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A Verissimo

Virginia Institute of Marine Science

D Grubbs

Jan McDowell

Virginia Institute of Marine Science

J Musick

Virginia Institute of Marine Science

D Portnoy

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Frequency of Multiple Paternity in the Spiny Dogfish *Squalus acanthias* in the Western North Atlantic

ANA VERÍSSIMO, DEAN GRUBBS, JAN McDOWELL, JOHN MUSICK, AND DAVID PORTNOY

From the Department of Fisheries Science, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062; the Florida State University Coastal and Marine Laboratory, Saint Teresa, FL; and the Department of Wildlife and Fisheries Sciences, Center for Biosystematics and Biodiversity, Texas A&M University, College Station, TX.

Address correspondence to A. Veríssimo at the address above, or e-mail: averissimo@vims.edu.

Abstract

Multiple paternity (MP) has been shown to be widespread in elasmobranch fishes although its prevalence and the number of sires per litter vary considerably among species. In the squaloid shark *Squalus acanthias*, MP has been reported, but whether it is a common feature of the species' reproductive strategy is unknown. In this study, we determined the frequency of MP in 29 litters of *S. acanthias* sampled from the lower Chesapeake Bay and coastal Virginia waters, using 7 highly polymorphic nuclear DNA microsatellite loci. Only 5 litters (17% of the total) were genetically polyandrous, with at least 2 sires per litter. Litter size increased with female size but was similar between polyandrous and monandrous females.

Key words: coercive mating, genetic polyandry, squaloid sharks, yolk-sac viviparity

Multiple paternity (MP) in elasmobranch fishes has been shown to be widespread across species. Multiply sired litters were detected in several galeoid species such as the lemon shark *Negaprion brevirostris* (Feldheim et al. 2002), the nurse shark *Ginglymostoma cirratum* (Saville et al. 2002), the bonnethead *Sphyrna tiburo* (Chapman et al. 2004), and the sandbar shark *Carcharhinus plumbeus* (Portnoy et al. 2007), as well as in squaloid species including the spiny dogfish *Squalus acanthias* (Lage et al. 2008) and the shortspine spurdog *S. mitsukurii* (Daly-Engel et al. 2010), and in the rajoid thornback ray *Raja clavata* (Chevolot et al. 2007). Fewer reports have been made on the prevalence and the levels of genetic polyandry, although estimates vary considerably among and within species. For instance, the lemon shark was dominantly polyandrous (81–87%) (Feldheim et al. 2004; DiBattista et al. 2008b), whereas the bonnethead and the shortspine spurdog were dominantly monandrous (18% and 11%, respectively) (Chapman et al. 2004; Daly-Engel et al. 2010). In the sandbar shark, polyandry was dominant in the western north Atlantic (85%, Portnoy et al. 2007) but not in the Central Pacific (40%, Daly-Engel et al. 2007).

Given the high physical stress associated with mating, particularly for females (e.g., mating wounds; Pratt and Carrier 2005), it is expected that females would either favor monogamous mating or obtain some benefit from engag-

ing in multiple matings (see Yasui 1998; Simmons 2005; DiBattista et al. 2008a; Karl 2008). However, no direct benefits to females, in the form of nuptial gifts or paternal care, are apparent in elasmobranchs (Chapman et al. 2004; Portnoy et al. 2007; DiBattista et al. 2008b). Likewise, there is little evidence of indirect benefits, such as increased juvenile survival, associated with MP (DiBattista et al. 2008b). If there are no benefits for females of polyandrous elasmobranch species, the patterns of female polyandry may be driven by male-associated benefits. For example, males might force females into multiple matings in order to maximize their reproductive success and thus bias the paternity of the litters (Portnoy et al. 2007).

