### Title

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# Multiple paternity in leopard shark (*Triakis semifasciata*) litters sampled from a predominantly female aggregation in La Jolla, California, USA

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**Abstract**

The number of sires per litter was determined for the leopard shark (Triakidae: *Triakis semifasciata*) to investigate the potential effect of female-biased aggregation behavior on the frequency of multiple paternity ($F_{MP}$). Four highly polymorphic microsatellite markers were developed and used to genotype 449 pups from 22 litters (20.4 ± 7.0 pups per litter) sampled from pregnant females collected from a female-dominated leopard shark aggregation in La Jolla, California, USA. Multiple paternity was detected in 8 of 22 litters ($F_{MP}$ = 36.4%), each having two sires per litter. The relatively low $F_{MP}$ (compared to other shark species) is generally consistent with the hypothesis that female aggregation behavior reduces mating attempts by males and thus limits genetic polyandry. Significant interannual variability in $F_{MP}$ observed between two years of the study (2010: $F_{MP}$ = 20.0%, $n$ = 10, and 2011: $F_{MP}$ = 83.3%, $n$ = 6) appears to be correlated with the frequency of males in the aggregation. Although females may benefit indirectly from mating with multiple males by promoting sperm competition and hedging against nonviable sperm, the most probable explanation for genetic polyandry in the leopard shark appears to be "convenience polyandry," where females acquiesce to superfluous mating attempts if the costs of resistance outweigh the costs of capitulation. Thus, $F_{MP}$ is expected to increase as the male-to-female ratio increases and the capacity of females to resist coercive males decreases at the time and place of mating.

**Keywords**

elasmobranch; microsatellite; multiple paternity; paternal skew; shark; sperm competition

## 1. Introduction

Multiply sired litters (multiple paternity) are common in elasmobranch fishes; however, the percentage of litters sired by multiple males (frequency of multiple paternity, $F_{MP}$) exhibits strong inter- and intraspecific variability (reviewed in Byrne and Avise, 2012). Recent work suggests $F_{MP}$ is related to the encounter rate between potential mates (Daly-Engel et al., 2010), which varies among and within species depending on the extent of sexual segregation (reviewed in Wearmouth and Sims, 2008). For example, protracted sexual segregation might account for the low $F_{MP}$ in the shortspine spurdog (*Squalus mitsukurii*, $F_{MP}$ = 11.1%; Daly-Engel et al., 2010) and the spiny dogfish (*S. acanthias*, $F_{MP}$ = 17.2%; Veríssimo et al., 2011), whereas the comparatively high $F_{MP}$ in the brown smoothhound (*Mustelus henlei*, $F_{MP}$ = 93.0%) might be due to the formation of dense mixed-sex mating aggregations (Byrne and Avise, 2012). Predominantly female aggregations are particularly common in sharks (reviewed in Jacoby et al., 2011) and are often hypothesized to function as a means of reducing male harassment in the form of potentially injurious mating attempts (Economakis and Lobel, 1998; Klimley, 1985; Sims et al., 2001). If this is true, then female avoidance of superfluous copulations should limit genetic polyandry and result in a low $F_{MP}$. The present study tests this prediction using the leopard shark (Triakidae: *Triakis semifasciata*) as a model.

The leopard shark is a nearshore benthic species that forms dense aggregations of mature females throughout California, USA (Ebert and Ebert, 2005; Hight and Lowe, 2007; Nosal et al., 2013) and has an annual reproductive cycle; females give birth to 6 – 36 pups in April – June following a gestation period of 10 – 11 months, and thus mating, ovulation, and fertilization is expected to occur within a narrow window (1 – 2 months) following parturition (Castro, 2011; Ebert, 2003). Sperm storage and the potential for multiply sired litters are likely in *T.*

*semifasciata*, given the well-developed oviducal gland in mature females (Ebert and Ebert, 2005), where sperm storage has been documented in other triakid sharks, including *Mustelus canis* (Conrath and Musick, 2002), *M. antarcticus* (Storrie et al., 2008), and *M. asterias* (Farrell et al., 2010).

The present study examines $F_{MP}$ for leopard shark litters acquired from pregnant females aggregating off La Jolla, California, USA. This site-specific aggregation, which forms annually in June – December and consists of > 95% mature females, has been hypothesized to function, in part, as a refuge from males after mating elsewhere (Nosal et al., 2013); thus, due to the local scarcity of males, $F_{MP}$ was hypothesized to be low.

