

# Is multiple mating beneficial or unavoidable? Low multiple paternity and genetic diversity in the shortspine spurdog *Squalus mitsukurii*

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**ABSTRACT:** Proposed benefits of multiple paternity include increased reproductive output, elevated fitness of progeny, and maintenance of population genetic diversity. However, another consideration is whether multiple paternity is simply an unavoidable byproduct of sexual conflict, with males seeking to maximize mating encounters while females seek to minimize the stress of copulation. Here we examined the polyandrous mating system in sharks, with a focus on the reproductive genetics of the shortspine spurdog *Squalus mitsukurii*. Members of the genus *Squalus* are long-lived, slow-growing, and employ among the longest gestation periods of any vertebrate. To evaluate multiple paternity and genetic diversity in *S. mitsukurii*, we genotyped 27 litters plus 96 individuals with 8 microsatellite loci. Further, 670 bp of the mtDNA control region were sequenced in 112 individuals to examine population structure. *S. mitsukurii* in Hawaii showed low genetic diversity relative to other sharks ( $\pi = 0.0010 \pm 0.0008$ ) and no significant population structure in the Hawaiian Archipelago. Direct allele counts and Bayesian approximations returned concordant estimates of 11 % multiple paternity, the lowest observed in sharks to date. Considering the protracted reproductive interval of *S. mitsukurii*, sexual conflict that results from differential male and female reproductive strategies may favor the development of female mating avoidance behavior to minimize trauma. In *S. mitsukurii* this behavior includes segregation of sexes and an asynchronous reproductive cycle.

**KEY WORDS:** Elasmobranch · Polyandry · Control region · Microsatellite DNA · Population structure · Sexual conflict · Sexual segregation · Reproductive strategy

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## INTRODUCTION

In sexually reproducing species, the existence of conflicting fitness strategies between sexes can lead to intense sexual selection and the establishment of sexual conflict, where coercive traits that arise in one sex are countered by the evolution of resistance traits in the other (Zeh & Zeh 2003). In the majority of verte-

brate mating systems, females bear the energetic burden of ova and parental care and are thus expected to be the more 'choosy' sex in regards to mate selection. Males, in contrast, are expected to be non-parental, sexually competitive, and promiscuous (Smith 1984, Birkhead 1998, Birkhead & Pizzari 2002). Contrary to the historical assumption of monogamy in the choosy sex, there is abundant evidence of multiple mating by

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females with conventional sex roles (reviewed by Zeh & Zeh 2003). Polyandry (females mating with more than one male) and multiple paternity (a single brood of offspring sired by multiple males) are now recognized as common strategies in widely divergent taxa including amphibians, mammals, reptiles, insects, crustaceans, and fishes (Evans & Magurran 2000, Toonen 2004, Adams et al. 2005, Bretman & Tregenza 2005, Daly-Engel et al. 2006, Dean et al. 2006, Jensen et al. 2006). It is still unclear, however, what roles sexual conflict and intersexual selection might play in polyandrous mating systems.

For males, the advantages to having multiple breeding partners are clear: the more females a male inseminates, the more offspring he fathers and the greater his reproductive fitness. The benefits of polyandry to females are less obvious. Potential direct benefits to the female include nuptial gifts or parental care on the part of the male. No direct benefits have been shown in shark mating systems, though there is potential for indirect or genetic benefits through polyandrous mating. If there is little or no opportunity to evaluate males prior to copulation, a female may hedge her bets by mating promiscuously and therefore increase her chances that one of these matings may lead to higher survivorship for offspring (genetic bet-hedging; Watson 1991, Madsen et al. 2005). Alternatively, polyandry may result in inbreeding avoidance or increase the likelihood that a female's offspring will be sired by a male whose genes are compatible with hers (genetic compatibility hypothesis; Zeh & Zeh 1997, 2001, Neff & Pitcher 2005). However, multiple mating can also be disadvantageous to females due to exposure to disease or risk of injury during mating events; female sharks may sustain serious injury or even die as a result of harm incurred during copulation (Pratt & Carrier 2001).

Apart from benefits to offspring, there is ongoing debate over whether multiple paternity might confer benefits to a population by maintaining genetic diversity, depending on whether it increases or decreases variance in reproductive success (Sugg & Chesser 1994, Zeh & Zeh 2003, Karl 2008). One school of thought maintains that multiple paternity may buffer against the loss of allelic diversity by increasing the effective population size (Sugg & Chesser 1994, Newcomer et al. 1999, Martinez et al. 2000, Hoekert et al. 2002). This is countered by theoretical results indicating that by increasing the variance in male reproductive success (because each mating may result in fewer offspring per male than with genetic monogamy), multiple paternity will reduce effective population size and, consequently, limit population genetic diversity (Nunney 1993, Ramakrishnan et al. 2004, Karl 2008). Reproductive strategy can have considerable effect on genetic diversity, which in turn affects the ability of

populations to respond to selection pressures like changes in environmental conditions (Rowe & Hutchings 2003, Frankham 2005). For this reason, loss of genetic diversity has been associated with increased vulnerability to population depletion and extinction (Dulvy et al. 2003, Rowe & Hutchings 2003, Frankham 2005). Though a speciose group, sharks in particular exhibit slower rates of genetic evolution than other vertebrates (Martin et al. 1992), as well as lower rates of growth and reproduction, which may limit their ability to recover from population depletion.

The frequency in a population with which a gravid female carries a brood sired by more than one male (multiple paternity) can be estimated by inferring the minimum number of fathers per brood from genotypes of mothers and their offspring. Previous work on multiple paternity in elasmobranchs has shown a large degree of inter- and intraspecific frequency variation (Ohta et al. 2000, Saville et al. 2002, Chapman et al. 2004, Feldheim et al. 2004, Daly-Engel et al. 2007, Lage et al. 2008). Given that Lage et al. (2008) found low (30%) multiple paternity in the congener *Squalus acanthias*, a low level of multiple paternity in *S. mitsukurii* could indicate genus-level concordance in squalid sharks. However, recent studies have further shown that rates of multiple paternity can vary even between populations of a single species (Daly-Engel et al. 2007, Portnoy et al. 2007), indicating high levels of behavioral trait plasticity. Though the number of studies on polyandrous mating in elasmobranchs continues to increase, multiple paternity has not yet been shown to confer either direct or indirect benefits to sharks (DiBattista et al. 2008a), leading some investigators to hypothesize that multiple paternity in elasmobranchs may be influenced by sexual conflict (Daly-Engel et al. 2007, Portnoy et al. 2007, DiBattista et al. 2008b).

