

Multiple Paternity of a Lemon Shark Litter (Chondrichthyes: Carcharhinidae)

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Despite the importance of sharks to marine ecosystems as top predators and growing concern over the conservation status of many shark species, surprisingly little is known about many aspects of their reproduction patterns and life history. Better knowledge of breeding biology and reproductive parameters will be important for designing appropriate management plans to protect dwindling populations of sharks. Here, we report new information regarding the mating system and reproductive cycle of a large coastal shark, the lemon shark *Negaprion brevirostris*, revealed through field observations and genetic analyses of an adult female and her offspring. Our findings demonstrate that this female exhibited philopatry to a nursery ground in Bimini, Bahamas, where she returned to give birth in both 1996 and 1998. Genetic analyses using DNA microsatellite loci developed for lemon sharks provided the first demonstration of polygamous mating and multiple paternity in a carcharhinid shark; at least three males had sired the litter she delivered in 1998.

RELATIVELY little information is available on breeding behavior, demographics, or population structure of most shark species and of elasmobranchs in general. Sharks generally grow very slowly and may take many years to reach maturity. Few young are born to each female at long interbirth intervals in contrast to most teleost fishes. Thus, maternal investment in each offspring is substantial, and recruitment rates will be low. Successful recruitment of some sharks seems to depend on the integrity of critical nursery grounds, specific areas where females go to give birth and where juvenile sharks may remain for extended periods.

Here we report findings regarding the mating system and breeding biology of the lemon shark, *Negaprion brevirostris*. Lemon sharks have a disjunct distribution and are found in western Atlantic waters from New Jersey to Brazil, in coastal Atlantic waters off west Africa, and in the eastern Pacific from Baja California to Colombia (Bigelow and Schroeder, 1948). Almost all carcharhinids, including lemon sharks, are viviparous, with transfer of nutrients from mother to embryo occurring through a yolk sac placenta. All sharks have internal fertilization; males possess paired claspers, intromittant organs for sperm transfer, but mating has rarely been observed in free-living sharks (Johnson and Nelson, 1978; Rouse, 1992; Carrier et al., 1994). Thus, information about shark behavioral mating systems is very limited, and genetic studies assigning paternity to offspring are completely lacking.

A long-term field and genetic study of a relatively undisturbed population of lemon sharks is in progress in Bimini, Bahamas, as well as sev-

eral other nursery sites in the eastern Atlantic. Bimini is a small, mangrove-fringed island cluster on the western edge of the Great Bahamas Bank that serves as a nursery ground for approximately 250 juvenile lemon sharks (Morrissey and Gruber, 1993). The objectives of our research are to characterize the use of the nursery grounds by female lemon sharks, to elucidate the genetic mating system and to characterize the population genetic structure of this species using genetic markers.

Studies on mating systems and stock structure in sharks have been hampered by the lack of genetic variability at allozyme (Smith, 1986; MacDonald, 1988; Lavery and Shaklee, 1989) and mitochondrial DNA loci (Heist et al., 1995, 1996), perhaps because sharks have a lower rate of molecular evolution than other vertebrates as suggested by Martin (1995, 1999). Therefore, we chose to develop a relatively new class of genetic markers, DNA microsatellites, for our lemon shark studies (Ashley, 1999). The usefulness of microsatellite loci for studies of population biology and mating systems has been widely demonstrated, although their application to studies of elasmobranchs has been extremely limited. With the exception of a recent paper by Heist and Gold (1999) reporting three microsatellite loci in the sandbar shark (*Carcharhinus plumbeus*), to our knowledge no studies have developed or applied microsatellite analysis to elasmobranch populations. This is surprising given the widespread and expanding use of microsatellites in studies of teleost fishes (e.g., McConnell et al., 1995; Pouyaud et al., 1999; Zane et al., 1999). We constructed a genomic library for lemon sharks, screened it for micro-

TABLE 1. DESCRIPTION OF THREE VARIABLE *Negaprion brevirostris* MICROSATELLITE LOCI INCLUDING REPEAT MOTIF, SIZE OF PCR PRODUCT, NUMBER OF ALLELES (A), PRIMER SEQUENCE, OBSERVED, AND EXPECTED HETEROZYGOSITIES, AND NUMBER OF INDIVIDUALS SCORED (n).