## 2. Methods

### *2.1. Sample Collection and DNA Extraction*

Seventeen dams were captured by hook and line from the La Jolla aggregation site (32.853°N, 117.263°W) between the months of September and November of 2007 – 2011 (well after ovulation and fertilization, so as to not artificially reduce the period in which additional copulations might have been procured) and transported to an open flow-through aquarium facility at Scripps Institution of Oceanography. In captivity, eight females gave birth naturally to litters of term pups, three were sacrificed, and six died of undetermined causes and prematurely delivered some or all of their pups shortly before death. Dead dams were dissected, whereas surviving dams and pups were donated to other laboratories, educational facilities, or released with permission from the California Department of Fish and Game. In addition to live-collected sharks, five pregnant females were found dead at the aggregation site during the course of the study (also between the months of September and November), salvaged, and included in the

multiple paternity analysis. Fin clips were collected from all dams and pups and preserved in 95% ethanol and stored at -80°C. To obtain baseline population genetic information, fin clips were collected from an additional 126 adult *T. semifasciata* at the aggregation site (tagged and released) during the same period. Total genomic DNA was extracted from each fin clip using a DNeasy Tissue Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions.

*2.2. Microsatellite Marker Development and Genotyping*

Four microsatellite markers (*Tse*01, *Tse*02, *Tse*03, and *Tse*04) were developed from enriched DNA libraries constructed specifically for *T. semifasciata* and screened according to the methods described by Jones et al. (2002). Briefly, total genomic DNA was digested with a cocktail of seven blunt-end restriction enzymes. Resulting fragments in the size range of 300 to 750 base pairs were hybridized to 5-prime-biotinylated oligonucleotides (microsatellite probes) and subjected to streptavidin magnetic bead capture (Millipore, Billerica, MA). Captured fragments were eluted and then amplified, cloned, and sequenced. Microsatellite-containing fragment sequences were identified by inspection and PCR primers were designed to anneal to flanking regions using DesignerPCR v1.03 (Research Genetics, Inc., Huntsville, AL). Forward primers were 5-prime-labeled with 6-FAM, HEX, or TET dyes for fluorescent visualization (Table 1).

Optimized PCR reactions for loci *Tse*01, *Tse*03, and *Tse*04 consisted of 25 µl of 1x GoTaq Green Master Mix (Promega Corporation, Fitchburg, WI), 10 µM each forward and reverse primer, and 50 – 100 ng of DNA template. For locus *Tse*02, the optimized PCR reaction consisted of 25 µl of 1x iTaq Buffer (Bio-Rad Laboratories, Inc., Hercules, CA), 1.5 mM $MgCl_2$, 200 µM each dNTP, 10 µM each forward and reverse primer, and contained 1.25 units iTaq

DNA Polymerase (Bio-Rad Laboratories, Inc., Hercules, CA) and 50 – 100 ng DNA template. PCR amplification on a MyCycler thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA) consisted of an initial denaturation at 95°C for 3 min, followed by 33, 28, 32, or 35 cycles (for loci *Tse*01, *Tse*02, *Tse*03, and *Tse*04, respectively) of 30 s at 95°C, 30 s at 56°C, and 1 min at 72°C, followed by a final extension at 72°C for 45 min. PCR products (including negative controls), ROX-labeled DNA ladder (MegaBACE ET550-R; GE Healthcare Life Sciences, Piscataway, NJ), and locus-specific allelic standards (positive controls) were resolved concurrently on 0.4 mm thick, large-format (33 x 39 cm) vertical polyacrylamide denaturing gels according to the methods described by Gruenthal and Burton (2008). Gels were electrophoresed at 60 W for 2 – 4 h and fluorescently scanned on a Typhoon 9410 Variable Mode Imager (Molecular Dynamics, Inc., Sunnyvale, CA). Allele sizes were determined using ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA) and manually scored, independently by two laboratory personnel. Discrepancies between scorers were rare, and settled by re-screening those particular individuals to confirm the genotype.

*2.3. Statistical Analyses*

For each microsatellite locus, the number of alleles, allele frequency distribution, observed and expected heterozygosity, and conformance to the expectations of Hardy-Weinberg equilibrium (HWE) were determined for the population sample ($n$ = 148, including the 22 dams) using Genepop v4.1 (Rousset, 2008). MicroChecker v1 (van Oosterhout et al., 2004) was used to infer potential genotyping errors due to null alleles and stutter peaks. Genotypic arrays of each litter were evaluated manually to ensure all progeny shared a maternal allele at each locus and to count the number of paternal alleles. A litter having three or more paternal alleles at one

or more loci was considered to be multiply sired. Gerud v2.0 (Jones, 2005) was used to estimate the minimum number of sires for each litter and determine the genotype of each sire. If no single solution of paternal genotypes could explain the progeny genotypic array, alternative solutions were ranked by relative probability based on patterns of Mendelian segregation and expected genotypic frequencies in the population (Jones, 2005). The most probable solution of paternal genotypes was used to assign a sire to each of the progeny and thus determine paternal skew for each litter.

