UNIVERSITY OF CALIFORNIA, SAN DIEGO

The influence of multiple paternity on genetic and morphological variation in leatherback hatchlings (*Dermochelys coriacea*) at Sandy Point National Wildlife Refuge

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in

Marine Biology

by

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The Thesis of Shreya Munshi Banerjee is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE THESIS

The influence of multiple paternity on genetic and morphological variation in leatherback hatchlings (*Dermochelys coriacea*) at Sandy Point National Wildlife Refuge

by

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Master of Science in Marine Biology

University of California, San Diego, 2017

Professor Carolyn Kurle, Chair

Molecular techniques can reveal information about mating systems and paternal identity in cryptic species. Leatherback turtle hatchlings exhibit variation in body size at Sandy Point National Wildlife Refuge (SPNWR), St. Croix, USVI, sometimes within one clutch. When maternal identity is known, microsatellites can be used to determine the number of fathers

contributing to a nest and to assign paternal identity to hatchlings. I measured body size and collected skin from leatherback hatchlings at SPNWR and found that hatchlings weighed 45.3 ± 3.5 g, were 59.1 ± 2.4 mm long (SCL), and had an average body condition index (BCI) of 2.2 x 10^{-4} ± 2.4 x 10^{-5} g/mm^3 (n =3,293). I used maternal and hatchling genotypes to reconstruct paternal genotypes, assigning fathers to each hatchling. I found multiple paternity in five of 16 nests, and compared mass, SCL, and BCI of hatchlings from different fathers and the same mothers. I found no significant differences between sizes of hatchlings based on paternal identity. I compared hatchling size variation for nests with and without multiple paternity and found a tendency for multiple paternity nests to have greater hatchling size variation, although this tendency was not statistically significant. Therefore, I found no direct evidence for paternal genetic influence on body size within a clutch. I also examined opportunistically collected dead embryonic twins and found they were genetically identical. Understanding factors affecting hatchling body size, and other possible measures of fitness, may reveal insights into the reproductive biology and development of cryptic leatherbacks.

Introduction

Body size is a trait commonly used to assess the quality of offspring, likely because it is easily measured across multiple species. Some evidence supports the "bigger is better hypothesis," indicating that larger offspring have a higher chance of survival and reproduction, or in other words, a higher fitness (Bobyn & Brooks, 1994; Janzen & Tucker, 2000; Janzen et al., 2007; Cornioley et al., 2017). For example, red-eared slider turtle hatchlings (*Trachemys scripta elegans)* experience natural selection for larger body size (Janzen & Tucker, 2000; Janzen et al., 2007) and hatchling body size in snapping turtles (*Chelydra serpentina)* may be used as an index for post-hatching success if variation between clutches and populations are taken into account (Bobyn & Brooks, 1994). In addition, paternal size has been linked to offspring body size and greater offspring fitness in wandering albatross (*Diomedea exulans*) for which fathers with larger body mass produced larger chicks, which then had higher survival rates than those from smaller fathers (Cornioley et al., 2017).

Investigating relationships between paternity and offspring fitness can be challenging as paternity can be difficult to assess. However, molecular techniques can provide insights into paternity and mating systems of cryptic species (Lee, 2008). These types of data indicate that multiple paternity is a common phenomenon across many taxa, including 50% of non-avian reptile clutches (Uller & Olsson, 2008). The prevalence of multiple paternity has led to several studies linking characteristics of offspring to multiple paternity and paternal identity (Kempenaers et al., 1997; Lee & Hays, 2004; Thonhauser et al., 2014). Some data show positive correlations between multiple paternity and traits used to determine fitness (e.g., blue tits (*Parus carrulus*; Kempenaers et al., 1997) and painted turtles (*Chrysemys picta*; Pearse et al., 2002), but there has been little evidence for widespread direct or indirect fitness advantages from multiple paternity across taxa (Uller & Olsson, 2008).

Molecular techniques are useful to infer the male mating behavior of cryptic marine species, which are difficult to observe in the open ocean. One such molecular technique is microsatellite analysis, which has revealed multiple paternity in green sea turtles (*Chelonia mydas;* Ireland et al., 2003), olive ridleys (*Lepidochelys olivaceae;* Jensen et al., 2006), Kemp's ridleys (*Lepidochelys kempii;* Kichler et al., 1999), hawksbills (*Eretmochelys imbricata;* Phillips et al., 2013), loggerheads (*Caretta caretta;* Moore & Ball, 2002), and leatherbacks (*Dermochelys coriacea;* Crim et al., 2002). Stewart and Dutton (2011, 2014) reexamined the occurrence of multiple paternity in leatherbacks and used multiple paternity analysis to reconstruct paternal genotypes and determine adult breeding sex ratios for the St. Croix leatherback population. Using molecular techniques to infer male behavior is critical because unlike some hard-shell sea turtle species, leatherback turtles are difficult to observe in the open ocean, and most assessments are made from nesting females (James et al., 2005). Male leatherbacks are less likely to be seen, and mating is rarely observed (Carr & Carr, 1986). Understanding male behavior and the mating systems of cryptic marine species is critical for determining demographic indices such as population growth and recovery rates.

Leatherback turtles exhibit phenotypic variation in reproductive traits that may be due to environmental or genetic factors (Wallace et al., 2007). For example, leatherback hatchlings from the same mother varied in mass by 0.2-6.4 g at Parque Nacional Marino Las Baulas, Costa Rica (Wallace et al., 2007), and embryonic twins attached at the yolk sac have been observed from nest excavations with some pairs of twins varying in size (Eckert, 1990). Hatchling size is positively correlated with egg mass in smooth softshell turtles (*Apalone mutica;* Janzen, 1993) and negatively correlated with incubation temperature in green sea turtles (Booth & Astill, 2001), and a combination of maternal and genetic components are thought to drive this size variation. Others found a 2-g increase in hatchling mass for every 10-g increase in egg mass for leatherbacks (significant positive correlation, $r^2 = 0.191$, $p < 0.001$), and suggested that variation

in the characteristics of hatchlings was due to interactions between hatchling genotype and the environment (Wallace et al., 2006, 2007).