We investigated MP in litters of the spiny dogfish *S. acanthias* Linnaeus, 1758, a coastal squaloid shark occurring in the temperate waters of the Atlantic and Pacific oceans (Compagno et al. 2005). Individuals commonly occur in large schools with immature fish segregating by size and mature fish segregating by size and sex (Templeman 1944; Nammack et al. 1985; Stenberg 2005). The spiny dogfish is a yolk-sac viviparous species with fecundity between 4 and 11 pups per litter (range: 1–20; Nammack et al. 1985; Menni 1986; Hanchet 1988; Tribuzio 2004; Ellis and Keable 2008). It has a synchronous reproductive cycle where 2 “gestational cohorts” of mature females can be found at any time during the year, exhibiting

pregnancy stages separated by roughly 12 months (Templeman 1944; Hisaw and Albert 1947; Hanchet 1988). Gestation has been estimated to last between 20 and 22 months, and most mature females begin a new gestational cycle as soon as the previous cycle has been completed, giving birth every 2 years (Hisaw and Albert 1947; Holden and Meadows 1964; Hanchet 1988). Spermatogenesis in *S. acanthias* is also synchronous in mature males and follows a well-defined annual cycle in which maturation and peak evacuation of sperm are coincident with the period between parturition and ovulation (Simpson and Wardle 1967; Hanchet 1988). Sperm storage has not been reported for *S. acanthias* but has been shown in other elasmobranchs, with sperm retention occurring in specialized tubules of the oviducal glands (Pratt 1993). However, the oviducal gland of spiny dogfish is structurally simple (Hamlett et al. 1998) and may not allow for long-term sperm storage.

Evidence of MP was previously found in 3 of 10 litters of *S. acanthias* from western North Atlantic and the Gulf of Maine, using 7 nuclear DNA microsatellite loci (Lage et al. 2008). But whether polyandry is a common feature of the spiny dogfish reproductive strategy is unknown. The major objective of the present study was to determine the frequency of MP in litters of spiny dogfish using highly polymorphic nuclear DNA microsatellite loci. In addition, the number of pups per litter and the female total length (TL) were compared between females with polyandrous and monandrous litters. The levels of inbreeding found across all litters of the spiny dogfish were compared with the levels of inbreeding found among full siblings in genetically polyandrous litters to test whether remating by the females might be a strategy of inbreeding avoidance. Also, as spiny dogfish have paired reproductive tracts with 2 functional uteri, special attention was paid to the uterine location (left or right) of full siblings.

Materials and Methods

Sample Collection and Genotyping

Twenty-nine pregnant females (TL: 80–110 cm) and respective litters (4–11 pups per litter) were sampled during the spring of 2006 in Chesapeake Bay and Virginia coastal waters. The uterine location (left or right) was recorded for each pup. Fin clips or muscle tissue samples from females and pups were collected and preserved in 20% dimethyl sulfoxide buffer saturated with NaCl (Seutin et al. 1991). Additional samples of spiny dogfish were obtained from off Cape Cod, MA, and off Virginia Beach, VA ($n = 48$ –55 per site) in order to provide estimates of the genetic diversity of spiny dogfish in the western North Atlantic. A study on the worldwide population structure of *S. acanthias* has found no significant differences in the genetic composition between the 2 locations (Veríssimo et al. 2010); therefore, these additional samples were pooled for more robust estimates of genetic diversity. Total genomic DNA (gDNA) was extracted from each individual sample using the Chelex

protocol (Estoup et al. 1996) or the QIAGEN DNeasy Tissue Kit according to the manufacturer's instructions (QIAGEN, Valencia, CA). Seven microsatellite loci available from the literature (*DF U285*, *DF T289*, *DF J451*, *DF J445*; McCauley et al. 2004; and *Saca GA11*, *Saca3853*, and *Saca6396*; Veríssimo et al. 2010) were screened in all samples. Polymerase chain reactions (PCRs) conditions for all 7 loci are described in detail in Veríssimo et al. (2010). Briefly, each PCR reaction contained 0.5 μ l of gDNA, taken directly from the supernatant of the Chelex DNA extractions or from the elutant of Qiagen DNA extractions, 37.5 nM T3-labeled forward primer, 150 nM reverse primer, 0.1 μ M fluorescent dye (NED, PET, VIC, or 6FAM; Applied Biosystems, Foster City, CA), 5 U *Taq* DNA polymerase, 0.2 mM each dNTP's, 1.5 mM $MgCl_2$, 0.5 μ l 10X Qiagen PCR buffer (without $MgCl_2$), and milli-Q water up to a final volume of 5 μ l. Temperature conditions included an initial denaturation period of 3 min at 94 °C, followed by 35–45 cycles of denaturation for 1 min at 94 °C, annealing for 35 s—1 min at 54–62 °C and extension for 1 min at 72 °C, with a final extension period of 2 min at 72 °C. The amplicons were run on an ABI Prism 3130x1 (Applied Biosystems) and with the size standard 500-Liz (Applied Biosystems), according to the manufacturer's protocol. Individual genotypes were scored manually aided by the software GeneMarker version 1.60 (Softgenetics LLC, State College, PA).

