



Within-season shifts in multiple paternity patterns in mass-nesting olive ridley sea turtles

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ABSTRACT: Multiple paternity is common to all sea turtle species, but its causes and consequences are hard to ascertain and the behaviors and success of males difficult to observe. This study aims to describe patterns of multiple paternity for olive ridley turtles *Lepidochelys olivacea* at Playa de Escobilla, an 'arribada' (mass-nesting) site on the Mexican Pacific coast with over a million clutches laid each reproductive season. A total of 15 females and their hatchlings were sampled during 3 arribada events which occurred over the 2016–2017 nesting season. Females and hatchlings (N = 329) were genotyped at 5 microsatellite loci, from which we inferred the alleles of 46 contributing males. Multiple paternity was detected in 60% of the analyzed clutches, which were sired by a range of 2 to 7 males. Multiple paternity rates differed significantly across arribada events, suggesting more males achieved fertilizations earlier in the breeding season. Paternal contribution in 6 of the clutches with multiple paternity was skewed towards a single male; the remaining clutches had a homogeneous male contribution. However, our results are based on relatively small within-arribada sample sizes. The frequency of multiple paternity among turtle clutches laid on this arribada beach could be related to the density of breeding individuals in the reproductive patch off Playa de Escobilla, rather than to the nesting population size or female size.

KEY WORDS: Polyandry · Arribada nesting beach · *Lepidochelys olivacea* · Mexican Pacific · Microsatellites

1. INTRODUCTION

In the last century, overexploitation of all sea turtle species has drastically reduced the size of their populations, resulting in their classification as threatened or endangered (Hays 2004). In the Mexican Pacific, olive ridley turtle (*Lepidochelys olivacea* Eschscholtz, 1829) populations were overfished due to an extensive industrial harvesting that emerged in the 1960s and continued until the

1980s, causing the decline of massive nesting colonies on the Mexican Pacific coast (Rodríguez-Zárte et al. 2013). In 1990, the Mexican government implemented a ban on all commercial sea turtle fishing (DOF 1990), which has enabled population recovery (Márquez et al. 2007). Although these results are encouraging, the assessment of the global olive ridley populations by the International Union for Conservation of Nature (IUCN) reports a reduction between 31 and 36% in the

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annual number of nesting females, which means that olive ridley turtles are facing a high risk of extinction in the medium- to long-term future (Abreu-Grobois & Plotkin 2008, Pasanisi et al. 2016).

Olive ridley turtle populations in the Mexican Pacific continue to be threatened by illegal harvesting of eggs and the incidental capture of turtles in shrimp trawl nets, and the species remains classified as endangered by the US federal government (Wallace et al. 2010, Rodríguez-Zárate et al. 2013). The severe decline in olive ridley turtle individuals in the Mexican Pacific has caused significant loss of genetic variability within the nesting colonies (Rodríguez-Zárate et al. 2013). Furthermore, it has been reported that population reduction in some nesting colonies has caused changes in nesting behavior (Rodríguez-Zárate et al. 2013).

For threatened species, reproduction is critical to population recovery (Comizzoli & Holt 2019). Unfortunately, direct observation of reproductive systems and mating patterns is difficult in sea turtles. Only partial information can be accessed from adult females on nesting beaches, and the adult males are even less accessible as they tend to remain in open water (Sari et al. 2017). However, molecular techniques have resolved many aspects of the reproductive biology of these species, which could greatly improve management and conservation strategies (Komoroske et al. 2017).

Molecular studies of microsatellite loci have shown that all 7 extant species of sea turtle practice polyandry (Lee et al. 2018). Female promiscuity is common in nature and has been analyzed in a wide variety of species, identifying potential direct and indirect benefits for females (Taylor et al. 2014). Direct benefits, in the form of paternal contribution to egg production or parental care, are unlikely to play an important role in many reptile species (Uller & Olsson 2008). On the other hand, there may be indirect benefits for promiscuous females such as greater genetic compatibility, increased genetic variation, and improved offspring quality and fitness (Uller & Olsson 2008, Meister et al. 2012).

In sea turtles, it has been demonstrated that polyandry generates within-clutch multiple paternity (MP), but the incidence varies by species and location (Lee et al. 2018). In addition, some studies have examined the contribution of each male to clutches with MP, concluding that parents did not contribute equally (Stewart & Dutton 2011, González-Garza et al. 2015). In leatherback (*Dermochelys coriacea* Vandelli, 1761) and hawksbill (*Eretmochelys imbricata* Linnaeus 1766) turtles, it has been shown that a 'sin-

gle, 'primary' male' can sire most of the hatchlings within a clutch (Stewart & Dutton 2011, Phillips et al. 2013, González-Garza et al. 2015). Another question that has been addressed regarding MP is the potential benefit to hatchling fitness. Most studies of marine turtles have found little evidence for this (e.g. Zbinden et al. 2007, Wright et al. 2013, Alfaro-Núñez et al. 2015; but see Phillips et al. 2017, Howe et al. 2018), although it is possible for such effects to exist but be almost undetectable, if they can recover fitness that might otherwise be lost (e.g. Price et al. 2010, Michalczyk et al. 2011).

