0.5 µL P7D F&R, 1 µL P7C F&R and 0.5 µL P8D F&R (10 µM). Thermocycling protocol was as follows: 95^oC for 5 min; 40 cycles of 95^oC for 20s; 60^oC for 30s and 72^oC for 30s; and 72^oC for 10 min.

PCR products were analyzed using an ABI 3500 Genetic Analyzer. Alleles were sized at each locus in relation to an internal size standard using GeneMapper 3.0 software;

also, sizing was checked by eye. Microsatellite loci were checked for null alleles using Micro-Checker 2.2.3 (Van Oosterhout et al., 2004). Probability of Identity (PI) values and probability of exclusion “when only one parent is known” were determined using GenAlEx 6.41 (Peakall and Smouse, 2006). Observed and expected heterozygosity, linkage equilibrium and deviations from Hardy-Weinberg Equilibrium were calculated and assessed using GenAlEx and GDA (Lewis and Zaykin, 2000) (Table 1).

Paternity Analysis

Analysis of paternity was evaluated with an exclusion paternity analysis. Each hatchling was determined to have its own multilocus genotype. When known maternal alleles were subtracted from each locus, the remaining paternal alleles formed suspected paternal genotypes. This analysis was performed using the programs GERUD 2.0 and COLONY 2.0. GERUD 2.0 assesses the minimum number of fathers per nest. GERUD 2.0 has been employed in a variety of paternity studies (Jones, 2005; Jones et al., 2010, Yue and Chang, 2010, Zbinden et al., 2007) and can be conservative in its estimates of the number of paternal contributions. COLONY 2.0 is a likelihood-based program that determines the maximum number of fathers per clutch. GERUD 2.0 fails if there are more than 6 fathers, so COLONY 2.0 was used to repeat the analysis on nests that GERUD 2.0 could not evaluate because more than 6 fathers were detected. Using both programs we approximated a range, where GERUD 2.0 was the minimum and COLONY 2.0 was the maximum.

COLONY 2.0 was also used to compare sibling relatedness for all the nests (Jones and Wang, 2010). In this analysis, the error rate of genotyping was set to 0.025 as suggested by Wang (Wang, 2004). The determination of multiple paternity within a nest was established by the occurrence of more than two paternal alleles over at least two loci–this allowed for the possibility of a mutation at one locus (Yue and Chang, 2010). To determine whether paternal contributions were significantly different from equality in each nest, goodness-of-fit χ^2-tests were run on all three years of data. When running χ^2-tests, the years were separated as we assume that each year is independent of one another.

The “Probability of Identity” (PI) was estimated using the method employed by GenAlEx within our three year data set.

$$PI = 2(\sum (p_i^2)^2) - \sum p_i^4$$

Where p_i is the frequency of the *i*th allele at a locus

PI provides an estimate of the average probability that two samples will have the exact same genotype given the estimated allele frequencies of the loci used. The Probability of Exclusion (when only one parent is known) was also determined using GenAlEx to estimate the statistical power of our individual loci and our combined loci (Table 1).

$$PE = 1 - 4\sum p_i^2 + 2(\sum p_i^2)^2 + 4\sum p_i^3 - 3\sum p_i^4$$

Where p_i is the frequency of the *i*th allele at a locus

Finally each paternal genotype was compared using COLONY 2.0 and GenAlEx to determine if any of the the estimated paternal genotypes were sampled more than once; furthermore using COLONY we could compare all the nests to these genotypes and determine how many nests an individual male contributed to.

Statistical Analysis

All three years were analyzed together (2008-2010). Every test that was performed using data from GERUD was also performed using data from COLONY. All analyses were carried out using the program SAS 9.3 (SAS Institute Inc).

The original assumption was that female characteristics should vary randomly by year and nesting events should be independent of one another: no repeat nesters were analyzed. We ran a generalized linear model with a gamma distribution. As our data was skewed to the left, the gamma distribution was the best model to compare variability within number of fathers due to year, season and female size. The model was run twice (once for each paternity program), with the number of fathers per nest as the dependent variable. Female size (Straight Carapace Length measured in centimeters), the period the nest was laid (early, middle or late), the Julian Date the nests were laid and the year the nest was laid (2008-2010) were all defined as independent variables. Initially I ran the model with all the variables and then removed the independent variable with the highest p-value, determining that it had no effect on the number of fathers per nest. Then I reran the model, removing the next highest and repeated until the only independent variable that remained had p-value less than 0.05.

I ran another generalized linear model (gamma distribution) to determine if the number of fathers per nest affected hatching success of each nest (percent of nest that did *not* emerge). Again, I ran two separate tests, the hatching success was defined as the dependent variable and the number of fathers per nest was defined as the independent variable.

Separately using the program JMP 9.0 (SAS Institute Inc) I performed a Wilcoxon/Kruskal-Wallis test to determine if there was a difference in the number of fathers between nesting periods. The nesting period (early, middle and late) was defined as the independent variable and the number of fathers as the dependent variable. This separate test was performed to determine the difference within years, whereas the model examined all nests over all years.

RESULTS

All five loci amplified consistently over the 92 mothers sampled. The program Pedant determined that the combined allelic dropout rate of the samples was 0.0254 and the false allele rate was 0.1070 (Johnson and Haydon, 2007). Allele number ranged from 12 for the locus *Cc*P7C06 to 27 for the locus *Cc*P8D06 (Table 2). Deviations from Hardy-Weinberg were not found. Combining all five loci produced an expected exclusion probability of 99.64%, assuming only one parental genotype is known. The combined probability of identity using the five loci (the likelihood that one independent individual will have the exact same genotype as another) was $1.5x10^{-6}$ (Table 2).

Of the original 92 mothers, 72 nests survived: 23 from 2008, 21 from 2009 and 28 from 2010. If we separate by nesting period, there are 23 early nests, 26 middle nests and 23 late nests. Nests were laid from as early as May 25 to as late as July 17. A total of 1282 hatchlings were sampled and analyzed. The average number of hatchlings genotyped per nest was 18.2 (SD = 4.3). Six nests had less than 10 hatchlings genotyped. Over the three-year period the average nest size was 114.7 (SD = 23.9) and the average percentage of a nest sampled from the nesting beach was 16.5% (SD = 5.7). The average percentage of hatchlings that did *not* emerge from a nest was 21.7% (SD = 13.7). Average female size (straight carapace length) over the three years was 98.6 cm (SD = 6.5).

In 2008, using both GERUD and COLONY multiple paternal contributions were found in 19 of 23 nests (82.6%), with an average of 3.00 (± 0.23) males per nest. In 2009, using GERUD multiple paternal contributions were found in 15 of 21 nests (71.4%) with an average of 2.62 (± 0.38) males per nest (COLONY: 18/21, 85.7%, 2.8 (± 0.35)), averaging 78.6% with 2.71 (± 0.25) fathers per nest. In 2010, using GERUD multiple paternal contributions were found in 18 of 28 nests (64.3%) with an average of 2.20 males per nest (COLONY: 19/28, 67.9%, 2.32 (± 0.25)). When all three years are combined, using GERUD multiple paternal contributions were found in 52 of 72 nests (72.2%) with an average of 2.58 (± 0.17) males per nest, 95% CI: 2.24 -2.93 (COLONY: 56/72, 77.8%, 2.72 (± 0.18), 95% CI: 2.36 – 3.09) (Figure 1).

Fathers per Nest

Our conservative estimate (GERUD) illustrated that over all three years 20 nests had 1 father, 17 nests had 2 fathers, 21 nests had 3 fathers, 7 nests had 4 fathers, 3 nests had 5 fathers, 2 nests had 6 fathers and finally 2 nests had more than 6 fathers (GERUD could not handle these data sets). Overall, 22 of 52 (42.3%) of nests with multiple fathers deviated significantly from equality. In 2008, 6 nests deviated significantly from equal contribution among fathers (χ^2- tests, p = 0.0001 – 0.0253), 13 nests with multiple fathers did not deviate significantly (p = 0.1651 – 0.9284) (Figure 2a). In 2009, 6 nests deviated significantly from equal contribution among fathers (χ^2- tests, p = 0.0006 – 0.0379), 9 nests with multiple fathers did not deviate significantly (p = 0.1651 – 0.9486) (Figure 3a). In 2010, 10 nests deviated significantly from equality (χ^2- tests, p = 0.0001 – 0.0492), 8 nests with multiple fathers did not deviate significantly (p = 0.0736 – 0.7212) (Figure 4a).

The upper range estimate of COLONY indicates 16 nests had 1 father, 23 nests had 2 fathers, 16 nests had 3 fathers, 7 nests had 4 fathers, 4 nests had 5 fathers, 4 nests had 6 fathers and finally 2 nests had 7 fathers. Overall, 23 of the 56 (41.1%) nests with multiple fathers deviated significantly from equality. In 2008, 9 nests with deviated significantly from equal contribution among fathers (χ^2- tests, p = 0.0001 – 0.0253), 10 nests with multiple fathers did not deviate significantly ($p = 0.1189 – 0.9735$) (Figure 2b). In 2009, 5 nests deviated significantly from equal contribution among fathers (χ^2- tests, $p = 0.0001 – 0.0384$), 13 nests with multiple fathers did not deviate significantly ($p = 0.0736 – 0.9486$) (Figure 3b). In 2010, 9 nests deviated significantly from equality (χ^2-tests, $p = 0.0017 – 0.0423$), 10 nests with multiple fathers did not deviate significantly ($p = 0.0736 – 0.9402$) (Figure 4b).