As a *post-hoc* power assessment, the probability of detecting multiple paternity (PrDM) was calculated in PrDM v1 (Neff and Pitcher, 2002). This program used a Monte Carlo simulation to generate 10,000 reconstructed genotypes of multiply sired litters based on the number of microsatellite loci, the number of alleles and allelic frequency distribution at each locus, maternal genotype, litter size, numbers of sires (at least two), and hypothetical skew of sire reproductive success in the litter (paternal skew; 50:50, 60:40, 70:30, 80:20, 90:10, and 95:5). PrDM is the proportion of 10,000 multiply sired litters that contain at least three unique paternal alleles at one or more loci and would therefore be correctly identified as multiply sired. This test was particularly relevant for apparently singly sired litters. The Bayesian program FMM v1 (Neff et al., 2002) was used to generate a 95% confidence interval (CI) for the observed frequency of multiple paternity ($F_{MP}$ = number of multiply sired litters / total number of litters analyzed * 100) based on the expected $F_{MP}$ given the allelic frequency distribution of the population.

Finally, to investigate why some litters might have exhibited multiple paternity, but not others, the following tests were conducted. A Mann-Whitney *U* test was used to determine whether dam total length (TL) and litter sizes were each significantly different between multiply

and singly sired litters. A chi-squared ($\chi^2$) test was used to determine if paternal skew in multiply sired litters was significantly different from 50:50, and whether paternal skew inside each uterine horn was significantly different from the overall paternal skew. Lastly, Fisher's Exact Tests were used to determine whether the number of singly and multiply sired litters differed significantly between dams collected in 2010 and 2011, and whether the number of litters containing unfertilized ova differed between those sired by single and multiple males.

## 3. Results

Mean TL ± SD of the dams ($n$ = 22) was 142.6 ± 7.0 cm (range: 130 – 156 cm). Litters of term pups ($n$ = 8) were born in captivity between 4 April and 1 July (mean date of birth ± SD = 16 May ± 34 d). Mean litter size ± SD ($n$ = 20) was 21.8 ± 5.6 pups (range: 11 – 33 pups); litters I and K were excluded from this calculation because each was partially consumed by several large swell sharks (*Cephaloscyllium ventriosum*) being held temporarily in the same tank at the time of birth. The surviving pups from litters I ($n$ = 9) and K ($n$ = 4) were nevertheless genotyped; thus, the mean number of progeny genotyped ± SD was 20.4 ± 7.0 pups (range: 4 – 33 pups). The ratio of male to female pups did not deviate from 50:50 (Wilcoxon Signed-Rank Test; $W$ = 4.0, $n$ = 18, $P$ = 0.924), nor did the ratio of pups found in the left to right horns of the uterus ($W$ = -4.0, $n$ = 11, $P$ = 0.857). These findings are summarized in Table 2. A positive linear relationship was observed between litter size and dam TL (excluding litters H and K; Fig. 1) with a slope ($b$) that was significantly different from zero ($b$ = 0.526, $r^2$ = 0.429, $n$ = 20, $P$ = 0.002). In each of eight litters (36.4%), 1 – 3 unfertilized eggs were found either at the anterior end of the uterine horns, or delivered along with the pups. Mating scars were not evident in any of the 148 females sampled.

185 The suite of four microsatellite markers exhibited moderate to high polymorphism (9 –
186 23 alleles per locus; Appendix 1) in the screened population sample ($n$ = 148, including the 22
187 dams) and conformed to the expectations of Hardy-Weinberg equilibrium (Table 1). There was
188 no evidence of linkage disequilibrium between the four loci (tested in Genepop v4.1; Rousset,
189 2008).