Locus	Repeat motif ^a	Primer sequence (5'-3') ^b	Size ^a	A ^c	N ^d	Ho	He
LS15	(CA)20	TGCGTGGGTTGTTGTTTGG GCACCTTGGATAGTTGAGCAGG	154	22	104	0.846	0.823
LS22	(CA)23	TTTCCTTGAGCCAGTTGTGGTG TTTGATACTGCTGGGGTCAGG	137	16	198	0.899	0.896
LS30	(CA)25	ACGTTAAATATCTCAGGCTCAGAG CCTTATTAACAACCTCAAACCTCGCC	196	13	181	0.718	0.725

^a Determined from sequenced clone.

^b Forward primer listed first. Reverse primer listed second.

^c Determined from juvenile and subadult lemon sharks caught at Bimini, Bahamas.

^d Juvenile and subadult lemon sharks caught at Bimini, Bahamas.

satellite repeats, and designed PCR primers from the flanking regions of several long dinucleotide repeats. Here, we report data from three of these loci that we show to be extremely variable among adult and juvenile lemon sharks. Thus, this class of markers can potentially provide an important new tool for population genetic studies of cartilaginous fishes. Further, we used these three microsatellite loci to genotype a mother lemon shark and 11 of her 13 pups to determine whether this litter had a single or multiple sires.

MATERIALS AND METHODS

Sampling.—As part of our larger population study, we captured a 2.4-m adult female lemon shark at Bimini in April of 1996, using long-line fishing. We measured her, injected her with a passive integrated transponder microtag (PIT 22423E172A), and took a small fin clip for DNA analysis. Field notes and video images indicated that this female was clearly gravid at the time of capture and likely gave birth soon after her release. We subsequently spotted this female at approximately the same location in Bimini lagoon in April 1998 and caught her by chasing her down with a skiff and netting her once she tired. At the time of her second capture, the female was again gravid, and we assisted her in giving birth to 13 lemon shark pups, of which 11 were tagged, measured, and sampled at the time of birth. After sampling, pups were brought to the nearest mangrove patch and released. Additional samples from Bimini, used to characterize genetic variability at microsatellite loci (Table 1), are comprised primarily of fin clips from juveniles collected using monofilament gill nets set up in the North Sound region of Bimini lagoon.

Genetic analyses.—Lemon shark microsatellite primer pairs were developed following a common screening protocol (Dow et al., 1995; Ashley and Dow, 1994). Briefly, DNA from a subadult lemon shark caught at Bimini, Bahamas, was extracted and used to construct a genomic library of small (200–1500 bp) fragments. Size-selected Sau3AI fragments were ligated into pUC18 vector and transformed into *Escherichia coli* competent cells. Bacterial colonies were lifted onto nylon membranes and screened with a cocktail of chemoluminescently labeled di- and trinucleotides following the manufacturer's protocol (Amersham ECL 3'-oligo-labeling and detection system). Positive colonies were grown overnight and plasmid DNA was extracted and used for sequencing (Amersham Thermo-Sequenase radiolabeled cycle sequencing kit). Sequencing reaction products were run on polyacrylamide gels and exposed to autoradiograph film. Primers were developed from the sequences flanking the core repeat with the aid of MacVector software (IBI). Primers were fluorescently labeled and used in PCR reactions.

Genomic DNA from the mother, pups, and many additional individuals from Bimini was extracted from fin clips following a salting-out protocol (Sunnucks and Hale, 1996). PCR conditions for the three primer pairs are described elsewhere (Feldheim et al., 2001). PCR products were electrophoresed on an ABI 373A automated sequencer along with a fluorescently labeled size standard (Tamra-350 PE, Biosystems). Genescan and Genotyper software (PE Biosystems) were used to score PCR products and to create electropherograms for each individual. Heterozygosities were calculated using the GDA computer program (vers. 1.0, P.O. Lewis and D. Zaykin, 2000, unpubl.). Pups were placed into full sib groups if they shared paternal alleles

TABLE 2. GENOTYPES OF A MOTHER LEMON SHARK, HER LITTER, AND THE THREE PUTATIVE MALES AT THREE MICROSATELLITE LOCI. Paternal alleles are in **bold**. ? indicates the male is either homozygous or has a second allele that was not inherited by the offspring.

