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Polyandry in *Gymnura marmorata* from the Upper Gulf of California

Poliandria en *Gymnura marmorata* del Alto Golfo de California

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ABSTRACT

Reproductive behavior in elasmobranchs is challenging to assess due to the few tools available to study their mating systems. Molecular techniques offer an indirect approach to examine paternity in pups and identify putative fathers. This study presents the first record of the mating system of the California butterfly ray (*Gymnura marmorata*) using microsatellite molecular markers. A pregnant female, with a disk width (DW) of 95 cm and carrying 13 pups, was collected in Puerto Libertad, Sonora, Mexico. Paternity analyses detected that the litter was sired by at least two males, with a clear bias towards one dominant male who fathered most of the pups. Additionally, embryos sired by this dominant male had the largest DWs, suggesting a possible influence of sexual selection in this species.

***Key words**:* polyandry, multiple paternity, paternity biasing, Chondrichthyes

RESUMEN

El comportamiento reproductivo en elasmobranquios es difícil de evaluar debido a la limitada disponibilidad de herramientas para estudiar sus sistemas de apareamiento. Las herramientas moleculares ofrecen una aproximación indirecta para examinar la paternidad en las crías e identificar los posibles padres. Este estudio representa el primer registro del sistema de apareamiento en la raya mariposa (*Gymnura marmorata*) utilizando marcadores microsatélites. Se colectó una hembra preñada con un ancho de disco (AD) de 95 cm y con 13 embriones en Puerto Libertad, Sonora, México. Los análisis de paternidad detectaron que la camada fue fecundada por al menos dos machos diferentes con sesgo en la paternidad, donde un macho fecundó la mayoría de las crías. Además, los embriones del padre dominante presentaron un mayor tamaño de AD, sugiriendo una posible influencia de selección sexual en esta especie.

Palabras clave*:* poliandria, paternidad múltiple, sesgo de paternidad, Chondrictios.

INTRODUCTION

The mating systems of elasmobranchs, particularly ray species, remain poorly understood (Chevolot, Ellis, Rijnsdorp, Stam, Olsen, 2007). To better assess the vulnerability of these species to fishing pressures and develop more effective management and conservation strategies, it is crucial to gather insights into their reproductive biology and life history (Rowe and Hutchings, 2003). Among Chondrichthyes, mating systems are usually complex, with many studies documenting the prevalence of multiple paternity (MP), with females exhibiting polyandrous behavior (Lamarca, Carvalho, Vilasboa, Netto-Ferreira, Vianna, 2020; Lyons, Kacev, Mull, 2021).

Reproductive behavior studies in sharks are inherently challenging due to the difficulty of direct observation in their natural habitats. Nevertheless, for several decades, paternity analysis has proven to be an invaluable tool for investigating aspects of reproductive behavior in wild organisms (Di Fiore, 2003; Uller and Olsson, 2008; Portnoy and Heist, 2012). In elasmobranch species, most insights into mating systems have been derived from paternity studies employing microsatellite markers (e.g. Chapman, Prodöhl, Gelsleichter, Manire, Shivji, 2004; Chevolot et al., 2007; Portnoy, Piercy, Musick, Burgess, Graves, 2007; Byrne and Avise 2012; Lyons, Chabot, Mull, Holder, Lowe, 2017). These markers are particularly useful for inferring paternal alleles when maternal genotypes are known. In Mexico, research on this topic remains limited; however, it represents a growing field of scientific interest (Tárula-Marín and Saavedra-Sotelo, 2021; Rendón-Herrera, Pérez-Jiménez, Saavedra-Sotelo, 2022; Armanda-Tapia, Castillo-Geniz, Victoria-Cota, Arce-Valdez, Enríquez-Paredes, 2023).

Fewer records of MP exist for rays compared to sharks (Chevolot et al., 2007; Lyons et al., 2017; Yano, Sato, Takahashi, 1999). Therefore, further studies on ray mating systems are needed to inform future fisheries management strategies. Current knowledge on ray mating systems is limited, making it challenging to assess the impacts of these systems on population recovery rates, particularly for species under fishing pressure. Reproductive behaviors, such as mate competition or choice, can have notable consequences for the recovery of commercially exploited marine species (Rowe and Hutchings, 2003). Understanding these dynamics is essential for improving conservation efforts.