Genetic Data Analyses

Allelic frequencies and allelic richness were calculated for each locus with FSTAT (Goudet 2002) using the 96 samples collected off Massachusetts and Virginia. Conformance to the expectations of Hardy–Weinberg equilibrium (HWE) was tested for each locus in GENEPOP v 1.2 (Raymond and Rousset 1995) using exact tests with 10 000 iterations (Guo and Thompson 1992). The probability of detecting MP (PrDM) in spiny dogfish litters using the above microsatellite loci was calculated with the PrDM software (Neff and Pitcher 2002).

Genotypic arrays were manually evaluated to ensure that all progeny shared at least one maternal allele at each locus. The number of paternal alleles for each locus was then summed. A litter that had 3 or more paternal alleles at one or more loci was considered polyandrous. Allele counts for each locus also allowed for an initial estimate of the number of contributing sires. For litters in which only 2 paternal alleles per locus were detected across all 7 loci, Fisher's Exact tests were used to determine whether loci conformed to the expectations of Mendelian segregation in a monogamous mating.

Gerud 2.0 (Jones 2005) was used to estimate the minimum number of fathers contributing to each multiply sired litter. The number of progeny per sire was also calculated from the array of genotypes expressed by the female and her progeny. For cases in which no unique sire-progeny solution was obtained, alternative solutions were ranked by relative probability (Jones 2005). Littermates were

also sorted by uterine position to allow for the detection of multiple sires within a single uterus.

Relationships between realized fecundity (litter size) and female TL were determined through linear regression analysis. Mean TL and mean litter size of genetically polyandrous and monogamous females were compared and tested under the null hypothesis of equality ($H_0: \mu_1 - \mu_2 = 0$) using a 2-tailed paired *t*-test assuming unequal variances. Levels of inbreeding in genetically polyandrous and monandrous litters were estimated with the internal relatedness (IR) metric (Amos et al. 2001). Mean IR values were obtained for each litter and for all full siblings in each polyandrous litter. Mean IR values per litter were compared with mean IR values for full siblings within genetically polyandrous litters using a one-tailed *t*-test. Assuming that females might remate as a means of avoiding inbreeding, the mean IR for full siblings in polyandrous litters should be greater than the mean IR in all litters. The most likely pattern of paternal contribution, as suggested by Gerud 2.0, was used to assign full siblings within genetically polyandrous litters.

Results

The number of alleles and the expected heterozygosity at each locus ranged between 6 and 16 and 0.40 and 0.90, respectively (Table 1). The distribution of genotypes at all loci conformed to the expectations of HWE (Table 1). The PrDM was lowest in genetically monogamous litters ($62 \pm 2\%$ vs. $89 \pm 1\%$ in polyandrous litters) and when reproductive skew was assumed to be high (3–1). In polyandrous litters, the PrDM increased as skew decreased.

