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Detecting patterns of fertilization and frequency of multiple paternity in *Chelonia mydas* of Colola (Michoacán, Mexico)

Detección de patrones de fertilización y frecuencia de paternidad múltiple en *Chelonia mydas* de Colola (Michoacán, México)

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ABSTRACT

We present a microsatellite analysis that allows an indirect determination of the process of fertilization in the genital tract of *Chelonia mydas.* The strategy was based in that the order of oviposition is related to fertilization order. Once the genotype of the offspring was obtained through microsatellite analyses, it was possible to determine the frequency of multiple paternity and to infer the presence of anatomical structures or physiological mechanisms that allow females to undergo cryptic choice processes, which allowed us to hypothesize the existence of post-copula factors that determine reproductive success in a polyandrous system. This paper does not show the presence of physiological mechanisms that allow control of fertilization order in polyandrous females of *Chelonia mydas*.

Key words: Multiple paternity, microsatellite, genetics.

RESUMEN

Presentamos un análisis con microsatélites para determinar de manera indirecta si existe un mecanismo de control del proceso de fertilización en el tracto genital de *Chelonia mydas*. La estrategia utilizada se basa en que el orden de oviposición está relacionado con el orden de fertilización. Determinando el genotipo de la progenie, fue posible determinar la frecuencia de paternidad múltiple e inferir la presencia de estructuras anatómicas o mecanismos fisiológicos, que actúen como factores post-copulatorios para regular los eventos reproductivos en un sistema poliándrico. El presente trabajo no muestra la presencia de mecanismos fisiológicos que permitan el control del orden de fertilización en hembras poliándricas de *Chelonia mydas.*

Palabras clave: Paternidad múltiple, microsatélites, genética

Five species of marine turtles have polyandrous mating systems (Uller & Olsson, 2008) where the percentage of clutches with multiple paternity (MP) can vary from 0 to 100% across species. There is no evident pattern that explains this variation, which may have implications on the genetic diversity and effective population size (Jennions & Petrie, 2000; Karl, 2008). It has been suggested that MP variation can be explained through different process. For *Lepidochelys olivacea* (Eschscholtz, 1829) Jensen *et al*. (2006) showed that in localities with massive nesting (arribadas) patterns, the frequency of multiple paternities, is higher than that of localities with solitary nesting females. This suggests that demographic conditions of a population affect MP frequency.

However, there may be other factors that determine MP frequency such as effects of the mating process, such as relative contribution of a male to the total pool of semen. In this scenario the amount of semen of each male in the genital tract of a female can be positively related with the time and/or number of couplings with a male as well as ejaculated semen volume per male (Otronen, 1997). Paternity bias can also be correlated to the structure of the genitals, corporal size of the male, or production of seminal chemicals (Jennions & Petrie, 2000) or sperm competition. It has been suggested that semen storage is a common event in reptiles (Girling, 2002) for up to seven years, as has been reported for the snake *Acrochordus javanicus* (Hornstedt, 1787) (Mangusson, 1979). The capacity to store semen obtained during copulation has been detected in *Chelonia mydas* (Linneo, 1758) (Chaves *et al*., 2000), even though structures that facilitate sperm storage have not been described in marine turtles (Wyneken, 2001). Stored semen has been found inside the reproductive tract of the Green Turtle (*C. mydas*), Kemp's Ridley Turtle *[Lepidochelys kempii* (Garman, 1880)] and Olive or Pacific Ridley Turtle (*L. olivacea)*, and it has been suggested that this storage is only present in short periods such as within one reproductive season (Miller, 1997).

Sperm competition might be present in turtles of the genus *Chelonia*, given the presence of a polyandrous system and semen storage within a nesting season. Birkhead & Pizzari (2002) suggested that females in polyandrous systems have the capacity to bias male participation in ovule fertilization after mating. It has been shown that females can identify the genotype of the sperm (Vaquier, 1998), allowing the possibility to choose the semen of a preferred male, and that there might even exist intracellular choice mechanisms.