The California butterfly ray, *Gymnura marmorata* (Cooper 1864), ranges from southern California to Mexico, including the Gulf of California (Yokota, White, De Carvalho, 2016). This species is aplacental viviparous, with matrotrophy and histotrophy, and uterine villi secrete lipid-rich histotroph to nourish embryos (Mossman, 1987; Wourms and Bodine, 1983). Such supplemental nutrition involves a high energetic investment from females, which is present before and after egg fertilization (Lyons et al., 2017). The California butterfly ray has a gestation period of 9 to 12 months, with fecundity ranging from 2 to 16 pups per litter, as well as an annual reproductive cycle (Burgos-Vázquez, 2013; Dávila-Ortiz, 2002; Yokota et al., 2016). These reproductive traits suggest that mate selection is crucial, as the energetic investment of females to produce high-quality offspring with greater chances of survival is likely notable (Lyons et al., 2021). The aim of this study was to identify the mating system of *G. marmorata* in the Gulf of California through paternity analyses.

MATERIAL AND METHODS

A gravid female with a disk width (DW) of 95 cm and bearing 13 pups was collected from an artisanal fishery in Puerto Libertad, Sonora, within the Gulf of California (29°54´15” N; 112°40´59” W). The organisms were collected in April 2017 using bottom-set gillnets operated by the artisanal fishery near Puerto Libertad. The DWs of the mother and pups were recorded, and tissue samples were collected for each individual. Samples were preserved in salt saturated 20% dymethilsulfoxide (DMSO) solution. Genomic DNA was isolated via proteinase K digestion, followed by a salting-out protocol using lithium chloride (Aljanabi and Martinez, 1997). The concentration and quality of DNA were verified by Nanodrop Lite (Thermo Fisher Scientiic, Waltham, USA) and agarose gel (1.5%) electrophoresis. Two microsatellite loci (SER61 and SER263) previously developed for *Aetobatus narinari* –(Sellas, Bassos-Hull, Hueter, Feldheim, 2011), were amplified for all samples. The amplicons were marked fluorescently with the universal primer M13 (De Arruda, Gonçalves, Schneider, Silva, Morielle-Versute, 2010).

The PCR profile consisted of two phases. The first phase consisted of 5 min at 94 ºC followed by 20 cycles of 94 ºC/15 s; 52 ºC/30 s (SER61), 53 ºC/30 s (SER263); and 72 ºC/30 s. To conclude the first phase, M13 primer (0.5 µM) was added to each reaction. The second phase was comprised of 10 cycles at 94 ºC/15 s; 53 ºC/30 s; 72 ºC/30 s; and 72 ºC for 15 min. Each 10 µl reaction contained 0.2 µM of each dNTP and 1X PCR buffer, 0.5 µM of each primer, ½ U *Taq* DNApol (NEB, Ipswich, MA), and 10 ng of template DNA. Allele separation was performed on the automatic sequencer ABI 3100 (Applied Biosystems Inc, CA), and genotypes were scored on GENEMARKER v. 2.7.0 (SoftGenetics®).

To evaluate the effectiveness of loci in detecting multiple paternity, we conducted simulations of multiple paternity probabilities using the PrDM program (Neff and Pitcher, 2002). These simulations varied parameters such as the number of sires, litter size, and the reproductive skew, of putative fathers. Putative father genotypes were inferred using the programs GERUD v. 2.0 (Jones, 2005) and COLONY2 (Jones and

Wang, 2010; Wang, 2004, 2010). GERUD estimates paternal genotypes and calculates the minimum number of sires per litter. In contrast, COLONY uses a maximum likelihood algorithm to assign embryos in full- or half-sibling groups based on inferred parent-offspring relationships. Finally, due to the lack of normality of the data, differences in embryo size between sires were evaluated using the Mann-Whitney U test.

RESULTS

Out of the 13 pups found, 6 pups were located in the left uterus and 7 pups in the right uterus (Table 1). The DW mean of embryos showed no significant differences between uteri ($p > 0.05$, Mann–Whitney U test; left uterus average DW = 17.67 cm, right uterus average DW= 16.82 cm). However, significant differences in embryo size were observed between sires ($p = 0.03$). Embryos sired by dominant male, defined as the father of the majority of the pups, were larger (n = 5, DW range = 17.2–18.2) compared to those sired by the second male (n = 3, DW range = 16.3–16.8).

Table 1. Summary of data. F: Female, M: Male, DW: Disc Width, DL: Disc Length, U: Uterus (L: Left, R: Right), Loci: Two microsatellites (SER61 and SER63) with allele sizes.

