

Assessment of multiple paternity in single litters from three species of carcharhinid sharks in Hawaii

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Abstract We tested for presence or absence of multiple paternity in single litters from each of three congeneric shark species in Hawaii: the sandbar shark, *Carcharhinus plumbeus*, bignose shark, *Carcharhinus altimus*, and Galapagos shark, *Carcharhinus galapagensis*. Based on eight polymorphic microsatellite loci, we excluded paternity by a single sire in sandbar and bignose sharks, but could not exclude a single sire for the litter from the Galapagos shark. This study doubles the number of shark species tested for multiple paternity, and is the first demonstration of multiple paternity in sandbar and bignose sharks.

Keywords Reproduction · Microsatellites · Polyandry · Conservation · Elasmobranch · Fisheries

Introduction

Mating in coastal sharks is seasonal and success may depend on the rate of encounter between potential

mates, especially in small populations. The likelihood of successful mating is increased by seasonal aggregations, but not all species aggregate. Commercial exploitation could decrease the rate of encounter due to declines in abundance or changes in the sex ratios of mature individuals. Life history traits in sharks, characterized by low fecundity and intrinsic rates of increase, are more comparable to those in cetaceans than to other fishes (Smith et al. 1998; Musick 1999). As a result, sharks are highly vulnerable to disturbance and particularly slow to rebound from population depletion (Smith et al. 1998). Therefore, insight into the mating systems of sharks likely has direct implications for understanding the reproductive consequences of exploitation.

Multiple paternity occurs when a single brood of offspring is fertilized by multiple males. Female sharks sometimes engage in multiple matings (polyandry) (Carrier et al. 1994), and have the ability to store viable sperm in the oviducal or shell gland for up to a year after mating (Pratt 1993). Sperm storage provides the potential for sperm mixing in the oviducal gland and for insemination of eggs by multiple sires. Multiple paternity has been reported previously in nurse sharks, *Ginglymostoma cirratum* (Saville et al. 2002), lemon sharks, *Negaprion brevirostris* (Feldheim et al. 2001; 2004), and bonnethead sharks, *Sphyrna tiburo* (Chapman et al. 2004). The occurrence of multiple paternity in all three species studied to date indicates that this strategy may be common in sharks. Further, the two studies that examined

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frequency of multiple paternity (Chapman et al. 2004; Feldheim et al. 2004) indicate that the prevalence may vary between species, populations and even between conspecific individuals.

Hawaii hosts one of the few unexploited habitats for coastal sharks, due in part to a ban on inshore longlining and to the status of the shark as an 'Aumakua, or traditional Hawaiian spirit guardian. The State of Hawaii conducted a number of shark control programs from 1959 to 1976. The goal of these programs was to cull large sharks from coastal waters in hopes of reducing the already rare occurrence of shark attacks. Attempts to re-establish the Hawaii Shark Control Program in the early 1990's were prevented by public pressure. This local concern fortified the perception that killing sharks is taboo in Hawaii. Cultural and legal mandates therefore provide a rare opportunity to gather data on these three species without the confounding effects of anthropogenic mortality.

From 2002 to 2005, a research program was conducted to survey sharks and other elasmobranchs outside Kaneohe Bay, Oahu. In the course of this survey, we collected single litters from each of three species of carcharhinid sharks and tested them for multiple paternity: the sandbar shark, *Carcharhinus plumbeus*, the bignose shark, *Carcharhinus altimus*, and the Galapagos shark, *Carcharhinus galapagensis*. The sandbar shark is a coastal species with a circumglobal distribution in tropical and warm temperate seas. It is targeted by large-scale fisheries throughout much of this range and was depleted in the western North Atlantic during the 1980s (Musick et al. 1993). Very little is known about the biology of the bignose shark. This species is easily confused with the sandbar shark and the dusky shark, *Carcharhinus obscurus* (Grace 2001), and misidentification is probably common. They are rarely reported in Hawaii, comprising 0.3% of the catch (or 1 out of 300) in the Hawaii Shark Control Program (Haight and Dalzell 2000). Bignose shark distribution is similar to that of the sandbar shark, though bignose typically inhabit deeper waters associated with the edges of continental shelves (Compagno 1988). The bignose shark is not targeted commercially, though it is taken as bycatch in longline fisheries (Berkeley and Campos 1988; Anderson and Ahmed 1993). The Galapagos shark is circumtropical in distribution and is usually associated with oceanic islands and sea-