We assessed the frequency of multiple paternity in 27 litters of the shortspine spurdog *Squalus mitsukurii* from throughout Hawaii using a suite of 8 polymorphic microsatellite DNA markers, including 6 novel species-specific markers developed for the present study and 2 previously published loci developed for *Squalus acanthias* (McCauley et al. 2004). In addition, we examined the link between genetic diversity and reproductive strategy by estimating genetic diversity using a 670 bp segment of the mitochondrial control region for comparison to other studies. We also calculated allelic richness for the microsatellite loci in all published surveys of shark multiple paternity to determine whether genetic diversity correlates with multiple paternity in sharks. This is the first estimation of genetic polyandry in *S. mitsukurii* coupled with one of the few direct measures of genetic diversity (allelic richness) in any squalid, a globally distributed family

of small sharks known collectively as dogfish. These data, generated from an unfished population, will serve as a foundation for future studies examining natural reproductive strategies and genetic diversity in both exploited and unexploited populations of elasmobranch fishes.

## MATERIALS AND METHODS

**Study species.** The shortspine spurdog *Squalus mitsukurii* aggregates on or near the bottom at a depth of 100 to 950 m in temperate, subtropical, and tropical seas, particularly along coastlines, continental shelves, and on seamounts (Wilson & Seki 1994, IUCN 2003). The species is ovoviviparous with low fecundity. Females give birth to an average of 6 pups ~25 cm in length at birth every 2 to 3 yr (IUCN 2003, Compagno et al. 2005). *S. mitsukurii* is widely distributed in the Pacific Ocean (Last & Stevens 1994) and is likely a species complex. Age at maturity is between 4 and 7 yr for males and between 14 and 16 yr for females (Wilson & Seki 1994, Taniuchi & Tachikawa 1999) and generation time is more than 25 yr (Compagno et al. 2005). *S. mitsukurii* has no known quiescent period between gestations, with ova maturing concurrently with embryos, such that when pups are at term the new ova are ready for fertilization (T. S. Daly-Engel & R. D. Grubbs pers. obs.).

*Squalus mitsukurii* is currently listed as endangered on the IUCN Red List (IUCN 2003), based primarily on data taken from the Australian population. There,

*S. mitsukurii* populations declined as much as 97% between 1976 and 1997 due to fishing mortality as bycatch from commercial trawling (Graham et al. 2001, IUCN 2003). The status of other populations of *S. mitsukurii* is unknown due to the high likelihood of misidentification and the lack of data from most of the world. In Hawaii, *S. mitsukurii* is rare as bycatch in the bottomfish fishery, and little is known about its range, population structure, or stock status. A study of large aggregations of *S. mitsukurii* from the Hancock seamount in the Hawaiian–Emperor seamount chain is the only published report of this species from the central Pacific (Wilson & Seki 1994). Anecdotal data and catch rates from the present study indicate a robust Hawaiian stock which is largely unaffected by fishing mortality, making Hawaii an ideal location to acquire a baseline understanding of the genetic mating system and allelic diversity of this species. A note on species identification: though the species of dogfish most common in Hawaii is widely accepted to be *S. mitsukurii*, recently published morphological keys (White et al. 2007) appear to exclude Hawaiian dogfish from *S. mitsukurii* (R. D. Grubbs unpubl. data). Until additional studies are done, however, we will continue to use accepted nomenclature.

**Sampling.** We collected sharks near Oahu and 5 other locations throughout the Hawaiian Archipelago between August 2005 and November 2008 (Fig. 1). Of the 27 litters collected, 4 were sampled from the newly established Papahānaumokuākea Marine National Monument in the Northwest Hawaiian Islands (NWHI). The NWHI includes 10 small atolls, pinnacles,

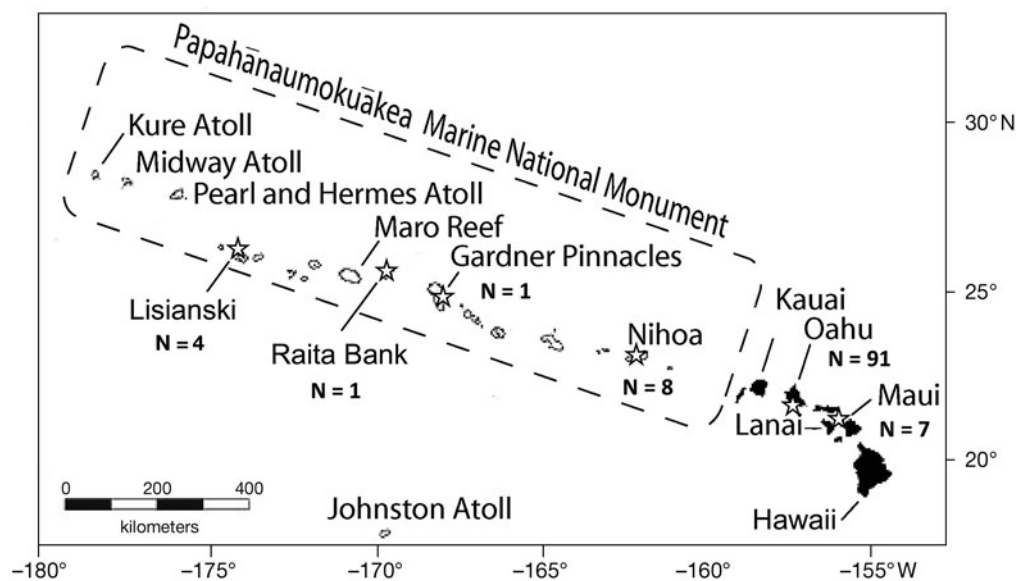


Fig. 1. Hawaiian Archipelago. Stars indicate the 6 sampling sites. N: corresponding sample sizes were analyzed for mitochondrial diversity. Base map reproduced from [www.oar.noaa.gov/spotlite/archive/images/bottomfishing\\_NWHI.jpg](http://www.oar.noaa.gov/spotlite/archive/images/bottomfishing_NWHI.jpg)

