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Multiple paternity in loggerhead turtle (*Caretta caretta*) nests on Melbourne Beach, Florida: a microsatellite analysis

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Abstract

Many aspects of sea turtle biology are difficult to measure in these enigmatic migratory species, and this lack of knowledge continues to hamper conservation efforts. The first study of paternity in a sea turtle species used allozyme analysis to suggest multiple paternity in loggerhead turtle (*Caretta caretta*) clutches in Australia. Subsequent studies indicated that the frequency of multiple paternity varies from species to species and perhaps location to location. This study examined fine-scale population structure and paternal contribution to loggerhead clutches on Melbourne Beach, FL, USA using microsatellite markers. Mothers and offspring from 70 nests collected at two locations were analysed using two to four polymorphic microsatellite loci. Fine-scale population differentiation was not evident between the sampled locations, separated by 8 km. Multiple paternity was common in loggerhead nests on Melbourne Beach; 22 of 70 clutches had more than one father, and six had more than two fathers. This is the first time that more than two fathers have been detected for offspring in individual sea turtle nests. Paternal genotypes could not be assigned with confidence in clutches with more than two fathers, leaving the question of male philopatry unanswered. Given the high incidence of multiple paternity, we conclude that males are not a limiting resource for this central Florida nesting aggregate.

Keywords: *Caretta caretta*, loggerhead sea turtle, mating systems, microsatellite, paternity, polyandry

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Introduction

Sea turtles are difficult to observe in coastal and oceanic habitats, and many aspects of their natural history are refractory to conventional scientific approaches. In most cases, migratory routes and reproductive behaviours are unknown, and these scientific gaps continue to hinder management goals for threatened and endangered populations. In recent years some aspects of reproductive behaviour, such as nest-site fidelity, have yielded to molecular genetic investigations (Bowen & Karl 1996). Other aspects remain understudied. Pedigree analyses of sea turtle clutches can reveal fundamental aspects of population biology, but most efforts to date have been hampered by small sample sizes and/or ambiguous markers, and it is not apparent whether multiple paternity is the exception or the rule (Table 1). Rieder *et al*. (1998) and Dutton & Davis (1998) found no evidence of multiple paternity in leatherback (*Dermochelys coriacea*) nests in Costa Rica or St. Croix, and FitzSimmons (1998) found a very low incidence of multiple paternity in green turtles (*Chelonia mydas*) nesting in the southern Great Barrier Reef. Olive ridleys (*Lepidochelys olivacea*) in Suriname showed multiple paternity in two of eight examined clutches (Hoekert *et al*. 2000). Multiple fathers have been provisionally detected in a study of green turtle clutches at Tortuguero, Costa Rica (Parker *et al*. 1996). Kemp's ridley (*Lepidochelys kempii*)

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Table 1 Previous studies of paternity in sea turtles

Study	Species	Location	Marker	MP?	N/F
Parker *et al*. 1996	*Chelonia mydas*	Tortuguero, Costa Rica	Jeffrey's Probes	50%	3/3
FitzSimmons 1998	*Chelonia mydas*	Queensland, Australia	Microsatellites	0.3%	22/13
Kichler *et al*. 1999	*Lepidochelys kempii*	Tamaulipas, Mexico	Microsatellites	56%	35/26
Hoekert *et al*. 2000	*Lepidochelys olivacea*	Suriname	Microsatellites	25%	8/8
Rieder *et al*. 1998	*Dermochelys coriacea*	Guanacaste, Costa Rica	Microsatellites	N	2/2
Dutton & Davis 1998	*Dermochelys coriacea*	St. Croix, USVI	Microsatellites	N	?/4
Harry & Briscoe 1988	*Caretta caretta*	Queensland, Australia	Allozymes	33%	45/24
Bollmer *et al*. 1999	*Caretta caretta*	Melbourne Beach, FL	Microsatellites	33%	3/3

MP? refers to the incidence of multiple paternity, and N/F refers to the total number of nests and females assayed (for example, 22/13 would be a total of 22 assayed nests, which were laid by 13 females).

clutches at Rancho Nuevo, Mexico had a high rate of multiple paternity (Kichler *et al*. 1999). Harry & Briscoe (1988) used allozymes to infer that at least some loggerhead (*Caretta caretta*) clutches in Queensland, Australia, had multiple paternity. Bollmer *et al*. (1999) found that one of three loggerhead clutches from Melbourne Beach, FL, had multiple fathers, with two fathers more likely than three.

