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# Paternity re-visited in a recovering population of Caribbean leatherback turtles (*Dermochelys coriacea*)

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ABSTRACT

Sea turtles in general are promiscuous breeders, but previous leatherback paternity studies found only a very low level of multiple paternity or none at all. Three highly polymorphic microsatellite markers (Dc99, Cc117, and Ei8) were used to investigate the paternity of a recovering population of leatherback turtles nesting at Playa Gandoca in Costa Rica, which is part of the Atlantic Costa Rican leatherback nesting population. The aim of this study was to (i) detect multiple paternity, (ii) compare the results to previous studies in the same and different nesting populations, (iii) consider the possibility of sperm storage, (iv) explore the possibility of successful inter-nesting mating taking place, and (v) determine the effect of small population size on mating patterns. Tissue samples from females and hatchlings were collected from one to three consecutive clutches (35 clutches total) of 18 nesting females included in the assay with an average sampling effort of 21.91% of offspring per clutch. Evidence of multiple paternity was found in four out of 18 females (22.22%), which had mated with two to three different males. The results from this study indicate that multiple paternity is more common than previously observed for the Atlantic Costa Rican leatherback nesting population. The analyses of successive clutches from the multiply mated females showed that paternal contribution varies between successive clutches and "new" fathers in consecutive clutches suggest the possibility of successful inter-nesting mating.

## 1. Introduction

The major problem for species threatened by extinction is small population size. Through the extinction vortex it can lead to a further rapid decline in population size, but low population densities might also lead to altered reproductive behaviours to accommodate for the reduced number of potential mates (Gilpin and Soulé, 1986).

The use of molecular techniques proves to be an important tool for the management and conservation of endangered species, with small population size, and which usually also suffer of diminished genetic diversity (O'Brien et al., 1985; Hedrick, 2001; Frankham, 2003). Genetic studies can provide new insights into the life cycles and mating patterns of species, which are otherwise difficult to study, e.g. due to wide distributions and small population size or elusive life-histories (Frankham, 2003).

Especially the reproductive biology and mating systems are important for many conservation issues, as they can greatly influence a species' genetic variation, and its resulting viability and fitness (Hedrick, 2001; Frankham, 2003). Small population size and a skewed operational sex ratio OSR (the proportion of males to females that are ready to mate at any given time) might decrease genetic variation and the ability to adapt to new environmental pressure (Kvarnemo and Ahnesjö, 1996; Montgomery et al., 2000).

The leatherback sea turtle (*Dermochelys coriacea*) has been listed as critically endangered species by the IUCN till 2000 (Sarti Martinez, 2000). It is a vastly pelagic species, which migrates long distances between its foraging and nesting grounds (Eckert et al., 2012). Very little is known about male leatherbacks, since most studies have focused on nesting females, which are easily accessible whilst on land (Eckert et al., 2012). Females migrate to their mating grounds, located in the tropics and subtropics, every two to three years to lay on average five to seven clutches of 40–120 eggs (Carr and Ogren, 1959; Eckert et al., 2012). In the Eastern Pacific Ocean, leatherback stocks have decreased in size by up to 95% during the past two decades (Spotila et al., 2000). These declines are the result of intense illegal egg poaching and incidental by-catch by commercial fisheries (Spotila et al., 1996, 2000; Sarti Martinez, 2000), and have put the Pacific Leatherback in high risk of extinction (Eckert et al., 2012). The Atlantic Ocean now holds about

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70% of the world population of this critically endangered species (Troeng et al., 2004). During the past 20 to 30 years extensive conservation measures have been put in place in the wider Caribbean region and the effects are such that the leatherback stocks of the wider Caribbean region are thought to be stable (Dutton et al., 2005; Girondot et al., 2007; Tiwari et al., 2007; Tiwari and Wallace, 2013; Eckert et al., 2012), and some nesting populations have even slightly increased in size (Dutton et al., 2005; Tiwari and Wallace, 2013).

Cheloniidae are promiscuous breeders and males engage in intense and aggressive courtship behaviour to gain access to females (Lee and Hays, 2004). For instance green turtle females are often surrounded by large groups of courting males and are known to mate with several males prior to nesting, as well as in between nesting events (FitzSimmons, 1998), which is called inter-nesting mating. All sea turtle species from the family of Cheloniidae have been shown to exhibit multiple paternity in their clutches (Kichler et al., 1999; Moore and Ball, 2002; Ireland et al., 2003; Jensen et al., 2006; Joseph and Shaw, 2011), with levels as high as 92% in synchronised-mass-nesting (*arribada*-nesting) olive ridley turtles (Jensen et al., 2006). Turtles, including sea turtles, are known for their ability to store sperm in their oviduct for at least the duration of a nesting season, as well as over extended periods of time (Gist and Jones, 1989; Gist and Congdon, 1998; Pearse and Avise, 2001).