Shark PIT#	<i>LS15</i>	<i>LS22</i>	<i>LS30</i>	Father
E172A-mother	154/197	147/147	206/206	
12549	154/ 199	125 /147	206 /206	Male1
34F26	154/ 199	125 /147	206/ 230	Male1
65C57	189 /197	141 /147	206/ 212	Male3
67205	154/ 219	125 /147	206/ 230	Male1
75747	154/ 217	141 /147	206 /206	Male3
7703A	154/ 219	147 /147	206 /206	Male1
97E52	197/ 199	125 /147	206 /206	Male1
B0338	154 /154	125 /147	196 /206	Male2
E006B	197/ 199	125 /147	206 /206	Male1
F0813	154 /154	147/ 165	196 /206	Male2
F5878	197/ 199	125 /147	206/ 230	Male1
Male1	199 / 219	125 / 147	206 / 230	
Male2	154 /?	125 / 165	196/?	
Male3	189 / 217	141 /?	206 / 212	

over the suite of loci. This grouping allowed partial genotypes of three putative males to be constructed (Table 2).

RESULTS

Developing useful microsatellite loci for lemon sharks proved to be an extremely laborious endeavor. We screened over 13,000 lemon shark genomic DNA colonies and found 82 microsatellites, a rate lower than typical for teleost fishes (Brooker et al., 1994; Colbourne et al., 1996). Through these efforts, however, we found several extremely long microsatellite repeats in the lemon shark genome. Of these, primers were developed and tested for several loci. Three loci, *LS15*, *LS22*, and *LS30* (Table 1) were determined to be highly variable. These three loci were comprised of (CA)_n repeats with *n* ranging from 20–25. For this study, over 100 juvenile lemon sharks from Bimini have been genotyped at these three loci (Table 1). The number of alleles ranged from 13–22 and heterozygosities (observed and expected) exceeded 0.70. The high heterozygosities and number of alleles make these three loci ideal for population and parentage studies in general and specifically for inferring paternities of the female and her litter.

Microsatellite genotypes of the female and her pups indicate that, although each pup had inherited one maternal allele at each locus, five paternal alleles were observed at *LS15* and four each at *LS22* and *LS30* (Table 2). Because any male will have only one allele (if homozygous) or two alleles (if heterozygous) at each locus,

these findings indicate that the female had mated with multiple males, and at least three males had sired her offspring. Reconstruction of the putative paternal genotypes (Table 2) indicates that one male had sired seven of the pups, and two different males had each sired two pups.

DISCUSSION

Our study provides the first demonstration of paternity analysis using highly variable microsatellite loci for an elasmobranch. We suspect that the previous paucity of shark microsatellite studies stems from two factors, a relatively small number of population geneticists working on sharks and technical difficulties associated with elasmobranch microsatellite development. With regard to the latter, evidence from our laboratory and that of Heist and Gold (1999) suggest that microsatellites are relatively rare in the genomes of carcharhinids and that relatively short microsatellites show low levels of polymorphism. Heist and Gold (1999) report that only 14 of 2880 clones of sandbar shark genomic DNA contained microsatellite sequences, and, of these, only three were suitable for primer design. These three were short (CA)_n repeats having only 6–9 repeat units in the cloned allele. These loci were only moderately variable, with 2–5 alleles and expected heterozygosities of 0.067–0.535. These authors report that microsatellites were similarly rare in blacktip sharks. They conclude that polymorphic microsatellite loci occur at low frequency in carcharhinids relative to teleosts and mammals and question

their utility for population genetic studies in such species.

Unlike Heist and Gold (1999), we were able to find extremely variable microsatellite loci in lemon sharks, although our results support their conclusion that microsatellites are relatively rare in carcharhinid genomes, and additional screening efforts are needed. For the long repeats used in this study, levels of allelic diversity and heterozygosity (Table 1) are as high as, if not higher than, that typically found in vertebrate microsatellite studies. Thus, the potential utility of microsatellite analysis for shark population studies has been demonstrated. Indeed, microsatellite data from a single female lemon shark and her litter reveal several important new findings about the breeding biology of this species and possibly other large sharks.