Though males could be limiting at Melbourne Beach (the sex ratio of loggerhead turtle hatchlings leaving the beach at Cape Canaveral, FL, has been reported at more than 9:1 female : male; Mrosovsky & Provancha 1989), sea turtles are unlikely to be monogamous. Like other turtles, sea turtles are likely to derive indirect genetic benefits from promiscuous matings (Pearse & Avise 2001). Benefits of polygyny could include bet-hedging (Watson 1991, 1998), avoidance of genetic incompatibility (reviewed in Zeh & Zeh 1996; Newcomer *et al*. 1999), avoidance of inbreeding (Madsen *et al*. 1992; Stockley *et al*. 1993), gaining 'good genes' (Keller & Reeve 1995), and increased genetic diversity among offspring (Watson 1991; Madsen *et al*. 1992; Baer & Schmid-Hempel 1999).

We used highly variable microsatellite loci to address the issue of multiple paternity for loggerhead turtles that nest in Melbourne Beach, FL, one of the largest nesting concentrations in the world for this species (Dodd 1988). Gravid females lumber onto these beaches to dig nest cavities, often using the same nesting beach in sequential nesting efforts to deposit up to seven clutches in a summer (Bjorndal *et al*. 1983; Dodd 1988; Van Buskirk & Crowder 1994). Mark–recapture data and radio and satellite tagging indicate that the interval between nesting seasons (typically 2–3 years) is spent on near-shore foraging grounds or in transit between foraging and mating/nesting beaches (Musick & Limpus 1997). Mating is believed to occur primarily in breeding aggregations near nesting beaches (Dodd 1988). While adult females regularly return to their natal beaches to nest, it is not known whether males also make philopatric migrations to mate, or whether resident males mate opportunistically with females passing through feeding grounds on their way to the beach (Dodd 1988; Miller 1997). Capture and stranding data, however, indicate that male loggerhead turtles increase in abundance in the near-shore waters of the southeastern US preceding the nesting season (Henwood 1987; S. Hopkins-Murphy, personal communication), and recent aerial observations of mating loggerheads suggest that mating occurs near the rookeries (Frick *et al*. 2000).

The mating system of loggerheads is relevant to conservation and recovery plans. Loggerhead turtles are listed as threatened under the US Endangered Species Act of 1973, and are restricted from international trade by Appendix I listing in the Convention on International Trade in Endangered Species (CITES). Polymerase chain reaction (PCR) studies allow analyses to be conducted from small amounts of blood and small, nonlethal tissue biopsies from hatchlings. Nonlethal retrieval of DNA from hatchlings allowed the collection of large sample sizes without adversely affecting this threatened species. We used these nondestructive technologies to assemble the largest data set to date on sea turtle reproductive genetics.

Materials and methods

Sampling from nesting females and hatchlings

Samples were collected at two locations on Melbourne Beach, FL. Each site covered about 1.5 km of beach, one immediately north of the Archie Carr National Wildlife Refuge (28°2.3′ N, 80°32.6′ W), and the other about 8 km south, within the Refuge (27°57.7′ N, 80°30.3′ W; hereafter described as the northern and southern sites). Using the method of Owens & Ruiz (1980), blood samples were collected from 75 nesting loggerheads at each site between 9 June and 22 June 1996. Blood was stored at room temperature in sodium dodecyl sulphate (SDS)–urea buffer, pH 6.8 [1% SDS, 8 M urea, 240 mM Na_2HPO_4,

1 mM ethylenediaminetetraacetic acid (EDTA)] in an approximately 1:10 ratio of blood : buffer.

Nests were marked after blood collection. Approximately 50 days later, nests were caged to detain emergent hatchlings and to deter predators (Moore 2000). Up to 20 hatchlings from each nest were sampled by biopsy of a rear marginal scute with a 2-mm disposable punch (Fray Products, #BP20). The resultant biopsy was stored at room temperature in 70% ethanol until DNA extraction.

Isolating DNA

Shell biopsies were rinsed with sterile water and dried briefly in a 37 °C oven to evaporate any remaining ethanol before digestion with proteinase K overnight (Palumbi *et al*. 1991). DNA was isolated using a standard phenol–chloroform extraction protocol and ethanol precipitation. Pellets were rehydrated in 10 mM Tris–HCl, pH 8.0, 1 mM EDTA. DNA yields were quantified with a fluorometer (Hoefer DyNAQuant 200, San Francisco, CA).