Olive ridley turtles show 2 strikingly contrasting nesting arrival strategies: arribada and solitary nesting (Abreu-Grobois & Plotkin 2008). Arribada nesting represents a synchronized and mass nesting behavior that includes hundreds to thousands of females emerging from the sea over several days. There are only a handful of beaches in the world where this is known to occur (Bernardo & Plotkin 2007). Solitary nesting is the most common behavior and consists of individual events where females emerge to lay their eggs without any apparent synchronicity (Jensen et al. 2006).

Throughout their geographical distribution, the incidence of MP in nesting populations of olive ridley turtles has been reported to vary according to nesting behavior. On arribada nesting beaches, levels of MP are often high. Jensen et al. (2006) estimated 92% incidence of MP in 13 nests at Ostional, Costa Rica, while at Playa de Escobilla, Mexico, studies have reported contrasting results: Villegas-Zurita (2008) observed a 50% MP rate in 16 nests collected during the 1999–2000 nesting season, while Cortés-Rodríguez et al. (2008) found 100% MP in 4 nests. For solitary nesting beaches, most studies have suggested a lower incidence of MP in comparison with arribada nesting beaches, e.g. 25% incidence was reported in Galibi, Suriname (Hoekert et al. 2002) and 30% at Playa Hermosa, Costa Rica (Jensen et al. 2006). However, a recent study demonstrated a high incidence of MP (75%) on a solitary nesting beach from Punta Raton and Punta Venado, Honduras (Duran et al. 2015). Kemp's ridley *Lepidochelys kempii* (Garman, 1880) is another species with mass nesting behavior (Kichler et al. 1999). The only known high-density rookery is located in Rancho Nuevo (Mexico), where 58% MP has been reported for 26 analyzed clutches (Kichler et al. 1999). Subsequent studies have re-analyzed this dataset, using 2 alternative methodologies that returned estimates of an 81% rate (Neff et al. 2002, Wang 2004). At a secondary reintroduced rookery on the coast of Texas, the

frequency of MP was estimated at 48% for 25 collected clutches (Frankel & Williams 2020).

In addition, assessing the incidence of MP in nesting populations is necessary to identify possible factors that may be influencing it. Numerous pressures may drive variation in rates of MP within and between marine turtle species (Jensen et al. 2006, Lasala et al. 2013, Lee et al. 2018). Several earlier studies have suggested that rookery size is a major driver, with larger rookeries having higher MP rates (Ireland et al. 2003, Jensen et al. 2006, Lee 2008). However, this model has since been refined to suggest that concentration of individuals on the approach to breeding grounds may be a more important driver (Lee et al. 2018). In Pacific olive ridley turtles, males and females assemble in large aggregations known as 'reproductive patches' near arribada nesting beaches (Kalb et al. 1992), creating an ideal environment for promiscuity.

MP may also be related to certain turtle reproductive strategies, such as the ability of females to store viable sperm in their oviducts (Pearse & Avise 2001). This strategy can allow females to avoid the energy cost of re-mating, ensuring fertilization in case they fail to find males for mating during their fertile period, and can also promote sexual selection through sperm competition and cryptic female choice (Phillips et al. 2014). In sea turtles, courtship and mating occur most frequently shortly before and during the early part of the nesting season, when pairs can easily be observed in nearshore waters (Plotkin et al. 1995). Males remain offshore near the nesting beaches for an extended time, but will drift away as the nesting season progresses; it is probable that this departure is associated with low male testosterone levels, while in females, the onset of mating activity and receptive period is associated with increased testosterone levels (Rostal 2007).

There are only a few nesting beaches in the world where olive ridley turtles still nest in arribadas, located in Mexico, Nicaragua, Costa Rica, Suriname, Panama, and India (Abreu-Grobois & Plotkin 2008). Playa de Escobilla, Mexico, has the largest world arribada rookery (>1 000 000 clutches per nesting season have been reported; Peralta & Luna 2016); thus, this nesting site has great importance for olive ridley turtle conservation, at national and international levels. This fact, and the overexploitation history of Oaxaca's olive ridley population, makes it imperative to generate new knowledge about its biology and ecology, in order to contribute to the improvement of restoration and conservation strategies for this population. Thus, the aims of the present

study were (1) to estimate the incidence of MP in the Playa de Escobilla olive ridley nesting population during the 2016–2017 breeding season, using microsatellites; (2) to identify the number of males involved in the fertilization of each clutch, as well as their paternal contribution to the offspring; and (3) to determine if there are correlations between the number of fathers per clutch and female size.