mounts. Galapagos sharks are taken as bycatch in many tropical fisheries and may be threatened with local extirpation in some regions (Compagno 1988).

Reproductive biology can have a major influence on the genetic diversity of a population and is therefore of particular interest to conservation biologists (reviewed by Neff and Pitcher 2005). The mating systems of sharks and other elasmobranchs is an area that has received growing attention in the past few years due to worldwide overexploitation and vulnerability. Despite the increased research effort, comparatively little is known about shark reproduction. The expected outcome of observed polyandrous behavior (Carrier et al. 1994) is multiple paternity, a common reproductive strategy in vertebrates and invertebrates (Neff and Pitcher 2005). Here we test for multiple paternity in three shark species. This preliminary study opens the door for further work on what appears to be a common reproductive strategy for sharks, and may have population-level consequences for shark species all over the world.

Materials and methods

Collections took place 5 km outside Kaneohe Bay on the Eastern coast of Oahu at approximately 80 m depth. During the sampling period (September 2003–September 2004) we captured one gravid female from each shark species and collected small samples of fin or muscle tissue from each mother and all pups. The sandbar shark measured 140 cm precaudal length (PCL) and was caught at 21°30' N, 157°47' W; the bignose shark was 175 cm PCL and was caught 21°30' N, 157°46' W; and the Galapagos shark was 194 cm PCL and captured at 21°32' N, 157°48' W.

Tissue was stored in 20% dimethylsulfoxide (DMSO)-saturated salt buffer (Seutin et al. 1991) or 75% ethanol (EtOH). DNA was extracted using a salting-out protocol adapted from Sunnucks and Hales (1996). Samples stored in EtOH were squeezed with a KimWipe and dried in a speed vacuum for 1 h at 55 °C before extraction. Unlabeled forward primers came from Integrated DNA Technologies, Inc. (Coralville, Indiana). Reverse primers from Applied Biosystems (Foster City, CA, USA) were labeled with 6-FAM, VIC, NED, and PET proprietary dyes (see Table 1). Microsatellite loci from pups (7 pups

for the sandbar shark, 9 in the bignose shark, and 7 in the Galapagos shark) were amplified by PCR and compared to those of the mother. We used eight microsatellite primer pairs (Cli-110, 108, 107, 106, 103, 102, 12, and 7) developed for the blacktip shark, *Carcharhinus limbatus*, by Keeney and Heist (2003). Keeney and Heist (2003) showed successful cross-amplification of their loci in a number of congeneric species, including those reported here, and we followed their published protocols. We then tested for heritability and quantified allelic variation at all eight loci for this study.

PCR reactions consisted of 0.1 U Biolase *Taq* DNA polymerase (Biolone; Randolph, MA, USA), 1× *Taq* buffer, 0.25–0.0625 μm of each primer (see Table 1), 200 μm each dNTP, and 2.0 mm MgCl₂. PCR amplification on a MyCycler (Bio-Rad; Hercules, CA, USA) consisted of an initial denaturation at 95 °C for 4 min, followed by 30 cycles of 1 min at 95 °C, 30 s at optimal annealing temperature (see Table 1), and 30 s at 72 °C, followed by a final extension at 72 °C for 20 min. PCR products were resolved with an ABI 3100 automated sequencer and visualized using ABI PRISM GeneMapper Software version 3.0 (Applied Biosystems, Foster City, CA, USA).