Generalized Linear Model

The conservative generalized linear model (Table 4a) shows that when all three variables (year, season and female size) are combined, the variation in the number of fathers per nest was not explained significantly by any of the independent variables ($p = 0.1303$, $0.7410 - 0.8275$, 0.2050 respectively). However when year and female size are run separately, both variables explained significant variation to the number of fathers per nest ($\chi^2 = 4.05$, $p = 0.0441$ and $\chi^2 = 5.02$, $p = 0.0251$ respectively). The number of fathers per nest decreases as the years go on (2008-2010, Figure 5), as female size increases, the number of fathers increases (Figure 6). The number of fathers per nest does not explain variation in nest hatching success ($\chi^2 = 1.93$, $p = 0.1645$) (Figure 7).

The less conservative (COLONY) generalized linear model (Table 4b) shows that when all three variables (year, season and female size) are combined, the variation in the number of fathers per nest was not explained significantly by any of the independent variables ($p = 0.1712$, $0.6610 - 0.7981$, 0.2091 respectively). However when year and female size are run separately, both variables explained significant variation to the number of fathers per nest ($\chi^2 = 3.96$, $p = 0.0465$ and $\chi^2 = 4.63$, $p = 0.0315$ respectively). As year decreases, the number of fathers per nest increases (Figure 5) and as female size increases, the number of fathers per nest increases (Figure 6). The number of fathers per nest explained no variation in nest hatching success ($\chi^2 = 0.97$, $p = 0.3258$) (Figure 7).

Qualitatively: there was a relationship between female size and nest size ($r^2 = 0.419$, $F_{(1, 64)(0.05)} = 46.26$, $p < 0.0001$) (Figure 8).

Season

When all three years were compared in the model, there was no variation in the number of fathers per nest due to the date the eggs were laid (nesting period: Julian Nest Date). A Wilcoxon/Kruskal-Wallis was performed to determine if the number of fathers per nest varied over the course of the nesting period within each year and between years (not over the course of all three years). Like the model however, there was no difference in the number of fathers due to the nesting period for any of the three years. In 2008: df =2, GERUD: $\chi^2 = 1.42$, $p = 0.4911$, COLONY: $\chi^2 = 0.48$, $p = 0.7867$) (Figure 9a); in 2009: (df = 2, GERUD: $\chi^2 = 5.33$, $p = 0.0696$, COLONY: $\chi^2 = 3.43$, $p = 0.1795$) (Figure 9b); finally in 2010: (df = 2, GERUD: $\chi^2 = 1.49$, $p = 0.4726$, COLONY: $\chi^2 = 0.72$, $p = 0.6976$) (Figure 9c). When all three years were combined, there was no difference in the number of fathers per nest due to the nesting season (df = 2, GERUD: $\chi^2 = 1.75$, $p = 0.4174$, COLONY: $\chi^2 = 2.12$, $p = 0.3459$) (Figure 9d). When comparing the number of fathers per nest directly to the Julian Nest Date (the date the nest was laid), there was no significant trend (Figure 10).

Paternal Genotype Analysis

One hundred and ninety five (195) individual male genotypes were inferred using COLONY over all three years. Figure 12 shows the relationship between nests and fathers, there are no half-sibs, suggesting that every father only contributed to one nest over all three years.

DISCUSSION

Multiple paternity has previously been documented in loggerhead sea turtles across the globe. Harry & Briscoe (1988) determined in Queensland, Australia that 8 of 21 nests samples (38%) had multiple paternal contributions. In Florida, two studies independently determined that just over 30% of nests showed multiple paternal contributions (Bollmer et al., 1999 and Moore & Ball, 2002). Lastly, in Greece, Zbinden et al. (2007) found multiple paternal contributions in 19 of 20 nests (95%) and found a maximum of 5 fathers per nest. These studies were all conducted at large nesting aggregations (greater than 1000 nests per nesting season) and with the exception of the study in Greece, did not take samples from more than one nesting season.

In my study, over the course of the three year sampling period, the average nest size of *C. caretta* on Wassaw Island was 114.7 (SD = 23.9) and emergence success was 0.773 (Caretta Research Project, personal correspondence). The average nest size is comparable to values suggested by NMFS and USFWS of 115, but is higher than those values for emergence of the Northwestern Atlantic Ocean DPS: 0.54 (Conant et al., 2009). It could be suggested that our presence on the small beach of Wassaw is positively influencing the emergence success (less predation due to patrols, caging of nests and people present on the beach). However this could just be a result of the smaller nesting count on a small island in relation to the rest of the DPS, as beaches throughout the more heavily nested areas (such as in Florida) would have more human traffic and lower percentages of nests caged. Also, emergence success of larger nesting aggregations are more likely to approach the mean.

More than one male sired 75% (72.2 – 77.8%) of nests on Wassaw Island during the three year period studied. The average minimum number of fathers per nest was 2.65 (2.58-2.72). Even though Wassaw is a much smaller nesting beach (less nesting females) than either Florida or Greece, this estimate is nearly double the findings in the large Florida rookery of 33% and 1.4 fathers per nest (Moore & Ball, 2002). But is less than estimates found in Greece of 95% and 3.5 fathers per nest (Zbinden et al., 2007) (Figure 11). Previous studies have suggested that larger nesting aggregations should increase the likelihood of multiple paternity (Jensen et al. 2006; Uller and Olsson, 2008; etc.) as there is a positive correlation between multiple paternity and the probability of mate encounters. Regarding the Greece data, this theory makes sense, but there must be something else limiting this theory with regards to Florida (one of the largest nesting aggregations in the world). Perhaps because there are less females nesting in Georgia and Greece, males are able to mate with as many females as possible, but in Florida there are so many nesting females that there is a limit to how many females males can actually mate with (eventually with 30,000 females, a males sperm will run out).

Multiple paternity may be favored if it increases the variability of the offspring (Uller and Olsson, 2008, Olsson et al., 1994), but these are not necessarily related. As I cannot directly study the fitness of all the hatchlings, an easy way to assess variability is by looking at the how many hatchlings actually pip out of the egg. There was no relationship between hatching success and the number of fathers per nest. In other words, the number of fathers per nest did not directly skew the hatchlings to be more or less likely to survive. This could suggest that more fathers do add to the *variability* of hatchlings, but not to the viability of the hatchlings. We posit that with more fathers there should be more variability, every additional male will have both “good alleles” and “bad alleles.” In this example, “good alleles” favor the hatchling survival (ie. pipping out of the egg) and “bad alleles” could hinder the hatchling survival. By adding variation to a nest it is unlikely every allele passed along will be “good” for the hatchlings. But because female turtles cannot prepare for what their young will face in their future, bet-hedging against an unpredictable environment could still provide some benefits.

A major goal of this study was to assess the full nesting population of Wassaw Island, GA in comparison to the distinct population segment as a whole. Previous studies within the distinct population segment have focused on the large rookery of Florida (Bollmer et al., 1999; Moore & Ball, 2002). These studies, as well as others outside of the distinct population segment, have focused on assessing how multiple paternity affects the nesting population directly. (Bollmer et al., 1999; Moore & Ball, 2002; Zbinden et al., 2007; etc.). None of these studies have looked at the trends of multiple paternity over the course of time.

There were three major reasons for us to look at changes in the frequency of multiple paternity in this nesting population over time. First, female turtles return to nest once every three years (Conant et al., 2009); by looking at three years of data, we are essentially looking at a snapshot of the “whole” nesting population on this island. Second, there was a steady decrease of nesting numbers from 1970's until 2008, with a rise of nesting numbers from 2009 to 2011 (Federal Register, 2011), we wanted to see if this sudden increase in nesting affect the number of nests with multiple fathers. Finally, there was an unseasonably cold and wet season in 2009 that invariably affected how many nests I could analyze (only 21 of 30 original nests). As females nest once every three years on Wassaw, the number of nests per year should be random, allowing our assessment of the number of fathers per nest to be unbiased. But in 2009 many nests were lost due to events outside of the experiment's control. By losing those nests, it is possible that my data has become skewed. There was significant variation in the number of fathers per nest due to the year: from 2008 – 2010, the number of fathers per nest did increase. However, this finding does not mean that the year caused this increase, the variation is only related to each year, suggesting that each year could affect the results (ie. the difference from 2009 could have been important, but I did not test for that specifically).

We found that the number of fathers per nest did vary with regards to female size (straight carapace length, cm), such that as female size rose, so did the number of fathers. This is most likely attributed to larger females being older (Casale et al., 2011). Males probably favor older females because they are likely more fecund, they have lived long enough that they have reproduced more than once and if they are larger they can hold more eggs. It is also possible that larger females can swim faster to mating grounds and would have a longer mating period (untested). The females varied significantly in 2010 in comparison to the other two years, the females were smaller, suggesting that these turtles were young. Regarding the difference in number of fathers per nest by year, this could be a difference in sample size: there are 5+ more nests analyzed in 2010 than from 2008 (23) or 2009 (21). Or there might not be any trend at all and 2010 was just an anomaly; the only way to tell for sure would be to continue sampling.

Coupled together, there was no difference in the number of fathers per nest in relation to year and female size. This could suggest that while the weather of 2009 might have affected the nests on the beach, there was no connection between the climate and the mating offshore. This also suggests that while females in 2010 were smaller and there were less fathers per nest, there was no real difference in the number of fathers per nest because of the smaller females.