2. MATERIAL AND METHODS

2.1. Study site and sampling

The samples were collected in Playa de Escobilla Sanctuary, Santa Maria Tonameca, Oaxaca (15° 43' 37.56" N, 96° 44' 49.23" W), located on the southwest Mexican Pacific coast. Playa de Escobilla Sanctuary is the most important arribada beach for olive ridley turtles in Mexico (Fig. 1). This nesting site is considered as an index beach for *L. olivacea*, due to its historic records and to the large quantity of clutches reported each reproductive season (Peralta & Luna 2016).

Sampling was performed during 3 arribada events (September, November, and February; Appendix, Table A1) of the 2016–2017 nesting season. In each arribada, 5 females were randomly selected and 0.5 ml of blood from the dorsal cervical sinus were obtained according to the protocol of Dutton (1996), and preserved in 96% ethanol at 4°C. All females were measured (curved carapace length; CCL) and tagged using Inconel tags to avoid duplicate sampling.

Each female's clutch was collected in a clean plastic bag within a bucket and transported (within 3 to 6 h after laying) to a site where it was incubated under artificial conditions until the emergence of the hatchlings. After the incubation period (approximately 45 d), 25 hatchlings from each clutch were randomly selected. For each hatchling, a small carapace tissue sample was obtained using sterile pliers according to the method described by González-Garza et al. (2015). All samples were preserved in 96% ethanol at 4°C.

2.2. DNA extraction and microsatellite genotyping

DNA extractions were performed using a Gentra® PureGene® Core Kit A (Qiagen) for the hatchling carapace samples and DNeasy Blood & Tissue Kit (Qiagen) for female blood samples. DNA quality and