DNA from 200–400 μL of blood diluted in SDS–urea buffer was extracted using a standard phenol–chloroform extraction and purified by dialysis (White & Densmore 1992; protocol 11). DNA was precipitated with ethanol, re-hydrated, and quantified as above.

Characterization of microsatellites

We constructed a microsatellite-enriched DNA library based on Armour *et al*. (1994) and Karagyozov *et al*. (1993), as modified by the University of Florida Education Core (Moore 2000). Over 300 putative microsatellite clones were sequenced, and many revealed long (> 400 bp) imperfect repeats, repeat sequences that were microsatellites within minisatellites, or microsatellites of a desirable size but with insufficient flanking sequence for primer design.

Because *Taq* polymerase often adds a nontemplated adenine at the end of a replicated strand of DNA, microsatellite PCR products can have additional bands at 1-base pair (bp) intervals from the nonadenylated allele, which can make it difficult to decide which peak to score (Smith *et al*. 1995). To achieve consistent scores, plus-A product formation was encouraged by PIGtailing one primer of each pair (adding 2–4 specific, nontemplated bases on the 5′ end of the primer, as described in Brownstein *et al*. 1996).

Using the computer program PRIMER 3 (Rozen & Skaletsky 1998) and the Operon oligo toolkit web page (http://www.operon.com/toolkit), primers were designed from 11 unique loci containing six or more repeat units and flanked by 20 or more base pairs of unique, nonrepetitive sequence. Primers were synthesized by Operon Technologies, Inc., with one primer of each pair labelled with a fluorescent phosphoramidite dye. Eight of the 11 amplified successfully, and seven were monomorphic in the four individuals tested. One clone, Ccar176, was polymorphic (176F: GGCTGGGTGTCCATAAAAGA; 176R: gtTTGATGCAGGAGTCACCAAG; lower case bases are the PIGtail)[1].

In addition to Ccar176, microsatellites for the mothers were amplified with radiolabelled primers Cc7 and Cc141 (FitzSimmons 1997) and fluorescently labelled primers CCM2 (CCM2F: gtttTGGCACTGGTGGAAT; CCM2R: TGACTCCCAAATACTGCT; Francisco 2001). Clutches with 10 or more sampled offspring were chosen for paternity analysis. Half of these clutches were analysed at all four loci, and half were analysed at only Ccar176 and CCM2. We amplified samples in 25 μL reactions containing 25–50 ng of turtle genomic DNA, 0.12 mM each dNTP, 1.0–1.5 mM $MgCl_2$, 1 × PCR buffer (20 mM Tris–HCl pH 8.4, 50 mM KCl), 0.35 units *Taq* DNA polymerase (Gibco Life Technologies, Rockville, MD), 0.42 μg bovine serum albumin, and 240 nM of each primer (Cc7 and Cc141 forward primers were radiolabelled). Cycling parameters consisted of an initial denaturation at 94 °C for 2 min, followed by 30 cycles of 30 s at 94 °C, 30 s at an optimal annealing temperature (58 °C for CCM2, Cc7, and Cc141; 60 °C for Ccar176), and 45 s at 72 °C, followed by a final 5 min extension at 72 °C. Radiolabelled PCR products from Cc7 and Cc141 were resolved on denaturing 6% acrylamide sequencing gels, and alleles were scored based on comparisons with a sequenced M13 size standard. For CCM2 and Ccar176, one primer of each pair was labelled with either 6-FAM or TET fluorescent dye (Applied Biosystems Inc., Foster City CA). We amplified approximately half of the samples using 6-FAM as the label for both loci, and the remaining samples using TET for both loci. Allele sizes were resolved on an ABI 377 automated sequencer at the Iowa State University Sequencing Facility. Because loci overlapped only slightly in size, they could be distinguished by size and (in the rare cases of size overlap) peak morphology, even if they were the same colour and were co-loaded. Allele sizes were assigned using GENOTYPER and GENESCAN ANALYSIS software (Perkin-Elmer Corp., Foster City, CA). DNA from mothers was run alongside DNA from their offspring. Offspring DNA that yielded novel alleles which indicated a second father was re-amplified and re-run alongside their mother's DNA to confirm results. Negative controls were run with each set of reactions, using 1 μL of sterile Milli-Q water in place of the template (all other reagent concentrations remained the same).

Data analysis

All raw allele scores were binned into named allele categories as described in Rosel *et al*. (1999). Alleles which could not be accounted for if only one mother and one

[1]The sequence of this clone is deposited in GenBank under accession number AF333763.

father contributed to a clutch were deemed to be 'extra' paternal alleles. Null alleles detected by parent–offspring genotype mismatches were assigned an arbitrary number (allele size of 100) and treated like any other allele for analysis.