There was no difference in the number of fathers per nest due to nesting period. This is consistent with significant sperm mixing at the beginning of the season. (Pearse et al., 2002; Uller and Olsson, 2008; etc.) Females store the sperm in their oviduct after mating and can potentially hold the sperm for at least a year. (Uller and Olsson, 2008) If the sperm from male to male was being packed in one after another, it could be possible to see a skew in the number of fathers per nest over the course of the season. However, because there was no pattern of multiple fathers or single fathers, sperm must mix.

Additionally, 21% of the nests sired by more than one male showed skewed paternal contributions (ie. proportions of the nest were biased to one of the fathers). When a nest is skewed to one father over another, it is typically hypothesized as evidence for cryptic female choice (allowing females to preferentially choose sperm) or for the promotion of “good alleles,” thus supporting sperm competition (Uller and Olsson, 2008). Furthermore, we found that there was no difference in hatching success due to the number of fathers per nest, suggesting that females do not mate repeatedly to guarantee fertilization.

This study estimated 195 unique paternal genotypes. No male sired more than one nest throughout the three years, we posit that this nesting population's mating system is polyandrous and not polygynous. It is probable that males are contributing to other nests, but our data does not support this.

Counting males is difficult as males rarely return to the beach and they return to their feeding grounds immediately after the mating season. By comparing male genotypes we can “count” without having to witness the males or the mating itself. As hatchling sex ratios are becoming increasingly female biased (Hanson et al., 1998; Delgado et al., 2010 etc.), it is important to determine how many males are actually contributing to this nesting population. If we assume that each year in this study is independent of one another, that the females nesting in 2008 are not nesting in 2009 and females in 2009 are not nesting in 2010, then we have to look at the ratio of males to nests individually (3 years, 3 sub-nesting-populations: 3.00, 2.71, 2.67). But because we didn't find any males that contributed to more than one year's nests we can take the average number of males per nests over all three years (2.65 males to nests).

The estimate in Florida, is that there is a 1:1 male to female adult ratio mating and contributing to the nesting beaches, in other words if there are 30,000 females nesting per year in Florida then there are 60,000 active adults (Federal Register, 2011). If the ratio of number of fathers to nests (ie. number of females) is correct and not just a remnant of this experiment's small sample size, Northern nesting beaches could be essential to the continuation of a healthy nesting population of the Northwestern Atlantic distinct population segment.

Table 1

Percentage of nests showing multiple paternity within sea turtles from previous studies using microsatellite markers.

Species	**# Clutches Analyzed**	**% Multiple Paternity**	**Citation**
Caretta caretta	70	31% (22/70)	Moore & Ball, 2002
C. caretta	20	95% (19/20)	Zbinden et al., 2007
Chelonia mydas	22	9% (2/22)	Fitzsimmons, 1998
C. mydas	18	61% (11/18)	Lee & Hays, 2004
Dermochelys coriacea	20	10% (2/20)	Crim et al., 2002
Lepidochelys olivacea	13	30% (4/13)	Jensen et al., 2006
L. olivacea	13	92% (12/13)	Jensen et al., 2006
Lepidochelys kempi	26	58% (15/26)	Kichler et al., 1999

Table 2

Descriptive statistics of the five polymorphic microsatellite markers used Number of alleles (A), expected heterozygosity (H_E) and observed heterozygosity (H_O).

Locus	**Size Range (bp)**	**Dye**	**A**	**H_E**	**H_O**	**Expected Exclusion Probability**
CcP7E05	164 – 236	6FAM	18	0.920	0.978	0.695
CcP2F11	252 – 308	6FAM	16	0.892	0.956	0.626
CcP7D04	320 – 376	6FAM	14	0.907	0.913	0.669
CcP7C06	256 – 296	HEX	12	0.864	0.858	0.541
CcP8D06	256 – 376	TAMRA	27	0.941	0.956	0.792

Table 3

Descriptive table of multiple paternity by year, using GERUD, COLONY and the average between the two programs. The percentage is determined by dividing the number of nests with multiple fathers by the total number of nests and multiplying by 100. The second number is the average number of fathers per nest and SE.

Year	GERUD	COLONY	Average
2008	19/23 = 82.6% 3.00 (±0.29)	19/23 = 82.6% 3.00 (±0.35)	19/23 = 82.6% 3.00 (±0.23)
2009	15/21 = 71.4% 2.62 (±0.38)	18/21 = 85.7% 2.80 (±0.35)	16.5/21 = 78.6% 2.71 (±0.25)
2010	18/28 = 64.3% 2.21 (±0.23)	19/28 = 67.9% 2.32 (±0.25)	18.5/28 = 66.1% 2.67 (±0.17)
Average	52/72 = 72.2% 2.58 (±0.17)	56/72 = 77.8% 2.72 (±0.18)	54/72 =75% 2.65 (±0.13)

Table 4a

Generalized Linear Model for GERUD, distribution gamma. All variables have 1 degree of freedom, N = 72, Chi-square values are first, followed by the p-value.

Parameter	**Initial Model**	**Step 1**	**Step 2**	**Run Alone**
Year (2008-2010)	2.29, 0.1303	2.34, 0.1259	2.18, 0.1394	**4.05, 0.0441**
Straight Carapace Length (cm)	1.61, 0.2050	2.17, 0.1408	3.34, 0.0676	**5.02, 0.0251**
Season (Early – Middle)	0.05, 0.8275	0.21, 0.6444		
Season (Middle – Late)	0.11, 0.7410			
Julian Nest Date	0.09, 0.7656			
Hatching Success				1.93, 0.1645

Table 4b

Generalized Linear Model for COLONY, distribution gamma. All variables have 1 degree of freedom, N = 72, Chi-square values are first, followed by the p-value.

Parameter	**Initial Model**	**Step 1**	**Step 2**	**Run Alone**
Year (2008-2010)	1.87, 0.1712	1.91, 0.1675	1.80, 0.1803	**3.96, 0.0465**
Straight Carapace Length (cm)	1.58, 0.2091	1.54, 0.2152	2.91, 0.0880	**4.63, 0.315**
Season (Early – Middle)	0.07, 0.7880	0.01, .9257		
Season (Middle – Late)	0.19, 0.6610	0.31, 0.5801		
Julian Nest Date	0.07, 0.7981			
Hatching Success				0.97, 0.3258

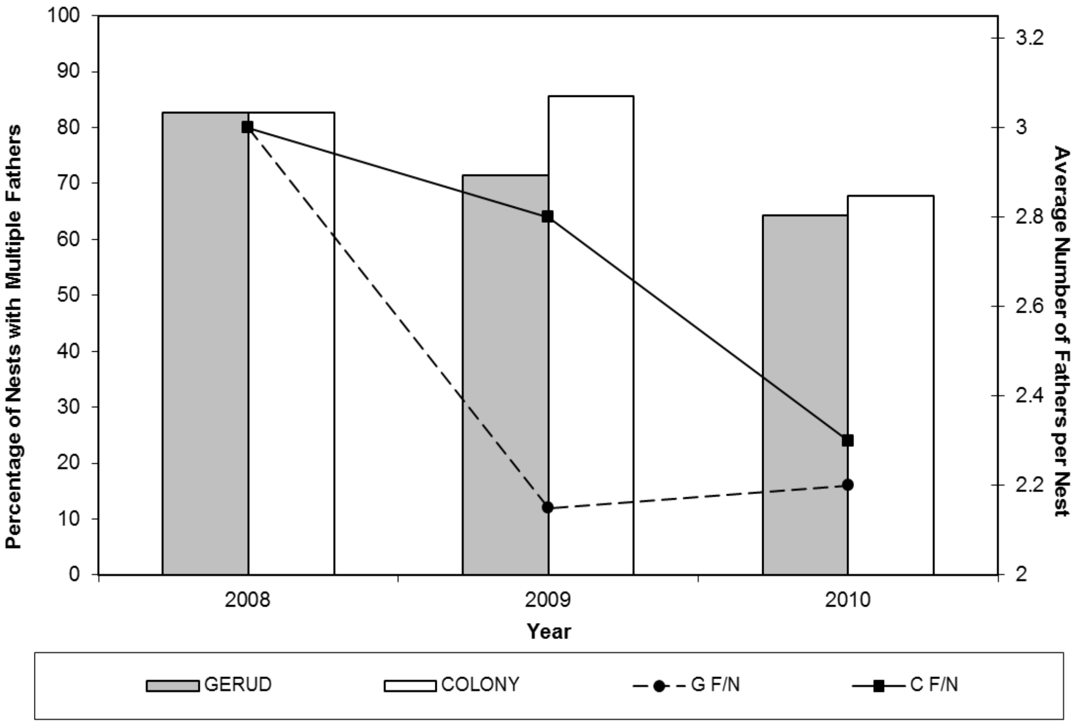


Figure 1: Graph showing the relationship between the two programs used and the three years analyzed. (N_{2008} = 23, N_{2009} = 21, N_{2010} = 28). The gray bars are GERUD by year, the white bars are COLONY by year, G F/N represents the number of fathers per nest according to GERUD and C F/N represents the number of fathers per nest according to COLONY