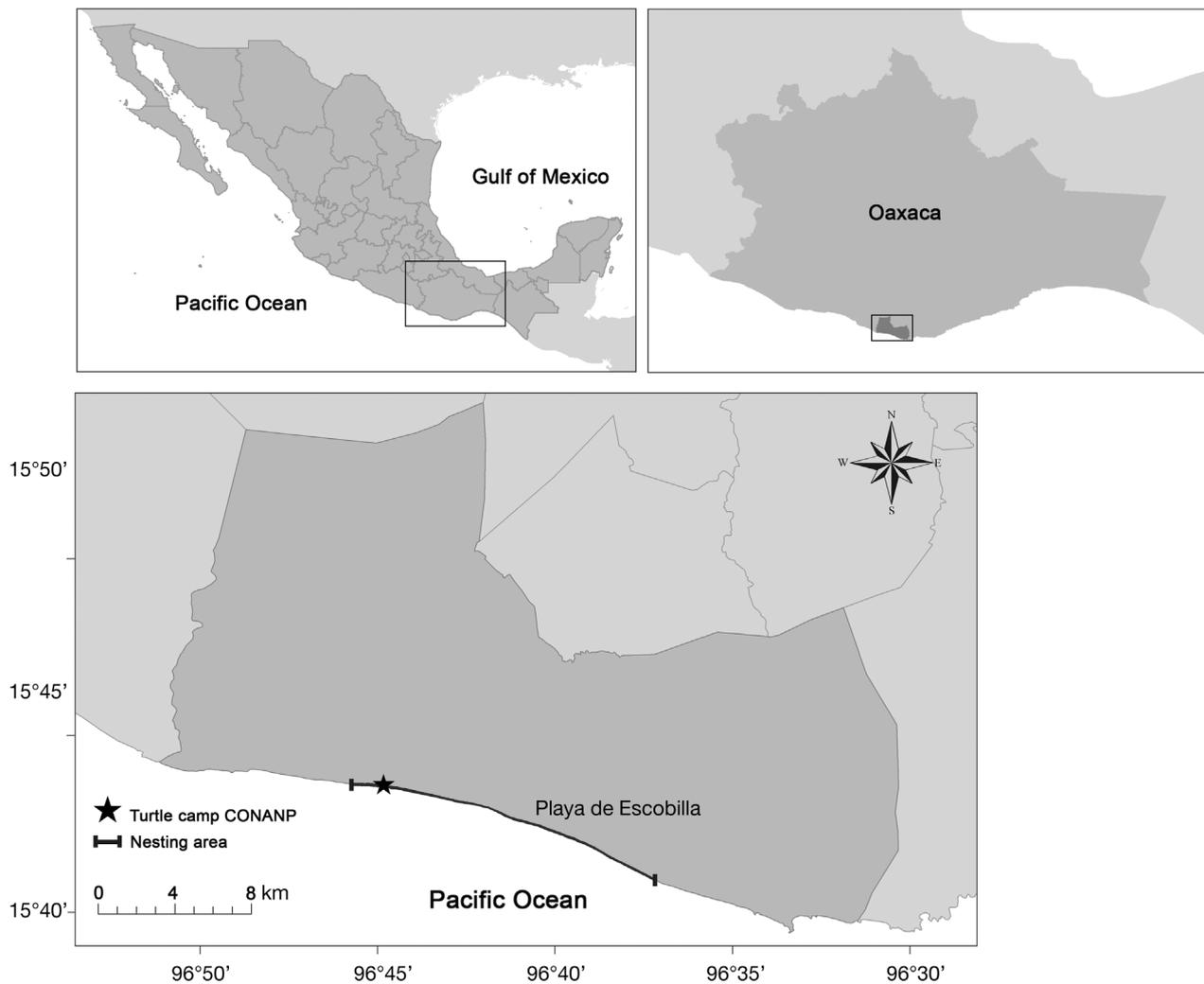


Fig. 1. Study site Playa de Escobilla, Mexico

integrity were verified by electrophoresis in 1% agarose gel using 1 kb Plus DNA ladder as reference. Five previously published nuclear microsatellites were genotyped for all females and hatchlings: OR-1, OR-11, OR-18, OR-20, and OR-22 (Aggarwal et al. 2004, 2008; Table A2). Each forward primer was labeled with a fluorescent dye (NED, VIC, and FAM; Applied Biosystems).

Microsatellites were multiplexed by polymerase chain reactions (PCR) using the Type-IT Microsatellite (Qiagen) protocol. In a 5 μ l reaction, 2.5 μ l of the Qiagen Multiplex PCR Master Mix, 1 μ l of 10 \times primer mix (2 μ M each primer), and 1.5 μ l of template DNA (~30 ng) were mixed. Thermal conditions for PCR were an initial activation of HotStarTaq polymerase for 5 minutes at 95°C, followed by 28 cycles of 95°C for 30 s, 57°C for 90 s, and 72°C for 30 s, with a final

extension at 60°C for 30 min. PCR products were analyzed using an Applied Biosystems 3730xl Genetic Analyzer and LIZ 600 as a size standard.

Allele scoring was carried out using GENEMARKER v. 1.71 (SoftGenetics). Peak sizing and allele calling were verified manually, and samples that failed to amplify at more than 2 loci were not considered in the analyses. Genotyping error rate (for all loci and each locus) was calculated based on approximately 20% of samples. Each microsatellite was analyzed for scoring errors and the presence of null alleles with MICRO-CHECKER v.2.2.3 (Van Oosterhout et al. 2004)

The allele frequencies, observed and expected heterozygosity, as well as the deviation from Hardy-Weinberg equilibrium, were calculated only for the females (N = 15), using GENALEX 6.5 (Peakall &

Smouse 2012). Allelic richness and inbreeding coefficient were estimated in FSTAT v2.9.3.2 (Goudet 1995).

Prior to the parentage analysis, the probability of exclusion (defined as the probability that a supposed male is excluded as a potential parent, assuming that this parent is a random individual) was estimated with GERUD 2.0 (Jones 2005).

2.3. Parentage analysis

The genotypes of the contributing males were identified by manual assignment (Feldheim et al. 2004), and using COLONY v.2.0.6.5 (Jones & Wang 2010). In manual assignment, paternal alleles were inferred through simple autosomal Mendelian inheritance, comparing the known genotypes of the offspring with the genotype of their mothers, excluding maternal alleles. The presence of 3 or more paternal alleles at 2 loci was assumed to be derived from MP (Sari et al. 2017). To avoid overestimation of the number of fathers contributing to each clutch, we conservatively assumed that sires are heterozygous at each locus (Bouchard et al. 2018). On the other hand, in COLONY v.2.0.6.5 (Jones & Wang 2010), the identification of paternal genotypes and paternity assignment were performed considering each nest as a single data matrix, and the options of excluded maternity and excluded maternal sibships were selected. The software was set to update allelic frequencies in 3 medium-length runs, under the principle of maximum likelihood with a medium likelihood precision (parameters as Phillips et al. 2013).

2.4. Statistical analysis

Differences in the average number of fathers per clutch between arribada events (September, November, and February) were tested using a 1-way permutation analysis of variance (PERMANOVA; Anderson 2001) based on Euclidean distance and 999 999 permutations using PAST v.4.03 (Hammer et al. 2001). Omega-squared was estimated in order to measure the impact of the 'arribada event' factor in the population (Hammer et al. 2001). Because the PERMANOVA was significant, post-hoc pairwise comparisons between each arribada event (September, November, and February) were performed with a Student's *t*-test with 'number of fathers per clutch' as a response variable.

To assess the effect of female size (CCL) and the arribada event (month) on the number of fathers per clutch, we performed a generalized linear model (GLM) with a gamma distribution and fit with a log link function in STATISTICA v.8 (StatSoft). Female size and the arribada events (month) were defined as independent variables, while the number of fathers per clutch was the dependent variable.