Genetic polyandry was detected in 5 of 29 litters (17%) with a maximum of 2 sires per litter being detected by allele count. Only 6 loci were used in litter U as locus *Saca6396* did not consistently amplify in the mother and all her offspring. Gerud 2.0 also produced an estimate of 2 fathers per litter but was only able to produce a unique paternity solution for litter L in which 5 of 6 pups shared a single sire. For the remaining 4 litters, priority scores produced by Gerud 2.0 were used to rank scenarios. Litter X had 3 solutions all of

Table 1 Summary statistics for 7 nuclear microsatellite loci, for a total of 103 individuals of *Squalus acanthias* from the western North Atlantic: total number of alleles (*A*); gene diversity (*b*); conformance to Hardy–Weinberg equilibrium [*p* (HW)] calculated in GENEPOP

Marker name	Repeat motif	<i>A</i>	<i>b</i>	<i>p</i> (HW)
<i>DF U285</i>	[CT] ₁₁	9	0.75	0.55
<i>DF T289</i>	[TCC] ₇	10	0.40	0.64
<i>DF J451</i>	[AC] ₁₀	6	0.68	0.71
<i>DF J445</i>	[AC] ₁₀	8	0.60	0.45
<i>Saca GA11</i>	[TG] ₉ C [TG]	7	0.73	0.19
<i>Saca 6396</i>	[CA] ₁₉	16	0.90	0.36
<i>Saca 3853</i>	[TG] ₉ GC [TC] ₂	6	0.58	0.33
	All loci	62	0.66	0.61

which suggested that 4 of 6 pups shared a single sire. For litters M, U, and AB multiple patterns of paternal skew were found (8, 3, and 9 solutions, respectively; Table 2). All polyandrous litters except litter AB had litter mates with different sires in the same uterus (Table 2).

In the 24 litters where polyandry was not detected, Fisher's Exact tests were nonsignificant, as such no deviation from expectations of Mendelian segregation was found at any of the loci used, except for one locus in litter A. In litter A, the presence of 3 homozygous offspring at locus *DF J445* that did not share an allele with their mother suggests the presence of a low frequency null allele. However, there were only 2 paternal alleles present in the progeny array across all other loci, suggesting a single sire for this litter. These results indicate that the above litters were genetically monogamous.

The regression of litter size as a function of maternal TL (Figure 1) had a slope (*b*) that was significantly different from zero ($b = 0.229$, $P = 0.0003$) with fairly tight 95% confidence intervals ($0.11 < b < 0.34$). No difference was detected in the mean litter size for multiply sired litters and single sired litters (6 and 5.3, respectively; *t*-test degrees of freedom [df]: 19, $P = 0.23$). The null hypothesis of no difference in female TL between genetically polyandrous and monogamous females was also not rejected (93.8 and 89.6 cm, respectively; df: 6, $P = 0.13$). Further support for these results is provided by the observations that the 2 largest litters had only one sire and the largest female was genetically monogamous (Figure 1). Full siblings in genetically polyandrous litters had greater mean IR values than all combined siblings in each litter although the difference was not significant (0.148 and 0.118, respectively; df: 24, $P = 0.21$).

Discussion

The major objective of this study was to estimate the frequency of occurrence of MP in litters of the spiny dogfish *S. acanthias* from Virginia waters. Thus, a large number of females and respective litters ($n = 29$) were sampled, and robust estimates of the allelic frequencies in the source

Table 2 Characteristics of the 5 polyandrous litters of spiny dogfish *Squalus acanthias* ($n = 29$ litters analyzed)

Litter	TL (cm)		PrDM	No. sires		Dom:Sec
	TL	No. pups		Skew	No. sires	
L	87	6	0.90	2	5:1 (NA)	2:1
M	91	7	0.88	2	4:3 (8)	1:3
U	95	6	0.87	2	5:1 (4)	2:1
X	98	6	0.90	2	4:2 (3)	1:1 and 2:1
AB	98	5	0.89	2	3:2 (9)	NA

Maternal total length (TL); litter size (No. pups); probability of detecting multiple paternity integrated over all loci (PrDM); minimum number of sires suggested by GERUD (No. sires); most likely ratio of paternal contribution (Skew) and number of correspondingly possible alternatives (in parentheses); ratio of pups sired by dominant versus secondary male in uteri with half siblings (Dom:Sec). NA, no alternative.