In this study, I used microsatellite analysis to better understand relationships between multiple paternity and potential paternal contribution to hatchling body size in leatherback sea turtle hatchlings. I also investigated the genetics of embryonic leatherback twins to determine if they are fraternal or identical. To my knowledge this is the first study to link paternal identity to hatchling size for leatherback turtles and determine the level of relatedness of embryonic leatherback twins. I addressed the following questions: (1) What is the variation in size for leatherback hatchlings at SPNWR, and has it changed over time? (2) Is body size variation within a clutch of leatherback sea turtle hatchlings related to paternal identity? (3) Do nests with multiple paternity have greater size variation among hatchlings? and (4) Are embryonic twins fraternal or identical? The answers to these questions further our understanding of factors that affect hatchling morphology and demonstrate the utility of reconstructing paternal genotypes using microsatellite markers.

Methods

Field methods

I collected skin samples from leatherback hatchlings at Sandy Point National Wildlife Refuge (SPNWR), a beach on a small peninsula at the southwestern end of St. Croix, US Virgin Islands (Figure 1), as part of an ongoing project conducted since 2009 to determine age at maturity for leatherbacks using genetic techniques (Dutton and Stewart 2013). This beach supports a nesting population of Atlantic leatherbacks that has been studied for several decades (Dutton et al., 1999; Roden & Dutton, 2011; Stewart & Dutton, 2014). The beach has been under federal protection since 1984, when it became a refuge, and since then has seen an increase in the

leatherback nesting population (Dutton et al., 2005), partially due to the high survival rate of females (90%; Dutton et al., 2005; Kendall et al., in prep), and the practice of moving nests from areas of the beach prone to erosion to more stable locations to increase hatchling production. Maternity data for each nest are very reliable as all nest locations are recorded and females are identified through PIT (Passive Integrated Transponder) tags, flipper tags, and genetic analysis.

I chose first emergence hatchlings from nests with known mothers and took skin samples from the trailing edge of the front flipper using a 2-mm biopsy punch and methods outlined in Dutton & Stewart (2013). I applied styptic pencil (aluminum sulfate 56%) to each biopsy site to prevent bleeding and released all hatchlings within two hours of collection (Dutton & Stewart, 2013). All activities were permitted under IACUC permit SWPI_2016-02 and Endangered Species Permit #DFW16030X (US Virgin Islands Department of Planning and Natural Resources). Skin samples were stored in a saturated salt (NaCl) solution in 96-well Sorensen PCR plates. I measured the mass (g) of each hatchling with a spring scale, and straight carapace length (SCL, mm), straight carapace width (SCW, mm), and body depth (mm) using Vernier calipers (SPI #6056449). I recorded each hatchling's sample location in the 96-well plate with its size measurements. Additionally, I collected dead embryonic twins from partially developed eggs when nests were excavated to assess hatching success and embryonic mortality. Samples were kept in a -20°C freezer in the Southwest Fisheries Science Center in La Jolla, CA until analysis. I collected samples during the summers of 2009, 2012, 2015, and 2016; and took size measurements in 2009, 2012, 2013, 2015, and 2016. For 2009, I had genetic samples and size measurements from the same nests, but the measurements were not paired with a specific individual. For 2012, 2013, and 2016 hatchlings were randomly selected and there were genetic samples paired with body size measurements. For 2015, hatchlings were selected by eye for size variation and there were genetic samples paired with measurements (see Table 1 for a summary of data collection).

Table 1. Data collection, sampling criteria, and statistical methods used to address each study question.

Question	Data collected	Sampling criteria	Analysis
What is the size variation in hatchlings at SPNWR; has it changed over time?	Size measurements (2009, 2012, 2013, 2015, 2016)	2009, 2012, 2013, and 2016 randomly selected hatchlings; 2015 nests selected for large variation by eye	Kruskal-Wallis tests
Is size variation within a nest related to paternal identity?	Paired size measurements and samples (2012, 2015)	Selected for greatest size range	Multiple paternity analysis, Kruskal-Wallis tests
Do nests with multiple paternity have greater size variation among hatchlings?	Paired size measurements and samples (2016), un-paired size measurements and samples (2009)	Randomly selected	Multiple paternity analysis, 2016 could not be used to answer question, results based on 2009 data
Are embryonic twins fraternal or identical?	Embryonic twin samples from nest excavations (2016)	As many twins as possible, opportunistic	Matching genotypes

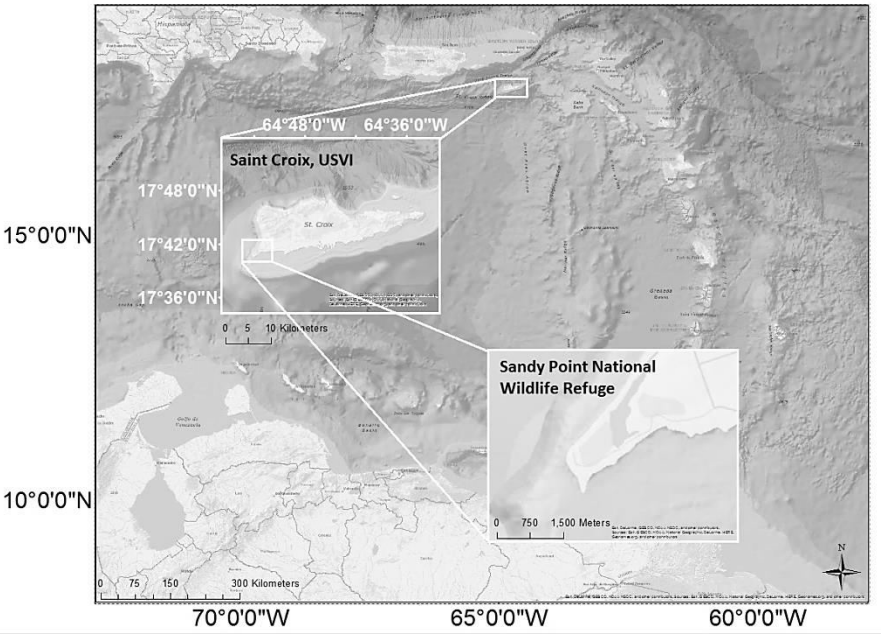

Figure 1. Sandy Point National Wildlife Refuge lies on the southwestern edge of St. Croix, USVI.