Samples exhibiting one or more of the following problems were eliminated from the analysis: (i) individuals with raw scores for both alleles that fell conspicuously outside of the observed allele size range (two hatchlings); (ii) in clutches analysed at four loci, if an 'extra' paternal allele indicating a second father appeared in only one hatchling at one locus, this allele was classified as a mutation (two hatchlings); and (iii) offspring lacking maternal alleles when the distribution of alleles could not be explained by the presence of null allele(s) in the mother (four hatchlings). This last problem could have been due to mutation of one of the maternal alleles, or it could provide evidence that these hatchlings were from a different nest (a potential hazard when cryptic nests could introduce hatchlings into our own sampled nests). The possibility remains that hatchlings from a different nest that shared an allele with the mother of a nest in question could have gone undetected and made it appear that there was a second father when there was none. The chances of this happening, however, were small: in addition to wayward offspring of another nest sharing an allele with the nest in question, the females would have had to nest very close to each other, temporally and geographically, for the nests to hatch at the same time and place.

Parental genotype and allele frequencies were analysed with an approximation of Fisher's exact test as implemented in GENEPOP (Raymond & Rousset 1995) to determine if: (i) maternal allele frequencies were in Hardy–Weinberg equilibrium; and (ii) maternal allele and genotype frequencies differed between sampled locations. F_{ST} was also estimated using GENEPOP (Raymond & Rousset 1995), and R_{ST} was estimated using FSTAT (Goudet 2000).

Mean paternity exclusion probabilities, or probabilities per individual of excluding putative fathers selected from the population at random, were calculated for each locus and across multiple loci as reviewed in JA DeWoody *et al*. (unpublished manuscript; formulae available at www.genetics.uga.edu/popgen/parentage.html). Since four hatchlings in our sample lacked maternal alleles, indicating that they may have hailed from different nests, exclusion probabilities were calculated with two different formulae: one assumed the mother was known with certainty (P_{EK} = per locus paternity exclusion probability, P_{CEK} = cumulative across loci) and another which assumed neither parent was known (P_{EU} = per locus parental exclusion probability, P_{CEU} = cumulative across loci; DeWoody *et al*. 2002; also see Westneat *et al*. 1987; Dodds *et al*. 1996). Pairs of loci were tested for linkage disequilibrium using a Markov chain method as implemented in GENEPOP (Raymond & Rousset 1995). Data from mothers from northern and southern sites were pooled for tests of deviation from Hardy–Weinberg equilibrium and linkage disequilibrium.

Results

Population genetic analysis

Of the 150 nests originally marked in this project, 88 nests were located again, caged, and produced hatchlings. Seventy nests were chosen for analysis because they each had at least 10 sampled offspring. From these nests, 683 hatchlings yielded DNA of sufficient quantity and quality to amplify at least one locus, with 95% of hatchlings amplifying at more than one locus. DNA from all 150 mothers amplified for at least two of the four tested loci, with 90% of maternal and hatchling samples amplifying at all assayed loci. Loci had 10–20 alleles and varied in observed heterozygosity from 0.73 to 0.90 (see Fig. 1 for allele frequency distributions). Analysis of variance revealed no population differentiation between mothers from northern and southern sites (overall $F_{ST} < 0.001$, $P = 0.23$; overall $R_{ST} = -0.001$); mothers were pooled for subsequent analyses. All loci were in Hardy–Weinberg equilibrium ($P = 0.23$ for Cc141, $P = 0.34$ for Cc7, $P = 0.61$ for Ccar176, and $P = 0.24$ for CCM2). Linkage disequilibrium was not detected for any pair of loci. The paternity exclusion probability with a single locus and one known parent varied from $P_{EK} = 0.55$ to $P_{EK} = 0.74$. With neither parent known, P_{EU} ranged from 0.37 to 0.58 (Fig. 1). The known-mother paternity exclusion (P_{CEK}) and unknown-parents parental exclusion (P_{CEU}) probabilities with two loci (CCM2 and Ccar176) were $P_{CEK} = 0.86$ and $P_{CEU} = 0.70$, and for all four loci, $P_{CEK} = 0.98$ and $P_{CEU} = 0.93$.