Unfortunately, sightings of courtship and mating behaviour in leatherback turtles have been rare and many facts still remain in the dark. The current knowledge of the mating behaviour and copulation choreography in leatherbacks are assumptions derived from observations made in other sea turtle species, as well as from testimonies of fishermen and scientists, with only a few peer-reviewed publications and no proof in pictures or video (Carr and Carr, 1986; Godfrey and Barreto, 1998; Reina et al., 2005). The leatherback turtle, however, is the sole surviving member of an ancient lineage of sea turtles (Dermochelyidae), which differs substantially from the family Cheloniidae in morphology, physiology, ecology and distribution (Hendrickson, 1980; Pritchard and Mortimer, 1999), and it is likely that the assumption that mating patterns are similar may be false.

Previous studies of the mating patterns of leatherback turtles found low levels of multiple paternity for Pacific leatherback turtles nesting at Playa Grande (10%) (Costa Rica) (Crim et al., 2002) and for Atlantic leatherback turtles nesting at Pacuare (8%) (Costa Rica) (Curtis, 1998), and Dutton et al. (1998) found no evidence of multiple paternity in St. Croix (US Virigin Islands).

A follow-up study of the nesting population in St. Croix was conducted in 2009, twelve years after the first paternity study, in a population which had increased in size (Dutton et al., 2005; Stewart and Dutton, 2011) and found a higher level of multiple paternity (41.7%). Stewart and Dutton (2011) suggested that the first paternity study in St. Croix, which did not detect multiple paternity, missed multiple paternity by chance, as a result of small sample size (four females and 174 hatchlings). Another possibility for these results might be small population size and a resulting lack of available mates, due to low densities, which might affect observed mating patterns (Courchamp et al., 1999; Møller and Legendre, 2001). Hence, the natural mating pattern of a stable leatherback population is possibly still unknown.

The genetically distinct Atlantic Costa Rica (ACR) nesting population (Dutton et al., 2013; Dutton, 2007) has an epicentre of nesting between the San Juan River in Nicaragua and Chiriquí Beach in Panama on the Caribbean coast (Dutton et al., 1999; Troeng et al., 2004; Dutton, 2007). Three major nesting beaches for this population (Tortuguero, Pacuare, and Gandoca) fall within the borders of Costa Rica (Troeng et al., 2004; Chacón-Chaverri and Eckert, 2007). A number of conservation projects were established throughout the 1990s to improve conservation efforts and continuous monitoring along the Caribbean coast of Costa Rica (Troeng et al., 2004). Up until these efforts were in place, illegal egg harvest had been intense for more than two decades and caused the nesting population to decline (Chacón-Chaverri and Eckert, 2007). The general population trend for the ACR nesting population of leatherbacks now is thought to be stable or slightly increasing (Tiwari et al., 2007; Tiwari and Wallace, 2013; Eckert et al., 2012).

When a leatherback paternity study was conducted in Pacuare (part of the ACR, Fig. 1) in 1996, analysing 12 nests of 12 females (Curtis, 1998), long-term conservation efforts might have been too recently established to have had an effect on the population size and population trend of the ACR nesting population. Especially, since leatherbacks are thought to reach sexual maturity earliest with 9 years, but probably even as late as with 20–30 year (Eckert et al., 2012). Likewise, the small sample size of clutches or offspring per clutch could have led to a random non-detection of multiple paternity, even though higher levels of multiple paternity were present within the studied population.

The present study was conducted at one of the three major nesting sites of the ACR located on the Caribbean coast of Costa Rica: Playa Gandoca, about 100 km south of Pacuare (Fig. 1). Playa Gandoca has 100–300 nesting leatherbacks and 582.9 (±303.3) clutches per year (Troeng et al., 2004; Chacón-Chaverri and Eckert, 2007; Turtle-Working-Expert-Group, 2007), and continuous conservation and monitoring efforts were established by a Costa Rican association (ANAI) in 1990.

The main objectives of this study were to (i) investigate the levels of multiple paternity of the leatherback population nesting at Playa Gandoca using microsatellite analysis; to (ii) compare the results to leatherback paternity studies conducted in other nesting populations, to (iii) test the hypothesis that sperm is stored during mating prior to nesting and used to fertilise eggs of all consecutive clutches, to (iv) see if inter-nesting mating might lead to successful fertilisation, and (v) to investigate the effect of small population size on the observed mating strategy.