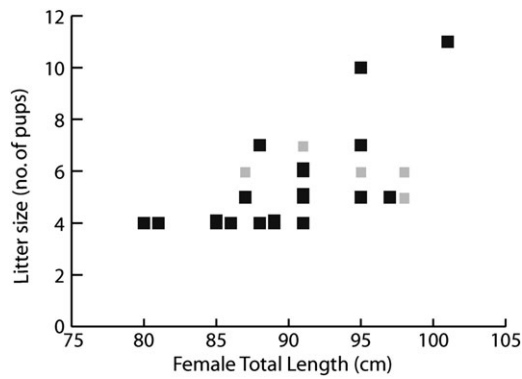


Figure 1. Linear regression of *Squalus acanthias* maternal TL versus litter size for the 29 litters analyzed (some data points may be overlapping). Black squares: females with monandrous litters; gray squares: females with polyandrous litters. Regression statistics: $R^2 = 0.396$, $P < 0.001$; litter size = $0.229(\text{TL}_F) - 15.44$.

population ($n = 103$) were calculated based on 7 nuclear microsatellite loci. Only 5 of 29 litters surveyed were polyandrous (17%) with probabilities of detecting MP in these 5 litters ranging from 87% to 90%. Our data indicate that the spiny dogfish *S. acanthias* is predominantly monandrous with a small percentage of the litters being sired by at least 2 males.

In another study, Lage et al. (2008) detected MP in 30% of 10 litters of *S. acanthias* sampled from off Massachusetts and the Gulf of Maine. These authors argued that their estimated frequency of MP was likely underestimated given the low probability of detecting MP in small litter sizes (e.g., 3–7 pups) and when alleles were common in the source population. However, the authors did not collect baseline allelic frequencies from their source population. The discrepancy of results between our study and that of Lage et al. (2008) is likely due to the difference in the number of litters surveyed (29 vs. 10, respectively). Although we cannot discard the possibility of regional differences in the frequency of MP in litters of the spiny dogfish, the evidence available is consistent with the presence of a single panmictic population throughout the western North Atlantic. (Hisaw and Albert 1947; Veríssimo et al. 2010).

The predominance of singly sired litters in the spiny dogfish off the western North Atlantic could result from monogamous mating, that is, most females mate with only one male per breeding cycle. This scenario is counterintuitive because the operational sex ratio during the mating season is expected to favor males over females as all mature males will be available for copulation in contrast to only half of the mature female population (i.e., only one “gestational cohort”). Nevertheless, monogamous mating in the spiny dogfish may be facilitated by the dissuasive effect that mature female aggregations may induce on aggressive and persistent male courtship behavior. By grouping together, receptive females may succeed in avoiding male harassment

and even be able to select a mate (Klimley 1985; Pratt and Carrier 2005).

Alternately, spiny dogfish females may mate with multiple males per breeding cycle but only one male sires all the offspring in the majority of the cases. This may result from postcopulatory sperm selection by either the females or the males. Female-mediated sperm selection is usually accepted as a mechanism to reduce inbreeding resulting from mating among closely related individuals (Simmons 2005). However, the wide distribution of the spiny dogfish along the western North Atlantic, its high dispersal ability, and the absence of geographically discrete mating grounds (Burgess 2002) render the possibility of inbreeding unlikely. The dominance of monandry in *S. acanthias* could be due to male-mediated sperm-selection mechanisms such as sperm precedence. In this case, the first or last mating male might fertilize all or most of the eggs (Jones et al. 2002; Kraaijeveld-Smit et al. 2002), thus increasing the chances of paternity for a single male and biasing the patterns of genetic polyandry. It is also possible that the number of males contributing sperm to egg fertilization is limited by the short time period between parturition and ovulation; in other words, there may not be enough time for multiple matings/inseminations before ovulation/fertilization takes place.