Multiple paternity

We assumed that clutches with three to four paternal alleles represented offspring from a mating between one female and two male loggerhead turtles. If more than four paternal alleles were present, the clutch was designated as having a minimum of three fathers. No clutch had more than five paternal alleles. Clutches that were determined to have three fathers often had fewer than five alleles, but in these cases the distribution of alleles across loci could not be attributed to just two fathers. Based on these criteria, 22 clutches had at least two fathers, and six of these 22 had at least three fathers (Table 2).

Due to alleles shared between mothers and fathers, we could not unequivocally assign paternal genotypes, so maternal and paternal allele frequencies could not be compared to determine if fathers and mothers represented one population. Likewise, we could not address the possibility of unequal paternal contribution.

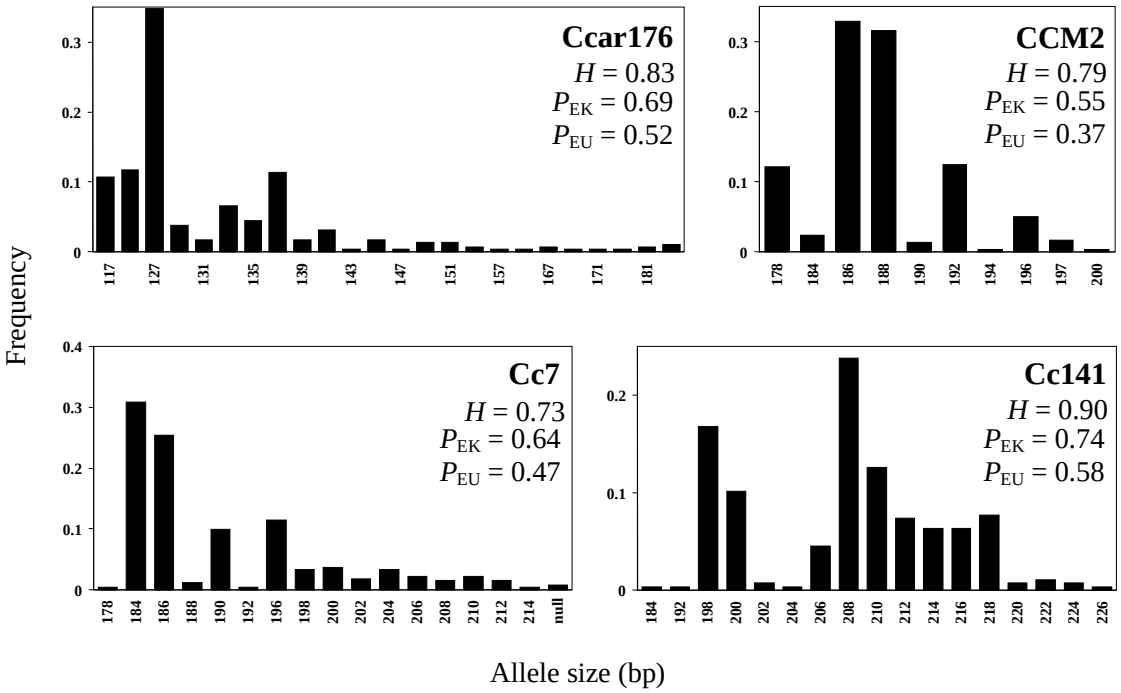

Fig. 1 Maternal allele frequencies, observed heterozygosities (H), per locus paternity exclusion probabilities (P_{EK}) and per locus parental exclusion probabilities (P_{EU}) for four microsatellite loci. Details of allele frequency distribution are available in Moore (2000) or from the lead author.

Table 2 Number of clutches with one, two, or three fathers

No. of fathers observed	No. of clutches	Percentage of total
1	48	68.6
2	16	22.8
3	6	8.6

Discussion

Multiple paternity was common, with 31% of all nests possessing multiple fathers. Almost 10% of the multiply fathered nests had three or more fathers — more fathers than have previously been reported for sea turtle species.

Population structure was not detected between female loggerheads nesting in the northern and southern sites of Melbourne Beach, FL. This was not surprising, as loggerheads are less site-specific than green turtles at this location (D. Bagley & L. Ehrhart, personal communication), and green turtles did not exhibit distance-related structure on this beach (Peare & Parker 1996). Also, recent work on loggerheads in Florida found that population structure in the nuclear genome was detectable only on a scale similar to that found in Mediterranean loggerheads (about 100 km; Francisco 2001). The mitochondrial DNA (mtDNA) structure in the Mediterranean may also be a remnant of recent colonization (< 12 000 years ago) by two different matrilines after the last glacial period, and philopatry may be slowing the decay of founder-induced structure (Schroth *et al*. 1996).