The adult female studied here was captured in April 1996 and 1998 in Bimini lagoon and was gravid both times. Therefore, we have demonstrated that this female shark exhibited site fidelity for parturition. The home range of adult lemon sharks is unknown, but tag returns demonstrate that it encompasses many tens of thousands of square kilometers (SG, unpubl. data). Therefore, we feel it is unlikely that site fidelity of this female is simply a result of a restricted home range. Because young lemon sharks are highly site attached and may remain in their natal lagoon for several years (Gruber et al., 1988), selection of sites for delivering pups is an important aspect of reproduction and recruitment. In parallel studies of marine turtles, it will be interesting to determine whether either of two behavioral models of female site fidelity are operating in lemon sharks: natal homing (where females return to their natal nursery to reproduce) or social facilitation (where first-time breeders follow experienced breeders to nursery sites and subsequently return; Fitz-Simmons et al., 1997). Long-term tagging studies, as well as examination of the genetic population structure for both nuclear and mitochondrial genes, should provide evidence for alternative models and whether site fidelity encompasses mating sites as well as parturition sites. Numerous females with fresh mating wounds have been observed at Bimini, providing evidence that it may also serve as mating grounds. Understanding these aspects of shark breeding biology will be important for management. Fidelity for mating sites could result in genetic structure and distinct stocks for management even in highly vagile marine species. Clearly, protection of nursery habitat will be vital for the maintenance of many shark species. For lemon sharks in the Caribbean, intact man-

grove lagoons appear critical for successful recruitment. The degree of site fidelity for nurseries will determine whether recovery must occur through intrinsic growth of populations using specific nursery areas, or alternatively, whether migration can replenish stocks of depleted populations.

The reproductive cycle for this female appeared to be biennial. Although an annual cycle cannot be ruled out (she was not captured in 1997; thus, her reproductive status for this year is unknown), the observation of two classes of females at Bimini, those with fresh mating wounds and intact gravid females, provides further support for biennial reproduction (SHG, pers. obs.). A two-year reproductive cycle comprised of alternating ovarian and gestational cycles may be typical of carcharhinids (Castro, 1996) and in lemon sharks likely consists of a spring and early summer mating period (Springer, 1950), a 10–12 month gestation (Springer, 1950), parturition of 4–17 young, followed by a year for oogenesis and vitellogenesis. Sexual maturity in lemon sharks is not reached until 12–16 years of age (Brown and Gruber, 1988). Given these life-history characteristics and low fecundity, it is clear that recovery of depleted stocks of large sharks will take many years, and recruitment rates may be insufficient to sustain current rates of directed and bycatch fishing mortalities (Manire and Gruber, 1990; Hueter, 1998).

A final important finding is that the litter of this female lemon shark had been sired by multiple males, indicating that female lemon sharks mate polygamously. By reconstructing putative paternal genotypes (Table 2), we found that one male had sired seven of the offspring, and two males had sired only two offspring each. The mating and courtship practices of elasmobranchs are poorly characterized, and the degree of mate choice that females are able to exercise is unknown. Shark mating involves biting by the male to gain physical purchase of the female, and thus females commonly exhibit tooth lacerations during mating season. In nurse sharks, *Ginglymostoma cirratum*, multiple males were observed pushing and grasping the same female during mating activities, although only a small percentage of mating attempts ended in copulation because of avoidance behavior by the female (Carrier et al., 1994). Perhaps males helping one another “roll” a female take turns mating with her. Alternatively, helper males may be younger males developing the necessary mating skills they need as they reach sexual maturity (J. C. Carrier, pers. comm.). Multiple paternity may not, however, result

from females copulating with more than one male during the same mating episode but may result from mating with different males over a protracted period. The oviducal gland of female sharks may allow for sperm storage; active spermatozoa have been reported in the oviducal glands of several shark species (Metten, 1941; Pratt, 1979; Pratt and Tanaka, 1994), and stored sperm may be viable for over a year (Castro et al., 1988; Pratt and Tanaka, 1994).

In conclusion, our findings demonstrate that microsatellite analysis combined with nondestructive field sample collections can provide answers to previously intractable questions of elasmobranch population biology and behavior. Female lemon sharks have the potential to breed with multiple males and can use the sperm of several males to produce litters comprised of both half-sib and full-sib offspring. Evidence for philopatry to specific nursery grounds is also reported. Further investigations are needed to determine whether the findings from this female are common within the species and whether they extend to other large coastal shark species.

Note added in proof.—On 10 May 2001, a gravid female shark was caught and sampled 1 km south of Bimini, Bahamas. She gave birth to 18 pups, which were also sampled, and these are currently being genotyped in Ashley's laboratory at UIC.

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