RESEARCH ARTICLE

Breeding sex ratio and population size of loggerhead turtles from Southwestern Florida

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Abstract

Species that display temperature-dependent sex determination are at risk as a result of increasing global temperatures. For marine turtles, high incubation temperatures can skew sex ratios towards females. There are concerns that temperature increases may result in highly female-biased offspring sex ratios, which would drive a future sex ratio skew. Studying the sex ratios of adults in the ocean is logistically very difficult because individuals are widely distributed and males are inaccessible because they remain in the ocean. Breeding sex ratios (BSR) are sought as a functional alternative to study adult sex ratios. One way to examine BSR is to determine the number of males that contribute to nests. Our goal was to evaluate the BSR for loggerhead turtles (*Caretta caretta*) nesting along the eastern Gulf of Mexico in Florida, from 2013–2015, encompassing three nesting seasons. We genotyped 64 nesting females (approximately 28% of all turtles nesting at that time) and up to 20 hatchlings from their nests (n = 989) using 7 polymorphic microsatellite markers. We identified multiple paternal contributions in 70% of the nests analyzed and 126 individual males. The breeding sex ratio was approximately 1 female for every 2.5 males. We did not find repeat males in any of our nests. The sex ratio and lack of repeating males was surprising because of female-biased primary sex ratios. We hypothesize that females mate offshore of their nesting beaches as well as en route. We recommend further comparisons of subsequent nesting events and of other beaches as it is imperative to establish baseline breeding sex ratios to understand how growing populations behave before extreme environmental effects are evident.

Introduction

Increasing global temperatures threaten marine turtle populations [1–4]. Most authors' concerns grow from consideration of temperature-dependent sex determination (TSD), the mechanism by which incubation temperature of the nest directly impacts the sex of the embryo [5,6]. In marine turtles, warmer incubation temperatures tend to produce females, whereas cooler temperatures tend to produce males [7, 8]. Authors are concerned that higher temperatures will cause such a female bias in sex ratios that populations will face extinction [9,10]. Currently however, the magnitude of the sex ratio skew in adults is unknown due to our limited

understanding of the proportion of adult males (and males approaching sexual maturity) [11]. Marine turtle individuals are often widely distributed geographically, outside of the nesting season. Dispersed members of populations make detecting sex ratio issues across populations challenging. In addition, adult males are very difficult to access because they rarely come to land. While a determination of adult sex ratio is beyond reach, a functional alternative, breeding sex ratios (BSR: the proportion of males and females that successfully mate at any time) [12] can be used to identify the minimum number of males and females contributing to populations. By estimating BSR at small, growing nesting aggregations a more thorough proportion of the nesting beach can be assessed than at large nesting beaches, and inferences can be made about the impact of climate change on the population as a whole [13, 14].

The loggerhead sea turtle (*Caretta caretta*) is listed globally as vulnerable by the International Union for the Conservation of Nature (IUCN) [15]. However, along the continental US and adjacent waters in the Northwest Atlantic Ocean, it is listed as threatened [16]. The Northwest Atlantic contains one of only two marine turtle nesting aggregations of greater than 10,000 individuals nesting annually [15, 17]. Florida nesting loggerheads make up approximately 90% of that aggregation [18–20]. Florida Fish and Wildlife Conservation Commission (FWC) estimated that 184,064 loggerhead nests were laid in the 2016 nesting season and the overall nesting trend is an increasing one across the state [21].

Due to their accessibility, nesting females, nest success, and hatchlings are frequently examined and used for demographic studies and population models [22–24]. Data on nesting females and hatchlings are supplemented with in-water capture/recapture and satellite tag studies, which provide additional information on the number of turtles [25,26]. The Turtle Expert Working Group estimated that the female loggerheads return to nest every 2.5 years on average [19]; however, using mark-recapture data over a 20-year data set, Phillips et al. estimated it at an average of 3.2 years for turtles nesting in Southwestern Florida [27]. From tagging and resighting data, it has been estimated that loggerheads lay on average from 3–4.1 nests per season [22, 28, 29] while satellite tagging suggests that within the Gulf of Mexico, the average is closer to 5.4 nests per season [30]. Nest frequency is an important metric because it can be used to calculate how many females nest each year. Unfortunately, information regarding adult male behavior and number is lacking. Many in-water capture studies do not identify the sex of the turtles [31]. Studies that do identify the sex of captured individuals tend to examine juvenile sex ratios [32–35]; or are focused on migration or distribution [36,37]. Consequently, male sea turtles' reproductive behavior is poorly understood and sex ratio cannot be estimated directly.