Size Comparison

I obtained body size measurements for 3,293 hatchlings during the summers of 2009, 2012, 2013, 2015, and 2016. I used these measurements to quantify the overall size and variation in size of leatherback hatchlings at SPNWR, and to calculate body condition index (BCI) as mass/SCL^3 (Bjorndal & Bolten, 2000) for each hatchling. I evaluated hatchling mass, SCL, and BCI for differences in means by year with non-parametric Kruskal-Wallis tests in the open source statistical software, R 3.3.2 (R Core Team, 2016).

DNA analysis

I selected the twenty nests with the widest ranges of mass (g) and SCL (mm) from 2012 and 2015 and randomly selected ten nests from 2016 for genetic analysis. I used the following methods for the 2012, 2015, and 2016 nests. I used manual salt (NaCl) extractions (Hillis & Davis, 1986; Dutton et al., 1999) to isolate DNA from the hatchling and female tissue samples and included negative control extractions (without tissue) in each extraction batch to ensure that the DNA extractions were not contaminated. I quantified the DNA using a Nanodrop Spectrometer or a Victor Fluorometer and then diluted the DNA to 4 ng/ul with milliQ water. For each sample, the DNA was amplified in Polymerase Chain Reactions (PCR) for four microsatellite markers in 25 ul reactions at temperatures and run times specific to those markers in a thermal cycler 2720 (PE Applied Biosystems, Foster City, CA, USA). The microsatellite markers used for the nests from 2012, 2015, and 2016 were DERM01, DERM02, DERM37, and DERM48 (Alstad et al., 2011). Microsatellite markers, LB142 (Roden & Dutton, 2011), Cc5C08t, and Cc5H07t (Shamblin et al., 2007), were only used for the 2012 nests. I performed PCR reactions for each of these primers without DNA (negatives) as controls to check for contamination between samples. The St. Croix leatherback population has eight or more alleles at each of these genetic markers, so these markers may be used to differentiate individuals and

assess relatedness within this nesting population. I determined the genotypes of each sample at each marker using an ABI Prism 3730 Genetic Analyzer with ROX500 fluorescent size standard (Applied Biosystems, Foster City, CA, USA), scored alleles using Genemapper 5.0 (Applied Biosystems, Foster City, CA, USA), and manually verified each allele call. I replicated fifty-one sample genotypes to assess genotyping error rate. I classified hatchling genotypes without a maternal allele as genotyping error. The nests from 2009 were analyzed with similar methods, as specified in Stewart & Dutton (2011).

Multiple Paternity Analysis

Paternal alleles in each of the hatchlings were determined visually by comparing each hatchling genotype to that of its mother for each nest analyzed. Clutches with more than two paternal alleles per locus, for at least two loci, were considered cases of multiple paternity (Stewart & Dutton, 2011). The computer program GERUD 1.0 was used to confirm paternal alleles and reconstruct paternal genotypes based on paternal alleles paired with each other at multiple loci (Jones, 2001). Nests whose paternal genotypes could not be confirmed by using 12 hatchlings had an additional 12 hatchlings genotyped to confirm paternal genotypes. All paternal genotypes used to assign hatchlings to a father in this study were fully resolved.

For the nests selected for size variation from 2012 and 2015, each hatchling's paternal alleles at one or more loci were used to assign it to a father in nests with multiple paternity once paternal genotypes were confirmed. All hatchlings should have one maternal allele. Those that did not were due to genotyping error, were not the mother's offspring, or had a mutation. By excluding a hatchling's maternal allele, one can identify the paternal allele and use it to match the hatchling to a father. This can be done at multiple loci to confirm paternity. Hatchlings with paternal alleles that did not clearly determine paternal identity were excluded from the size

comparison. I evaluated differences in mass, SCL, and BCI of hatchlings from the same mother but different fathers with non-parametric Kruskal-Wallis tests.

The means, standard deviations (SD), and variances of mass, SCL, and BCI of the five 2009 nests with multiple paternity and of five randomly selected single paternity 2009 nests were calculated (n = 10 hatchlings per nest). To compare the amount of variation between multiple and single paternity nests, we averaged the variances of the five nests of single and multiple paternity, and compared them with Kruskal-Wallis tests.

We calculated allele frequencies and assessed Hardy-Weinberg equilibrium and heterozygosity for each of the polymorphic loci from the 30 nesting females genotyped for this study with the program GENEPOP on the Web (Raymond and Rousset, 1995; Rousset, 2008). The probability of detection of multiple paternity (D) was calculated with the formula given in Westneat et al. (1987) using the allele frequencies from the Sandy Point nesting population. Additionally, the probability that two unrelated individuals shared the same multi-loci genotype due to chance (Q) was calculated with the formula given in Hanotte et al. (1991).

Results

Hatchlings on Sandy Point (n = 3,293) had a mean (± SD) mass of 45.3 ± 3.5 grams, SCL of 59.11 ± 2.41 mm, and BCI of $2.20 \times 10^{-5} \pm 2.43 \times 10^{-4}$ g/mm^3 (Figure 2).