190 In total, 22 dams and 449 pups were genotyped for paternity analysis. Multiple paternity
191 was detected in 8 of 22 litters ($F_{MP}$ = 36.4%, Bayesian estimate of 95% CI: 19 – 57%). No more
192 than four paternal alleles were observed at each locus, consistent with having one or two sires
193 per litter (Table 2). Paternal skew was high in the multiply sired litters; the dominant of the two
194 males (having higher reproductive success) sired up to 95.5% of the pups in a given litter (mean
195 ± SD = 76.0 ± 18.1%). Paternal skew deviated significantly from the expected ratio of 50:50 in
196 multiply sired litters R, T, U, and V ($\chi^2$ = 13.500 – 20.571, d.f. = 1, $n$ = 22 – 28, $p < 0.0002$), but
197 not in litters E, I, K, and S ($\chi^2 < 3.841$, d.f. = 1, $n$ = 4 – 30, $p > 0.05$). Of the eight multiply sired
198 litters, GERUD 2.0 produced a unique paternal skew solution for five litters (E, I, R, S, and U)
199 and two possible paternal skew solutions for three litters (K, T, and V). However, the most
200 likely (reported) solutions for two of these litters (T and V) were > 4 x $10^3$ times more probable
201 than their alternatives. Two nearly equally likely paternal skew solutions (3:1 and 2:2) were
202 returned for litter K. Given that high paternal skew decreases PrDM, the empirically derived
203 $F_{MP}$ of 36.4% might underestimate the true $F_{MP}$. For example, if one half of the apparently
204 singly sired litters ($n$ = 7) had paternal skews of 90:10 (two thirds, $n$ = 4.67) or 90:5 (one third, $n$
205 = 2.33), and given a mean *post-hoc* PrDM of 0.865 (90:10) and 0.647 (95:5) for the apparently
206 singly sired litters, then 1 – 2 of these might actually have been sired by multiple males, and not
207 detected. Thus, $F_{MP}$ for *T. semifasciata* might be as high as 40.9 – 45.5%, which still falls within

the 95% CI. Finally, significant interannual variability in $F_{MP}$ was observed between two years of the study (2010: $F_{MP}$ = 20.0%, $n$ = 10, and 2011: $F_{MP}$ = 83.3%, $n$ = 6; Fisher's Exact Test, $n$ = 16, $P < 0.035$) (Table 2).

In comparing singly and multiply sired litters, no significant differences were found in dam TL (Mann-Whitney Test, $U$ = 73, $n$ = 22, $P$ = 0.259) or litter size (excluding litters I and K; Mann-Whitney Test, $U$ = 52, $n$ = 20, $P$ = 0.435). In litter S, the paternal skew was significantly different from the overall skew of 17:13 in both the left (13:1, $\chi^2$ = 7.476, $n$ = 14, d.f. = 1, $P$ = 0.006) and right uterine horns (4:12, $\chi^2$ = 6.543, $n$ = 16, d.f. = 1, $P$ = 0.011); however, no other litter exhibited this pattern.

## 4. Discussion

The present study is the first to demonstrate multiple paternity and estimate $F_{MP}$ for *T. semifasciata*. The observed $F_{MP}$ of 36.4% is not the lowest reported for sharks (*S. mitsukurii*, $F_{MP}$ = 11.1%; Daly-Engel et al., 2010); however, it certainly falls to the lower end of the spectrum of $F_{MP}$ reported for other species (reviewed in Byrne and Avise, 2012) and thus generally supports the hypothesis that female aggregation behavior limits genetic polyandry. Nevertheless, multiply sired litters were detected; the simplest explanation for these is that they arose as a consequence of sexual conflict without any benefit to the females (Daly-Engel et al., 2010). However, it is important to address alternative explanations for these multiply sired litters and for the relatively low observed $F_{MP}$.

Pratt and Carrier (2001) suggested repeated copulations might be required in some shark species to ensure complete fertilization of ova; thus, multiple mating could increase fecundity. Consistent with this hypothesis, unfertilized ova were present in eight of the 22 leopard shark

litters examined (36.4%), which, in dissected dams ($n$ = 6), were located at the anterior end of either or both uterine horns, nearest the oviducal gland. Assuming conservation of uterine position throughout gestation, thus indicative of ovulation sequence (Smale and Compagno, 1997; Smale and Goosen, 1999), these unfertilized ova were last to pass through the sites of fertilization, when sperm had perhaps already been depleted. However, because unfertilized ova were found in both singly and multiply sired litters with no significant difference between the two, multiple mating clearly did not ensure complete fertilization. Moreover, there was no significant difference in litter size between multiply and singly sired litters, suggesting mating multiply does not increase fecundity in *T. semifasciata*.