We analyzed paternity keeping the order of egg arrangement in a nest, making inferences about how those eggs were fertilized in the genital tract of female marine turtles. We inferred the order of fertilization of two turtle's eggs nests assuming one of the two following possible scenarios: 1) egg fertilization by the semen of at least two males followed copulation order, or 2) egg fertilization by the semen of at least two males did not follow copulation order. To assess the multiple paternity, we used standard molecular tools, as has been done by other authors working with marine turtles (Uller & Olsson, 2008) and most importantly, we complemented this study by recording the oviposition order, that allowed us to describe for the first time, fine-grained aspects of egg fertilization in marine turtles.

We followed the oviposition process in two females of Eastern Pacific Green Turtle, known locally as Tortuga Prieta or Black turtle (*C. mydas*) in the beach of Colola, Michoacán, collecting the eggs directly from female cloaca and maintaining the order with a plastic extruded net. We transported and planted the eggs in a hatchery, maintaining the oviposition order until eclosion. Immediately after oviposition, we collected 500 µl of blood from adult females. We also collected 50µl of blood from all hatchlings after eclosion (54 and 55 days of incubation respectively for each nest). We used the protocol described by Owens & Ruiz (1980), resuspending samples in a lythic solution (Dutton, 1996). To comply with local regulations, we obtained a collecting permit from the General Direction for Wildlife-SEMARNAT (SGPA/SGVS/12409). We obtained total DNA following the protocol described by FitzSimmons (1997), and with the AquaPure Genomic DNA Isolation Kit (Bio-Rad Laboratories, CA, USA). We amplified two oligonucleotides, OR1 (Aggarwal *et al*., 2004) with the 6-FAM, and Ei8 (FitzSimmons, 1997) VIC modified oligonucleotide forward. Both oligonucleotides were amplified in 25µl reactions: Buffer 1X, 0.2mM DNTPs; 2.4 mM $MgCl_2$; 0.5 µM of each primer; 20 µg/ml BSA; 1U Taq polimerase; 20- 50 ng of DNA, with the following program: 94°C 5 minutes, 30 cycles of 94°C 10 seconds, 45°C 10 seconds, 72°C 30 seconds and a final step of 72°C 7 minutes. We carried out electrophoresis with an AB3100 Avant unit in 0.2 µl of each PCR product and 0.25 ml of LIZ-500. We obtained the genotypes with Genotyper 4.0 from Applied Biosystems (Applied Biosystems Inc. CA, USA). Finally we identified the paternal genotypes with GERUD 2.0 (Jones, 2005).

The Ei8 oligonucleotide was monomorphic, and was eliminated from both analyses. We found 8 alleles with the OR1 oligonucleotide. In nest one, 40 hatchlings were released before we were able to collect samples, and DNA samples from 11 hatchlings were not large enough to obtain material to purify or amplify. We obtained genotypes from 50 hatchlings, which represents 49.5% of the total progeny, following the order of oviposition (sampling 50% of each nest is preferable according to FitzSimmons (1996)). We found at least two paternal genotypes. The most frequent paternal genotype fertilized 49 of the analyzed hatchlings (98% of analyzed hatchlings) and a second genotype fertilized one hatchling (2% of analyzed hatchlings).

For female two, we obtained the genotype of 65 of 69 eggs, or 94.2% of clutch size. We detected the participation of three paternal genotypes. The first fertilized 59 hatchlings (90.8% of analyzed hatchlings), the second fertilized four hatchlings (6.1%

Table 1. Genotypes of female I nest. Egg number represents oviposition order. Different gray shade represents, different male genotypes. White spaces are for unknown hatchling genotype due to DNA purification or PCR problems. (G) genotype, (EN) egg number.