The minimum number of mates for each female was inferred from the number of non-maternal alleles

detected among the pups (after Toonen 2004). Mendelian inheritance of maternal alleles was tested in each litter using a χ^2 -goodness of fit test against an expected 1:1 inheritance ratio of maternal alleles. Although the genotypes of the sires for each litter are unknown in these field-collected animals, we assume conservatively that every female mated with only heterozygous males. Given this assumption, the minimum number of sires required to generate the allelic distributions detected in each litter is estimated by one-half the number of non-maternal alleles. For cases in which there were an odd number of non-maternal alleles detected among the pups, estimates of the minimum number of males were rounded up. For example, if three non-maternal alleles are detected, the minimum estimated number of mates must be rounded up from 1.5 to 2 males.

Results

The alleles detected in this study did not differ from those reported by Keeney and Heist (2003), and we found at least three non-maternal alleles at each of two loci (four separate loci in total) in the litters of sandbar and bignose sharks. Therefore single paternity was rejected by at least two independent loci for these litters (Tables 2 and 3). We found no such

Table 1 Microsatellite loci from Keeney and Heist (2003), including locus name and dye label

Primer name	Sequence	<i>T_a</i> (°C)	Primer concentration (μm)	Dye label
Cli-112	F: AGAGAGGGAGGAAGGTGGGC R: TCACGGTTTCTTCAACTCTCCC	66	0.125	PET
Cli-110	F: GAGGGAAGACTTAAACACAAGG R: TTTCTTTGGCTGTCGCTG	62.3	0.25	NED
Cli-108	F: TCACTGGGTTAGACACTTCC R: CCACAGTCAGAAAACAAATTG	56.3	0.125	PET
Cli-107	F: GGATTCACAACACAGGGAAC R: CTCATTCTTAGTTGCTCTCG	56.3	0.25	PET
Cli-106	F: GATTCTACAACCGCAACATTTCG R: GCTCCCCTAACTATTCCACGC	52.3	0.0625	6-FAM
Cli-103	F: GCTTCATTCCATGAGAG R: TTTCTCTGCTCGGTGTTTC	46.9	0.25	VIC
Cli-102	F: GACTGGCTGACCTAACTAAGC R: ATCCTGTGGTCCTTCTATC	50.8	0.0625	6-FAM
Cli-12	F: TCCCAGTCACATTTACACATGC R: GGAAGACCATTGAACCCAATC	54.4	0.0625	VIC
Cli-7	F: TAGCACACAGCCCCATCACT R: TGTGGTGTGAGCCTCATTC	59.6	0.25	NED

Our optimal annealing temperature (*T_a*) and optimal primer concentration are also shown. In all cases the reverse primer was labeled

evidence in the Galapagos litter and were therefore unable to reject single paternity in this sample (data not shown). Two to seven alleles were detected at each locus in the 23 total pups among the three species. Each pup possessed at least one of the alleles detected in the mother, and χ^2 -tests confirmed that inheritance of these alleles did not vary from predicted 1:1 Mendelian inheritance ratios within each litter (df=1, $P > 0.05$). Although our power with a single litter is very low, we did not see any evidence for null alleles at any locus for any of the three litters.

Our estimate of the minimum number of sires per litter is conservative because this method will underestimate the genetic contribution of homozygous sires, or those which have an allele in common with the mother shark (Toonen 2004). Because heterozygosity is low in sharks compared to other taxa (Heist 2004), the likelihood of shared alleles between parents is high. We found three paternal alleles present in both the sandbar and bignose shark litters; therefore at least two male sharks must have fertilized these litters.

Discussion

After genotyping a single litter from each species, we can reject single paternity for sandbar and bignose sharks, but not for the Galapagos shark. However, it remains possible that multiple paternity occurs in all three congeners, but either did not occur or escaped detection in this particular Galapagos shark litter.