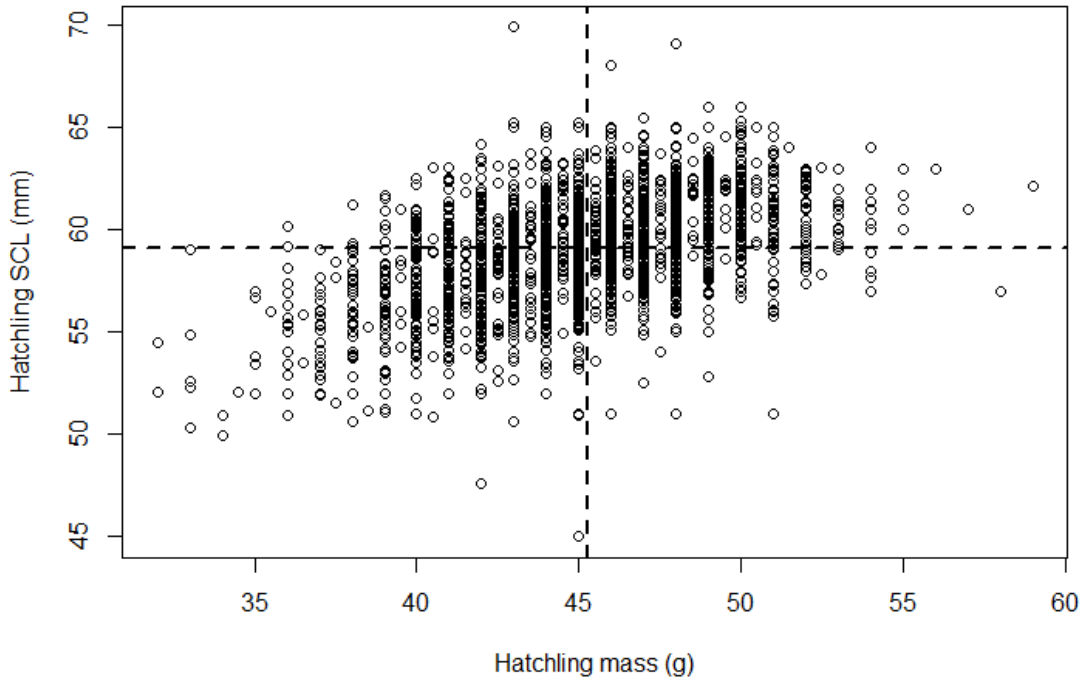

Figure 2. Body size of leatherback hatchlings at SPNWR, n = 3,293. Each point represents one hatchling. The dashed lines represent mean mass (45.3 g) and mean SCL (59.1 mm).

There were significant differences between the mean mass (Kruskal-Wallis test, $p < 2.0 \times 10^{-6}$), SCLs (Kruskal-Wallis, $p = 0.0004$), and BCIs (Kruskal-Wallis test, $p < 2.0 \times 10^{-6}$) of hatchlings grouped by year (Figure 3). However, the overall measures of hatchling size variation are relatively low over the timescale of this study (Figure 3).

