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Genetic mating system of the brown smoothhound shark (*Mustelus henlei*), including a literature review of multiple paternity in other elasmobranch species

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Abstract Although an understanding of mating systems is thought to be an important component of long-term population management, these life history characteristics are poorly known in sharks. Here, we employ polymorphic microsatellite markers to test for the occurrence and prevalence of multiple paternity in a population of the brown smoothhound shark, *Mustelus henlei*. We analyzed litters from 14 females sampled from the Pacific coast of Baja California Sur. The minimum number of sires ranged from one to three with an average of 2.3 sires per litter. Regression analyses did not indicate a relationship between female body size and number of sires, or female body size and size of the litter. A review of the existing literature on genetic mating systems in sharks suggests that polyandry may be common and that reproductive behavior may have evolved from conflicting selection pressures between the sexes.

Introduction

Unlike most teleosts, sharks generally are characterized by slow growth rate, late age of sexual maturation, and low fecundity. These ‘K-selected’ life history traits render sharks acutely vulnerable to fishing exploitation and make them slow to recover from population depletion (Smith et al. 1998; Musick et al. 2000). Despite this knowledge, levels of exploitation over the last few decades have grossly

exceeded the reproductive capacity of many sharks, resulting in catastrophic declines that have put many species at risk of extirpation (Manire and Gruber 1990; Baum et al. 2003; Myers and Worm 2003). The development of effective, long-term conservation plans will benefit from knowledge of shark demographics and behavior, including mating systems that now are recognized as an important component of population assessments (Pratt and Carrier 2001; Rowe and Hutchings 2003).

In addition, sharks display a suite of reproductive modes, including oviparity, aplacental viviparity, and placental viviparity (Wourms 1977), that make these animals especially appropriate for testing hypotheses regarding mating systems and sexual selection. While the patterns of structure and function represented in these diverse modes of reproduction and development are well characterized (see reviews by Wourms 1977; Dodd 1983), the occurrence and prevalence of multiple mating and multiple paternity among these species are poorly known.

Direct observations of shark reproductive morphology and mating behavior suggest that multiple paternity may be common in some species. The ability to store sperm in oviducal glands, in some cases for more than a year, has been documented in numerous species, thus opening the possibility that ova from single or repeated ovulations are fertilized by multiple males (Pratt 1993). In addition, multiple mating by females (polyandry) has been observed directly in some species (Carrier et al. 1994; Pratt and Carrier 2001). On the other hand, genetic analyses of many animal taxa have illustrated that such observations of social mating systems do not always reflect realized genetic contributions (Avise 2004).

Contrary to these life history traits that may promote polyandry, physical wounds incurred by females during mating encounters can be severe and may discourage

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multiple mating by females. Males are known to bite the fins and flanks of females, sometimes resulting in substantial open wounds, and physical evidence shows that females may suffer from lacerations and hematoma within the vaginal canal as a result of insertion of the male clasper through the cloaca (Pratt and Carrier 2001). Such occurrences render the female vulnerable during and after copulatory events to predation, blood loss, and infection; and, therefore, polyandrous behavior may in effect decrease female fitness.

At present, genetic analyses of shark mating systems are scarce, consisting of 13 reported studies on a total of 9 species. Furthermore, five of these studies were based on the analysis of only a single litter. Here, we use polymorphic microsatellite markers to analyze the genetic mating system of the brown smoothhound shark, *Mustelus henlei*, a viviparous ground shark of the family Triakidae. Brown smoothhounds are known to range from northern California (where they are commonly taken for sport) to the Gulf of California (where they are commercially harvested), and from Ecuador and Peru, although they are infrequently reported south of the Gulf of California (Castro 1983). The distribution of *M. henlei* is thought to be discontinuous along its range, but the species is usually abundant where it does occur (Compagno 1984), potentially allowing females to encounter multiple mating partners during each mating season. In addition, sperm storage has been reported in several closely related species of this genus (Conrath and Musick 2002; Storrie et al. 2008; Farrell et al. 2010), leading us to hypothesize that *M. henlei* also may possess this capability and thus perhaps exhibit multiple paternity within particular litters. We also compare our results to those of other studied elasmobranchs and investigate whether female reproductive success is correlated with the size of the female.