and islands and encompasses 360 000 km<sup>2</sup> of ocean water northwest of Kauai. The remote location of the NWHI combined with high level of protection made it difficult to acquire specimens, which were opportunistic bycatch from bottom fishing vessels. The remaining 23 litters were obtained from the Main Hawaiian Islands (MHI) off the islands of Oahu and Maui (Penguin Banks, Table 1) using monofilament research lines (shortened longlines, ~0.8 to 1.2 km) anchored at each end and marked with buoys. Approximately 17% of the sharks caught by longlining were pregnant females. We used branch lines or gangions 4 m in length composed of stainless steel tuna clips attached to 2.5 m of 250 kg monofilament line. The line was attached to 1.5 m of stainless steel aircraft cable with 8/0 stainless steel swivel and 11/0 circle hooks baited with Japanese mackerel *Scomber japonicus* or squid (*Loligo* spp.). Each line consisted of 50 to 80 gangions spaced approximately 15 m apart. Sharks were measured and weighed, litter size was recorded, and small samples of fin or muscle tissue were taken using scissors from mothers and pups. Tissue was stored in 20%

dimethylsulfoxide (DMSO) saturated salt buffer (Seutin et al. 1991) or >75% ethanol (EtOH). DNA was extracted from tissue using a salting-out protocol adapted from Sunnucks & Hales (1996). Samples stored in EtOH were dried in a speed vacuum for 30 min at 55°C before extraction.

**Microsatellite fragment analysis.** We developed microsatellite markers using an enrichment protocol (Glenn & Schable 2005). This protocol, which employs streptavidin-coated magnetic beads and biotin-labeled repetitive probes—here, (AGAT)<sub>8</sub>, (AAAG)<sub>8</sub>, and (AAAC)<sub>6</sub>—was followed as described previously (Feldheim et al. 2007). Six species-specific primers were developed using the default settings in OPrimer3 ([http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)). These 6 plus 2 primer pairs developed for *Squalus acanthias* (T289 and U285; McCauley et al. 2004) were found to be highly variable and therefore informative for parentage analysis (Table 2). Following optimization, unlabeled reverse primers were obtained from Integrated DNA Technologies. Forward primers were labeled with 6-FAM, VIC, NED, and PET proprietary dyes (Applied Biosystems). PCR reactions consisted of 0.1 U Biolase *Taq* DNA polymerase (Bioline), 1× *Taq* buffer, 0.25 to 0.0625 μm of each primer (see Table 2), 200 μm each dNTP, and 2.0 mm MgCl<sub>2</sub>. PCR amplification on a MyCycler (Bio-Rad) consisted of an initial denaturation at 95°C for 4 min followed by 35 cycles of 1 min at 95°C, 30 s at optimal annealing temperature (*T<sub>a</sub>*) (Table 2), 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 3.0 (Applied Biosystems). Negative and positive controls consisted of extraction and amplification of known samples and DNA sequencing of randomly selected individuals.

Table 1. *Squalus mitsukurii*. Date of capture and location is shown for each of 27 litters, as well as the size of the mother (TL: total length, cm), number of pups per litter, average TL of pups, maximum number of paternal alleles detected across 8 microsatellite loci, and minimum number of sires indicated by the presence of these alleles in each litter. PB: Penguin Banks; nd: not determined

Litter ID	Capture date	Capture location	Maternal TL	No. pups in litter	Mean TL of pups	Max. no. paternal alleles	Min. no. sires
B	Aug 2005	Oahu	60.0	6	21.5	2	1
C	Aug 2005	Oahu	66.5	7	14.6	2	1
D	Sep 2005	Oahu	68.5	5	23.6	2	1
E	Feb 2006	Oahu	78.5	8	16.3	2	1
F	Feb 2006	Oahu	78.0	5	12.7	2	1
G	Feb 2006	Oahu	84.0	5	10.3	2	1
H	Feb 2006	Oahu	74.0	5	24	2	1
I	Feb 2006	Oahu	77.0	5	22.3	2	1
J	May 2007	Oahu	nd	3	7.9	2	1
K	Feb 2008	Oahu	87.5	10	nd	2	1
L	Mar 2008	PB	72.5	4	17.4	2	1
N	Mar 2008	PB	79.0	7	3.5	2	1
O	Mar 2008	PB	81.0	6	4.2	2	1
P	Mar 2008	PB	70.0	5	2.3	2	1
Q	Apr 2008	Oahu	nd	7	nd	2	1
R	Nov 2008	Oahu	88.0	9	12.8	2	1
S	Nov 2008	Oahu	87.5	10	2.5	2	1
T	Nov 2008	Oahu	84.5	10	9.3	2	1
U	Nov 2008	Oahu	79.5	5	8.4	2	1
V	Nov 2008	Oahu	88.0	9	14.7	4	2
W	Nov 2008	Oahu	92.0	9	1.9	2	1
X	Nov 2008	Oahu	86.0	10	18.4	2	1
Y	Nov 2008	Oahu	83.0	6	4.2	2	1
NA	Jun 2006	Lisianski	90.0	6	20.2	4	2
NB	Jun 2006	Lisianski	101.0	7	10.9	2	1
NC	Oct 2006	Gardner	84.5	5	7.6	2	1
ND	Dec 2007	Nihoa	78.5	4	11.9	4	2

We estimated heterozygosity and tested for deviation from Hardy-Weinberg Equilibrium (HWE) in 96 unrelated individuals, including mothers and all individuals from the NWHI, using Genepop 3.4 (Raymond & Rousset 1995), and tested for linkage disequilibrium using Arlequin 3.11 (Excoffier et al. 2005). We used MicroChecker 1 (van Oosterhout et al. 2004) to infer genotyping errors due to null alleles, short PCR dominance (large

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allele dropout), the scoring of stutter peaks, and typographic errors. We inferred the minimum number of sires from the number of non-maternal alleles detected among all pups following the methods of Neff et al. (2002). For each litter, we removed the maternal alleles and counted the number of unique non-maternal alleles (Toonen 2004). Since the genotypes of the sires are unknown in these field-collected animals, we used the conservative assumption that every female mated with only heterozygous males. Given this assumption, the minimum number of sires per litter is one-half the number of non-maternal alleles. If an odd number of non-maternal alleles were detected among the pups, the minimum number of males was rounded up. For example, if 3 non-maternal alleles were detected, the minimum estimated number of sires was rounded up from 1.5 to 2 males. Mendelian inheritance of maternal alleles was tested in each litter using a chi-squared goodness-of-fit test against an expected 1:1 inheritance ratio.