		G
	Female I	150/150
	Male I	158/146
	Male II	134/134

EN	G	EN	G	EN	G	EN	G
1		27		53	150/158	79	150/158
2		28		54	150/158	80	
3		29		55	146/150	81	150/158
4		30		56	146/150	82	150/158
5		31		57	146/150	83	150/158
6		32		58	150/158	84	146/150
7		33		59		85	150/158
8		34		60	150/158	86	146/150
9		35		61	146/150	87	150/158
10		36		62	146/150	88	146/150
11		37		63	146/150	89	146/150
12		38		64		90	
13		39		65	146/150	91	146/150
14		40		66	146/150	92	150/158
15		41	150/158	67	146/150	93	146/150
16		42	150/158	68		94	150/158
17		43	146/150	69	150/158	95	146/150
18		44		70	146/150	96	
19		45	146/150	71	146/150	97	
20		46	150/158	72	150/158	98	150/158
21		47		73	146/150	99	146/150
22		48		74	146/150	100	146/150
23		49		75	150/158	101	146/150
24		50	134/150	76	146/150		
25		51	146/150	77	150/158		
26		52	150/158	78	146/150		

of analyzed hatchlings) and a third fertilized two hatchlings (3.1% of analyzed hatchlings).

The genotypes of males involved in the fertilization process and fertilization order for both nests are shown in Table 1 and 2. Upon analysis of the paternal genotypes we infer that there is no paternal genotype shared between the two analyzed nests. In both nests a male fertilized more than 95% of the progeny (male 158/146 and 158/150 for nests one and two, respectively), with a minor contribution from a second male for the first nest and from two additional males for the second nest.

Despite the importance of participation of one male in both nests, we did not suppose that the genital tract of the female was capable of separating semen from each copulation (through peristaltic movements). The order of fertilization was less pronounced in the second nest, where three males participated. In this nest, the second male fertilized eggs 5, 19, 20 and 68, while the third male fertilized eggs 9 and 61. Overall, the fertilization pattern could be explained through a cryptic choice mechanism. We cannot eliminate the possibility that a cryptic choice mechanism in the female is driving the proportion of the progeny fertili-

Table 2. Genotypes of female II nest. Egg number represents oviposition order. Different gray shade represents, different male genotypes. White spaces are for unknown hatchling genotype due to DNA purification or PCR problems. (G) genotype, (EN) egg number.

		G
	Female II	146/150
	Male I	158/150
	Male II	136/168
	Male III	156/120

EN	G	EN	G	EN	G	EN	G
1	150/158	19	136/146	37	150/158	55	150/158
2	150/158	20	136/146	38		56	150/158
3	150/158	21	146/150	39	150/158	57	150/158
4		22	146/150	40		58	150/158
5	136/150	23	146/150	41	150/158	59	150/158
6	146/150	24	146/150	42	150/158	60	150/158
7	146/150	25	146/150	43	150/158	61	120/150
8	146/150	26	150/158	44	150/158	62	150/158
9	150/156	27	150/158	45	150/158	63	146/150
10	146/150	28	146/150	46		64	150/158
11	146/150	29	150/158	47	146/150	65	150/158
12	150/158	30	150/158	48	146/150	66	150/158
13	146/150	31	150/158	49	146/150	67	146/150
14	146/150	32	150/158	50	150/158	68	150/168
15	146/150	33	150/158	51	146/150	69	150/150
16	146/150	34	150/158	52	146/150		
17	146/150	35	150/158	53	146/150		
18	146/150	36	150/158	54	150/158		

zed by each male, favoring a genotype in particular. However, a few scattered genotypes within a dominant background suggest, an imperfect cryptic choice mechanism. Alternatively spermatic competition processes could also affect the frequency of multiple paternity, but our observations do not suggest that they determine the order of fertilization.

Even though this study has a small simple size, we present an innovative strategy that allows to study the order of fertilization in an indirect form. This strategy can be used for oviparous organisms for which there are reports of polyandrous mating systems where oviposition order can be maintained and associated to fertilization processes.