Individuals	Sex	DW	DL	U	Loci			
					SER61		SER63	
Mother	F	95	55.7		156	160	246	248
Embryo 1	M	17.2	10.2	L	148	160	246	248
Embryo 2	F	17.7	10.1	L	156	148	246	246
Embryo 3	M	17.4	10	L	-	-	-	-
Embryo 4	M	17.6	10.2	L	160	160	246	248
Embryo 5	M	17.2	9.9	L	-	-	-	-
Embryo 6	F	18.2	10.2	L	160	160	246	248
Embryo 7	M	17.1	10.4	R	-	-	-	-
Embryo 8	M	17.5	10.1	R	160	160	246	248
Embryo 9	M	16.8	10.1	R	156	156	246	248
Embryo 10	M	16.7	9.9	R	156	160	246	248
Embryo 11	M	16.3	9.8	R	156	160	246	248
Embryo 12	M	17.4	10.2	R	-	-	-	-
Embryo 13	H	17.6	10	R	-	-	-	-

Due to DNA quality constraints, only eight offspring could be successfully genotyped. The genetic diversity was moderate to high in both loci (SER63 *Ho*: 0.875, *He*: 0.492; SER61 *Ho*: 0.500, *He*: 0.557). Prior to any analyses, we compared embryo genotypes with the mother genotype to identify at least one shared allele per locus (Table 1). Three paternal alleles were identified at the SER61 locus and two at the SER63 locus. The probability of detecting multiple paternity (PrDM) using two microsatellite loci was low under different scenarios (Table 2).

Table 2. Probability of detecting multiple paternity with PrDM using two microsatellite loci in *Gymnura marmorata* under different scenarios of multiple mating.

No. Sires (Reproductive skew)	**Litter size**					
	5	**10**	**15**	**16**	**17**	**18**
2 (0.5:0.5)	0.055	0.154	0.204	0.216	0.211	0.219
2 (0.067:0.333)	0.049	0.14	0.192	0.197	0.202	0.207
2 (0.75:0.25)	0.042	0.122	0.17	0.177	0.18	0.187
3 (0.333:0.333:0.333)	0.085	0.252	0.349	0.357	0.369	0.377
3 (0.57:0.285:0.145)	0.069	0.211	0.296	0.308	0.32	0.331
4 (0.25:0.25:0.25:0.25)	0.104	0.315	0.435	0.452	0.465	0.478
4 (0.52:0.27:0.14:0.07)	0.081	0.248	0.354	0.373	0.383	0.399
5 (0.20:0.20:0.20:0.20:0.20)	0.112	0.358	0.499	0.515	0.529	0.545
5 (0.50:0.26:0.13:0.07:0.04)	0.087	0.266	0.386	0.398	0.413	0.428

GERUD indicated a minimum number of two sires, while COLONY2 suggested between one and six sires using under various mating scenarios, including monogamy, polyandry, polygyny, and promiscuity. These results were then used in COLONY2 to assign paternity and sibling relationships among pups based on their genotypes using a maximum likelihood framework (Figure 1).