Table 2 Allelic evidence of multiple paternity at two loci (Cli-108 and Cli-106) in the sandbar shark, *C. plumbeus*

	Cli-108	Cli-106
Maternal alleles	118, 118	187, 187
CPB19D	118, 134	181, 187
CPB20D	118, 118	183, 187
CPB21D	118, 122	181, 187
CPB22D	118, 118	173, 187
CPB23D	118, 122	181, 187
CPB24D	118, 118	183, 187
CPB25D	118, 118	173, 187
Total paternal alleles	118, 122, 134	173, 181, 183
Minimum number of sires	2	2

Alleles from the mother are shown at the top in bold. Alleles from seven offspring (CPB19D–CPB25D) are shown in plain type. Total non-maternal alleles and minimum number of sires are listed below

The mating systems of sharks have received growing attention in recent years due to increasing awareness of worldwide overexploitation and vulnerability of shark populations (Musick 1999). To date, few studies have addressed this issue. Here we report the first evidence for multiple paternity in Hawaiian carcharhinid sharks, and show clear evidence that multiple paternity occurs in at least two of the three species we examined. This study doubles the number of shark species investigated for the occurrence of multiple paternity to date, and opens the door for more comprehensive work on the role of this strategy in shark reproduction.

Although we have shown that multiple paternity is possible in these species, we lack any inference on the frequency of this mating strategy in the population. Thus, we now require much larger sample sizes to determine the frequency and consequences of multiple mating in Hawaiian populations of carcharhinid sharks. Multiple paternity as a reproductive strategy has numerous potential advantages (reviewed by Reynolds 1996; Newcomer et al. 1999; Jennions and Petrie 2000; Neff and Pitcher 2005). For example, under certain conditions multiple paternity can maintain genetic diversity of a population or increase the effective population size (Hoekert et al. 2002). Multiple paternity can also increase the number of viable offspring in a single brood (Newcomer et al. 1999). Because multiple paternity is frequently expected to increase individual fitness (Neff and Pitcher 2005), it should be particularly common in

Table 3 Allelic evidence of multiple paternity at two loci (Cli-107 and Cli-12) in the bignose shark, *C. altimus*

	Cli-107	Cli-12
Maternal alleles	90, 94	196, 196
CAB01A	88, 94	194, 196
CAB02A	82, 94	194, 196
CAB03A	82, 90	194, 196
CAB04A	82, 94	190, 196
CAB05A	82, 90	194, 196
CAB06A	82, 90	194, 196
CAB07A	82, 94	194, 196
CAB08A	82, 90	190, 196
CAB09A	90, 90	196, 204
Total paternal alleles	82, 88, 90	190, 194, 204
Minimum number of sires	2	2

Alleles from the mother are shown at the top in bold. Alleles from nine offspring (CAB01A–CAB09A) are shown in plain type. Total non-maternal alleles and minimum number of sires are listed below

taxa where females have little opportunity to evaluate males, such as in the brief copulatory encounters typical of most elasmobranchs (Carrier et al. 1994). Where female mate choice occurs, multiple paternity may also result from active female preference. Although little is known about female mate choice in sharks, Whitney et al. (2004) recently showed that female whitetip reef sharks, *Triaenodon obesus*, actively avoid copulation with some males and permit it with others during competitive group-courtship events. We lack such information about mating in wild carcharhinid sharks, but if females cannot always choose the males with whom they copulate, then multiple paternity may be the result of some post-copulatory process, such as sperm utilization or differential investment (reviewed by Neff and Pitcher 2005).

Genetic diversity is critical to the long-term viability of populations and species by enabling adaptations to changing conditions in the environment (Frankel 1974; Lande and Shannon 1996, reviewed by Moritz 2002). Reproductive strategies can have a fundamental influence on fitness and genetic diversity, especially in relatively small populations of top predators. Therefore, mating strategies are of particular interest to conservation biologists (Neff and Pitcher 2005). Hawaii hosts one of the few known unexploited populations of these three coastal shark species. If multiple paternity proves to be a common reproductive mode in sharks, there may be population-level consequences that need to be considered in any management strategy. Information on multiple paternity from a population that suffers little unnatural mortality is therefore vital to understanding how exploitation affects shark reproduction and the transmission of genetic diversity.

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