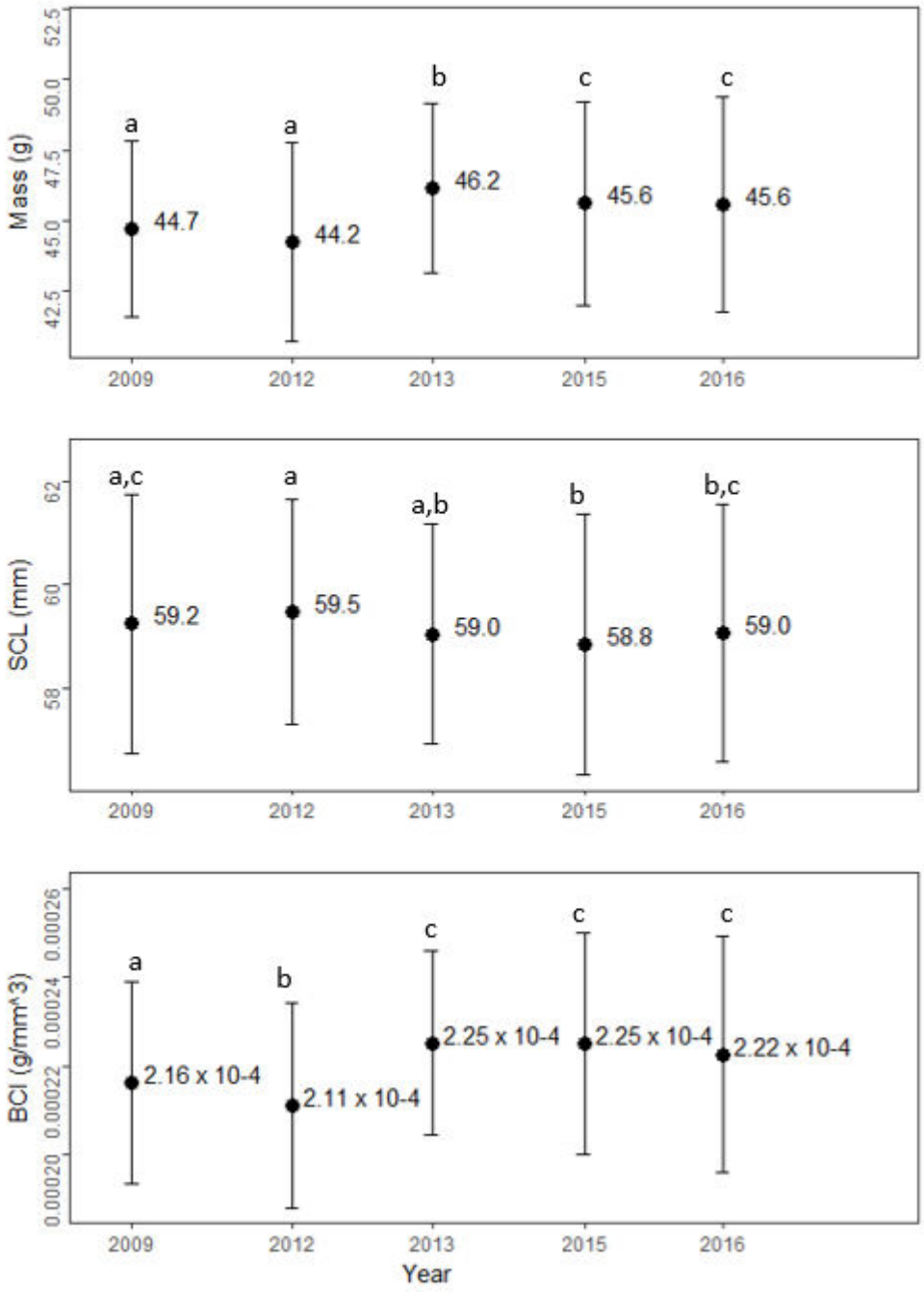

Figure 3. Hatchling size variation by year: (top) mass, (middle) SCL, (bottom) BCI. Means and standard deviations are shown. There are small but significant differences between years for all size measurements (Kruskal-Wallis test, $p < 0.01$). Letters represent significant differences between yearly means (Tukey test, $p < 0.05$).

The four leatherback-specific markers used were polymorphic in the population of nesting females at SPNWR. These markers had from 10 to 15 alleles each. The probability of

detecting multiple paternity in this population with these four markers (D) was 0.993 (Westneat, 1987). Genotypes for the 30 nesting females were used to calculate Hardy-Weinberg expected and observed values. These, along with the number of alleles and size ranges for each locus, are displayed in Table 2. The genotyping error rate of this study was 3.6% (n = 1,571 single locus genotypes).

Table 2. Characteristics of the four polymorphic microsatellite markers used in this study. H_e = expected heterozygosity, H_o = observed heterozygosity, p = the p value generated using a chi square test for Hardy-Weinberg equilibrium, q = probability of two unrelated individuals sharing a genotype at each locus, and d = probability of detecting multiple paternity at each locus.

Locus	Range	Number of alleles (A)	H_e	H_o	p (HW)	q	d
DERM01	215-263	11	0.89	0.83	0.17	0.03	0.75
DERM02	166-222	15	0.88	0.96	0.34	0.03	0.74
DERM37	145-199	13	0.88	0.81	0.54	0.03	0.74
DERM48	338-374	10	0.83	0.93	0.47	0.04	0.63

I generated genotypes for 16 nesting mothers and 12 hatchlings each from 2012 and 2015 nests. For five nests from 2012 and 2015, I needed to genotype 12 extra hatchlings to fully resolve reconstructed paternal genotypes. I genotyped a total of 276 hatchlings. The program GERUD 1.0 requires hatchlings with genotypes at every marker so I used at least seven hatchlings per mother in the multiple paternity analysis. I considered two or more paternal genotypes given by GERUD 1.0 that differed at two or more loci as evidence for multiple paternity, which I found in five out of 16 nests. For the five nests with multiple paternity, I assigned each hatchling, including those missing a genotype at a locus, to a father based on its paternal alleles. The females, as identified by flipper tag, who mated with more than one male were XXZ168, YYL884, XXZ059, SPP073, and SPP088. Turtle SPP088 mated with three males, whereas turtles XXZ059, YYL884, and SPP073 all clearly mated with two males each. Analysis

of XXZ168 and her hatchlings with GERUD 1.0 revealed three paternal genotypes, but one of the potential fathers had only one offspring and shared alleles with the other paternal genotypes at each locus, therefore this potential third father was dismissed as genotyping error. From the 16 nests used for this portion of the study, 22 distinct paternal genotypes in total were reconstructed. Additionally, ten paternal genotypes were reconstructed from the ten nests sampled in 2016, which I did not use in any further analysis of hatchling size comparisons. None of the 32 males identified mated with more than one of the 26 females genotyped in this study. However, I was unable to fully resolve the genotype for the male who mated with the female TTZ345 in 2012, who may be the same male who mated with the female SPP102 in 2015, depending on his second allele at one locus. The probability of unrelated individuals sharing a genotype at all four loci used in this study is 8.81×10^{-7} (Hanotte et al., 1991), and thus I am confident that there were at least 32 distinct breeding males identified with fully resolved genotypes.

There were no significant relationships between paternal identity and three metrics of hatchling body size. Differences in the masses of hatchlings sired by different fathers ranged from 0.5 g in YYL884's and XXZ059's clutches to 4.3 g in SPP088's clutch, but there were no significant differences between the mean mass of hatchlings from each of distinct fathers in any of the nests analyzed (Kruskal-Wallis, $p>0.05$; Figure 4, Table 3). Differences in SCL for hatchlings with paternity from different fathers ranged from 0.16 mm in XXZ059's clutch to 2.66 mm in SPP088's clutch, but there were no significant differences between the mean SCLs of hatchlings sired from different fathers (Kruskal-Wallis, $p>0.05$; Figure 4, Table 3). Differences in BCI from hatchlings from different fathers ranged from 2.33×10^{-7} g/mm^3 in XXZ059's clutch to 7.00×10^{-6} g/mm^3 in SPP088's clutch, but, again, these differences were not significant for any of the five nests with multiple paternity (Kruskal-Wallis, $p>0.05$; Figure 4, Table 3).