Methods and materials

Sampling and genotyping

Muscle tissue from 22 deceased specimens of *M. henlei*, as well as 14 whole uteruses containing unborn pups from gravid females, was obtained from local fishermen at Las Barrancas (25°59'N, 112°11'W) on the western coast of Baja California Sur, Mexico, in September 2007. The total length (TL) of all adult sharks was measured, and all tissues were immediately packed on ice and transported to a –80°C freezer. Unborn pups later were removed from the uteri, muscle tissue was sampled from each, and the pups were refrozen at –80°C.

Genomic DNA was extracted from individual pups and adults using Qiagen DNeasy kits (animal tissue protocol).

An enriched microsatellite library then was created and screened for desirable loci following a protocol by Hamilton et al. (1999) as modified by Hauswaldt and Glenn (2003), using DNA from a single adult. Primer sets for four informative microsatellite markers were designed, and one primer from each set was fluorescently labeled with FAM (Mh5 and Mh9), or HEX (Mh2 and Mh17).

Single-locus polymerase chain reaction (PCR) amplifications were carried out in 15 µL reactions composed of the following: 0.2 mM of each dNTP, 0.2 µM each of forward and reverse primer, 1.5 mM MgCl₂, and 0.75 units GoTaq DNA Polymerase in a buffer supplied by the manufacturer (Promega). PCR cycling conditions consisted of an initial 2-min denaturation at 95°C, followed by 35 cycles of 30 s at 95°C, 1 min at 56°C, and 2 min at 72°C, and a final 5-min extension at 72°C. Following PCR, 1 µL of diluted PCR product was mixed with 9.55 µL Hi-Di Formamide (Applied Biosystems) and 0.45 µL of GeneScan™ 1000 ROX™ size standard (Applied Biosystems). These mixtures subsequently were denatured at 95°C for 3 min and electrophoresed on an ABI 3100 Genetic Analyzer in multiplex loading groups. Allelic scoring was performed automatically with GeneMapper 4.0 software (Applied Biosystems) with verification accomplished by eye.

Genotyping errors can greatly affect the conclusions drawn from studies based on individual genetic identification (Bonin et al. 2004; Hoffman and Amos 2005). Therefore, effort should be made to quantify and reduce the occurrence of such errors. To address these concerns, we rescored a random subset (20%) of the samples by eye and compared the findings to the original results. No discrepancies were found. Finally, alleles were inspected visually to ensure that all progeny shared at least one allele with their known mother.

Statistical analyses

We tested for deviation from Hardy–Weinberg equilibrium (HWE) at each locus and for linkage disequilibrium between pairs of loci, and we also estimated observed and expected heterozygosities, counted the number of alleles, and calculated allele frequencies from all adult individuals using GENEPOP 4.0.10 (Raymond and Rousset 1995). Bonferroni corrections were applied for multiple tests where appropriate. The program MICRO-CHECKER (Van Oosterhout et al. 2004) then was employed to check for the presence of null alleles, large allele drop out, and other genotyping errors using all microsatellite data. Expected exclusion probabilities were calculated using GERUD 2.0 (Jones 2005).

The probability of detecting and correctly quantifying multiple paternity within a brood depends not just only on the polymorphism of the microsatellite loci but also on the

clutch size and the number of putative fathers and their corresponding reproductive success (Neff and Pitcher 2002). Here, we used the program PrDM (Neff and Pitcher 2002) to run a number of simulations to test the power afforded by our suite of microsatellite markers to unveil multiple paternity under several different hypothetical scenarios. Previous research on sharks has detected a range of 1–4 sires per brood (Saville et al. 2002; Chapman et al. 2004; Feldheim et al. 2004; Daly-Engel et al. 2006; Portnoy et al. 2007; DiBattista et al. 2008b; Lage et al. 2008; Daly-Engel et al. 2010), so here we assume a conservative range of 2–5 sires under equal, as well as skewed, reproductive success. Each simulation was run with litter sizes ranging from 4 to 17 (the minimum and maximum clutch sizes observed in the present study).