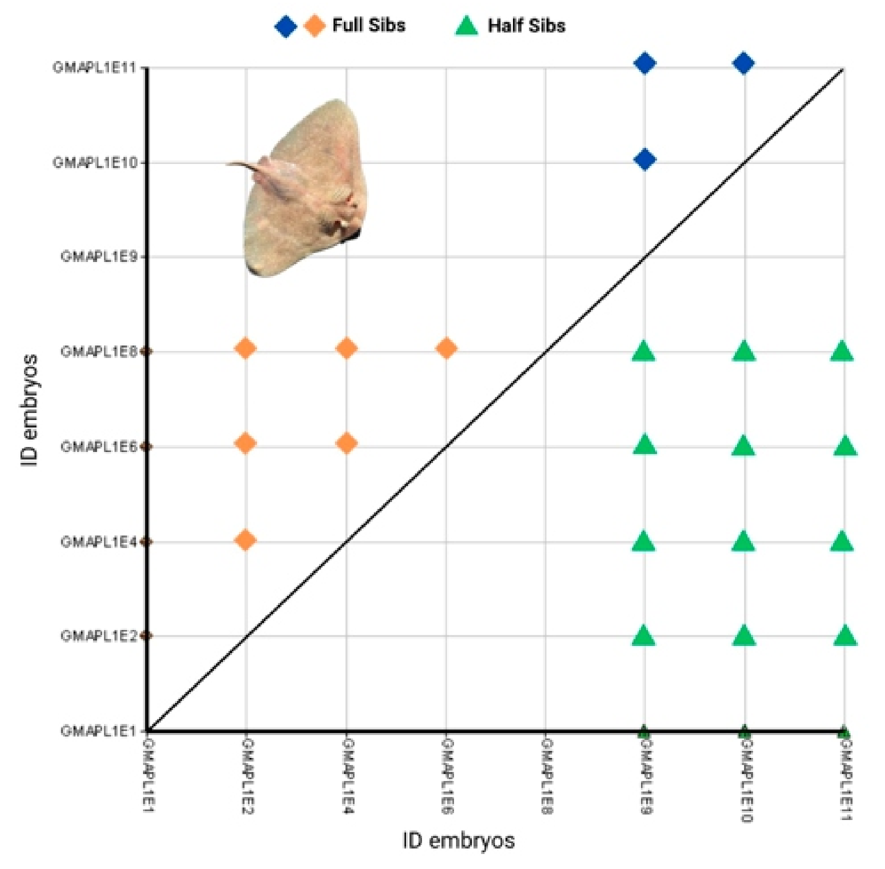

Figure 1. Kinship analyses among pups of the litter of the sampled *Gymnura marmorata* female. Axes show each pup.

DISCUSSION

Two males were identified as likely sires of the litter, establishing the first confirmed case of multiple paternity (MP) in *G. marmorata*. We prioritized the number of sires and genotypes estimated by GERUD, given that COLONY2 has been known to overestimate the number of putative sires due to over-split –(Sefc and Koblmüller, 2009). Over-split sibships occur if the probability of exclusion (0.0063–0.0901) is low despite the high probability of inclusion (0.490–1), which results in an overestimated number of sires.

Despite the limited number of loci used in this analysis, it was possible to detect the number of sires. The lower amplification and genotyping success in this study could be attributed to the use of primers developed for another species and the low quality of DNA in some samples.

Although only two microsatellite loci were analyzed, the genetic variability detected was sufficient to identify multiple sires (*Ho*: 0.875 and 0.500). However, it is acknowledged that in the multiple paternity simulations, the probability of detecting multiple sires in a scenario involving 13 embryos and two males was low with the available dataset (Table 2). Ongoing research aims to apply high-coverage genetic analysis (SNPs) to a larger sample size, allowing us to further validate these preliminary findings.

As highlighted earlier, elasmobranchs invest substantial energy in producing offspring, which necessitates a mechanism to ensure high quality progeny with an enhanced chance of survival (Lyons et al., 2021). This is often achieved through cryptic sexual selection mechanisms in both sexes, a potent selective force driving the evolution of diverse sexual traits (Birkhead, Hosken, Pitnick, 2009; Birkhead and Møller, 1998). Studies on cryptic sexual selection in elasmobranchs have focused mainly on females, as evaluating direct reproductive traits like embryo size, weight, and positioning provides insight into potential female-controlled aspects of selection (Chapman et al., 2013; Farrell, O'Sullivan, Sacchi, Mariani, 2014; Lyons et al., 2017; Rendón-Herrera, 2023). Statistical differences in these traits are commonly attributed to cryptic female choice, an aspect of sexual selection (Lyons et al., 2017; Rendón-Herrera, 2023). Our findings suggest male skewed paternity, as the largest embryos were sire by the dominant male, indicating potential cryptic sexual selection in the female favoring this sire. Nevertheless, abortions can occur in rays upon capture; therefore, this result could be biased due to the loss of embryos (Adams, Fetterplace, Davis, 2018).

On the other hand, polyandry can drive intersexual conflicts that result in antagonistic coevolution, where female sexual selection mechanisms are countered by opposing male mechanisms (Zeh and Zeh, 2003). Sperm competition, a male reproductive strategy, arises due to the wide variation in multiple mating rates among females (Parker, 1970). In some species of elasmobranchs, sperm competition serves as a selective force influencing sperm morphology (e.g., sperm flagellum length), which can prove advantageous during competitive fertilizations (Rowley et al., 2019). Although it was not possible to evaluate these traits in the current study, the potential role of sperm competition in *G. marmorata* cannot be ruled out.

This study has certain limitations, particularly the use of only two molecular markers, which restricts the resolution or analysis of the mating system in *G. marmorata*. Increasing the number of molecular markers is essential for achieving a more comprehensive understanding of this species. Currently, genomic population analysis is underway to address this limitation. Despite these constraints, our findings suggest polyandry in *G. marmorata* with skewed male paternity, likely influenced by cryptic sexual selection in the female. Further research on sexual selection mechanisms in elasmobranchs is essential to identify the factors promoting multiple paternity in rays, which may range from female control over oocyte fertilization to selective nutrient allocation to specific offspring. Understanding these dynamics is crucial to uncovering the evolution of mating systems in elasmobranchs.

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