3. RESULTS

3.1. Microsatellite analysis

Genomic DNA was obtained from a total of 426 samples: 15 females and 329 hatchlings, an average of 21.9 ± 2.3 (mean \pm SD) hatchlings per clutch, and 82 randomly selected replicates. All individuals were successfully genotyped at 5 microsatellite loci. The 5 loci were highly polymorphic, identifying 56 alleles in the whole dataset (females and hatchlings, Table 1). OR-11 was the most polymorphic locus ($N_a = 19$), whereas OR-18 presented the least number of alleles ($N_a = 6$). No evidence of allele dropout, stuttering, or null alleles was found. The genotyping error rate was 0.0097 considering all loci, and 0.0048 for loci OR-11 and OR-22 when individually considered.

3.2. Genetic diversity and Hardy-Weinberg deviation

For the 15 females analyzed, 44 alleles were identified at 5 loci. The observed heterozygosity (H_o) was 0.787 ± 0.10 (mean \pm SD) and the maximum value was detected for loci OR-11 and OR-22 ($H_o = 0.933$, for each locus). The expected heterozygosity values

Table 1. Descriptive information for the microsatellites used in females and hatchlings of *Lepidochelys olivacea* from Playa de Escobilla, Mexico ($N = 344$). N_a : number of alleles. Size range is that identified in this population. The most common allele is shown in parentheses

Locus	N_a	Size range (bp)	Genotyping error
OR-1	12	145–197 (149)	0
OR-11	19	192–234 (222)	0.0048
OR-18	6	115–127 (121)	0
OR-20	8	99–121 (115)	0
OR-22	11	217–243 (221)	0.0048
All loci	56		0.0097

were slightly lower at all loci, but no significant deviations from Hardy-Weinberg equilibrium were found for any loci. The probability of exclusion for the 5 loci combined was 0.990 (Table 2).

3.3. Parentage analysis

Considering the genotypes of the mothers and their offspring, 46 fathers were successfully identified by manual assignment, and 51 contributing males were identified by COLONY. For the paternity assignment using COLONY, only those multilocus genotypes with a confidence value ≥ 0.90 were considered, as suggested by Phillips et al. (2013). Paternity results from both manual assignment and COLONY showed that 9 of the 15 clutches (60%) showed MP. The number of fathers identified per MP nest ranged from 2 to 7 in the manual assignment, and from 2 to 9 in COLONY (Table 3). For this reason, we considered that the results from the manual assignment method were the most conservative for the identification of contributing males, and these numbers were used for all subsequent analyses, showing no evidence that any male sired offspring in more than 1 nest.

For clutches in which we detected MP, the males that sired the highest number of offspring were classified as the primary sire (Table 3, Fig. 2). The nests sired by 2 to 4 males showed a tendency to present a primary sire: this male sired between 56 and 85% of

Table 2. Genetic variation per locus for the subsample of *Lepidochelys olivacea* from Playa de Escobilla, Mexico (N = 15). N_a : number of alleles; H_o : observed heterozygosity; H_e : expected heterozygosity; F_{IS} : inbreeding coefficient; EP: exclusion probability; HWE: Hardy-Weinberg equilibrium deviation significance (ns: not significant; $p > 0.05$); \bar{x} : mean; SD: standard deviation

Locus	N_a	H_o	H_e	F_{IS}	EP	HWE
OR-1	10	0.867	0.800	-0.049	0.630	ns
OR-11	15	0.933	0.904	0.003	0.809	ns
OR-18	4	0.400	0.340	-0.143	0.178	ns
OR-20	5	0.800	0.776	0.003	0.557	ns
OR-22	10	0.933	0.807	-0.123	0.634	ns
\bar{x}	8.800	0.787	0.725	-0.050	0.990	
(\pm SD)	(± 1.95)	(± 0.10)	(± 0.09)			

the hatchlings from each clutch, while the secondary sire fertilized between 10 and 38% and the third and fourth males sired <8% of the offspring. Of the 2 clutches fertilized by 7 males, one (N07) was fertilized by a primary father (45% of the hatchlings), the secondary sire fertilized 18%, and each of the remaining males (N = 5) sired between 5 and 9% of the offspring. While in the other clutch sired by 7 fathers (N08), the individual male contribution was homogeneous, so there was no dominant sire; this same tendency was observed in the clutches sired by 6 males (N09 and N10; Table 3, Fig. 2).

Significant differences in the average number of fathers between the 3 arribada events were found (PERMANOVA: $F_{2,12} = 7.114$, $p = 0.011$), with an

Table 3. Olive ridley nests analyzed from Playa de Escobilla, Mexico. The arribada event denotes the month in which the female and her clutch were sampled during the 2016–2017 nesting season. Number of fathers identified using manual assignment and COLONY. Primary sires were indicated by the male-ID in corresponding nests, na: not applicable; – no evidence of primary sire