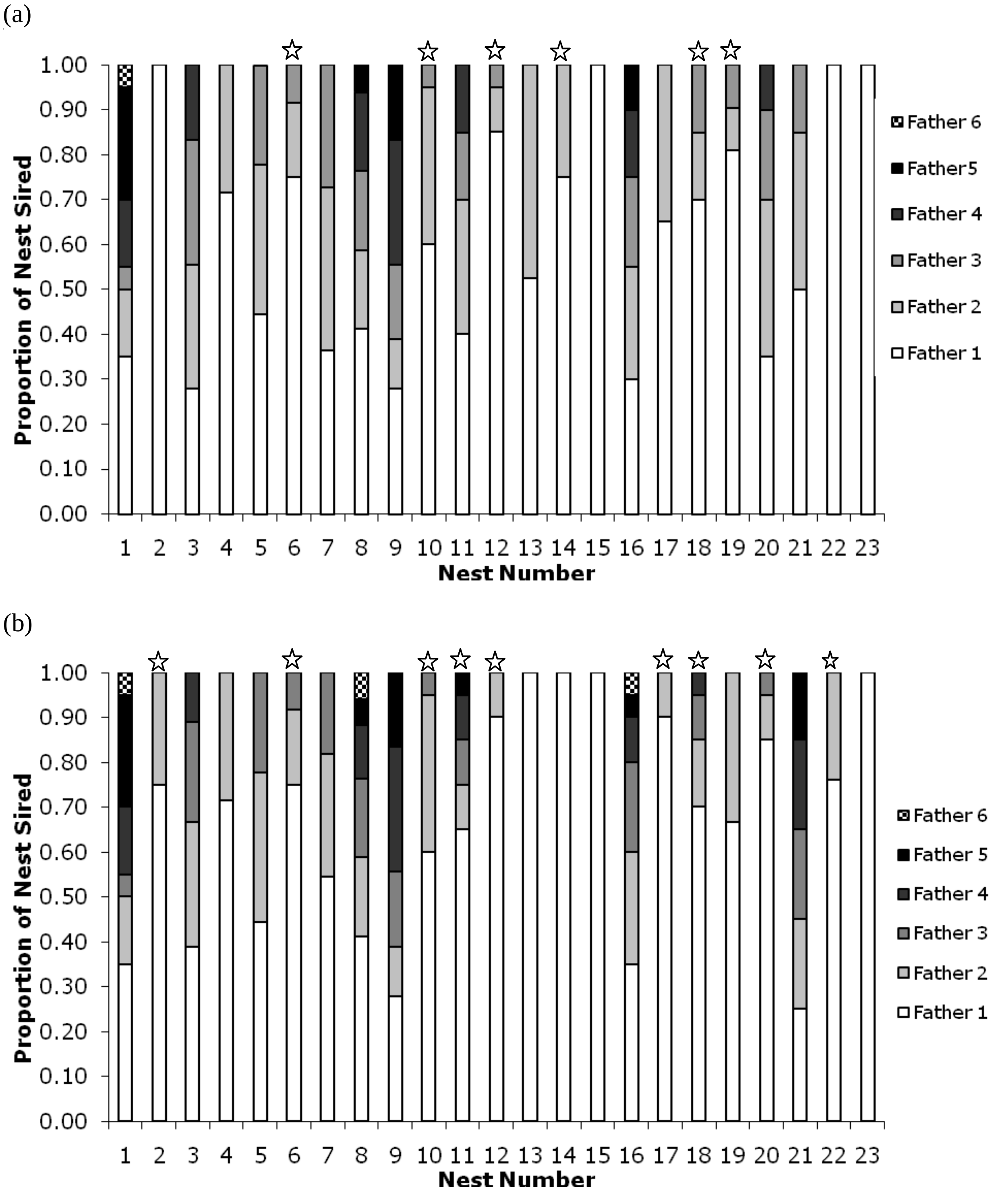


Figure 2: Relative contribution of fathers to clutches sired by multiple fathers from 2008. (a): Data estimated using GERUD. (b): Data estimated using COLONY. Stars indicate nests that paternal contributions did deviate significantly from equality (goodness of fit χ^2-tests, *P*>0.05) in a nest.

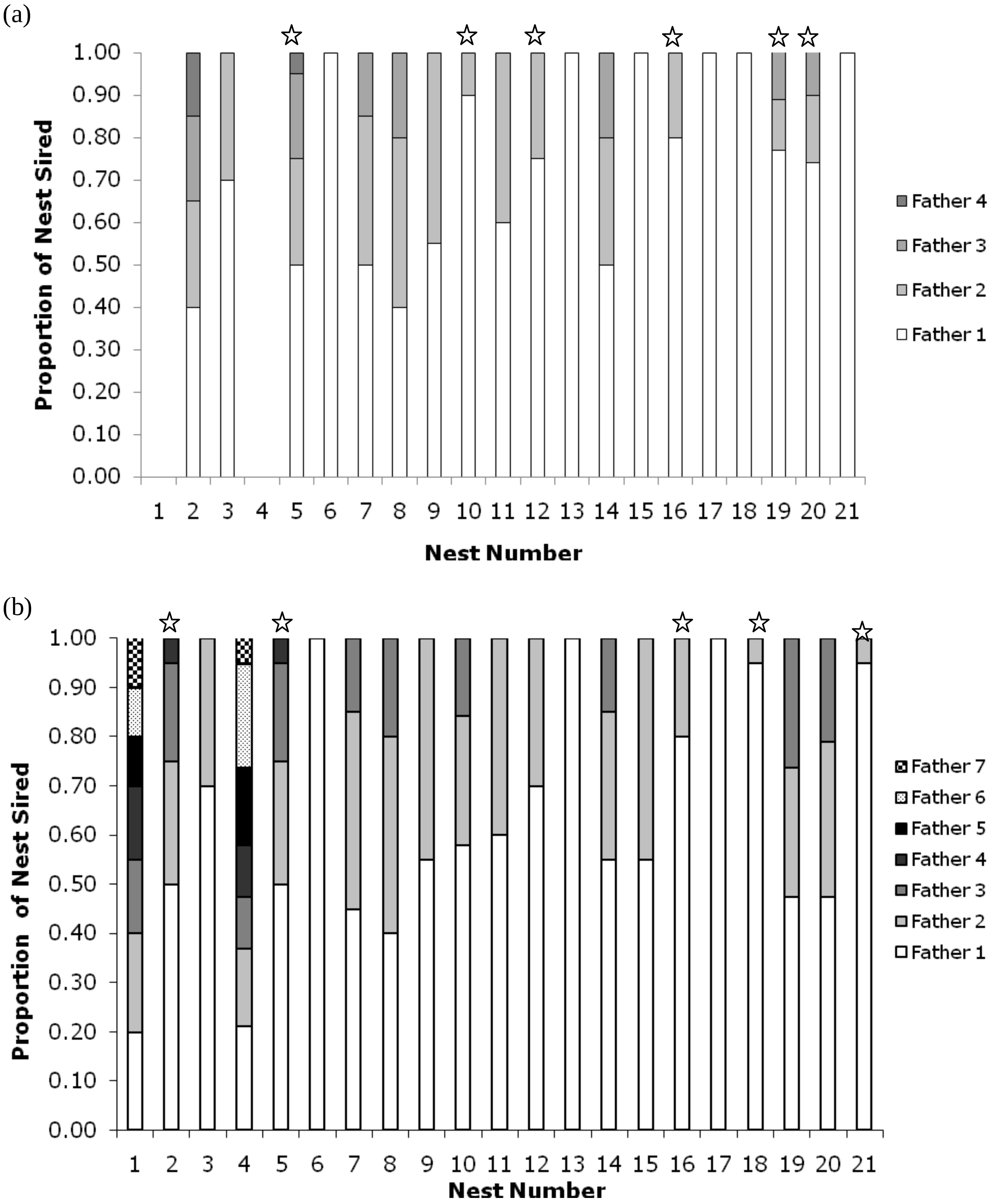


Figure 3: Relative contribution of fathers to clutches sired by multiple fathers from 2009. (a): Data estimated using GERUD. (b): Data estimated using COLONY. Stars indicate nests that paternal contributions did deviate significantly from equality (goodness of fit χ^2-tests, *P*>0.05) in a nest.