Analysis of paternity was conducted in two ways. First, we subtracted the mother's known genetic contribution from the genotype of the young at each locus. The number of paternal alleles for each locus was then summed (and rounded up for uneven numbers) and divided by 2. These numbers were compared across loci within clutches to determine the minimum number of sires that could explain the observed paternal alleles. It should be noted, however, that this method will often underestimate the true number of sires since it does not take into account multi-locus genotypes. Second, we applied the program GERUD 2.0 (Jones 2005) to the multi-locus genotypes to estimate the minimum number of sires through an exhaustive search.

Physical measurements

To address whether either brood size or the number of sires per clutch correlates with the mothers' total lengths (straight-line measurement taken from the tip of the snout to the tip of the longer lobe of the caudal fin), data were visualized with regression analysis.

Results

Results from the program MICRO-CHECKER did not detect genotyping errors in our data set, and visual reviews of the data confirmed that each of the pups shared an allele with its known mother. These results led us to believe that non-amplifying (null) alleles, which tend to be common in microsatellite data and can confound results from parentage analyses (Pemberton et al. 1995; Dakin and Avise 2004), were not of concern with our present suite of markers. In addition, all microsatellite loci were found to conform to expectations of HWE and exhibited between 4 and 12 alleles per locus (mean = 8; Table 1). Pairwise combinations showed no evidence of linkage disequilibrium (all $P > 0.05$ after Bonferroni correction), and the combined expected exclusion probability for the four loci was high (0.93; Table 1).

As anticipated, simulations indicated that the probability to detect multiple matings using our suite of microsatellite markers increased with both clutch size and the number of fathers (Table 2). Power was moderate (57–82%) with an indicated clutch size of 4 but quickly increased to high power (93–100%) with a clutch size of 10. Since 13 of the 14 clutches in our data set had at least 9 embryos (average = 13.2, SD = 3.55), we consider these markers to be highly reliable in estimating the minimum number of sires that contributed to each clutch.

The minimum number of sires determined by both allele counting, as well as with the program GERUD, ranged from 1 to 3 per clutch (average = 2.1 and 2.3, respectively) with a mode of 2 (Table 3). Thirteen of our 14 analyzed clutches were determined to have multiple sires (93%).

We did not observe a significant relationship between the total length (TL) of the mother and the number of sires that contributed to her clutch, or between the mother's TL and the size of her brood ($r^2 = 0.099$ and 0.005, respectively; Fig. 1).

Table 1 Characterization of microsatellite loci developed in *Mustelus henlei*

Locus	Primer sequence (5'–3')	Repeat motif	T_A (°C)	Size range (bp)	N_A	H_O	H_E	HWE (P value)	P_E
<i>Mh2</i>	F: HEX-ACTACACTGCATATAAACAGGC R: TTTTCAGAGGGCATAACTCAC	(GA) ₉	56	591–599	5	0.73	0.69	0.63	0.45
<i>Mh5</i>	F: GTGACTCTAACC GTTGAAG R: FAM-CATTGCTGCCTTGGGATCTT	Imperfect	56	451–471	11	0.80	0.77	0.60	0.65
<i>Mh9</i>	F: FAM-CAACCATCTTTACTACACTG R: GATGGACCTCACATTTAACAC	(GA) ₉	56	327–333	4	0.80	0.69	0.88	0.36
<i>Mh17</i>	F: HEX-CCTGTCTCAGTTCAGTCTTG R: TCAAACACATACTCCGTC	Imperfect	56	731–801	12	0.80	0.63	1.00	0.43
Combined								0.93	0.93

T_A annealing temperature, N_A number of observed alleles, H_O observed heterozygosity, H_E expected heterozygosity, HWE P value for Hardy–Weinberg equilibrium, P_E exclusion probabilities

Table 2 Probabilities to detect multiple matings (PRDM) using the present microsatellite markers under eight hypothesized mating scenarios