We used the program PrDM 1 (Neff & Pitcher 2002) to calculate the probability of detecting multiple mating (PrDM) in a sample of offspring based on (1) the number of loci, (2) the number of alleles per locus, (3) allele frequencies in the natural population (obtained from the 96 unrelated individuals), (4) the conservative estimate of number of sires contributing to each brood, and (5) reproductive skew of each sire (Vieites et al.

2004). The model assumes single-sex multiple mating (polygyny or polyandry), where all offspring in a brood were either full-siblings or half-siblings. We used an initial model of only 2 sires, each with the probability of mating equal to 0.5, because this is the most conservative estimate. Adding sires to the model would increase the statistical power to detect multiple paternity, but could lead to false overestimation of multiple matings (Neff et al. 2002). We performed 8 replicates of the analysis for the range observed in our samples (3 to 10 pups).

The Bayesian program FMM 1 (Neff et al. 2002) was used to estimate expected frequency of multiple paternity in this population. Because not all of the males in the population are heterozygous for alleles other than those carried by the mother, an estimate based solely on the observed number of non-maternal alleles may underestimate the true frequency of multiple paternity (Neff et al. 2002, Toonen 2004). The Bayesian method used in FMM takes the allele frequency distribution of the population into consideration when calculating the most likely frequency of multiple paternity, and assigns a 95% confidence interval to that estimate. Statistical correlations between the total length (TL) of the mother, number of pups per litter, and number of paternal alleles detected were tested with Minitab 14.

**Genetic structure and diversity.** Because we sampled across a broad geographic range (2000 km), we needed

Table 2. *Squalus mitsukurii*. Details on microsatellite loci used in the present study. Locus name, primer sequence (F: forward; R: reverse), repeat motif, and size (bp) of the allele from which primers were developed, plus annealing temperature ( $T_a$ , °C) and primer concentration ( $\mu\text{mol reaction}^{-1}$ ). Also shown are allelic diversity ( $k$ ), allelic richness ( $A$ ), observed and expected heterozygosities ( $H_{\text{obs}}$  and  $H_{\text{exp}}$ ), probabilities (p-values) from Hardy-Weinberg Equilibrium tests for homozygote excess based on multi-locus genotypes from 96 unrelated *S. mitsukurii*, and values of Jost's estimated  $D$  ( $D_{\text{est}}$ ). Dye labels were applied to forward primers

Locus	Primer sequence	Motif	Size	$T_a$	Primer conc.	$k$	$A$	$H_{\text{obs}}$	$H_{\text{exp}}$	p	$D_{\text{est}}$
Smi033	F: GAAAGCAGAAATGCCACAT R: GGGATATATGAACCCCTTTAAGTCA	(AC) <sub>22</sub>	223	62	0.5	13	11.20	0.311	0.352	0.229	-0.003
Smi063	F: GGACAATTCAAACAATCTAAACAATG R: AGTGCTGGACCATCATAGCC	Imperfect	191	62	0.125	21	19.12	0.756	0.806	0.352	-0.034
Smi242	F: CATGTTTCAAGGAAGGATGG R: TAGTTGGGCACATGCAAGAA	Imp. AAAG/ AAGG repeat	286	62	0.25	2	1.93	0.607	0.514	0.000 <sup>a</sup>	-0.007
Smi292	F: TATATGGGGAATGASATTAAG R: AAAAGGAGATGGAATAACTATGGTG	Imperfect	249	56	0.15	8	8.00	0.385	0.373	0.097	-0.005
Smi294	F: AACATAGCCACCCAATCACC R: TTCAATGCACGTCAACAAGG	Imperfect	158	62	0.15	2	2.00	0.674	0.502	0.000 <sup>a</sup>	-0.007
Smi327	F: CCGCTTCAGATCAGCTTTTT R: CCAAGGATTTGTACGGCATC	(TAGA) <sub>17</sub>	202	62	0.125	13	11.64	0.846	0.866	0.574	-0.044
T289 <sup>a</sup>	F: GGGCGTCTGTGAACGCAGAC R: ATAGTCCAGTAACATAACCTG	(TCC) <sub>7</sub>	191	56	0.25	6	5.39	0.489	0.519	0.556	-0.011
U285 <sup>a</sup>	F: CTGTCCATGGTCACTTTT R: GATACTTTTGTTCAGAGC	(CT) <sub>11</sub>	240	56	0.125	8	7.56	0.551	0.602	0.138	-0.016

<sup>a</sup>McCauley et al. (2004)



to first confirm that we were assaying a single breeding population. To this end, mitochondrial haplotype diversity was calculated in all 112 unrelated individuals collected across the sampling locations represented in the present study (Fig. 1). A fragment of the control region (670 bp) was amplified from each sample using the ProL2 (5'-CTG CCC TTG GTC CCC AAA GC-3') and PheCaCaH2 (5'-CTT AGC ATC TTC AGT GCC AT-3') primers (Pardini et al. 2001). Target DNA was amplified using the protocol outlined above, with a  $T_a$  of 60°C. PCR products were cycle sequenced using Big Dye chemistry on an ABI 3100 automated sequencer (Applied Biosystems) at the Hawaii Institute of Marine Biology EPSCoR Sequencing Facility, aligned by eye, and edited using Sequencher 4.6 (Gene Codes Corporation). Arlequin was used to generate nucleotide and haplotype diversities. PAUP\* 4.0b10 (Swofford 2000) was used to calculate genetic distance and Structure 2.2 (Pritchard et al. 2000) was used to calculate the likely number of distinct populations ( $K$ ) using microsatellite data. In Structure we used the admixture model with a 10 000 burn-in length and 10 000 simulations to test  $K = 1 - 5$  with 10 repetitions each. The relationships between haplotypes are described with a parsimony network based on TCS 1.21 (Clement et al. 2000) (see Fig. 2). We also used SMOGD 1.2.0 (Crawford 2009) to calculate Jost's  $D$  for unrelated individuals at 8 microsatellite loci. Jost's  $D$  is a measure of genetic differentiation that is independent of within-subpopulation heterozygosity (Jost 2008).