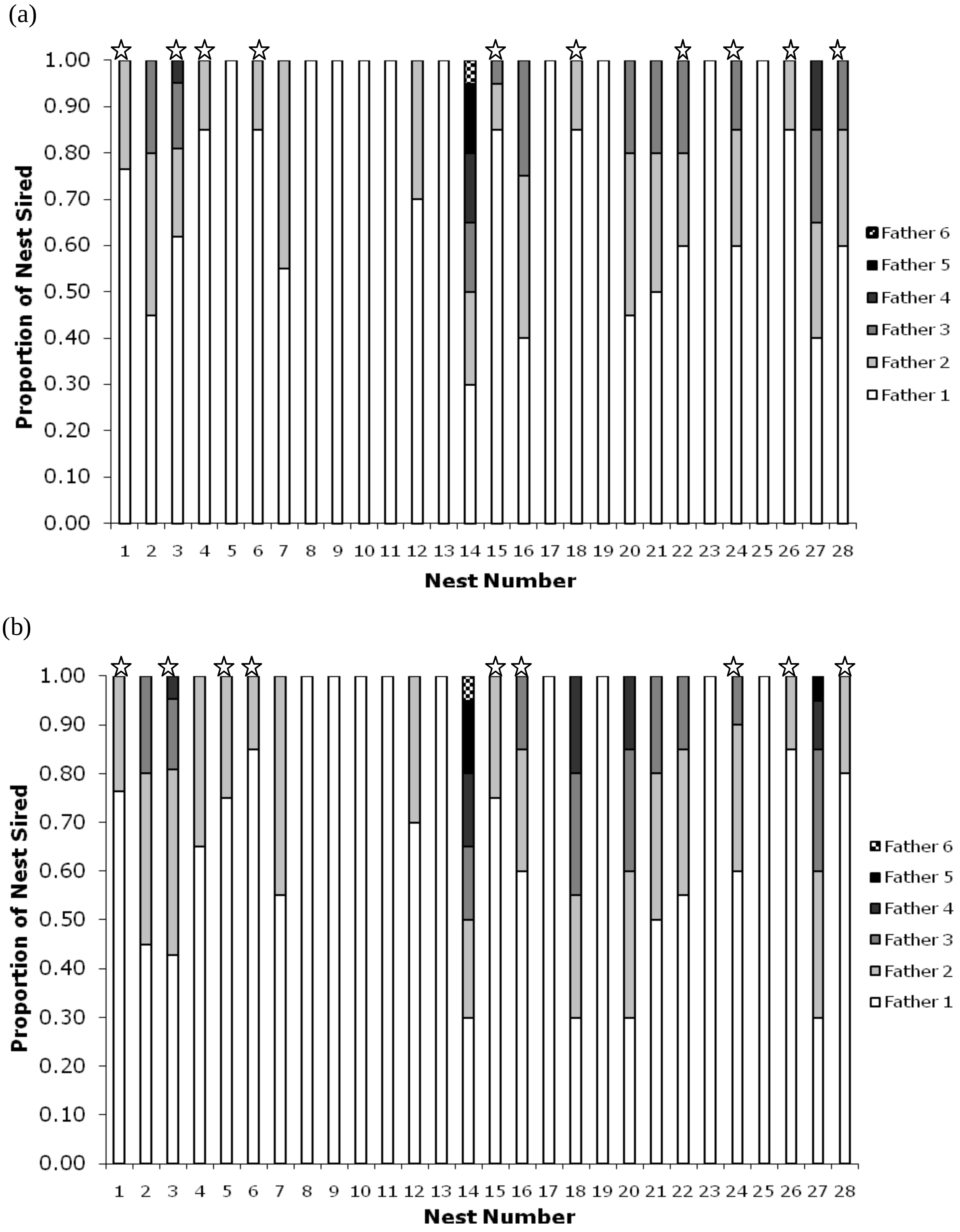


Figure 4: Relative contribution of fathers to clutches sired by multiple fathers from 2010. (a): Data estimated using GERUD. (b): Data estimated using COLONY. Stars indicate nests that paternal contributions did not deviate significantly from equality (goodness of fit χ^2-tests, $P>0.05$) in a nest.

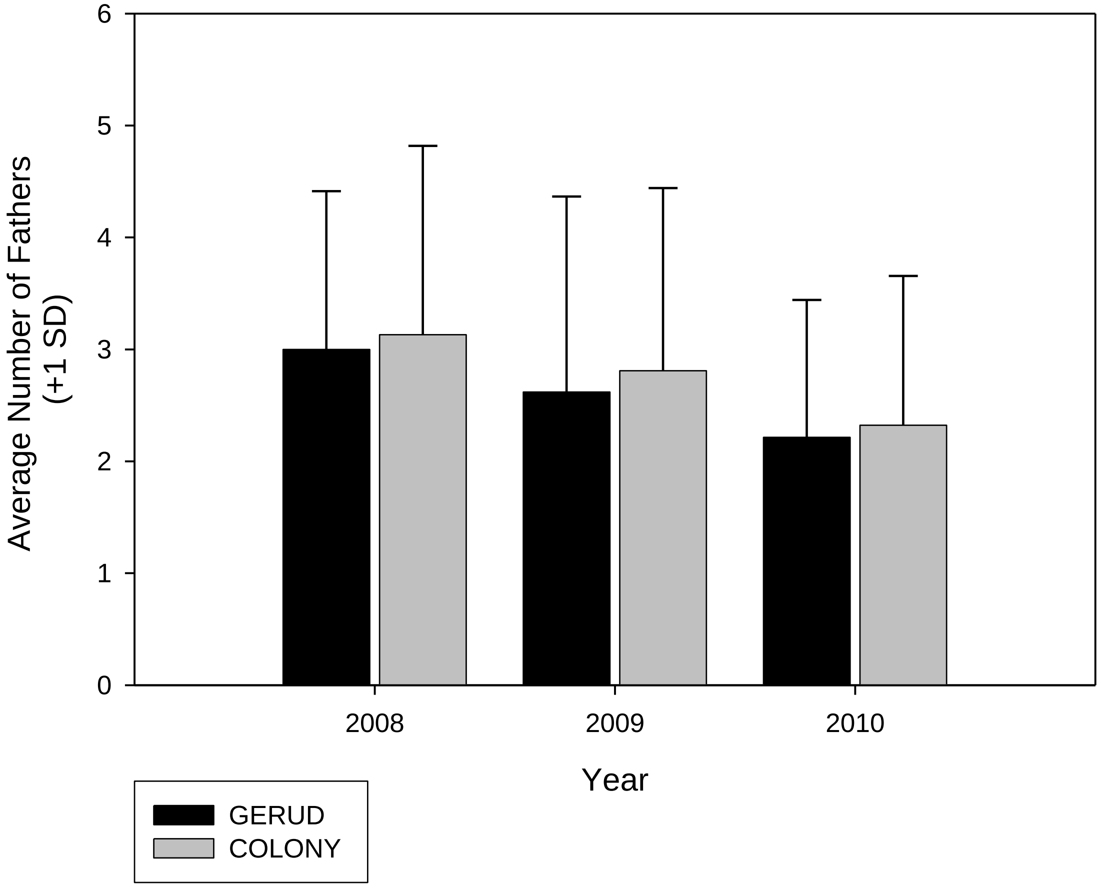


Figure 5: This graph shows the difference between the average number of fathers by year. There is a visually negative trend as the years increase and using the generalized linear model the trend is significant (G: χ^2 =4.05, p = 0.0441; C: $|^2$ =3.96, p = 0.0465).

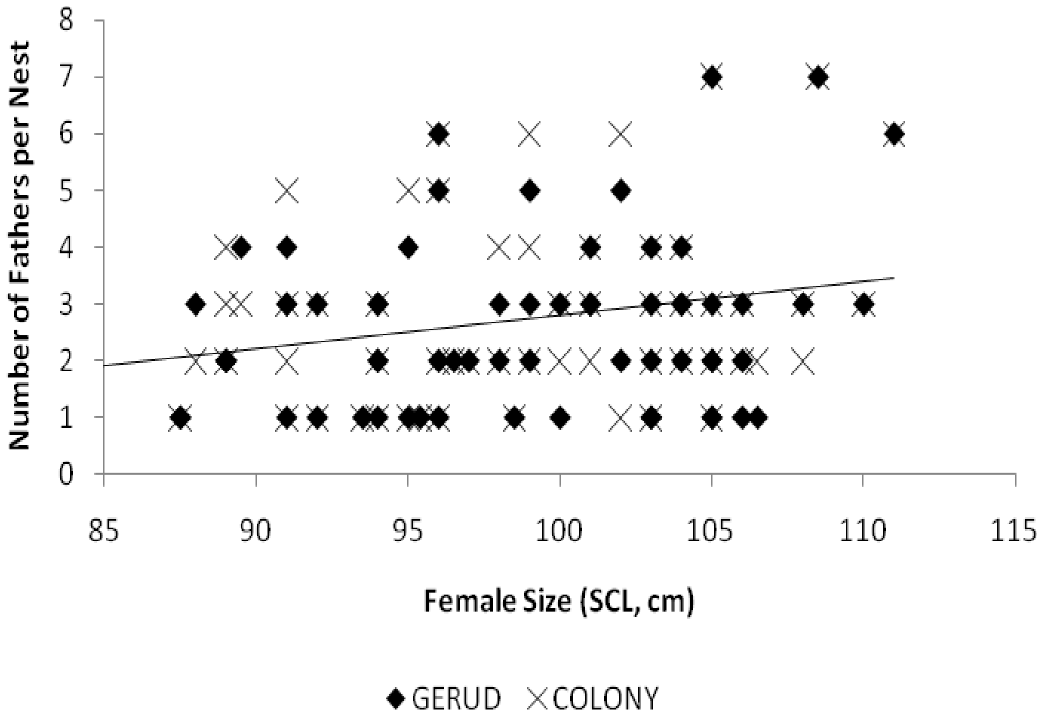


Figure 6: The relationship between female size (Straight Carapace Length, cm) and the number of fathers per nest. There is a significant difference in number of fathers due to female size: as female size increases, so does the number of fathers (G: $|^2$ = 5.02, p = 0.0251; C: $|^2$ = 4.63, p = 0.0315).