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REFERENCES

Aggarwal, R. K., T. P. Velavan, D. Udaykumar, P. S. Hendre, K. Shanker, B. C. Choudhury & L. Singh. 2004. Development and characterization of novel microsatellite markers from the olive ridley sea turtle (*Lepidochelys olivacea*). *Molecular Ecology Notes* 4: 77-79.

Birkhead, T. R. & T. Pizzari. 2002. Postcopulatory sexual selection. *Nature Review Genetics* 3: 262-273.

Chaves, A., M. Arana & L. Du Toit. 2000. Primary evidence of sperm storage in the ovaries of the olive ridley marine turtle (Lepidochelys olivacea). Proceedings of the eighteenth international sea turtle symposium. U.S. Dept. of Commerce. Edited by NOAA Technical Memorandum NMFS-SEFSC-436. 123 p.

DUTTON, P. H. 1996. Methods for collection and preservation of samples for sea turtle genetic studies. *In: Proceedings of the International Symposium on Sea Turtle Conservation Genetics.* B.W. Bowen & W.N. Witzel (Eds.). Edited by NOAA Technical Memorandum NMFS-SEFSC-396, pp. 17-24.

FITZSIMMONS, N. 1996. Use of microsatellite loci to investigate multiple paternity in marine turtles. *In: Proceedings of the International Symposium on sea turtle conservation genetics. B. W. Bowen & W. N. Witzel (Eds.).* Edited by NOAA Technical Memorandum NMFS-SEFSC-396, pp. 69-77.

FITZSIMMONS, N. 1997. Male marine turtles: gene flow, philopatry and mating systems of the green turtle *Chelonia mydas*. Ph D Thesis. Queensland University Australia. 241 p.

GIRLING, J. E. 2002. The reptilian oviduct: a review of structure and function and directions for future research. *The Journal of Experimental Zoology* 293: 141-170.

JENNIONS, M. D. & M. PETRIE. 2000. Why do females mate multiply? A review of the genetic benefits. *Biological Reviews of the Cambridge Philosophical Society* 75: 21-64.

JENSEN, M. P., A. ABREU-GROBOIS, J. FRYDENBERG & V. LOESCHCKE. 2006. Microsatellites provide insight into contrasting mating patterns in arribada vs. non-arribada olive ridley sea turtle rookeries. *Molecular Ecology* 15: 2567-2575.

JONES, A. G. 2005. Gerud 2.0: a computer program for the reconstruction of parental genotypes from half-sib progeny arrays with known or unknown parents. *Molecular Ecology Notes* 5: 708-711.

KARL, S. A. 2008. The effect of multiple paternity on the genetically effective size of a population. *Molecular Ecology* 17: 3973-3977.

MANGUSSON, W. E. 1979. Production of an embryo by *Acrochordus javanicus* isolated for seven years. *Copeia* 1979: 744-745.

MILLER, J. D. 1997. Reproduction in sea turtles. *In: The Biology of Sea Turtles.* P .L. Lutz & J. A. Musick (Eds.). CRC Press, Inc., Boca Raton, Florida, pp. 51-81.

OTRONEN, M. 1997. Sperm numbers, their storage and usage in the fly *Drymomysa anilis. Proceedings of the Royal Society of London. Series B: Biological Sciences* 264: 777-782.

OWENS, W. D. & G. J. RUIZ. 1980. New methods of obtaining blood and cerebrospinal fluid from marine turtles. *Herpetologica* 36: 17-20.

ULLER, T. & M. OLSSON. 2008. Multiple paternity in reptiles: patterns and processes. *Molecular Ecology* 17: 2566-2580.

VAQUIER, V. D. 1998. Evolution of Gamete Recognition Proteins. *Science* 281: 1995-1998.

WYNEKEN, J. 2001. *The Anatomy of Sea Turtles.* U. S. Department of Commerce NOAA Technical Memorandum NMFS-SEFSC. 172 p.

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