Number of males (paternal skew)	Number of young				
	4	7	10	14	17
2 (50:50)	0.573	0.863	0.929	0.951	0.956
2 (66.7:33.3)	0.569	0.858	0.929	0.953	0.956
3 (33.3:33.3:33.3)	0.723	0.953	0.987	0.995	0.998
3 (57:28.5:14.5)	0.573	0.855	0.93	0.953	0.9555
4 (25:25:25:25)	0.786	0.976	0.996	0.999	1.000
4 (52:27:14:7)	0.682	0.931	0.978	0.994	0.997
5 (20:20:20:20:20)	0.823	0.984	0.998	1.000	1.000
5 (50:26:13:7:4)	0.709	0.941	0.983	0.996	0.998

Table 3 Summary of analyzed clutches including clutch size, total length (TL) of mother, and results regarding paternity estimated by allele counting (Min. no of sires) and inferred via the program GERUD

Mother	Clutch size	TL of mother (cm)	Min. no. of sires	Inferred no. of sires
6	13	94	2	2
12	17	–	2	2
34	15	77	2	2
36	17	98	3	3
38	17	87	2	2
39	4	82	1	1
40	9	94	2	2
43	16	87	2	2
45	11	99	2	3
46	15	89	2	3
57	14	76	2	2
58	10	83	3	3
59	14	81	2	3
61	13	91	2	2

“–” indicates missing data

Discussion

Here, we provide the first evidence of multiple paternity in the brown smoothhound shark. As hypothesized, multiple paternity was common, occurring in 13 of the 14 litters surveyed (93%). We observed evidence for monandry in only one litter, which was the smallest of our litters with only 4 pups. However, one would expect that the power to detect multiple paternity decreases with clutch size, as is shown through simulation events (Table 2). Therefore, while it may be the case that this litter was singly sired, this may also reflect our lack of power to detect multiple paternity in this specific case. Our power to detect multiple paternity in

Fig. 1 Correlation between **a** total length (TL) of mother and number of sires estimated in GERUD ($r^2 = 0.099$) and **b** TL of mother and brood size ($r^2 = 0.005$)

our other 13 clutches was high (93–100%; Table 2), giving us strong reason to believe that our estimates for these litters in each case reflect the true number of genetic paternal contributions.

We were unable to detect a correlation between female TL and the number of sires per litter, or between female TL and brood size (Fig. 1). This may be explained by the small sample size investigated here, considering that a previous analysis of 219 females did find a strong correlation between female TL and litter size (Pérez-Jiménez and Sosa-Nishizaki 2008). If the lack of correlation between TL and number of sires reflects a true biological pattern, this would indicate that females either do not show a preference in their number of mates, are unable to control how often they are inseminated, or that females mate opportunistically when they encounter males such that mate numbers might vary among females in a manner independent of body size. The latter explanation is unlikely to apply in the present case, however, because brown smoothhounds are known to school in large numbers, potentially creating many opportunities for multiple matings.

Multiple paternity in elasmobranchs

The existing literature on genetic mating systems in elasmobranchs consists of 15 studies (present study included) spanning 10 species of viviparous sharks and 1 species of an oviparous skate (Table 4). The percentage of litters with multiple paternity in the current study is the highest reported so far in a species of shark. Our observed range of 1–3 sires per brood is consistent with that seen for other genetically surveyed shark species, where observations ranged from 1 to a maximum of 4 fathers per litter. Of note, all of the studies available to date that involve analysis of more than one litter have shown evidence of multiple paternity within the species, indicating that polyandry may be common among sharks.

Interestingly, among all studied elasmobranchs, the highest percentage of litters with multiple paternity within a species, as well as the greatest maximum number of sires, is seen in the thornback ray, an oviparous species of

skate. While the paucity of studies restricts our ability to draw conclusions from these data, it is possible that the observations reflect a greater readiness by female skates to mate with multiple partners when compared to viviparous sharks. This outcome might be in response to reduced maternal energy expenditure and a reduced opportunity for genomic conflicts in species for which the female lacks prolonged internal gestation (see below), as well as perhaps a reduced risk of physical injury during mating events. Much more research is needed, especially among oviparous species, to see whether this pattern holds true, or whether the observed difference is due to species-specific life history characteristics.