A variety of methods have been used to infer aspects of male reproductive behavior. In all seven-extant species of marine turtles, it has been shown that sperm from more than one male can fertilize a single clutch (multiple paternity) [12, 38–43]. Furthermore, in at least one species, a single male may mate with more than one female [44]. Little is known about mate choice, and while direct observations of multiple matings occur [45, 46], assigning which male (s) successfully father young from observed copulations may not be accurate. Hormonal studies suggest that loggerhead males could mate annually [47] and satellite tracking of adult males suggests that about 40% remain close to nesting beaches during a breeding season and may therefore mate more than once [11, 37]. Together, these findings suggest that males contribute to multiple nests during a nesting season and might breed more frequently than females. The number of males fathering each clutch can be determined genetically and used to estimate the minimum BSR [12, 48]. Whether the BSR or reproductive behavior vary among populations is unknown.

The primary goal of this study was to estimate the breeding sex ratio for the loggerhead turtle nesting on a small nesting beach on the southwestern coast of Florida. To this end, paternal

genotypes were identified through exclusion analysis and were used to estimate the number of males contributing to this population.

Materials and methods

All marine turtle sampling techniques were reviewed and approved by the Florida Fish and Wildlife Conservation Commission (Marine Turtle Permits 13-073A, 14-073D and 15–216) and by the Florida Atlantic University's Institutional Animal Care and Use Committee (IACUC A13-04). All efforts were made to minimize pain. Nesting loggerhead turtles and their offspring were sampled over three consecutive nesting seasons (late May through July: 2013–2015) on Sanibel Island, in Lee County, Florida. The areas sampled include 10.3 km of beach from J.N. Ding Darling National Wildlife Refuge (26.46719, -82.17030) to Tarpon Bay Road (26.42215, -82.08013) (Fig 1). All nests were monitored by the Sanibel Captiva Conservation Foundation (SCCF) and nesting females were found with their assistance.

Nesting females were identified, measured and tagged during night patrols. To ensure unbiased sample collection from females, nesting turtles were sampled only once. Sampling locations were disinfected with 0.02% chlorohexidine gluconate solution. Blood samples (1 mL per adult turtle) were taken from the external jugular vein [48–50] using a 21G x 1½ inch (3.8 cm) needle and drawn into 4 mL sodium heparin vacutainers (BD Vacutainer Blood Collection Needles/Vacutainers, Franklin Lakes, NJ USA). Skin biopsies, as a backup source of DNA, were taken from the soft tissue along the trailing edge of the pectoral limbs, using either a disposable 6mm biopsy punch (Integra® Miltex®, York, Pennsylvania USA) or a sterile one-use razor blade and forceps to collect approximately 6mm² of skin. Skin samples were stored in 70% ethanol until needed for analysis. In rare instances, the female was found returning to the ocean and a blood or skin sample could not be taken. In these cases, one viable egg was collected from her nest immediately. The yolk and albumin were discarded and the eggshell and its eggshell membrane (which are maternally derived) were stored in 70% ethanol (extraction based upon protocols established by [51]).

Nest locations of the sampled females were recorded via GPS and marked following SCCF's protocols. Screens were placed over research nests to deter predators. Restraining cages were placed over the nest chamber approximately 45 days after oviposition. These cages were used to further prevent predation and ensure an adequate sample of the hatchlings could be obtained upon emergence. Hatchling samples were collected from July through September. Up to twenty hatchlings per nest were collected indiscriminately upon emergence. Blood samples (up to 100 µL per turtle) were taken from the external jugular vein using heparinized syringes (Allergy Syringe with PrecisionGlide™ Needle [26G ½ inch] Becton Dickinson, Franklin Lakes, NJ, USA). Skin samples of approximately 1 mm x 3 mm were taken from the trailing edge of one of the flippers using a sterilized scalpel blade. Blood and skin were treated as for nesting females.