For the analysis comparing allelism at microsatellite loci to frequency of multiple paternity, results from 7 studies were compared: Chapman et al. (2004), Feldheim et al. (2004), Daly-Engel et al. (2007), Portnoy et al. (2007), DiBattista et al. (2008b), Lage et al. (2008), and the present study. Allelic richness was calculated using FSTAT 2.9.3.2 (Goudet 1995). FSTAT applies a rarefaction method to standardize alleles per locus to a uniform sample size, in this case, 60 to 70 individuals. In studies where the number of unrelated individuals genotyped was already 60 to 70 individuals, rarefaction was not performed. Percent multiple paternity was arcsine square root-transformed for linearity, and Pearson correlation on these data was done using Minitab 14.

## RESULTS

Using the program Structure, we found no evidence for more than one population within Hawaii ( $K = 1$ ) with estimates of posterior probability approaching 1, which is consistent with a lack of genetic structure. Similarly, within-population tests of genetic differentiation showed little differentiation across loci (average

$D_{est} = 0.016$ , Table 2). The TCS parsimony network of haplotypes (Fig. 2) showed 11 variable sites and 6 haplotypes (GenBank accession no. GU192450–GU192455). Two of these were exhibited among the vast majority of individuals (107 out of 112), with the other 4 haplotypes distributed among 5 remaining individuals. No more than 2 mutational steps separated any haplotype from another except for the divergent type found in a single specimen from Gardner Pinnacles, which was separated from the ancestral type by 8 mutations (a genetic distance of  $d = 1.2\%$ ). The parsimony network (Fig. 2) shows that the 2 most common haplotypes were observed at every sampling site where more than 1 sample was obtained, indicating high maternal gene flow throughout the sampling range.

MicroChecker detected no microsatellite scoring errors resulting from DNA degradation, low DNA concentrations, or primer-site mutations. There was evidence of deviation from HWE at Smi242 and Smi294, which showed significant heterozygote excess in the sample of 96 unrelated individuals (Table 2). Maternal

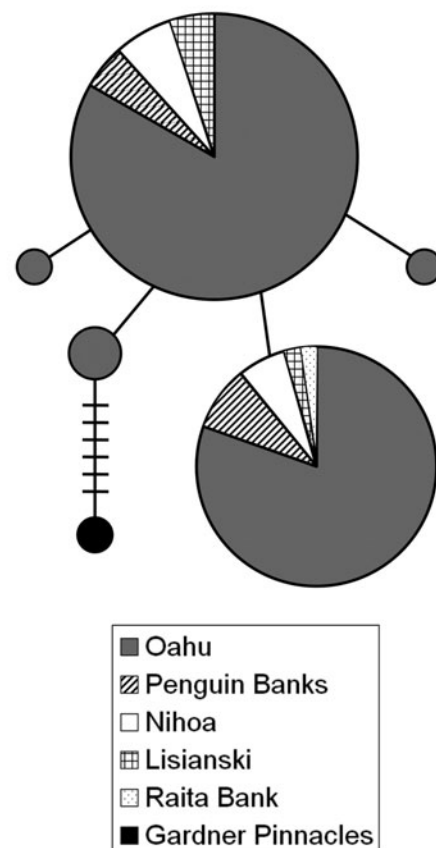


Fig. 2. *Squalus mitsukurii*. Parsimony network of control region haplotypes from 112 unrelated individuals. Size of circles or wedges represents the number of samples within each haplotype, and uninterrupted branches represent single mutational steps

alleles at these loci were inherited in expected 50:50 ratios in all offspring of the 27 litters, so heterozygote excess at these loci did not affect our estimate of multiple paternity. Smi033 was out of HWE due to heterozygote deficiency until we excluded the specimens from Lisianski and Raita Banks (the 2 atolls at the distal northwest end of our sampling range), possibly indicating a null allele at these locations. Though heterozygote deficiency may indicate a Wahlund effect, we eliminated this possibility because the discrepancy in HWE was limited to a single locus. Exclusion of the 5 individuals or this locus from any of our analyses did not significantly change our results, so we retained them in our analysis. HWE at Smi033 was based on 91 rather than 96 unrelated individuals. There was no evidence of linkage disequilibrium among pairs of loci after Bonferroni correction.

We found evidence of multiple paternity (3 or 4 paternal alleles at each of 2 to 3 loci) in 11% of the litters sampled (3 of 27 litters; Table 1). Each of the 178 pups had at least one maternal allele, and chi-squared tests confirmed that inheritance of these alleles did not vary from predicted 1:1 Mendelian inheritance ratios within each litter ( $df = 1$ ,  $p > 0.05$ ). The program FMM estimated the expected Bayesian frequency of multiple mating to be 9% in this population (excluding Smi242 and 294; Neff et al. 2002), which closely approximated our estimate of 11% based on direct count of non-maternal alleles. The 95% confidence interval (CI) was 1 to 24% mixed paternity. When we removed Smi033 from this analysis, the results were essentially unchanged (expected frequency of multiple mating = 12%, 95% CI = 2 to 27%).

Litters of *Squalus mitsukurii* ranged in size from 3 to 10 pups, and mean litter size was 6.6 (Table 2). The program PrDM (Neff & Pitcher 2002) assigned a 90%

probability of detecting multiple paternity in litters of this size (Neff & Pitcher 2002), hence we had good power to detect multiple paternity in *S. mitsukurii*. If we adjusted our calculation of multiple paternity to conservatively assume that it occurred in the 10% of cases where we lacked statistical power to detect it, then the frequency of multiple paternity in this population was approximately 12%, well within the 95% CI calculated by FMM. Among these 27 litters we found a significant correlation (Spearman's test,  $df = 1$ ,  $\alpha = 0.05$ ) between the TL of the mother and the number of pups per litter ( $R^2 = 0.34$ ,  $\rho = 0.59$ ,  $p = 0.002$ ). There was no significant correlation between the TL of the mother and the number of paternal alleles found among the pups ( $R^2 = 0.06$ ,  $\rho = 0.26$ ,  $p = 0.216$ ), or between litter size and the number of paternal alleles detected ( $R^2 = 0.01$ ,  $\rho = -0.06$ ,  $p = 0.76$ ).

Arlequin yielded a haplotype diversity value of  $h = 0.5412 \pm 0.0221$  and nucleotide diversity value of  $\pi = 0.0010 \pm 0.0008$ . Table 3 shows the results of all known studies documenting nucleotide and haplotype diversities in the mitochondrial control region for elasmobranch species. Haplotype diversity in *Squalus mitsukurii* is the third lowest among sharks to date, and nucleotide diversity was the second lowest measured in an elasmobranch.