A predominance of singly sired litters in other elasmobranchs was found only in the bonnethead shark *S. tiburo* and in the shortspine spurdog *S. mitsukurii* (Chapman et al. 2004; Daly-Engel et al. 2010). The bonnethead has a highly synchronized annual reproductive cycle, a short gestation period (4–5 months), and sperm storage (Parsons 1993; Manire et al. 1995). It is also a social shark, commonly found in groups of 3–15 individuals where sexes are segregated (Compagno et al. 2005; Pratt and Carrier 2005). Thus, the aggregation of mature females, seen in both *S. tiburo* and *S. acanthias*, may reduce the potential for multiple mating and genetic polyandry (Klimley 1985), although postcopulatory sperm selection mechanisms cannot be dismissed (Chapman et al. 2004). On the other hand, the shortspine spurdog has an asynchronous cycle, a 2-year gestation time and limited ability for sperm storage (Compagno et al. 2005; Daly-Engel et al. 2010). Moreover, sexes are also segregated with depth (Wilson and Seki 1994). The dominance of genetically monandrous litters in the shortspine spurdog could result from the bathymetric segregation of the sexes in addition to the absence of breeding aggregations due to the asynchronous reproductive cycle (Daly-Engel et al. 2010). This combination of features eliminates the potential for a male-biased operational sex ratio at the time of copulation, allowing for a more effective mating avoidance strategy by females.

In contrast to the above condition, predominance of polyandry in litters of elasmobranch taxa was reported for the lemon shark *N. brevirostris* (Feldheim et al. 2002, 2004; DiBattista et al. 2008b) and for the sandbar shark *C. plumbeus* off the western North Atlantic (Portnoy et al. 2007). Convenience polyandry, whereby females subject to

multiple matings as a way to avoid or reduce male harassment (Portnoy et al. 2007; DiBattista et al. 2008a), was suggested as a possible explanation in both cases. All the above studies suggest that the frequency of MP in elasmobranchs may be a function of the females' ability to avoid aggressive and persistent mating attempts by the males, lending support to the hypothesis that genetic monandry in *S. acanthias* may be due to an effective male-avoidance strategy by the females.

In this study, dominant male contribution was found in both uteri, whereas secondary male contribution was confined to one uterus in 4 of 5 genetically polyandrous litters. In elasmobranch taxa where both oviducts are functional, sperm delivery to the oviducts is supposedly independent, that is, the clasper must be inserted into each oviduct separately (Pratt and Carrier 2005). Thus, in species where only one clasper is inserted during mating, multiple matings may be required to successfully inseminate both oviducts. If this is the case for *S. acanthias*, then confinement of secondary male contributions to one uterus may simply be due to limited mating opportunities. When considering only the uteri containing half siblings, the ratio of pups sired by the dominant versus the secondary male is roughly equivalent (Table 2), suggesting that sperm from different males may have an equal probability of contributing to the progeny. These observations need further testing with larger sample sizes, both in terms of number of litters and litter size. Future studies should aim at exploring this issue in more detail.

No benefits concerning increased reproductive success in polyandrous females were detected with our data, though the *t*-tests used for this purpose had low power given the small sample sizes. On the other hand, mean IR values for full siblings within genetically polyandrous litters were higher than for siblings in all litters, consistent with the idea that females might remate to avoid inbreeding. However, the mean IR values were not significantly different between the 2 groups of siblings. This result is far from conclusive given the small sample sizes involved in the comparison and the possibility of incorrect grouping of full siblings within polyandrous litters. Indirect genetic benefits through increased genetic diversity of the offspring therefore cannot be dismissed, though one would expect a higher frequency of multiply sired litters in *S. acanthias* if there were clear benefits to the female and/or offspring. On the contrary, males can potentially benefit from coercing females to multiple matings even if on a strictly opportunistic basis (Portnoy et al. 2007; DiBattista et al. 2008a): the chances of siring some fraction of that female's offspring will provide direct benefits for the male through increased reproductive potential, irrespective of the number of times each male copulates per mating season.

In summary, our results indicate that MP in the spiny dogfish is the exception rather than the rule, occurring only in a small percentage of the sampled litters. Although other mechanisms cannot be discounted (e.g., male-mediated postcopulatory sperm selection), the low levels of polyandry detected in this study may be the result of limited mating opportunities for males due to an effective male-avoidance

strategy through aggregation of mature females coupled with a short period between mating and ovulation/fertilization.

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Corresponding Editor: Brian W Bowen