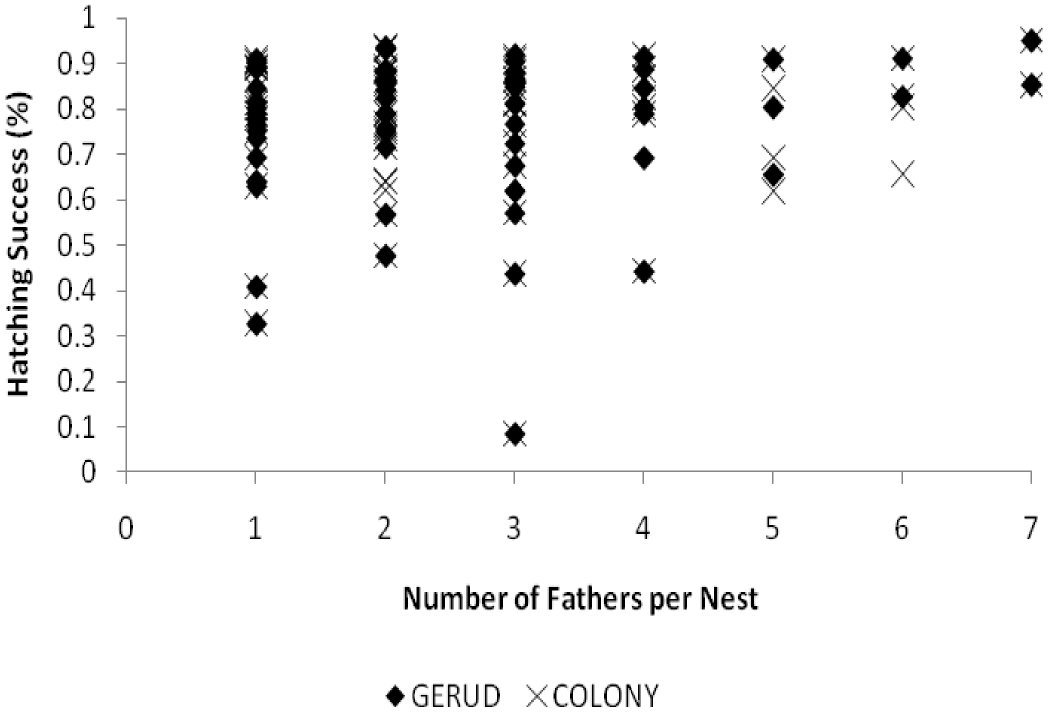


Figure 7: The relationship between the number of fathers per nest and the hatching success (%) of each nest, using both programs. The generalized linear model determined there was no significant relationship.

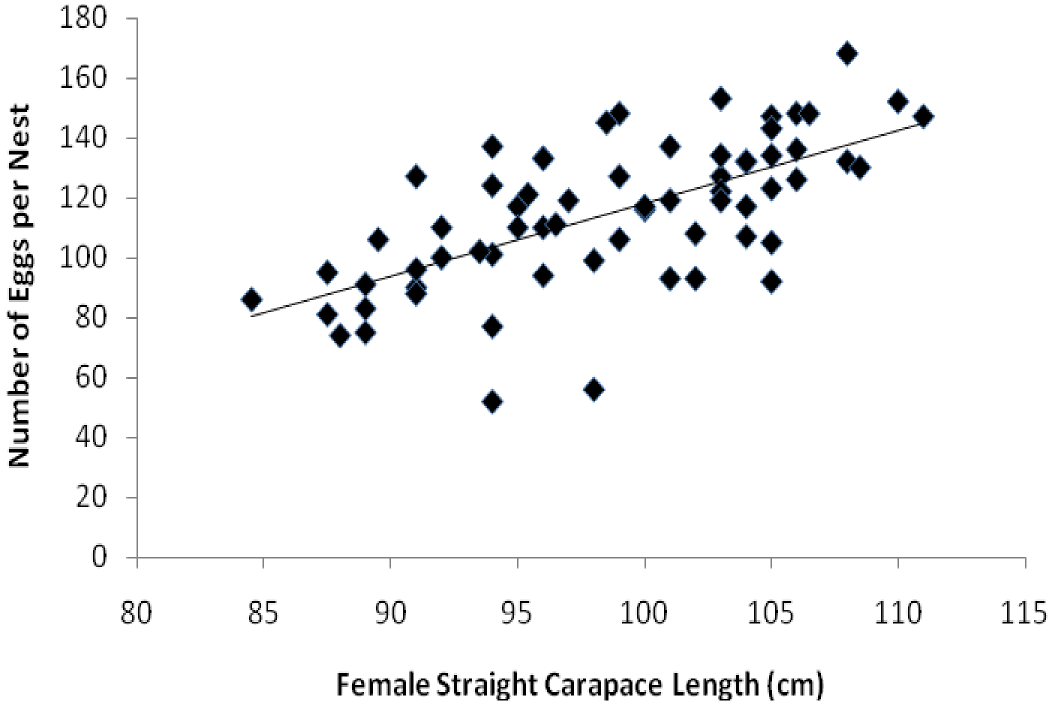
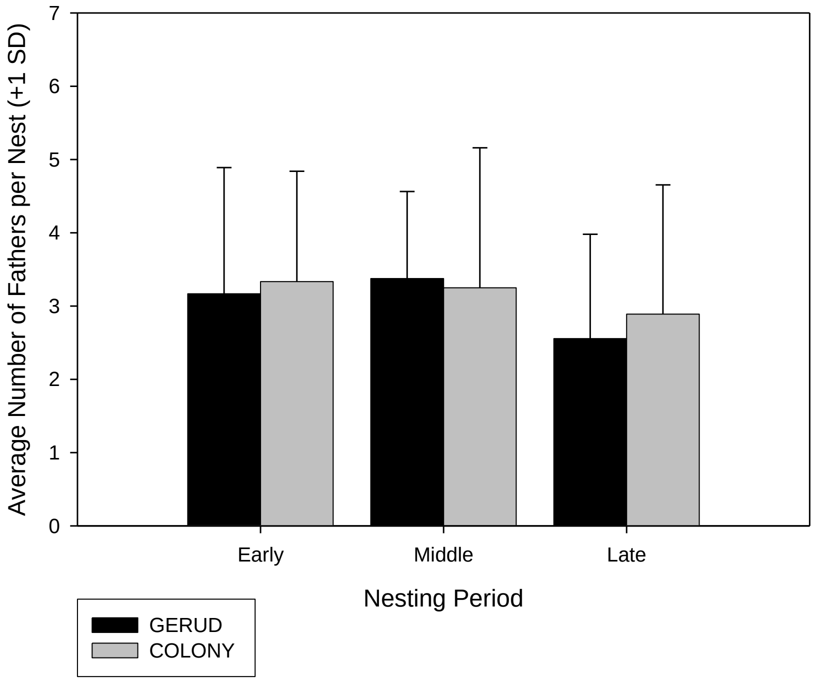
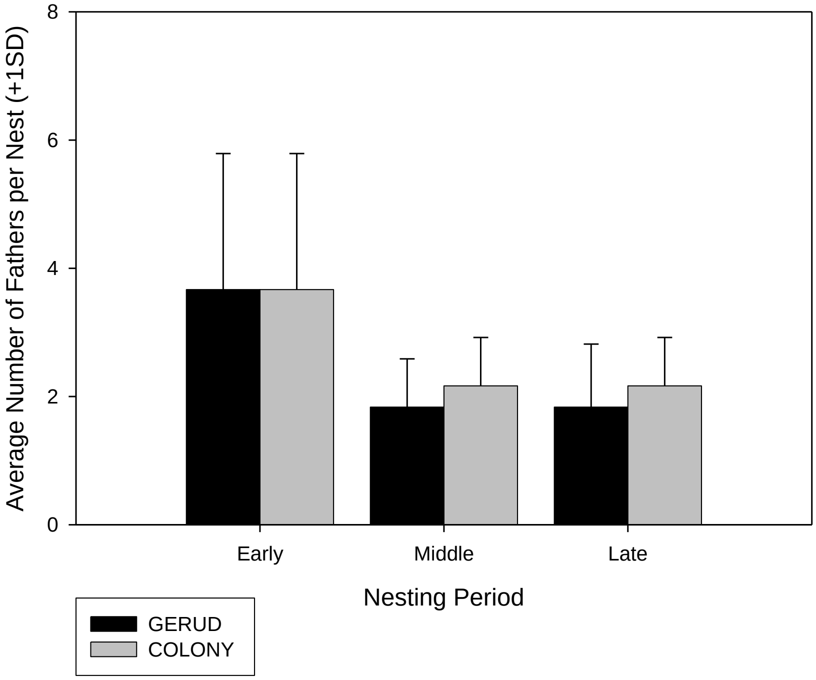


Figure 8: Relationship between Female Straight Carapace Length (cm) and the number of eggs per nest (ie. nest size). There is a positive correlation between the two, as females get larger, the number eggs per nest increases (r^2 = 0.419, $F_{(1,64)(0.05)}$= 46.26, p<0.001)

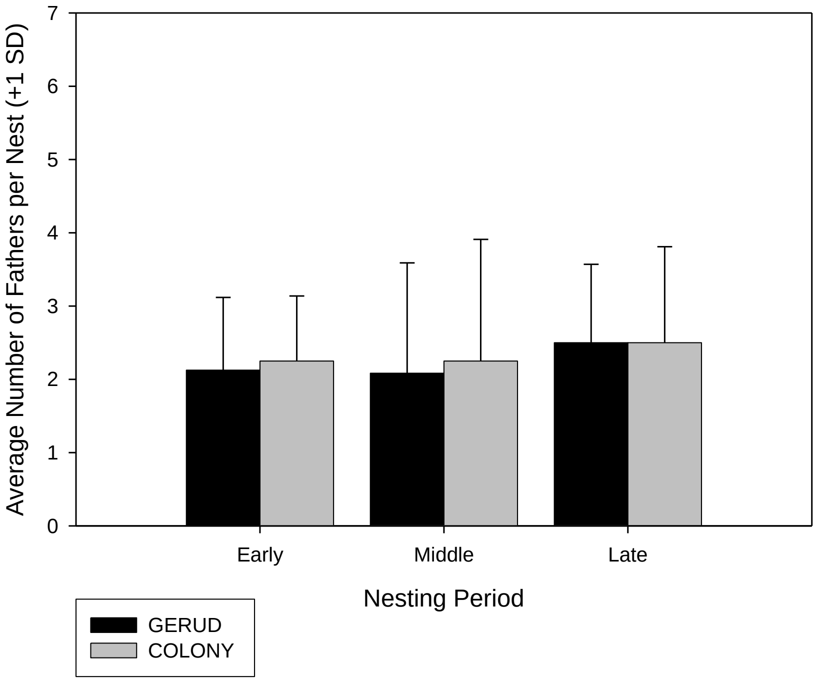
(a)