To examine the relationship between mating strategy and genetic diversity we performed correlation analysis on all 7 data points from shark paternity studies published to date. Fig. 3A reports the results from the 5 studies that used only species-specific microsatellite loci (Feldheim et al. 2004, Portnoy et al. 2007, DiBattista et al. 2008b, Lage et al. 2008, present study), and Fig. 3B reflects the same analysis of these 5 studies plus 2 that did not use species-specific loci (Chapman et al. 2004, Daly-Engel et al. 2007). Microsatellite loci that

Table 3. Genetic diversity in the mitochondrial control region among 13 elasmobranch species. Nucleotide diversity ( $\pi$ ), haplotype diversity ( $h$ ), sequence length (bp), and sample sizes (N) are shown. nd: not determined. \*Studies encompassing more than one geographic region. SA: South Africa; WA: Western Australia

Species	$\pi \pm SD$	$h \pm SE$	Sequence length	N	Source
<i>Squalus mitsukurii</i>	0.0010 $\pm$ 0.0008	0.541 $\pm$ 0.022	670	112	Present study
<i>Galeorhinus galeus</i> *	0.0025	0.805	~990	116	Chabot & Allen (2009)
<i>Negaprion brevirostris</i>	0.0059	0.780	1090	80	Schultz et al. (2008)
<i>Negaprion acutidens</i>	0.0006	0.280	1090	58	Schultz et al. (2008)
<i>Rhincodon typus</i>	0.0110 $\pm$ 0.006	0.974 $\pm$ 0.008	1236	70	Castro et al. (2007)
<i>Cetorhinus maximus</i> *	0.0013 $\pm$ 0.0009	0.720 $\pm$ 0.028	1085	62	Hoelzel et al. (2006)
<i>Carcharias taurus</i> (SA)	0.0030 $\pm$ 0.0001	0.717 $\pm$ 0.010	700	26	Stow et al. (2006)
<i>Carcharias taurus</i> (WA)	0.0031 $\pm$ 0.0001	0.458 $\pm$ 0.024	700	16	Stow et al. (2006)
<i>Sphyrna lewini</i> *	0.0130 $\pm$ 0.0068	0.800 $\pm$ 0.020	548	271	Duncan et al. (2006)
<i>Carcharhinus limbatus</i>	0.0021 $\pm$ 0.0013	0.805 $\pm$ 0.018	1070	323	Keeney et al. (2005)
<i>Raja clavata</i>	0.0072	0.610	335	26	Valsecchi et al. (2005)
<i>Raja miraletus</i>	0.0031	0.170	330	12	Valsecchi et al. (2005)
<i>Raja asterius</i>	0.0092	0.290	329	18	Valsecchi et al. (2005)
<i>Carcharodon carcharias</i>	0.0203	nd	nd	88	Pardini et al. (2001)

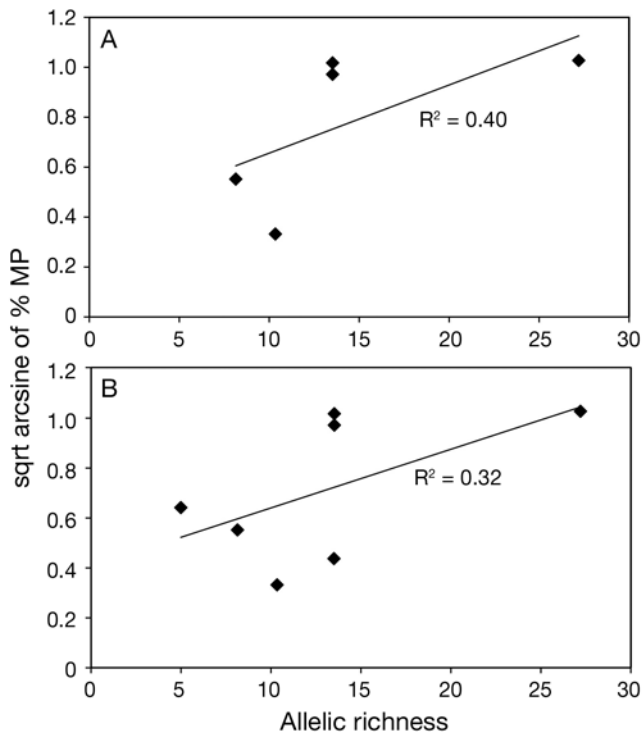


Fig. 3. Correlation of allelic richness with percent multiple paternity (% MP; square root-arc sine transformed) in all elasmobranch multiple paternity studies to date for which allele frequency data was available. (A) Includes data points from 5 studies with species-specific microsatellite markers (Feldheim et al. 2004, Portnoy et al. 2007, DiBattista et al. 2008b, Lage et al. 2008, present study); (B) shows the same correlation including 2 studies that used non-species-specific loci (Chapman et al. 2004, Daly-Engel et al. 2007)

are cross-amplified across species may be less polymorphic than they are in target species, though the number of loci that successfully cross-amplify in sharks is often higher than in other taxa, presumably due to their slower rate of nucleotide mutation (Martin et al. 1992). Although the removal of 2 studies leaves us with only 5 data points in Fig. 3A, we chose to present both sets of data because we thought that the effect of this variable cannot be sufficiently resolved within the scope of the present study (though the present study used 2 loci developed for a congener, these loci were not considered when calculating allelic richness). The correlation between multiple paternity and genetic diversity in the 5 species-specific studies returned an  $R^2$  value of 0.40 ( $p = 0.184$ ; Fig. 3A). When we included the 2 studies that did not use species-specific markers (Chapman et al. 2004, Daly-Engel et al. 2007), the  $R^2$  dropped only slightly, to 0.32 ( $p = 0.249$ ; Fig. 3B). While preliminary and not statistically significant, these data indicate that a relationship may exist between allelic richness and multiple paternity in sharks, though more data points are needed to provide thorough analysis.