Females might benefit indirectly from multiple mating because the simultaneous presence of sperm from multiple males could promote sperm competition and cryptic female sperm choice (Fitzpatrick et al., 2012). If sperm competitiveness is heritable, then male progeny of males with competitive sperm should have greater reproductive success (Keller and Reeve, 1995). Alternatively, if sperm competitiveness is related to genetic quality of the corresponding male more generally, then promoting sperm competition could increase the chances that offspring will inherit "good genes" (Jennions and Petrie, 2000; Sheldon, 1994; Yasui, 1997). Finally, cryptic female choice among ejaculates may similarly increase offspring genetic quality or complementarity (Fitzpatrick et al., 2012; Olsson and Madsen, 2001; Simmons, 2005). Consistent with these hypotheses are the high paternal skews observed in four multiply sired *T. semifasciata* litters (mean = 91.7:8.3), which suggest some form of post-copulatory sperm selection, or else are an artifact of mating order. However, direct evidence supporting "good genes" hypotheses (e.g., trading up, genetic bet-hedging) is lacking in elasmobranch fishes (Daly-Engel et al., 2007; DiBattista et al., 2008).

Finally, interspecific differences in oviducal gland ultrastructure could generate variation in sperm storage capability, and perhaps affect $F_{MP}$ (Pratt, 1993). For example, Daly-Engel et al. (2010) suggested the underdeveloped oviducal gland in the family Squalidae could limit sperm storage (Hamlett, 2005; Hamlett et al., 1998), which could at least partly explain the low $F_{MP}$ in *Squalus mitsukurii*. Comparatively low $F_{MP}$ in *S. acanthias* supports this hypothesis (Lage et al., 2008; Veríssimo et al., 2011), as does the high $F_{MP}$ for *M. henlei* (Byrne and Avise, 2012), a member of the family Triakidae, in which complex partitioning and sperm storage in the oviducal gland have been documented (Conrath and Musick, 2002; Farrell et al., 2010; Storrie et al., 2008). Given the apparent conservation of oviducal gland ultrastructure within taxonomic families, *T. semifasciata* is expected to have a structurally complex oviducal gland; thus, limited sperm storage does not readily explain the comparatively low $F_{MP}$ observed for *T. semifasciata*.

The most likely explanation for the interannual variability in $F_{MP}$ for *T. semifasciata* is interannual variability in the mate encounter rate. During a concurrent study of leopard shark movement patterns using passive acoustic telemetry (Nosal, 2013), the mean number of tagged males detected $d^{-1}$ at the La Jolla aggregation (all males were tagged in July 2009 at a site 12 km north of the La Jolla aggregation) was 0.009 (range: 0 – 1 male detected $d^{-1}$) in 2010 ($F_{MP}$ = 20.0%) and 0.784 (range: 0 – 5 males detected $d^{-1}$) in 2011 ($F_{MP}$ = 83.3%). The increased male presence at the La Jolla aggregation site in 2011 suggests the mate encounter rate was also likely higher during this year, which in turn led to increased mating activity and the higher $F_{MP}$ that year. Given this interannual variability in $F_{MP}$, multiple paternity likely arises in *T. semifasciata* as a consequence of sexual conflict; despite the need for cooperation for successful intromission, female sharks may acquiesce to most, if not all, mating attempts if the costs of resistance (e.g., incurring injury) are expected to exceed the costs of capitulation (Daly-Engel et al., 2010;

DiBattista et al., 2008; Portnoy et al., 2007). Such "convenience polyandry" does not necessarily exclude more elaborate evolutionary explanations of multiple paternity (e.g., genetic bet-hedging, trading up, hedging against insufficient or nonviable sperm); however, it would seem unlikely, for example, that female leopard sharks would hedge their bets or trade up to more attractive males more in one year than another.

In conclusion, the results of this study generally support the hypothesis that genetic polyandry is limited by female aggregation behavior and that $F_{MP}$ should largely reflect the probability of encountering mates during each reproductive cycle (Daly-Engel et al., 2010). However, the lack of mating scars in sampled females suggests mating may be less violent than in other species, and that, at least for *T. semifasciata*, avoiding mating-related injuries *per se* may not be the primary incentive for aggregation. Alternatively, avoiding male harassment may be an additional benefit to aggregating near favorable resources such as foraging grounds and warm, calm water (Nosal et al., 2013).