Multiple paternity and sexual conflict

Due to anisogamy, sexual selection gradients often differ between the sexes (Bateman 1948), with a male's reproductive success increasing with additional matings while a

Table 4 Reports on genetic mating system analyses in elasmobranchs

Species	No. of litters analyzed	Avg. no. of progeny per litter	No. of progeny analyzed	Average no. of sires per litter	Range of sires per litter	No. of microsat. markers	% litters with MP	References
<i>Carcharhinus altimus</i> bignose shark	1	NA	9	NA	1	8	NA	Daly-Engel et al. (2006)
<i>Carcharhinus galapagensis</i> Galapagos shark	1	NA	7	NA	1	8	NA	Daly-Engel et al. (2006)
<i>Carcharhinus plumbeus</i> sandbar shark	1	NA	7	NA	1	8	NA	Daly-Engel et al. (2006)
<i>Carcharhinus plumbeus</i> sandbar shark	20	9.4	187	2.3	1–4	5	85	Portnoy et al. (2007)
<i>Ginglymostoma cirratum</i> nurse shark	1	NA	32	NA	4	1	NA	Saville et al. (2002)
<i>Mustelus henlei</i> brown smoothhound	14	13.2	185	2.3	1–3	4	93	Present Study
<i>Negaprion brevirostris</i> lemon shark	1	NA	11	NA	3	3	NA	Feldheim et al. (2001)
<i>Negaprion brevirostris</i> lemon shark	97	6.7	707	2	1–4	9	87	Feldheim et al. (2004)
<i>Negaprion brevirostris</i> lemon shark	85	4.3	408	2	1–4	11	81	DiBattista et al. 2008b
<i>Raja clavata</i> L. thornback ray	4	43.3	173	4.5	4–6	5	100	Chevolot et al. (2007)
<i>Rhincodon typus</i> whale shark	1	NA	29	NA	1	9	NA	Schmid et al. (2010)
<i>Sphyrna tiburo</i> bonnethead shark	22	8.5	188	1.2	1–3	4	19	Chapman et al. (2004)
<i>Squalus acanthias</i> spiny dogfish	10	5	50	1.3	1–2	7	30	Lage et al. (2008)
<i>Squalus acanthias</i> spiny dogfish	29	5.4	NR	1.2	1–2	7	17	Veríssimo et al. (2011)
<i>Squalus mitsukurii</i> shortspine spurdog	27	6.6	178	1.1	1–2	8	11	Daly-Engel et al. (2010)

MP multiple paternity, NA calculation not applicable due to analysis of single litter, NR data not reported in study

female's reproductive success usually is limited by her fecundity. Therefore, all else being equal, males may be under much greater selection pressure to pursue multiple matings relative to females.

In addition, polyandry may well be costly to females for several reasons. The production of ova and internal gestation of the young, when present, are energetically expensive for the mother. A female may be exposed to predation while searching for and copulating with males, and each mating also exposes the female to the risk of sexually transmitted diseases. For sharks in particular, mating events can be violent. Males have been known to bite the fins and flanks of their mates, seemingly to coerce them into mating behavior and also to stabilize the female in a physical position suitable for successful mating. Such biting behavior often leaves large open wounds on a female's body, and internal injury also may be incurred when a male's claspers sometimes lacerate a female's reproductive tract and cause hematomas (Pratt and Carrier 2001). The blood loss and exposure to infection characterized by these events may significantly decrease female fitness with each subsequent mating event.

Other researchers have suggested that sexual conflict between the male and female genomes may influence female fitness and reproductive behaviors (Chapman et al. 2003; Zeh and Zeh 2003). For example, females may mate polyandrously to reduce fertilizations by genetically incompatible males (Zeh and Zeh 1996, 1997), which may in some cases include their own relatives and result in inbred young (Zeh and Zeh 2001). Such genomic conflicts may be especially prevalent in viviparous species (such as the brown smoothhound shark) with internal offspring development (Zeh and Zeh 2001), and in which contact between the mother and each of her young occurs directly through yolk-sac placentas.