## DISCUSSION

### Population structure

We analyzed 670 bp of the mitochondrial control region to characterize population structure and nucleotide diversity in the shortspine spurdog *Squalus mitsukurii* in Hawaii. Overall, our observation of several common haplotypes distributed among nearly all sampling sites indicates that *S. mitsukurii* throughout the Hawaiian Archipelago is composed of a single breeding population ( $K = 1$ ). Given the low mtDNA diversity and low sample sizes at most locations, however, the conclusion of no population structure in *S. mitsukurii* from Hawaii must be regarded as provisional. Although a robust test of this conclusion would require larger sample sizes, the finding of no genetic structure is consistent with reef fish studies that show high connectivity across the Hawaiian Archipelago (Craig et al. 2007, Eble et al. 2009). Interestingly, the single specimen obtained from Gardner Pinnacles had the most divergent haplotype, 1.2% from the nearest related haplotype. This divergence is notable because Gardner Pinnacles in the central Hawaiian Archipelago is near Johnston Atoll, a suspected entry point for colonization into Hawaii (Gosline 1955). These data indicate that dispersal in *S. mitsukurii* is greater than their known habitats would indicate (see Schultz et al. 2008), because maternal gene flow appears to occur across depths greater than the maximum depth (954 m) reported for this species (Compagno et al. 2005).

### Multiple paternity in sharks

Our observation of 11% multiple paternity (3/27) in Hawaiian *Squalus mitsukurii* is the lowest level estimated in an elasmobranch species to date, with a maximum of 4 paternal alleles found at any single locus. Number of paternal alleles detected per litter was not correlated with TL of the mother or number of pups per litter, though significant correlation was found between TL of the mother and number of offspring, a finding consistent with other shark species (Cortes 2000). Estimates of the frequency of multiple paternity in natural shark populations have included a predominance of genetic monogamy in the present study, as well as in the bonnethead shark *Sphyrna tiburo* (18%; Chapman et al. 2004). Two studies have returned intermediate values of multiple paternity, for the spiny dogfish shark *Squalus acanthias* (30%; Lage et al. 2008) and the Hawaiian population of sandbar sharks *Carcharhinus plumbeus* (40%; Daly-Engel et al. 2007). Very high prevalence of multiple paternity was reported in lemon sharks *Negaprion brevirostris* from the



Bahamas (87%; Feldheim et al. 2004) and Florida (85%; DiBattista et al. 2008a), and in the Northwest Atlantic population of sandbar sharks *C. plumbeus* (86%; Portnoy et al. 2007). No shark species examined to date has shown a complete absence of multiple paternity. The ubiquity of multiple paternity in sharks indicates that this strategy is beneficial or unavoidable, or possibly both.

Portnoy et al. (2007) proposed that females with longer reproductive cycles may employ polyandrous mating behavior, effectively increasing the cumulative genetic variation in progeny. Lemon sharks and Northwest Atlantic sandbar sharks, which show a high rate of multiple paternity, mate once every 2 yr, while the predominantly monogamous bonnethead sharks have an annual reproductive cycle (Chapman et al. 2004, Compagno et al. 2005). Our current results and those from a previous paper (Daly-Engel et al. 2007) do not support the long reproductive cycle–high multiple paternity hypothesis, since sandbar sharks in Hawaii have the same reproductive cycle as those in the Atlantic, but a much lower rate of multiple paternity.

#### Encounter rate theory and sexual conflict

The simplest explanation for the multiple paternity observed in natural populations is the encounter rate theory (Lopez-Leon et al. 1993, Daly-Engel et al. 2007), which holds that rate of multiple mating should depend on the number of male conspecifics a female encounters over the course of a breeding season. In high density populations, therefore, a female should have more opportunities to encounter males, and the rate of multiple paternity should increase (Kokko & Rankin 2006, Daly-Engel et al. 2007). For example, in nesting populations of olive ridley sea turtles *Lepidochelys olivacea*, Jensen et al. (2006) found that the frequency of multiple paternity was highly correlated with the density of reproductive adults.

In sexually reproducing species, differing fitness strategies can lead to conflict between males and females. Though we did not directly measure sexual conflict, the frequency of multiple paternity may be determined not only by the ecological conditions that affect encounter rate, but the sex ratios under which those encounters occur. Among roving predators such as sharks, the social interplay between the sexes can strongly influence encounter rate. Though mating behavior in sharks is difficult to observe, female sharks do exert mate choice in the wild, largely through mating avoidance (Pratt & Carrier 2001, Whitney et al. 2004). In contrast, male sharks are expected to exhibit a fitness strategy that favors promiscuity. Because many sharks exhibit sexual segregation as well as sex-

ually differential migration, the conflict between the sexes is played out largely during mating encounters. Shark mating is usually characterized by the male biting the female, especially around the base of the fins and flank, until he succeeds in grasping one of her pectoral fins, wrapping his body around her, and inserting 1 of 2 intromittent organs (claspers) into her cloaca for insemination (Pratt & Carrier 2001, Hamlett 2005). Though mortality is rare, it is common for females to incur serious injury during mating (Carrier et al. 2004) and to be more vulnerable to predation during and immediately after mating attempts. In sharks, the female is the larger of the 2 sexes, and could theoretically avoid mating with a conspecific male in a one-on-one encounter. In encounters where males outnumber females, which may occur within a mating aggregation, males can overcome the size disadvantage with cooperative behavior (mobbing or herding) to induce otherwise unwilling females to mate (Pratt & Carrier 2001, Whitney et al. 2004). In the case of coercive mating, a female may capitulate to avoid incurring more harm, resulting in convenience polyandry (Thornhill & Alcock 1983, Lee & Hayes 2004, DiBattista et al. 2008a). For example, populations of sandbar sharks *Carcharhinus plumbeus* in the Northwest Atlantic are sexually segregated throughout much of the year, but aggregate in the warmer water of the Gulf of Mexico in the winter (Musick 1999). These aggregations may create opportunities for cooperative behavior on the part of the males to induce mating. In the Hawaiian sandbar shark population, males and females mix throughout the year (Daly-Engel et al. 2006, 2007) and no large aggregations for the purposes of mating have been observed. The encounter rate theory predicts that because sexual segregation is less stringent in Hawaii than in the Atlantic, there should be a higher rate of multiple paternity in Hawaii. Instead, the rate of multiple paternity in Hawaii is about half that observed the Northwest Atlantic (Daly-Engel et al. 2007, Portnoy et al. 2007), indicating that aggregative behavior which facilitates male coercion may have a disproportionately large effect on rate of multiple paternity.