All blood samples were stored at -80˚C until preparation for DNA extraction. DNA extraction method depended on type of tissue. Blood: 5 µL of whole blood was added to 50 µL of lysis buffer (10mM TRIS, pH8.3, 40mM KCl, 0.5% Tween20 and 200 µg/mL Proteinase K) and incubated at 65˚C for 1 h, followed by 100˚C for 15 min. Skin/eggshell: a DNEasy blood and tissue kit (Qiagen, Valencia, CA USA) was used following manufacturer's protocol (for eggshell, incubation was longer than manufacturer's instructions per [51]). Samples were genotyped with 7 microsatellite loci following published protocols: CcP7E05, CcP2F11, CcP7D04, CcP5H07, CcP7C06, CcP7B07 and CcP8D06 [52]. PCR products were multiplexed together and analyzed with GeneScan500 fluorescent size standard (Applied Biosystems, Foster City, CA USA) using an ABI 3730 DNA Analyzer. Positive and negative
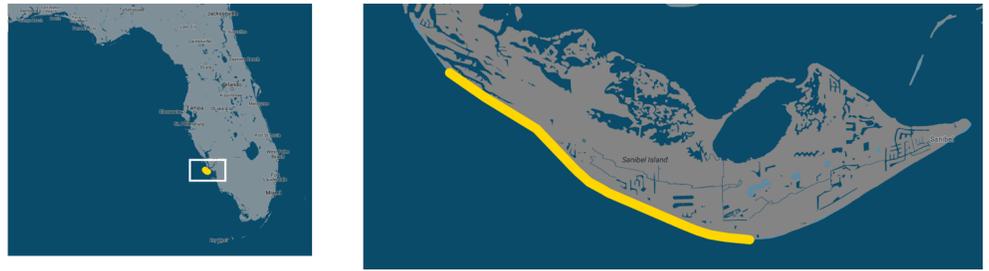
Fig 1. Sanibel Island, Florida. The yellow line notes the 10.3 km (6.4 miles) of beach that is covered in the sampling.

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controls were run with every PCR plate to identify if there was contamination. Geneious R10 (Biomatters Inc., Newark, NJ, USA) was used to identify alleles. A subset of samples was re-run to identify genotyping error rate. Loci were checked for allelic dropout, stutter and null alleles using MicroChecker 2.2.3 [53].

We assigned paternity through exclusion analysis. Known maternal alleles are "subtracted" from hatchling genotypes and the remaining alleles are assigned to the most likely paternal genotypes. Each female's alleles and those of her hatchlings were analyzed using COLONY 2.0 [54], a maximum likelihood-based program that determines the maximum number of fathers per clutch based on the genotypes available. Multiple paternity was identified from the presence of three or more paternal alleles identified at each of two or more loci. This approach allows for the possibility of a mutation at one locus [55].

We used GenAlEx [56] to quantify: (1) the observed and expected heterozygosity of alleles and deviations from Hardy-Weinberg Equilibrium (HWE) of the maternal genotypes for each locus, (2) the probability of identity (PI), (3) the probability of exclusion (PE) and (4) F-statistics (F_{IS} and F_{ST}). The PI provides the likelihood that two samples will have the exact same genotype given the estimated allelic frequencies at that locus and when all loci are combined in a mixed profile. The PE is the proportion of the population, which has a genotype that contains at least one allele not observed in the mixed profile. The PE value depends on the number of parental genotypes that are known, if only one parent is known (PE_1), if both parents are known (PE_2), or if no parents are known (PE_3). All nests had maternal samples, so we provide PE_1 for all primers. F_{IS} is equal to the reduction in heterozygosity due to non-random mating within its local population (inbreeding). A positive F_{IS} indicates inbreeding and a negative indicates outbreeding. F_{ST} provides a series of values to identify the genetic structure of a population. Briefly, a value of 0 suggests that the subpopulations are interbreeding freely (panmixis), whereas a value of 1 suggests that the compared subpopulations are genetically distinct.