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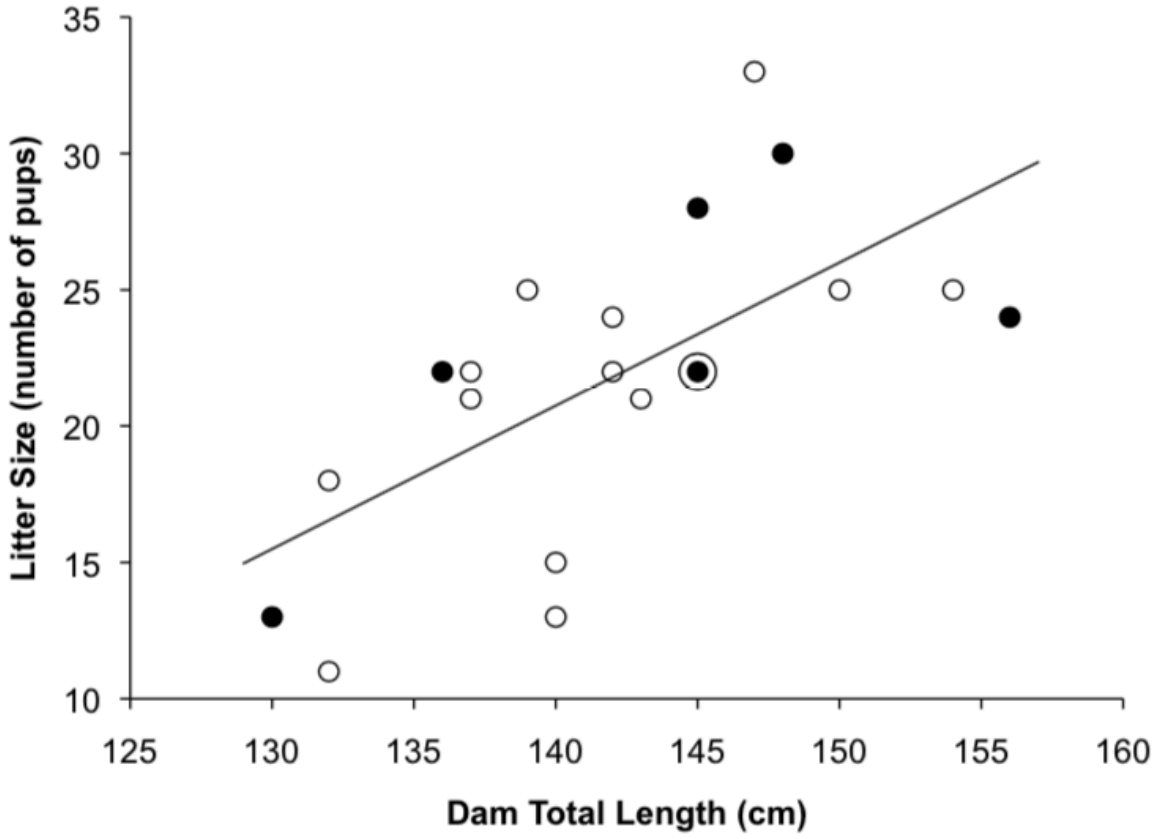


**Figure 1.** Linear regression of *Triakis semifasciata* litter size versus dam total length. Litters H and K are excluded because these litter sizes were artificially low (see Section 3). Singly sired litters appear as open circles and multiply sired litters appear as closed circles. Both a singly sired and a multiply sired litter occur at (145, 22). Regression statistics: $r^2 = 0.429$, $p = 0.002$; litter size = 0.526*(dam total length) – 52.826

488 **Tables**

489

490 **Table 1**

491 Summary of microsatellite characteristics for 148 presumably unrelated *Triakis semifasciata*. Annealing temperature ($T_a$); number of
492 PCR cycles (Cycl. #); size in base-pairs of the cloned allele from which the primers were designed (Size); number of individuals
493 screened ($N_i$); allelic diversity ($N_a$); expected heterozygosity ($H_E$); observed heterozygosity ($H_O$); *p*-values from Hardy-Weinberg
494 exact tests for homozygote excess ($P_{HW}$); exclusion probability ($P_E$)