Average Number of Fathers per Nest (+1 SD)
0
1
2
3
4
5
6
7
Early
Middle
Late
Nesting Period
GERUD
COLONY

(b)


Average Number of Fathers per Nest (+1SD)
0
2
4
6
8
Early
Middle
Late
Nesting Period
GERUD
COLONY

(c)



(d)

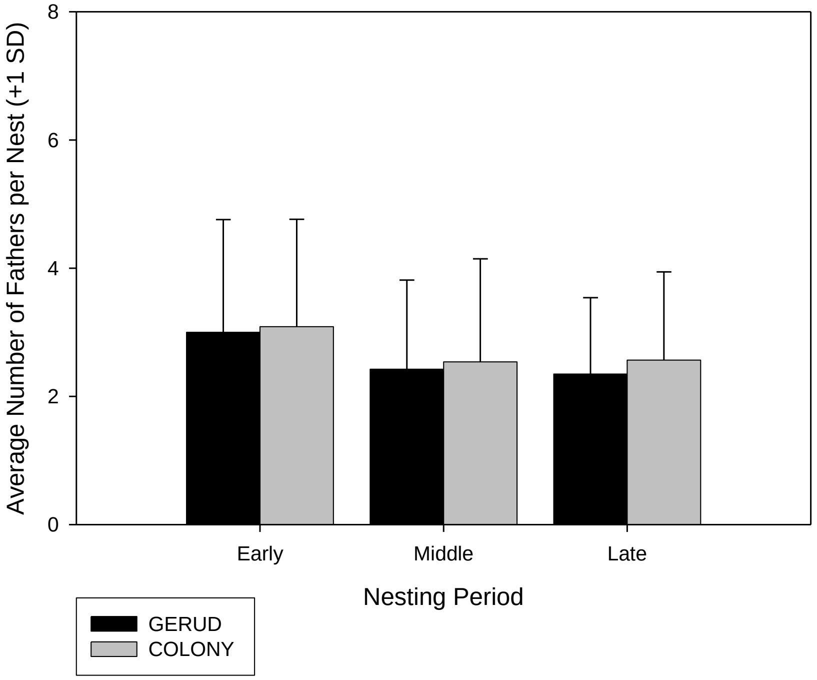


Figure 9: Nesting period (early, middle and late) versus the average number of fathers, (a): 2008, (b): 2009, (c): 2010 and (d): all years combined. The bars represent +1 Standard Deviation. There is no significant difference in the number of fathers due to nesting period across all years.

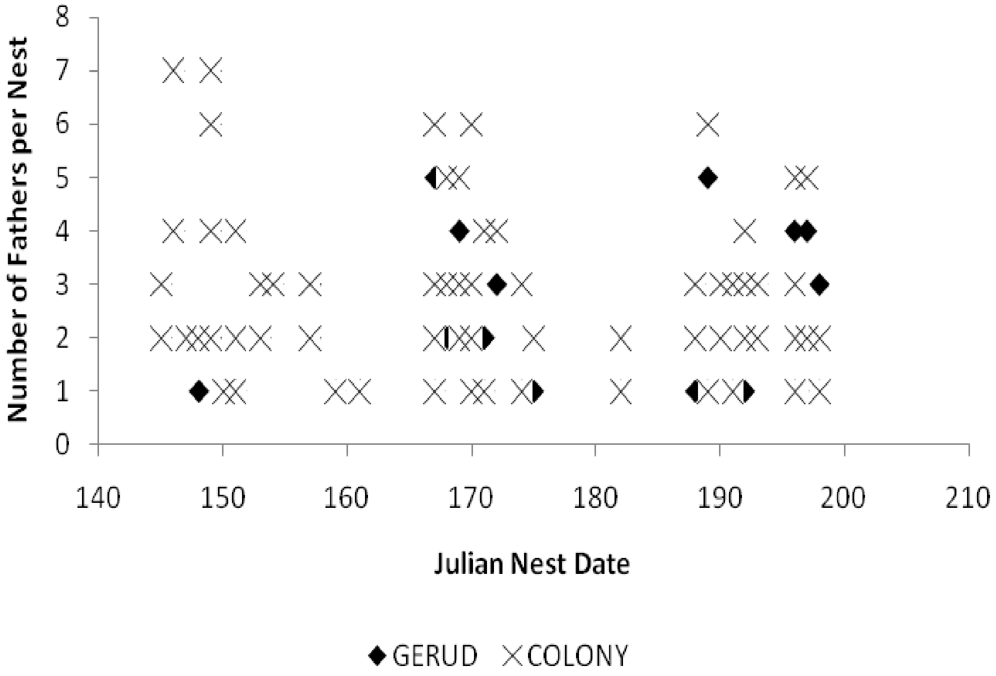


Figure 10: Julian Nest Date compared to number of fathers per nest. There is a visual negative trend: as the nesting season progresses, the number of fathers decreases (not significant).

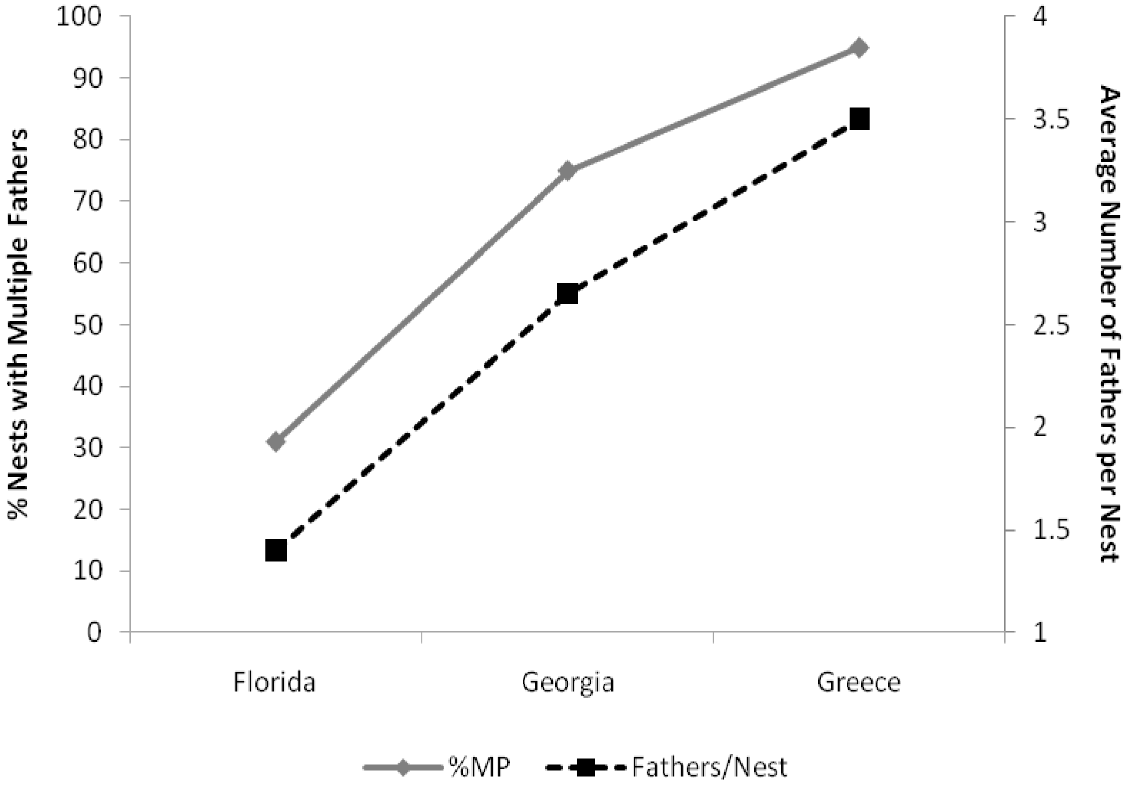


Figure 11: Relationship between location, percentage of nests sired by multiple males and the average number of fathers per nest by location. Adapted using data from Moore and Ball, 2002 and Zbinden et al., 2007.