We modify the calculation of breeding sex ratio [57] using the paternal genotypes (i.e., numbers of males identified = successful males) to estimate the number of breeding males during each year (Eq 1). The equation is used for individual years to compensate for different cohorts of mating and nesting turtles. The typical female loggerhead does not nest annually (usually every 2–3 years), thus, each year of sampling represents a portion of the overall breeding population and the equation can be used for each year separately.

$$Total \ \# \ Breeding \ Males_n = \left[\left(\frac{total \ \# \ nests_n}{Avg \ \# \ nests \ per \ female} \right) x \ (successful \ males_n) \right] \quad (1)$$

Eq 1. Equation to determine the minimum number of breeding males. Where *n* is the year sampled, using the total number (#) of nests, literature values for average number nests/female, and data from this study for the number of males per clutch.

The total number of nests per year is the total number of loggerhead nests laid on Sanibel Island in that year. We include two estimations of the average number of nests per female. The minimum value (3.9) is based off of tagging returns on Keewaydin, a nesting beach south of Sanibel Island [22]. The maximum value (5.4) is based on satellite tag tracking of turtles who returned to nest on beaches north of Sanibel; that value suggests that night patrols are missing re-nesters and underestimate nests/female[30]. Finally, both the estimated number of males and females was rounded to the nearest whole number.

Results

Over three nesting seasons, 64 females were sampled (2013: 25; 204:16; 2015:23). Thirteen nests were lost due to flooding, predation or did not develop. In those cases, we included the nesting female's genotype in the allelic frequencies but not in the resulting breeding sex ratios bringing our total to 51 females. In total, 989 hatchlings were sampled: 350 in 2013, 276 in 2014 and 363 in 2015. Of those 51 females, two had been tagged elsewhere (Casey Key and Manasota Key) and four were tagged in Sanibel and returned but we did not resample them.

When the three years were compared, the F_{IS} value was -0.051 (SE: 0.026) and the F_{ST} value was 0.031 (SE: 0.007). These values suggest that there is no evidence of genetic differentiation among yearly cohorts, and therefore they can be considered a single population.

Combining all seven loci resulted in a PI of 7.3 x 10^{-13} and a PE of 1.0. Micro-Checker detected no evidence of scoring error due to stutter, allele dropout, or null alleles. The number of alleles present ranged from 12–24 and there were no deviations from Hardy-Weinberg (p > 0.05). (Table 1).

In 70% of the clutches, more than one male fertilized the clutch (one father per egg so no polyspermy was detected). We identified the genotypes of 126 distinct males. No male fertilized eggs in more than one nest. The maximum number of fathers per nest was 7, mean = 2.51. In 2013, 17 nests were analyzed and 44 fathers were found; in 2014, 14 nests were analyzed and 37 fathers were found and in 2015, 20 nests were analyzed and 45 fathers were found.

Using total numbers of nests (Table 2) and estimates of 3.9–5.4 clutches/female/season [22, 30], we estimate that there were at least 230–318 females nesting on Sanibel from 2013–2015. Based upon these estimates we sampled 20.1–27.8% of the nesting population. Further, our model estimates that there were between 571–829 males mating during that time period. We identified genotypes of 15.2–22.1% of the likely mating males, as male genotypes were unique

Table 1. Descriptive statistics for each locus.

Locus	N_A	H_O	H_E	PI	PE_1
P7E	17	0.893	0.913	0.014	0.785
P2F	13	0.889	0.885	0.024	0.726
P7D	14	0.905	0.900	0.018	0.744
P5H	12	0.841	0.858	0.036	0.707
P7C	13	0.952	0.896	0.020	0.726
P7B	18	0.841	0.887	0.021	0.796
P8D	24	0.921	0.943	0.008	0.844

Data from nesting females only. N_A is the number of alleles per locus, H_O and H_E are observed and expected heterozygosities respectively. PI is the probability of identity for each locus, and PE_1 is the probability of exclusion when one parent is known at each locus.

Table 2. Breakdown of breeding sex ratio (BSR) calculations.