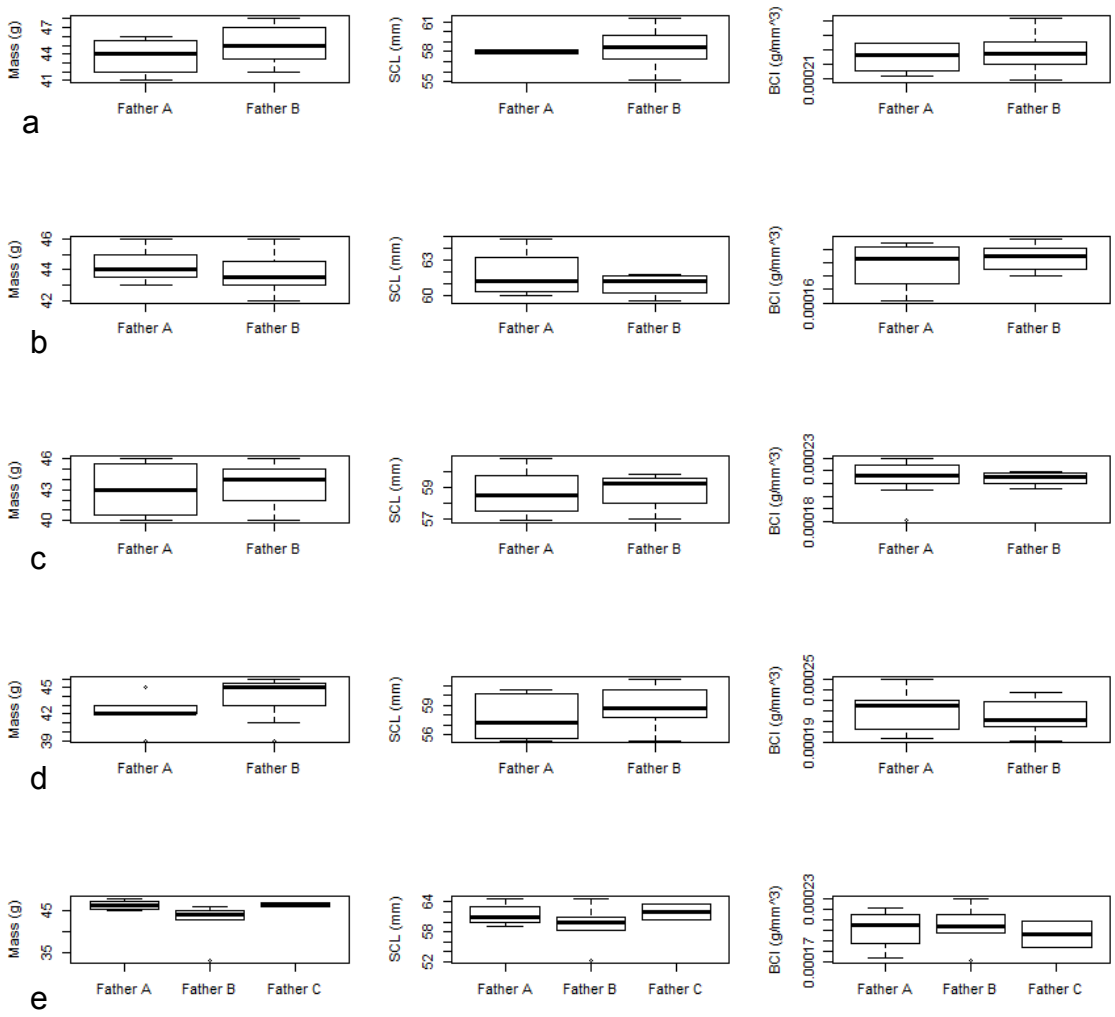

Figure 4. The mass, SCL, and BCI of hatchlings grouped by father for each of the nests with multiple paternity from the females: (a) XXZ168 (b) YYL884 (c) XXZ059 (d) SPP073 and (e) SPP088 from top to bottom. There were no significant differences in any of the body size metrics from hatchlings among different fathers. Boxplots display the minimum, first quartile, median, third quartile, maximum (from bottom to top), and outliers (points).

Table 3. The mean values (± SD) for mass, SCL, and BCI separated by paternal identity for hatchlings from five female leatherbacks who mated with multiple males in 2012 and 2015. There were no significant differences in any of the body size metrics from hatchlings among different fathers.

Hatchling parameter	Paternal identity	XXZ168	YYL884	XXZ059	SPP073	SPP088
Mass	Father A (g)	43.8 ± 2.2 (n=4)	44.3 ± 1.3 (n=4)	43.0 ± 2.6 (n=8)	42.2 ± 2.2 (n=5)	46.5 ± 1.3 (n=4)
	Father B (g)	45.1 ± 2.3 (n=7)	43.8 ± 1.3 (n=8)	43.5 ± 2.5 (n=4)	43.9 ± 2.7 (n=7)	42.2 ± 5.3 (n=5)
	Father C (g)	----	----	----	----	46.5 ± 0.7 (n=2)
SCL	Father A (mm)	58.0 ± 0.2 (n=4)	61.8 ± 2.1 (n=4)	58.6 ± 1.4 (n=8)	57.8 ± 2.5 (n=5)	61.5 ± 2.3 (n=4)
	Father B (mm)	58.3 ± 2.1 (n=7)	60.9 ± 0.8 (n=8)	58.8 ± 1.2 (n=4)	58.9 ± 2.2 (n=7)	59.2 ± 4.5 (n=5)
	Father C (mm)	----	----	----	----	61.9 ± 2.3 (n=2)
BCI	Father A (g/mm^3)	$2.2 \times 10^{-4} \pm 1.1 \times 10^{-5}$ (n=4)	$1.8 \times 10^{-4} \pm 1.9 \times 10^{-5}$ (n=4)	$2.1 \times 10^{-4} \pm 1.5 \times 10^{-5}$ (n=8)	$2.2 \times 10^{-4} \pm 2.3 \times 10^{-5}$ (n=5)	$2.0 \times 10^{-4} \pm 2.0 \times 10^{-5}$ (n=4)
	Father B (g/mm^3)	$2.3 \times 10^{-4} \pm 1.5 \times 10^{-5}$ (n=7)	$1.9 \times 10^{-4} \pm 9.7 \times 10^{-6}$ (n=8)	$2.1 \times 10^{-4} \pm 5.8 \times 10^{-6}$ (n=4)	$2.2 \times 10^{-4} \pm 1.7 \times 10^{-5}$ (n=7)	$2.0 \times 10^{-4} \pm 2.2 \times 10^{-5}$ (n=5)
	Father C (g/mm^3)	----	----	----	----	$2.0 \times 10^{-4} \pm 1.9 \times 10^{-5}$ (n=2)