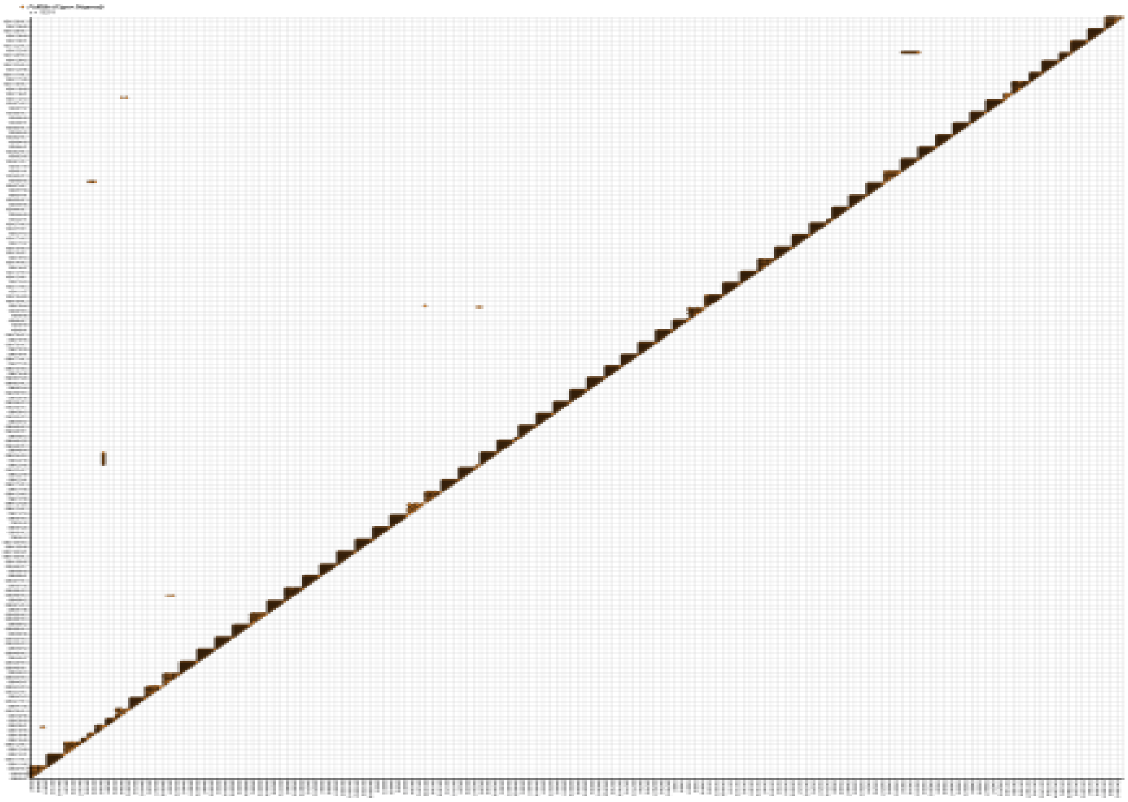


Figure 12: Relatedness of siblings. Estimate of fathers contributing to each nest over all three years. Triangles above the transect represent individual nests (fully related siblings). Triangles below the transect represent individuals who are related to each other (half-siblings).

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APPENDIX A: FULL NEST DATA

Season	Date Laid	JND	Nest # (CRP)	Year	Mother SCL	Mother SCW	N	Nest Total	% Nest Sampled	Hatching Success	HS%	Gerud	Colony
Early	28-May	149	9	2008	111	99.5	20	147	13.60544218	91.16	0.9116	6	6
Early	30-May	151	11	2008	106	97	20	136	14.70588235	63.97	0.6397	1	2
Early	30-May	151	12	2008			20	149	13.42281879	44.3	0.443	4	4
Early	1-Jun	153	15	2008	105	97	7	147	4.761904762	93.2	0.932	2	2
Early	1-Jun	153	16	2008	104	99	9	132	6.818181818	84.85	0.8485	3	3
Early	2-Jun	154	18	2008	103	95.5	12	127	9.448818898	81.1	0.811	3	3
Middle	15-Jun	167	38	2008	94	83	11	101	10.89108911	88.12	0.8812	3	3
Middle	15-Jun	167	39	2008	99	92	17	148	11.48648649	80.41	0.8041	5	6
Middle	16-Jun	168	41	2008	96	95	20	133	15.03759399	90.98	0.9098	5	5
Middle	17-Jun	169	43	2008	106	101	20	126	15.87301587	86.51	0.8651	3	3
Middle	17-Jun	169	45	2008	95	91	20	117	17.09401709	84.62	0.8462	4	5
Middle	18-Jun	170	46	2008	91	85	20	90	22.22222222	85.56	0.8556	3	2
Middle	19-Jun	171	49	2008	102	94	21	108	19.44444444	84.26	0.8426	2	1
Middle	19-Jun	171	50	2008			20	110	18.18181818	86.36	0.8636	2	1
Late	7-Jul	189	86	2008	96	82	20	94	21.27659575	90.43	0.9043	1	1
Late	7-Jul	189	88	2008	102	92.5	20	93	21.50537634	65.59	0.6559	5	6
Late	8-Jul	190	91	2008	97	90	20	119	16.80672269	78.99	0.7899	2	2
Late	10-Jul	192	94	2008	99	95	20	106	18.86792453	86.79	0.8679	3	4

Late	11-Jul	193	96	2008	108	97.5	21	132	15.90909091	85.61	0.8561	3	2
Late	14-Jul	196	97	2008	89.5	79.5	20	106	18.86792453	91.51	0.9151	4	3
Late	14-Jul	196	99	2008			20	113	17.69911504	61.95	0.6195	3	5
Late	14-Jul	196	100	2008	105	95.5	21	105	20	84.62	0.8462	1	2
Late	16-Jul	198	105	2008	105	99	20	92	21.73913044	63.04	0.6304	1	1
Early	26-May	146	5	2009	108.5	93	20	130	15.38461539	85.38	0.8538	7	7
Early	26-May	146	6	2009	103	97	20	153	13.07189543	80.39	0.8039	4	4
Early	27-May	147	8	2009	106	94	20	148	13.51351351	88.51	0.8851	2	2
Early	29-May	149	12	2009	105	99	19	143	13.28671329	95.1	0.951	7	7
Early	29-May	149	13	2009	104	97	20	107	18.69158879	88.79	0.8879	4	4
Early	31-May	151	17	2009	98.5	94.5	20	145	13.79310345	88.97	0.8897	1	1
Early	6-Jun	157	22	2009	108	102	20	168	11.90476191	57.14	0.5714	3	3
Early	6-Jun	157	23	2009	100	90.5	5	116	4.310344828	76.72	0.7672	3	3
Early	6-Jun	157	24	2009	96	90	20	110	18.18181818	93.64	0.9364	2	2
Middle	17-Jun	168	46	2009	89	84	19	75	25.33333333	88.67	0.8867	2	3
Middle	18-Jun	169	47	2009	98	94	5	56	8.928571429	75	0.75	2	2
Middle	18-Jun	169	48	2009	104	93.5	20	132	15.15151515	56.82	0.5682	2	2
Middle	23-Jun	174	55	2009	94	86.5	20	137	14.59854015	69.34	0.6934	1	1
Middle	23-Jun	174	56	2009	101	93	20	137	14.59854015	90.51	0.9051	3	3

Middle	24-Jun	175	58	2009	106.5	99.5	20	148	13.51351351	89.19	0.8919	1	2
Late	1-Jul	182	63	2009	94	81	20	52	38.46153846	86.54	0.8654	2	2
Late	1-Jul	182	64	2009	103	97.5	4	122	3.278688525	90.16	0.9016	1	1
Late	7-Jul	188	74	2009			20	110	18.18181818	73.64	0.7364	1	2
Late	9-Jul	190	77	2009	91	86	19	96	19.79166667	43.75	0.4375	3	3
Late	11-Jul	192	78	2009			19	87	21.83908046	72.41	0.7241	3	3
Late	11-Jul	192	79	2009			20	111	18.01801802	76.58	0.7658	1	2
Early	25-May	145	6	2010	99	95	17	127	13.38582677	88.19	0.8819	2	2
Early	25-May	145	8	2010	110	100	20	152	13.15789474	88.16	0.8816	3	3
Early	26-May	146	10	2010	101	98	21	119	17.64705882	78.99	0.7899	4	4
Early	27-May	147	11	2010	103	94	20	134	14.92537313	47.76	0.4776	2	2
Early	28-May	148	13	2010	100	92	20	117	17.09401709	64.1	0.641	1	2
Early	29-May	149	14	2010	94	89	20	124	16.12903226	79.03	0.7903	2	2
Early	29-May	149	15	2010	105	94.5	20	134	14.92537313	71.64	0.7164	2	2
Early	30-May	150	17	2010	87.5	79	20	81	24.69135803	80.25	0.8025	1	1
Middle	8-Jun	159	37	2010	91	87	20	88	22.72727273	90.91	0.9091	1	1
Middle	10-Jun	161	42	2010	84.5	77	6	86	6.976744186	81.4	0.814	1	1
Middle	10-Jun	161	44	2010	92	86	20	100	20	79	0.79	1	1
Middle	16-Jun	167	55	2010	96.5	93.5	20	111	18.01801802	75.68	0.7568	2	2
Middle	16-Jun	167	57	2010	93.5	85	20	102	19.60784314	89.22	0.8922	1	1
Middle	19-Jun	170	60	2010	96	92	20	133	15.03759399	82.71	0.8271	6	6

Middle	19-Jun	170	61	2010	88	77.5	20	74	27.02702703	86.49	0.8649	3	2
Middle	19-Jun	170	62	2010	92	81	20	110	18.18181818	86.36	0.8636	3	3
Middle	19-Jun	170	64	2010	87.5	79	20	95	21.05263158	77.89	0.7789	1	1
Middle	20-Jun	171	65	2010	89	84	20	91	21.97802198	82.42	0.8242	2	4
Middle	20-Jun	171	66	2010	95.4	90.4	18	121	14.87603306	75.21	0.7521	1	1
Middle	21-Jun	172	67	2010	98	88	20	99	20.2020202	91.92	0.9192	3	4
Late	7-Jul	188	114	2010	104	92	10	117	8.547008547	80	0.8	3	3
Late	10-Jul	191	116	2010	105	97	20	123	16.2601626	81.3	0.813	3	3
Late	10-Jul	191	117	2010	95	89	15	110	13.63636364	40.91	0.4091	1	1
Late	12-Jul	193	123	2010	94	81	20	77	25.97402597	67.53	0.6753	3	3
Late	15-Jul	196	129	2010	103	94	13	119	10.92436975	32.77	0.3277	1	1
Late	16-Jul	197	132	2010	89	82	20	83	24.09638554	85.54	0.8554	2	2
Late	16-Jul	197	136	2010	91	79	20	127	15.7480315	69.29	0.6929	4	5
Late	17-Jul	198	138	2010	101	91	20	93	21.50537634	62.07	0.6207	3	2