Nest ID	Arribada event	Clutch size	Hatchling success (%)	Genotyped hatchlings	Manual assignment	COLONY assignment	Primary sire
N06	Sep	91	58.24	22	1	1	na
N07	Sep	109	22.02	22	7	8	M05
N08	Sep	92	26.09	23	7	9	–
N09	Sep	40	57.50	18	6	6	–
N10	Sep	105	45.71	22	6	7	–
N11	Nov	109	82.57	25	1	1	na
N12	Nov	101	88.12	25	4	4	M18
N13	Nov	108	84.26	18	2	2	M30
N14	Nov	68	98.53	19	1	1	na
N15	Nov	95	88.42	24	3	4	M03
N16	Feb	85	87.06	20	3	3	M39
N17	Feb	109	85.32	23	1	1	na
N18	Feb	111	89.19	25	1	1	na
N19	Feb	87	81.61	21	2	2	M15
N20	Feb	47	91.49	22	1	1	na

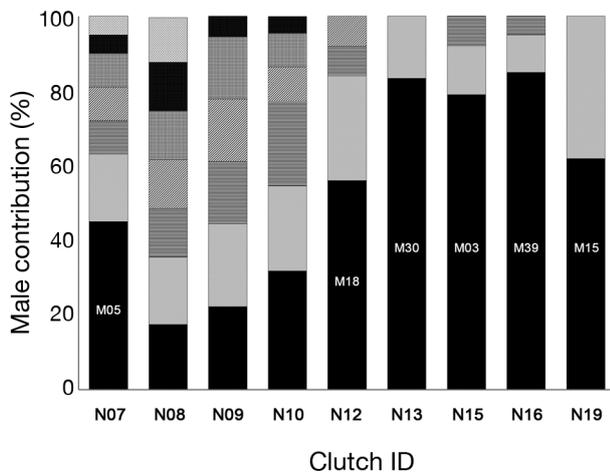


Fig. 2. Relative contribution of different males to multiple-sired olive ridley clutches from Playa de Escobilla, Mexico. The different fill styles in the bars indicate the contribution of each father to the clutch, and the primary father (contribution >45% offspring; see Section 4) in each clutch is indicated with the male's ID

omega-squared value of 0.449, suggesting a moderate effect of the 'arribada event' factor. The post hoc test (*t*-distribution) revealed significant differences between September and November ($p = 0.048$) and September and February ($p = 0.048$), but there was no significant difference between November and February ($p = 0.603$). These results show that the clutches fertilized by a higher number of males correspond to females that nested in the first months of the nesting season, and that the number of fathers identified decreased as the breeding season progressed. The GLM showed that a significant amount of the variation in number of fathers per clutch was explained by the arribada event factor (month) ($p = 0.001$), while there was no significant effect of female size on number of fathers ($p = 0.271$) (Table A3).

4. DISCUSSION

4.1. Multiple paternity and male contribution

MP studies on olive ridley turtle arribada beaches have only been carried out in Ostional, Costa Rica (Jensen et al. 2006) and Playa de Escobilla, Mexico (Villegas-Zurita 2008, Cortés-Rodríguez et al. 2008, this study), and have reported different patterns. The present study reports a frequency of 60% MP, which is lower than the 92% reported by Jensen et al. (2006) in Ostional, Costa Rica. Differences in the incidence of MP between these 2 populations may be due to various local factors, including density and

movement of individuals within breeding areas, sex ratio differences, reproductive behavioral strategies (Sari et al. 2017, Lee et al. 2018), or timing of the survey in the nesting season.

Methodological aspects could explain the contrasting results between MP studies for Playa de Escobilla (DeWoody et al. 2000, Alfaro-Núñez et al. 2015). Our data show an incidence slightly higher than that reported by Villegas-Zurita (2008), considering a similar sample size (he analyzed 16 females and 319 hatchlings). However, the differences between these 2 studies could be due to: (1) number of microsatellites analyzed (he only used 2, Cm84 and Ei8); (2) mistyping of 1 bp alleles of the Cm84 microsatellite, that can generate genotyping errors from band overlap (Duran et al. 2015, Hoekert et al. 2002), especially when genotyping with silver-stained PAGE gels (as he did). By contrast, the present study shows a lower incidence of MP than that reported by Cortés-Rodríguez et al. (2008); however, their results should be interpreted with caution, given their very low sample size (4 females and 103 hatchlings) in a single arribada event, which could have led to an overestimation of MP frequency. Additionally, a substantial difference from previous studies of MP in Playa de Escobilla is the temporal scope analyzed. The present study is the first to sample 3 arribada events during a nesting season, allowing hypotheses to be tested.

In nests with MP, the number of fathers identified per clutch ranged from 2 to 7, including the highest number of fathers ever detected in an olive ridley clutch. Previous studies of *Lepidochelys olivacea* have reported a minimum of 2 fathers and a maximum of 5 on arribada nesting beaches (Jensen et al. 2006, Villegas-Zurita 2008), and between 2 and 3 fathers in solitary nesting populations (Hoekert et al. 2002, Jensen et al. 2006, Duran et al. 2015). This observed variation could be due to the methods employed, which could underestimate or overestimate the number of contributing males. In this study, 2 methods for paternity assignment were compared, COLONY provided a higher estimate than manual assignment. Some studies have found that COLONY can overestimate when the molecular data does not fit the following criteria: (1) use of 5 to 7 loci and (2) expected heterozygosity values (H_e) larger than 0.84 for each locus (Sefc & Koblmüller 2009, Bouchard et al. 2018).