None of the 2016 nests had evidence of multiple paternity, and thus were not used to evaluate relationships between the variations in hatchling sizes and number of contributing fathers. For the five nests with multiple paternity sampled in 2009, there were no significant differences (Kruskal-Wallis, all $p > 0.05$) in the variations around the means for hatchling mass, SCL, and BCI between the five nests with multiple paternity and the five randomly selected nests with single paternity. Although not statistically significant, there was a tendency for nests with multiple paternity to have greater size variation than those with single paternity.

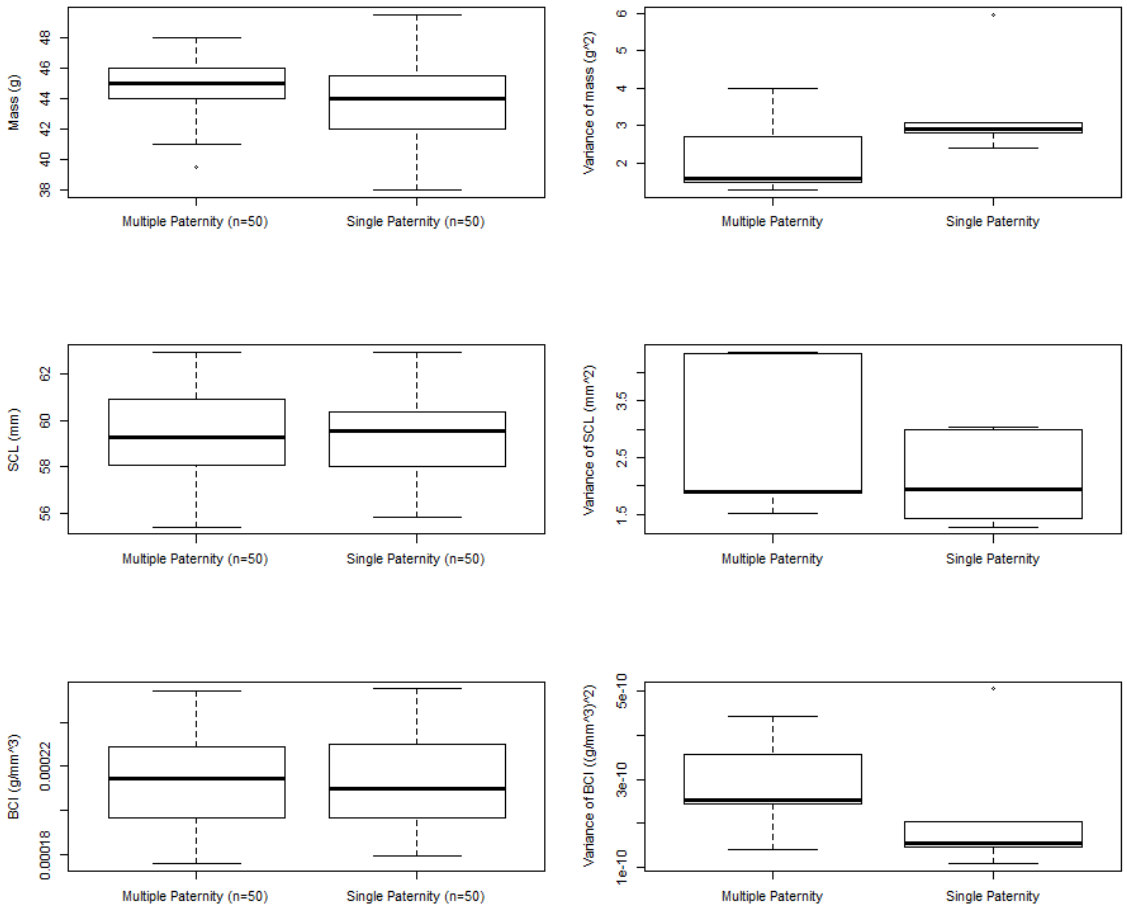

Figure 5. The mean values and the variation around the means for mass, SCL, and BCI for 50 hatchlings from five nests with multiple paternity and five nests with single paternity. The graphs on the left are the means and variance for each metric and those on the right are the variances around the means. There were no significant differences in the means or in the variation around the means, but there was a tendency toward higher variances among the size metrics for hatchlings from nests with multiple paternity. Boxplots display the minimum, first quartile, median, third quartile, maximum (from bottom to top), and outliers (points).

I collected six sets of embryonic twins and successfully genotyped four sets (8 embryos total) for the four markers used for the multiple paternity analysis component of this study. All four sets were identical at each genotyped locus. Females with flipper tags SPP327 and SPP254, and an unknown female with two sets of twins in the same nest, all produced more than one set of twins within the season.

Table 4. The mothers of and the genotypes for each set of embryonic twins analyzed at four polymorphic loci. Each pair was genetically identical.

Mother	Twin	DERM01	DERM02	DERM37	DERM48
SPP327 (Yonce)	Twin 1	235/243	186/198	175/179	362/362
	Twin 2	235/243	186/198	175/179	362/362
SPP327 (Yonce)	Twin 1	215/235	186/198	179/183	362/362
	Twin 2	215/235	186/198	179/183	362/362
SPP254 (Flounder)	Twin 1	215/243	182/186	167/167	342/342
	Twin 2	215/243	182/186	167/167	342/342
Unknown	Twin 1	243/255	190/194	175/179	362/362
	Twin 2	243/255	190/194	175/179	362/362

Discussion

I characterized the variation in leatherback hatchling morphology on Sandy Point National Wildlife Refuge, St. Croix, USVI and established a baseline of hatchling morphometrics for future studies at Sandy Point and on populations at other locations. I found no evidence that paternal identity is linked to hatchling size and that nests with multiple paternity had a tendency toward increased size variation within a clutch, although this trend was not significant. I also demonstrated the application of assigning hatchlings to fathers with reconstructed genotypes, which is useful as there are few published studies using this technique to assess the quality of offspring from different males.