#### Genetic polyandry and mating avoidance

The discrepancy between predictions based on the encounter rate theory and observations from Pacific and Atlantic sandbar sharks indicates that the sex ratio during mating encounters (male-biased aggregations versus one-on-one encounters) may play a role in determining the prevalence of multiple paternity. Even when a population does not include mating aggregations, predictability in the mating behavior of one sex (e.g. female dependence on coastal nursery grounds,

or philopatry; Feldheim et al. 2004, Grubbs et al. 2007) may create the opportunity for seasonally elevated density. Such predictable behavior may account for the high (81 to 87%) multiple paternity observed among populations of philopatric lemon sharks *Negaprion brevirostris*. Feldheim et al. (2004) and DiBattista et al. (2008a) suggest that high multiple paternity in lemon sharks is more likely a result of convenience polyandry than of indirect genetic benefits such as inbreeding avoidance.

*Squalus mitsukurii* in Hawaii have a number of physiological and life history traits which, taken together, may reduce genetic polyandry. Compared with oviparous sharks, the squalid oviducal gland (the organ of elasmobranch sperm storage) is relatively reduced (Hamlett 2005), suggesting that long-term sperm storage may not play a large role in the squalid mating system. Ecologically, *S. mitsukurii* inhabit a slope habitat (100 to 950 m depth), aggregating around pinnacles, canyons, and seamounts. Within these aggregations, males segregate from females, and adults from both subadults and juveniles (Wilson & Seki 1994). This sexual segregation is common to almost every shark species examined to date, and is thought to be a mechanism for both mating and cannibalism avoidance (Cortes 2000). Mating aggregations which facilitate convenience polyandry are unlikely in species like *S. mitsukurii*, whose asynchronous, ovoviparous reproductive strategy makes it difficult for males to predict when females might be receptive to mating. Lack of opportunity for male coercion could lead to potentially low rates of multiple paternity in species that demonstrate asynchronous reproduction. For example, Lage et al. (2008) recently estimated the rate of multiple mating to be 30% in 10 litters of the congener species *S. acanthias*, which has the same asynchronous, ovoviviparous reproductive strategy as *S. mitsukurii*.

The protracted reproductive cycle may provide further incentive for female *Squalus mitsukurii* to avoid incurring harm from multiple copulations (Siva-Jothy 2006). *S. mitsukurii* gestate their young for 24 mo (Compagno et al. 2005) and give birth to average of 6 pups per litter (Table 1). Every mature female *S. mitsukurii* caught for the present study had either fertilized ova or embryos; like the congener *S. acanthias*, *S. mitsukurii* appears to have little or no quiescent period between pregnancies (Fischer et al. 2006, T. S. Daly-Engel & R. D. Grubbs unpubl. data), such that successful copulation most likely occurs very soon following parturition. In squalid sharks, all fertilized ova in each uterus are encased in a single membranous casing or 'candle' that fills the uterus. The distal ends of this candle plug the oviduct cranially and the uterine sphincter caudally, such that any copulation following

fertilization would be unsuccessful, likely causing a rupture in the candle leading to the death of the existing embryos. This physiology likely results in decreased opportunity for multiple mating in squalid compared to carcharhinid sharks, which can mate while gravid over a period of several months without harming the embryos, and added incentive for male avoidance in female *S. mitsukurii*.

### Genetic diversity and multiple paternity

A frequently proposed benefit of multiple paternity is its potential for increasing effective population size by increasing the number of males that mate successfully, thereby maintaining population genetic diversity (Nunney 1996, Ramakrishnan et al. 2004, Frankham 2005). Elasmobranchs have lower genetic diversity than most other taxa (Hoelzel et al. 2006), perhaps because of their slow rate of molecular evolution (Martin et al. 1992). Multiple paternity at some frequency has been observed in every elasmobranch species examined to date, indicating that multiple paternity may serve as a stable evolutionary strategy to maintain genetic diversity in elasmobranch populations. Metabolism might also play a role in lowering genetic diversity in *Squalus mitsukurii*, which inhabits deeper, cooler waters than the other species examined, and whose correspondingly slower metabolic rate may confer a lower than normal rate of genetic evolution (Brown et al. 1979).

Multiple paternity may result in increased genetic diversity in a single litter, but at the population level, this effect is likely to be mitigated by a corresponding increased variance in male reproductive success (Karl 2008). Our comparison of published estimates of multiple paternity in sharks (Fig. 3) yielded a non-significant correlation of  $R^2 = 0.40$  between genetic diversity and multiple paternity. Though this test has arguably low power because of the sample size of only 5 studies, as more of these studies are done, the relatively high  $R^2$  value indicates that there may well be a relationship between these 2 variables and that further investigation is warranted. It is possible that allelic richness itself might account for some of this pattern, since increased allelic diversity enhances the probability of detecting multiple paternity across loci (Neff & Pitcher 2002). However, the ability to detect multiple paternity in most of these studies is quite good (>90%). It is possible that even in studies reporting a high PrDM, multiple paternity may be underestimated due to lack of allelic diversity across loci or sampling sites, but most studies incorporate an interpretation of allelism and PrDM in their discussions when reporting on rate of multiple paternity.

## CONCLUSIONS

Here we report the lowest level of multiple paternity (11%) observed to date in an elasmobranch, the short-spine spurdog *Squalus mitsukurii*. This is the first survey of genetic polyandry in a deep-water vertebrate. While frequency of genetic polyandry in shark populations is likely influenced by sexual conflict, the findings for *S. mitsukurii* also indicate a potential role for physiology and encounter rate in determining the frequency of multiple paternity. Under this hypothesis, the predominance of genetic monogamy in this species results from life history characters such as asynchronous reproduction, lack of mating aggregations, and an ovoviviparous reproductive mode where all embryos initially develop in a common casing. *S. mitsukurii* also exhibited low nucleotide and haplotype diversity relative to other elasmobranchs ( $\pi = 0.0010 \pm 0.0008$ ,  $h = 0.5412 \pm 0.0221$ ). Given that the *S. mitsukurii* in Hawaii represent a healthy, unfished population yet show low levels of genetic diversity, it is possible that populations elsewhere may experience low levels of diversity made even lower by exploitation. Though the case for a causative relationship between polyandry and genetic diversity has yet to be made, it is known that both reproductive strategy and genetic diversity can influence a species' ability to rebound from population depletion, and these factors should be considered in efforts to conserve and manage these taxa.

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