495

| Locus | Primer Sequences | 5' Label | $T_a$ (°C) | Cycl. # | Repeat Motif | Size (bp) | $N_i$ | $N_a$ | $H_E$ | $H_O$ | $P_{HW}$ | $P_E$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Tse*01 | F: 5'-TGTGCTTTTGTATTCCTAATCC-3'<br>R: 5'-CGGGAGTATGGTGGTATTC-3' | HEX | 56 | 33 | $(CA)_{14}$ | 239 | 148 | 9 | 0.785 | 0.791 | 0.502 | 0.580 |
| *Tse*02 | F: 5'-CACCAGCAATCTGTCACTTG-3'<br>R: 5'-CTGTCTTAGCAATGGGTCTGT-3' | 6-FAM | 56 | 28 | $(CA)_{10}C(CA)_{18}$ | 123 | 148 | 23 | 0.868 | 0.885 | 0.674 | 0.735 |
| *Tse*03 | F: 5'-CAGTATTCTGGGATGGACTCTA-3'<br>R: 5'-AAGCAGTGTCAGTGGTAGTAGG-3' | TET | 56 | 32 | $(GATA)_{15}$ | 287 | 148 | 17 | 0.878 | 0.872 | 0.541 | 0.763 |
| *Tse*04 | F: 5'-CCTGCCTGGTTATTGACC-3'<br>R: 5'-CCTGACTGAGGTGTGTAAGATT-3' | HEX | 56 | 35 | $(CTAT)_{16}$ | 140 | 148 | 18 | 0.873 | 0.885 | 0.780 | 0.757 |

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**Table 2**

Summary information for litters of *Triakis semifasciata*. Multiply sired litters are indicated in bold. Dam identification letter (ID); dam total length (TL) in cm; litter size (SIZE) in number of pups; ratio of female to male pups in a given litter (F:M); mean total length of pups in a given litter ± standard deviation (MEAN TL ± SD) in cm; minimum number of sires suggested by GERUD (# SIRES) and skew of male reproductive success (in parentheses; SKEW); probability of detecting multiple paternity (PrDM) for six paternal skew scenarios

^ Litter size artificially low because some pups were consumed by swell sharks (see Section 3)

* Significant departure from expected 50:50 ratio ($\chi^2 = 4.545$, n = 22, d.f. = 1, p = 0.033)

| DAM | | | LITTER | | | | PrDM | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ID | TL (cm) | YEAR ACQUIRED | SIZE | F:M | MEAN TL ± SD (cm) | # SIRES (SKEW) | 0.50: 0.50 | 0.60: 0.40 | 0.70: 0.30 | 0.80: 0.20 | 0.90: 0.10 | 0.95: 0.05 |
| A | 150 | 2007 | 25 | 15:10 | 20.1 ± 0.6 | 1 (25:0) | 1.000 | 1.000 | 0.999 | 0.995 | 0.922 | 0.714 |
| B | 142 | 2007 | 22 | 10:12 | 21.5 ± 0.7 | 1 (22:0) | 1.000 | 0.999 | 0.999 | 0.991 | 0.895 | 0.667 |
| C | 132 | 2007 | 11 | 7:4 | 21.2 ± 0.6 | 1 (11:0) | 0.997 | 0.994 | 0.975 | 0.907 | 0.675 | 0.423 |
| D | 140 | 2009 | 15 | n.d. | 4.1 ± 0.7 | 1 (15:0) | 0.999 | 0.998 | 0.993 | 0.959 | 0.784 | 0.526 |
| E | 130 | 2009 | 13 | 5:8 | 13.5 ± 0.8 | 2 (7:6) | 0.999 | 0.997 | 0.987 | 0.938 | 0.734 | 0.477 |
| F | 147 | 2009 | 33 | 22:11 | 21.0 ± 0.5 | 1 (33:0) | 1.000 | 1.000 | 0.999 | 0.999 | 0.965 | 0.809 |
| G | 145 | 2010 | 22 | n.d. | 4.3 ± 0.8 | 1 (22:0) | 0.999 | 0.999 | 0.999 | 0.989 | 0.891 | 0.664 |
| H | 154 | 2010 | 25 | n.d. | 4.9 ± 0.9 | 1 (25:0) | 1.000 | 1.000 | 0.999 | 0.995 | 0.921 | 0.713 |
| I | 147 | 2010 | 9^ | 4:5 | 12.8 ± 0.6 | 2 (5:4) | 0.993 | 0.986 | 0.953 | 0.855 | 0.603 | 0.362 |
| J | 142 | 2010 | 24 | n.d. | 3.8 ± 0.7 | 1 (24:0) | 1.000 | 0.999 | 0.999 | 0.993 | 0.913 | 0.699 |
| K | 150 | 2010 | 4^ | 2:2 | 15.8 ± 0.6 | 2 (3:1) | 0.801 | 0.772 | 0.685 | 0.536 | 0.313 | 0.167 |
| L | 140 | 2010 | 13 | 7:6 | 13.0 ± 0.9 | 1 (13:0) | 0.999 | 0.987 | 0.988 | 0.939 | 0.735 | 0.477 |
| M | 137 | 2010 | 21 | 10:11 | 21.8 ± 0.7 | 1 (21:0) | 0.999 | 0.999 | 0.998 | 0.988 | 0.882 | 0.645 |
| N | 137 | 2010 | 22 | 7:15 | 19.5 ± 2.0 | 1 (22:0) | 1.000 | 0.999 | 0.999 | 0.992 | 0.903 | 0.681 |
| O | 132 | 2010 | 18 | 7:11 | 20.2 ± 0.7 | 1 (18:0) | 0.999 | 0.999 | 0.997 | 0.973 | 0.825 | 0.574 |
| P | 143 | 2010 | 21 | 13:8 | 20.7 ± 0.5 | 1 (21:0) | 0.999 | 0.999 | 0.998 | 0.988 | 0.883 | 0.652 |
| Q | 139 | 2011 | 25 | 9:16 | 15.5 ± 0.6 | 1 (25:0) | 1.000 | 0.999 | 0.999 | 0.994 | 0.922 | 0.713 |
| R | 156 | 2011 | 24 | 12:12 | 20.6 ± 0.6 | 2 (21:3) | 1.000 | 0.999 | 0.999 | 0.994 | 0.914 | 0.700 |
| S | 148 | 2011 | 30 | 17:13 | 20.2 ± 0.4 | 2 (17:13) | 1.000 | 0.999 | 0.999 | 0.998 | 0.953 | 0.777 |
| T | 145 | 2011 | 22 | 6:16* | 20.6 ± 0.5 | 2 (20:2) | 1.000 | 0.999 | 0.999 | 0.990 | 0.893 | 0.667 |
| U | 136 | 2011 | 22 | 12:10 | 18.3 ± 1.1 | 2 (21:1) | 0.999 | 0.999 | 0.999 | 0.990 | 0.893 | 0.668 |
| V | 145 | 2011 | 28 | 16:12 | 21.6 ± 0.4 | 2 (26:2) | 1.000 | 0.999 | 0.999 | 0.997 | 0.941 | 0.752 |