The hatchlings from Sandy Point National Wildlife Refuge were larger than hatchlings in the Pacific Costa Rican population from Parque las Baulas (Wallace et al., 2007). Sandy Point hatchlings were 5.2 g heavier (45.3 ± 3.5g vs 40.1 ±2.7g) and 2.2 mm longer (SCL; 59.1 ±2.4 vs. 56.9 ± 2.1 mm), than those from Parque las Baulas (Wallace et al., 2007). These differences in size by population are consistent with size differences in nesting females reported in previous studies (Stewart et al., 2007; Robinson et al., 2017). Sandy Point nesting females and turtles from

other Caribbean nesting populations had longer curved carapace lengths (153.6 cm vs 147.0 cm) and exhibited more size variation than Las Baulas nesting females (Stewart et al., 2007; Robinson et al., 2017). In addition, by including measurements from many years, I was able to describe changes in hatchling size over time. The largest differences in means between years were 1.9 g (mass), 0.6 mm (SCL), and 1.4 x 10^{-5} g/mm^3 (BCI). The overall variation between years is low, but statistically significant. The differences among years support findings from previous studies that show that hatchling body size fluctuates with environmental conditions such as temperature and moisture (McGehee, 1990; Booth & Astill, 2001). However, because the differences observed were so small, it is possible that differences in hatchling body size are related to maternal investment, as shown by Wallace et al. (2007), or genetic factors. Hatchling variation is likely due to some combination of environmental factors, maternal investment, and genetics, which may include paternal contribution, although not detected in this sample set (Janzen, 1993; Bobyn & Brooks, 1994; Booth & Astill, 2001; Wallace et al., 2006, 2007).

I did not find a direct effect of differential paternity on leatherback hatchling body size, although there may still be some genetic component to hatchling body size contributed by the father. However, Cornioley et al. (2017) were able to detect a relationship between paternal identity and offspring size in wandering albatross chicks. My sample size for the paternal analysis was small and hatchling morphology may not be a strong indicator of male contribution in sea turtles. Future studies, for example, Booth (in press, 2017), relating other potential measures of hatchling fitness with multiple paternity studies may be better for assessing qualities of males' offspring.

The finding that multiple paternity does not increase size variation in leatherback nests compared to single paternity suggests that paternity does not have a direct effect on hatchling size. Previous studies on frogs (*Crinia georgiana;* Bryne & Roberts, 2002) and wild house mice

(*Mus musculus musculus*; Thonhauser et al., 2014) also found that multiple paternity does not increase variation in offspring size. While it is widely assumed that increasing genetic variation increases morphological variation, this may not be reflected in hatchling body size. It is possible that my small sample size may have prevented me from detecting a significant trend or that body size is not a strongly heritable trait in leatherbacks. Lee & Hayes (2004) found that green turtle nests with multiple paternity did not have greater hatching success than nests with single paternity, demonstrating no clear benefit to multiple paternity in that instance.

Female turtle reproductive success is often measured by hatching success rates or quantity of eggs or hatchlings (Wallace et al., 2006), which is possible because biologists can confirm the maternal identity associated with a nest via PIT (Passive Integrated Transponder) tags, flipper tags, or genetic identity. However, paternity of turtle hatchlings is difficult to measure as are potential relationships between hatchling fitness and paternity. I demonstrated that multiple paternity analyses allowed for genetic identification of leatherback hatchlings to assess the quantity and quality of a male's offspring without the need to observe cryptic mating events. I also contributed to an understanding about paternal contribution to potential measures of hatchling fitness. In addition, the genotypes generated by multiple paternity studies contribute to an understanding of potential mating patterns such as relative paternal contribution to each clutch, and the breeding sex ratios of leatherback populations. I identified 32 distinct males that were active members of the breeding population (mating with 26 females) during the three years of sampling. Although my yearly sample sizes were small, my data show yearly variation in male to female ratios. I found the lowest rate of multiple paternity in 2016 for the nests I tested, which may reflect the low number of females that nested that year. Jensen et al. (2006) showed that rates of multiple paternity in olive ridley turtles were lower when population density was lower. I showed it is possible to assign hatchlings to a father with confidence, which may allow me to assess male reproductive success (quantity and quality of offspring) and to compare

characteristics of their offspring in future studies. Using multiple paternity analysis to reconstruct male genotypes may help increase our understanding of the behavior and biology cryptic male leatherbacks.

Conclusion

In summary, my results indicate that paternal identity does not have a direct effect on leatherback hatchling body size as I found no significant differences in measures of three body metrics between leatherback sea turtle hatchlings from two different fathers but the same mother. Additionally, multiple paternity did not have a clear impact on the variations for within-clutch hatchling size. While a paternal genetic component to hatchling size may still exist, I was unable to detect a relationship with my limited sample size. Based on the findings of this study, further research into genetic and environmental factors that affect hatchling body size, and other measures of hatchling quality, would provide insight into conditions affecting endangered leatherback sea turtle populations.

This thesis uses material currently being prepared for submission for publication as The influence of multiple paternity on genetic and morphological variation in leatherback hatchlings (*Dermochelys coriacea*) at Sandy Point National Wildlife Refuge. Banerjee, Shreya; Frey, Amy; Kurle, Carolyn; Stewart, Kelly. The thesis author was the primary investigator and author of this paper.

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