Year	Total # Nests	Estimated # Females	Average # of Males	Estimated # Males
2013	334	62–86	2.58	160–222
2014	411	76–105	3.00	204–321
2015	496	92–127	2.25	207–286
		Σ = 230, 318		Σ = 571, 829
	BSR =	1 Female:		2.48–2.61 Males

Total numbers of nests based upon morning turtle activity censuses, estimated number of females (the minimum values from an average of 5.4 nests per female and the high values from 3.9 nests per female) rounded to the closest individual, average number of males per female (paternity analysis) and the estimated number of males rounded to the closest individual. The number of females and males are summed to reach the BSR value.

to each nest analyzed. The BSR for Sanibel Island from 2013–2015 is 1 female: 2.48–2.61 males (Table 2).

In 2015, there were more nests in which a single male fertilized all hatchlings sampled (Fig 2), but we found no statistical difference in number of fathers among years [χ^2 (df = 2) = 0.905, p = 0.636].

Discussion

This is the first evidence of multiple paternity in loggerhead sea turtle nests on the Gulf of Mexico coastline. Such behavior is well documented for the loggerheads nesting on the eastern coast of Florida and Georgia [48, 58, 59], as well as other ocean basins [43, 60–62]. To our surprise though, we found that no male had fertilized eggs in more than one clutch within or across years. If males move to breeding areas every year, and some remain for the season [11, 37], it is reasonable to think that a single male would have offspring in more than one female's
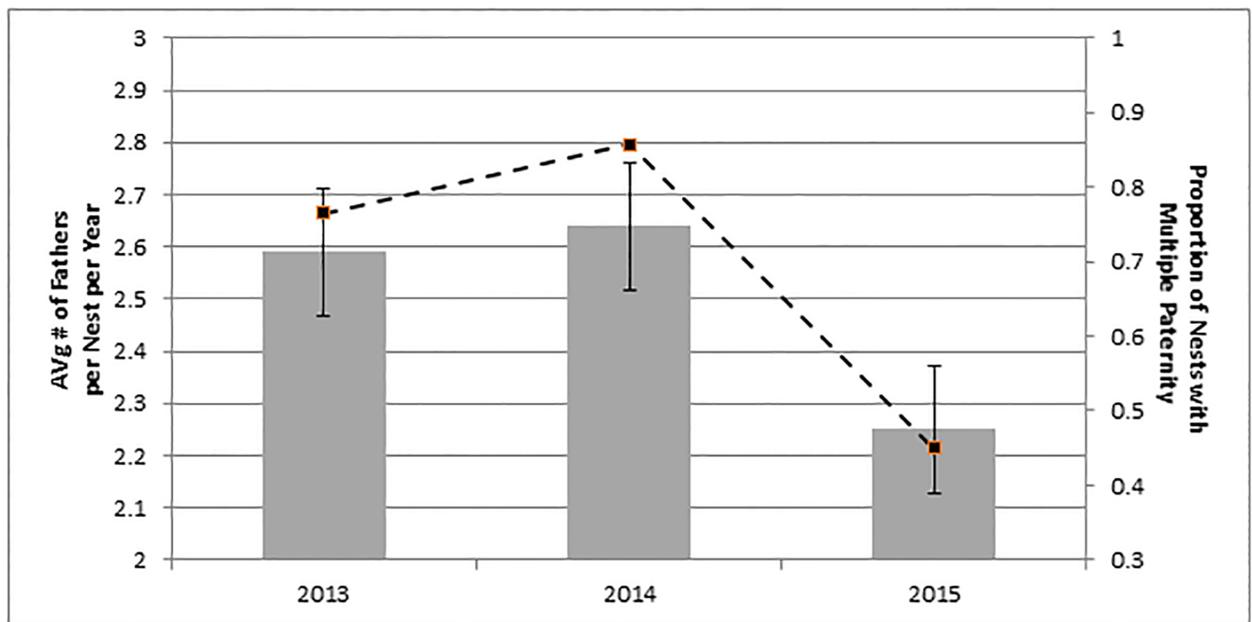
Fig 2. Multiple paternity across seasons. Mean number of fathers/nest/year is depicted by the vertical bars (±standard error of the mean). The proportions of nests with multiple paternity are plotted by the solid dots connected by dashed lines for emphasis (scale on the secondary vertical axis).

nests within a year, even across sequential years. Our result indicates that the number of males contributing to this population of nesting females is higher than would be expected from female-biased primary sex ratios alone [63–65].