Despite what appears to be many disadvantages to polyandry, multiple mating by females with different males is nearly ubiquitous in nature and phylogenetically widespread (Birkhead and Moller 1998; Birkhead 2000). Why then, might females take multiple mates?

Multiple paternity and genetic diversity

Many hypotheses have been put forth to answer this question for species such as sharks in which females receive no direct benefits from multiple mating (e.g. nuptial gifts or paternal care). Some have posited that multiple paternity increases the genetic variation in the young (Sugg and Chesser 1994; Chesser and Baker 1996), and hence increases the probability that at least some offspring in a litter will be viable in unpredictable environmental conditions (Yasui 1998). This might be especially applicable in sharks, which generally exhibit a slower rate of molecular evolu-

tion than other vertebrates (Martin et al. 1992). However, evidence for such indirect genetic benefits is lacking in some animals (Jennions and Petrie 2000), including at least one shark species (DiBattista et al. 2008a). Indeed, theoretical results suggest that in most natural conditions an increase in multiple paternity may result in a reduced effective population size and limited genetic diversity, since each mating may result in increased variation in male reproductive success when compared to monandry (Nunney 1993; Ramakrishnan et al. 2004; Karl 2008). However, under some conditions, such as multiple mating and sperm storage events followed by a population bottleneck, multiple mating would clearly increase the post-bottleneck effective population size (Karl 2008). Therefore, what effect polyandry has on the genetic variability of the young should be considered on a species-specific basis in conjunction with population demographic, behavioral, and physiological data.

Still other indirect genetic benefits to multiple paternity may apply. A few researchers have proposed hypotheses such as: the "sexy sperm hypothesis" where females that take multiple partners create an environment that promotes sperm competition, which ultimately might lead either to increased fertilization success or perhaps better genes for their zygotes (Harvey and May 1989; Keller and Reeve 1995; Simmons 2003); that multiply-mated females tend to achieve fertilizations by higher quality males (bet-hedging; Watson 1991); and/or that females take multiple mates in an effort to "trade-up" to better males (Petrie and Kempenaers 1998; Jennions and Petrie 2000). Several informative reviews debate these subjects (see Jennions and Petrie 2000; Zeh and Zeh 2001; Tregenza and Wedell 2000). Ideally, such hypotheses could be tested by following females, and the viability and fertility of their young, over the females' lifespans, a task that is prohibitively difficult for many systems.

Multiple paternity and convenience polyandry

Recently, several authors have suggested that multiple mating in sharks may reflect convenience polyandry, whereby females mate multiply only when the costs of mating avoidance (male harassment and injury) are greater than those incurred during mating events (Feldheim et al. 2004; DiBattista et al. 2008a). Females are the larger of the two sexes in sharks (Cortés 2000) and therefore may be in a position to thwart male advances at their choosing. Indeed, some shark females seem able either to avoid or actively allow copulations (Whitney et al. 2004), although this ability may be limited when several males attempt to mate with a single female at one time (Pratt and Carrier 2001). Many sharks appear to segregate into sex-specific schools. For example, mature females of *Sphyrna tiburo* form large

aggregations, which may limit the potential for multiple mating by each female (Klimley 1985). In addition, the possibility also exists that the number of sires contributing to a litter is limited by post-copulatory sperm selection (Jennions and Petrie 2000). At the least, it seems likely that convenience polyandry offers one of perhaps several plausible explanations for multiple mating in sharks.

Conclusions

In summary, we provide conclusive evidence that multiple paternity occurs with high prevalence in the sampled population of the brown smoothhound shark. Additional analyses across the species range would be informative as to whether this pattern varies with population sizes or mate encounter rates. Notably, our findings of multiple paternity are consistent with those for other multi-litter analyses of elasmobranchs, indicating that multiple paternity may be the norm in sharks, as it is in many other vertebrate groups.

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