**Appendix 1**

Allele frequency distributions of four microsatellite loci for 148 presumably unrelated *Triakis semifasciata* individuals. Allele size (SIZE) in bp; allele frequency (FREQ)

| *Tse*01 | | *Tse*02 | | *Tse*03 | | *Tse*04 | |
|---|---|---|---|---|---|---|---|
| SIZE | FREQ | SIZE | FREQ | SIZE | FREQ | SIZE | FREQ |
| **217** | 0.226 | **66** | 0.101 | **259** | 0.101 | **112** | 0.003 |
| **229** | 0.041 | **68** | 0.010 | **263** | 0.280 | **116** | 0.064 |
| **237** | 0.307 | **72** | 0.010 | **267** | 0.057 | **120** | 0.304 |
| **239** | 0.243 | **86** | 0.037 | **271** | 0.074 | **124** | 0.068 |
| **241** | 0.054 | **88** | 0.010 | **275** | 0.034 | **128** | 0.061 |
| **243** | 0.020 | **96** | 0.003 | **279** | 0.017 | **132** | 0.024 |
| **245** | 0.010 | **99** | 0.014 | **283** | 0.007 | **136** | 0.064 |
| **251** | 0.088 | **100** | 0.003 | **287** | 0.034 | **140** | 0.051 |
| **253** | 0.010 | **104** | 0.074 | **291** | 0.084 | **144** | 0.054 |
| | | **106** | 0.007 | **295** | 0.088 | **148** | 0.071 |
| | | **107** | 0.010 | **299** | 0.051 | **152** | 0.017 |
| | | **108** | 0.014 | **303** | 0.034 | **156** | 0.074 |
| | | **109** | 0.115 | **307** | 0.030 | **160** | 0.030 |
| | | **110** | 0.003 | **311** | 0.061 | **164** | 0.037 |
| | | **111** | 0.216 | **315** | 0.027 | **168** | 0.027 |
| | | **113** | 0.226 | **319** | 0.007 | **172** | 0.007 |
| | | **115** | 0.024 | **323** | 0.014 | **176** | 0.037 |
| | | **117** | 0.003 | | | **180** | 0.007 |
| | | **119** | 0.064 | | | | |
| | | **120** | 0.003 | | | | |
| | | **121** | 0.014 | | | | |
| | | **123** | 0.034 | | | | |
| | | **125** | 0.003 | | | | |