The current hypothesis of marine turtle breeding behavior is that females travel to their natal region to mate [66, 67]. Male loggerheads are thought to move close to nesting beaches and compete there to mate with females [68]. Following breeding, males return to foraging grounds and the females remain in the region to nest [69]. In this hypothesis, nesting population structure is driven by natal philopatry of females and gene flow occurs from males that travel along the coast to and from breeding areas [11, 33, 70]. This hypothesis assumes there are female "mating windows" during which males search and compete for females [71] and longer male mating windows. A male that mates earlier therefore, can mate again as soon as opportunity, sperm load, and energy resources allow. If males and female hatchlings and juveniles survive equally, and females return every 2–3 years while males return annually, over a three-year study, at minimum the BSR should approach 1 female for every one male (1:1). Our data do not support this hypothesis because of the numbers of individual males found. By sampling turtles over three years, we were able to estimate the population BSR independent of the complications created by females nesting every second or third year, and males breeding every year.

Here we propose an alternative hypothesis of loggerhead breeding. Female turtles can store sperm after mating events but prior to ovulation; sperm are sequestered in specialized sperm storage tubules [72, 73]. The literature for marine turtles suggests that sperm can be stored for over 3 months [74, 75]. If females mate as they travel from their feeding grounds to their natal regions, they could increase the number of potential mates that they could encounter. In theory, females can be more choosy, without decreasing the likelihood of successful fertilization [76] and the breeding sex ratio will rise as the total number of matings increases. For example, if a female had access to 2 males along the way from her forging area to her nesting grounds, as well as 2 more males in the natal region, the BSR would be 4:1 instead of 2:1. This hypothesis is supported by (i) the large numbers of distinct males found in our study, and (ii) that each males' genotype occurred just once with and across years. If the pool of available breeding males includes all those that a female encounters along her route to her nesting ground, there is a much-diminished chance of finding one male's offspring in multiple nests.

Two other kinds of behavior could lead to a larger number of individual males contributing to the population than expected. It is possible that males, like females, do not mate annually [70]; metabolic factors (foraging quality, number of competing individuals, etc.) may make annual breeding impossible. If so, each year's samples would be a new set of males. Determining whether male behavior changes among years, however, requires formidable efforts beyond the scope of this study (such as coordinated satellite tagging of reproductively active males and subsequent determination of paternity in the offspring of their mates). Alternatively, females may mate throughout their nesting season with a succession of males that arrive at different times. If such were the case, we would not detect that as we did not look at consecutive nests for this study. Future studies will be needed to identify if paternal contributions differ in subsequent nests.

In Florida, the primary sex ratio is already highly female biased, with over 90% female hatchlings produced in some highly productive areas [63, 65, 77], though the effect on adult sex ratio is difficult to predict. As temperatures rise we expect to see more female hatchlings leaving the beach. If those hatchlings survive to adulthood in equal proportions, then the adult sex ratio will skew [23, 78]. It is possible however, that there is differential survival between sexes. One study examined hatchlings that died en route to the ocean from the nest and found that females were four times more likely to perish than their male siblings [79]. Another study examined in-water juvenile sex ratios of loggerheads and found a 2:1 female to male ratio,

which was lower than the expected hatchling sex ratio [80]. Further, warmer temperatures produce females, but high temperatures can be fatal to developing embryos and hatching eggs. If temperatures continue to increase, feminized turtles may not survive to leave the nest [4]. Sea turtles are late maturing organisms, so it will take 20 or 30 years of observations to see how breeding sex ratios are affected by current skewed hatchling sex ratios, embryo mortality, and differential hatchling mortality. This study presents results against which those future sex ratios can be compared. If loggerhead hatchling sex ratios have skewed the adult sex ratios, we are not yet seeing the effects in the breeding sex ratio for the Gulf of Mexico. Rather, it seems that females have access to a large number of males, perhaps as they migrate from feeding grounds to the nesting beaches. There is no indication from our data that sex ratio bias has become so severe that extinction risk is elevated.

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