The resolution of multiple paternity using molecular techniques is important in elucidating the basic biology of this little-known species. Although mating aggregations of loggerheads, including 'courting groups', have been reported by fishermen in Australian waters, this behaviour is not commonly observed (Limpus 1985). Researchers have sighted isolated mounted pairs of loggerheads in Australia (Limpus 1985), and recent aerial observations of mating loggerheads in the southeastern USA reported only solitary courting pairs (Frick *et al*. 2000). This is a notable absence in a region that may host 70 000 nests per year (Florida Marine Research Institute 2000). Aggregations and competition for females may be reduced in the Melbourne Beach population because of a highly female-biased sex ratio. Hatchlings leaving beaches at Cape Canaveral, FL (40 km north of our study site) were 93% female due to temperature-dependent sex determination and the feminizing effects of warm sands (Mrosovsky & Provancha 1989). Sex ratios of juvenile loggerheads captured at Cape Canaveral were less skewed (63% female; Wibbels *et al*. 1987), but these animals probably originated from rookeries in North Carolina and South Carolina as well as Florida, and the more northern (cooler) rookeries probably contributed a higher proportion of males. The sex

ratio of breeding adults contributing to Melbourne Beach nests is unknown. However, if male loggerheads are philopatric like Australian green turtles (FitzSimmons *et al*. 1997), and survivorship of male and female hatchlings is approximately equal, then female turtles should far outnumber male turtles on the breeding grounds (even if male turtles return to the rookery area more frequently than the average 2–3-year interval for females).

Female loggerheads probably mate at the beginning of the nesting season and store sperm to fertilize the clutches for that season (as female green turtles do), allowing ample opportunity for sperm mixing and competition (Miller 1997; FitzSimmons 1998). Allozyme data suggest that multiple paternity is common in Australian loggerheads (33% of clutches examined), but because of the low level of detected polymorphisms, this inference is based on deviations from expected Mendelian patterns of inheritance, which can be attributed to other factors as well; in only one clutch was multiple paternity verified by the presence of an 'extra' paternal allele (Harry & Briscoe 1988). Loggerhead clutches from Melbourne Beach have a high incidence (at least 31%) of multiple paternity, second only to that of Kemp's ridleys (≥ 58%; Kichler *et al*. 1999). This study shows, for the first time, strong evidence for the genetic contributions of more than two fathers to a clutch. There were certainly fathers that went undetected, either because their offspring were not sampled (10 eggs were analysed from clutches that average about 113 eggs; Van Buskirk & Crowder 1994), or because they shared alleles with other fathers. Therefore, 31% is a conservative estimate of the number of multi-fathered clutches.

The advantages of multiple matings are many. If the adult sex ratio is skewed in this population, it may be that females choose to mate with the first male that they encounter to ensure fertilization, and then they can 'upgrade' if they happen upon a better male later on (Birkhead *et al*. 1993; Evans & Magurran 2000). Avoidance of genetic incompatibility (Zeh & Zeh 1996; Newcomer *et al*. 1999; Vala *et al*. 2000) and selection of competitive sperm (Smith 1984; Watson 1991; Madsen *et al*. 1992; Baer & Schmid-Hempel 1999) are also plausible hypotheses for maintaining polyandry in sea turtles. However, among the arguments for polyandry, the genetic diversity hypothesis is particularly attractive when considered in the light of known life history patterns. Although females are philopatric, they must occasionally deviate from natal homing in order to colonize new beaches, as appropriate nesting habitats come and go over evolutionary time (Bowen *et al*. 1989, 1992). These wayward, polyandrous, gravid females who colonize new nesting beaches carry with them much more of the diversity of their natal rookery than they would had they mated with only one male. This is particularly important in light of the theory that new nesting beaches are colonized by just a few gravid females (Bowen *et al*. 1992).

Whichever mechanism is operating to maintain polyandry in loggerheads, there is increasing evidence across taxa that sperm competition and postcopulatory female choice can bias paternity in the offspring of polyandrous females, and that their offspring gain fitness benefits (Birkhead *et al*. 1993; LaMunyon & Eisner 1993; Olsson *et al*. 1996; Zeh *et al*. 1998; Evans & Magurran 2000; also see Zeh & Zeh 1996; Jennions & Petrie 2000 for reviews). Whether these benefits were hypothesized to be due to mate choice, genetic diversity, or avoidance of genetic incompatibility or inbreeding, the positive effects of polyandry on the offspring were demonstrable, and there is no reason to suspect that loggerheads would not likewise benefit from multiple matings.

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