Paternal contribution in 6 of the clutches with MP was biased towards a single male (primary father), and the remaining clutches had a homogeneous male contribution. These differences could reflect the relative contribution of a male to the semen pool which can be influenced by a combination of factors

(Lara-De La Cruz et al. 2010). In clutches with a dominant (>45% offspring) father, reproductive behavior during mating could favor the reproductive success of a single male ensuring the fertilization of more hatchlings (Fitzsimmons 1998). In green turtles, it has been shown that successful males have a characteristic behavior where they bite the competing (mounted) male's tail or hind flippers, or ram the mated couple to try to drive away the competing male. Such behavior can create a dominance hierarchy, increasing mating opportunities and mechanisms to ensure paternity for the dominant male (Fitzsimmons 1998).

Another mechanism that could promote paternal contribution bias is sperm competition (Adams et al. 2005). Sea turtles have the ability to store viable sperm obtained during multiple matings at the beginning of the reproductive season in their oviducts, creating the opportunity for sperm competition between multiple males (Gist & Jones 1989, Adams et al. 2005, Phillips et al. 2014, Lasala et al. 2020). Low male contribution to a clutch could be the result of less competitive sperm or residual sperm stored from previous nesting season (Stewart & Dutton, 2011).

Some studies have also suggested additional mechanisms associated with sperm competition (Jones et al. 2002, Meister et al. 2012). Although these mechanisms have not been reported in sea turtles, they could explain the paternal contribution bias. One of these is the 'topping off' hypothesis, which proposes that the female may accept a large amount of sperm from the first mate, but if there is additional space left in her oviducts, additional mates provide sperm until she no longer needs more (Jones et al. 2002). Fertilization largely corresponds to the first male, but sperm from consecutive mates can be mixed and also used to fertilize the eggs as they are laid (Meister et al. 2012). Female characteristics can also influence a non-homogeneous contribution of fathers within a clutch. In this context, cryptic female choice can regulate the fertilization success through processes or structures that favor the paternity of specific males with particular traits (Meister et al. 2012, Eberhard 2015).

Finally, homogeneous male contribution could be explained by bet-hedging benefits such as protection against the risk of failed reproduction, male infertility, and low genetic quality or genetic compatibility. These bet-hedging benefits do not imply mate selection or sperm competition, just multiple mating (Garcia-Gonzalez et al. 2015). Mixing sperm from different males may generate increased genetic diversity within the clutch (Yasui 1998, McLeod & Marshall 2009). Although the aforementioned factors

could influence the differences in paternal contribution within clutches, it is not clear from our results which of them could explain the patterns observed in our study. However, it is possible that our within-arribada sample sizes are too small to detect such nuanced effects.

4.2. Factors associated with multiple paternity incidence in Playa de Escobilla

Sea turtle species show different MP patterns, even among nesting populations (Lee et al. 2018). MP studies in olive ridley turtles have found contrasting patterns. Jensen et al. (2006) suggested that MP incidence in olive ridley turtle rookeries varies considerably between solitary and arribada nesting beaches, and they found a significant positive correlation between MP incidence and the logarithm of rookery size. Another study reported a high MP incidence (75%) in a small solitary nesting colony composed of around 500 female olive ridley turtles (Duran et al. 2015). In other species such as leatherback and hawksbill turtles, MP patterns tend to be more consistent between different nesting populations, which maintain low MP rates regardless of rookery size (Lee et al. 2018).

Some studies suggest that a high density of individuals in mating areas generates a greater probability of male–female encounters, which could be the most influential factor in explaining MP incidence (Zbinden et al. 2007, Duran et al. 2015, Lee et al. 2018). Within the reproductive cycle of sea turtles, courtship and mating have been poorly studied. However, mated couples have been observed near feeding areas, along migratory corridors and in aggregations in nearshore waters off the nesting beaches (Meylan & Meylan 1994, Bevan et al. 2016, Pilcher et al. 2020). Olive ridley turtles are characterized by forming large reproductive groups both on nesting beaches and in nearshore waters during mating and before the arribada (Pandav & Choudhury 2000). Kalb et al. (1992) defined these large turtle aggregations in nearshore waters of nesting beaches as reproductive patches. These reproductive patches are made up of sexually mature individuals, whose residence is transitory during the reproductive season (Kalb et al. 1992, Pandav & Choudhury 2000), facilitating encounters between females and males and consequently multiple matings (Hoekert et al. 2002). Such reproductive patches have been identified in the vicinity of 2 important olive ridley turtle beaches: Nancite, Costa Rica (Kalb et al. 1992) and

Orissa, India (Gahirmatha and Rushikulya; Pandav & Choudhury 2000, Tripathy 2013, 2016). Although no monitoring has been carried out in nearshore waters from Playa de Escobilla to identify these reproductive patches, both mating couples and individuals gathered off the beach have been observed before and during the nesting season (Peralta & Luna 2016, E. Peralta-Buendía pers. comm.), which suggests the existence of a reproductive patch near Playa de Escobilla.

The temporal dynamics of reproductive patches can also influence MP. A study carried out in Papagayo Gulf off Nancite, Costa Rica, reported that pairs of olive ridley turtles are most often observed in the nearshore breeding ground from July through September (Plotkin et al. 1996). After the mid-season (late September) most females have mated and a great number of them emerge to nest at the beach, coinciding with the departure of males from the breeding ground (Plotkin et al. 1996). The energetic cost, limited foraging opportunities, and low probabilities of a male encountering a receptive female could be factors that explain the notable decrease in the number of males at the breeding ground (Plotkin et al. 1996). Another study in Gahirmatha, India, recorded a higher number of males in comparison to females at the beginning of the reproductive season, which could increase the proportion of multiple matings earlier in the season (Pandav & Choudhury 2000). These temporal patterns are supported by the results of the present study, as we show significant differences in the average number of fathers between the 3 arribada events, suggesting that a higher number of males are involved in the fertilization of clutches collected at the beginning of the nesting season at Playa de Escobilla. However, our results are caveated by the relatively small within-arribada sample sizes. Hence, we suggest that future studies should increase the number of clutches collected in each arribada event, as well as performing sampling across the entire nesting season to validate this pattern in the MP frequency at Playa de Escobilla. Similarly, the estimation of the operational sex ratio, which is the relative number of females and males in the breeding grounds, could strengthen the results about the incidence of MP in these nesting colonies (Lee et al. 2018).

It is necessary to monitor the marine area adjacent to Playa de Escobilla in order to verify the existence of a reproductive patch of *L. olivacea*. The cost of monitoring studies can be reduced through the use of unmanned aerial vehicles; these can be an excellent tool for identifying courtship and mating areas, as

well as estimating population size and determining the operational sex ratio at Playa de Escobilla (Bevan et al. 2016, Schofield et al. 2017, Rees et al. 2018). Identifying and understanding this important reproductive aggregation will improve conservation strategies against threats such as bycatch and meat and egg consumption, with the aim of reducing the mortality of reproductive adults, and ensuring the long-term persistence of this vulnerable species.

Ethics statement. All capturing, tagging, and sampling of biological samples performed during this study were authorized by permit SGPA/DGVS/09320/16/ from the Dirección General de Vida Silvestre (DGVS) de la Secretaría de Medio Ambiente y Recursos Naturales.

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APPENDIX. Additional data

Table A1. Arribada events during the nesting season 2016–2017. *The arribadas sampled for this study

Arribada number	Month	Collected clutches
1	July 2016	
2	August 2016	
3*	September 2016	N06–N10
4	Early November 2016	
5*	Late November 2016	N11–N15
6	December 2016	
7*	February 2017	N16–N20

Table A2. Microsatellite markers used in this study. Tag: fluorescence label at 5'-end; size range: size range (bp) reported in the reference study

Locus	Repeat motif	Primer sequence (5'–3')	Reference study	Tag	Size range (bp)
OR-1	(CAAA) ₁₆	F: CCC CTT GTG TTC TGA AAT CCT ATG A R: CAG GCA TAG GGA AAA ATC AGA GGT A	Aggarwal et al. (2004)	NED	150–202
OR-11	(GA) ₂₂	F: TGA GCA CTG CAA ATG GAG GAT GGT R: AGT GCC TGA TTC TTC GAG TTG CTG AG	Aggarwal et al. (2008)	FAM	182–242
OR-18	(AC) ₁₁	F: AAA CAC CAG AAT AGA GGC TCA AAC T R: TCT CTG GGC TGC CTA CTT TAT TC	Aggarwal et al. (2008)	FAM	108–120
OR-20	(TG) ₁₄	F: TCA CGA ACA TCA GCA AAA TTA TT R: GTC CCA TCC CCT CCA CCA	Aggarwal et al. (2008)	VIC	110–124
OR-22	(CT) ₇ (CA) ₆	F: AAG TCC TGT TGA ATC CTG CCA TAG R: GGT TAG ATA TAG GAG GTG CTG ATG TTA	Aggarwal et al. (2008)	VIC	217–247

Table A3. Results of Generalized Linear Model using gamma distribution and fit with log link function for curved carapace length (CCL; cm) and arribada event (month). Significant values ($p < 0.05$) in **bold**

	Effect	df	Wald	p-level	Score	p-level	Variable status
Step 1	CCL	1	1.2105	0.271			Removed
	Arribada event	2	12.4963	0.002			In
Step 2	Arribada event	2	13.7210	0.001			In
	CCL	1			0.203	0.653	Out