## 2. Material and methods

### 2.1. Study site

The present study was done at Playa Gandoca (9°59.972'N, 82°60.530'W), located in the Gandoca-Manzanillo-National-Wildlife-Refuge (REGAMA) on the Caribbean coast of Costa Rica. The main nesting of leatherbacks occurs along a 9 km stretch of the beach, which is monitored continuously from 8 pm to 4 am during the nesting seasons from February to August. Turtles were tagged with two external metal tags (Monel No.49) in their rear flippers and a PIT-tag (AVID) into the fatty tissue of their right shoulder. These tags are used to identify individual turtles. Beach-coverage is high in Gandoca and along the entire Caribbean coast of Costa Rica. All turtles are usually detected and tagged during their first nesting season after reaching sexual maturity, because they are re-nesting on average five to seven times within one season, and thus are likely to be encountered at least once during a given season. Hence, turtles encountered without tags are likely first-time nesters (neophytes) (Chacón-Chaverri and Eckert, 2007). The Leatherback project in Gandoca started sharing and compiling tagging data via the WIDECAST Database in 1991, which has been used as a reliable source for leatherback nesting history of turtles nesting in Gandoca and the Wider Caribbean.

### 2.2. Field sampling

Samples were collected throughout the entire nesting season of 2008. To obtain DNA, tissue was collected from nesting leatherbacks and their offspring following an established protocol (Dutton, 1996; FitzSimmons et al., 1999). For the paternity analysis, 18 females were sampled and one to three of their clutches ($n = 35$ clutches) with an average sampling effort of 21.91% (range 8–80%) hatchlings per clutch ($n = 373$). Additionally, 38 females were sampled from the Gandoca

**Fig. 1.** Map highlighting the boundaries of the Atlantic Costa Rican Rookery (ACR, shaded area), according to Dutton (2007) and Dutton et al. (2013), as well as the location of the study site (Gandoca) and the previously conducted paternity studies in Leatherback turtles in the U.S. Virgin Islands by Dutton et al. (1998), and Stewart and Dutton (2011), and in Costa Rica in Playa Grande by Crim et al. (2002), and in Pacuare by Curtis (1998).

population to establish a genetic baseline for the nesting population, and tissue samples ($n = 7$) from the Eastern Pacific nesting population were used as a comparison for the population baseline (provided by the *Baulas y Negras Ostional* project in Costa Rica). Tissue was collected during oviposition, while females were in their high disturbance threshold (HDT)-phase (Ehrenfeld, 1979), by removing a half circle of tissue of approximately 2–5 mm in diameter from the trailing edge of the rear flipper with a sterile surgical-blade (Dutton, 1996; FitzSimmons et al., 1999). The sampling area was disinfected with iodine to prevent infections, before and after the sampling procedure (Dutton, 1996; FitzSimmons et al., 1999).

Each clutch of females sampled was relocated to a protected hatchery, and reburied in the sand. Baskets, made of chicken-wire and anti-aphid mesh, were placed on top of egg chambers to protect clutches from crabs and fly larvae and also to ensure that emerging hatchlings remained with their siblings. Hatchlings were sampled after emergence, and released on the beach after a short observation time to ensure health and normal activity. Sampling procedures on hatchlings were the same as with females except that tissue pieces taken were smaller (1–2 mm). Tissue samples were preserved in 99.8% analytical Alcohol (EtOH p.A.) to prevent denaturation of DNA and to ensure a high PCR amplification.

### 2.3. DNA extraction and microsatellite genotyping

Genomic DNA was extracted from tissue using the PUREGENE Tissue Kit (Gentra) with the DNA purification protocol. After isolation, the DNA pellet was re-suspended in 50 μl sterile double distilled water and stored it at −20 °C.

Five dye-labelled (IRDye) microsatellite loci Dc99, Cc117, Ei8, Cm3, Cm58, published by FitzSimmons et al. (1999), were used to genotype each mother and its offspring by amplifying their DNA through a Polymerase Chain Reaction (PCR). The reaction volume of 12.5 μl contained 0.5 units of *Taq* DNA polymerase (Fermentas), 1 μl of 10× PCR buffer [750 mM Tris–HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20] (Fermentas), 2.5 mM of $MgCl_2$, 200 μM of dNTPs (desoxynucleoside triphosphates), 200 nM of each of a corresponding unlabelled forward- and a labelled reverse-primer, and 2 μl of template DNA diluted 1:10. After an initial denaturation step of 3 min at 94 °C, 30 cycles of PCR were performed, each cycle consisted of a 30 s denaturation at 95 °C, 30 s annealing (see Table 1 for specific annealing temperatures) at 55 °C (for primer Cc117), 58 °C (for primer Cm3 and Ei8), and 60 °C (for primer Cm58 and Dc99), 30 s extension at 72 °C. A final elongation step of 15 min was performed at 72 °C to complete the extension and to reduce stutter. The PCR was performed in an Eppendorf Mastercycler®.

**Table 1**
Microsatellite loci used to investigate paternity in 18 adult female leatherbacks at Playa Gandoca, Costa Rica during the 2008 nesting season. PCR primer sequences, annealing temperature (AT), number of observed alleles, and product sizes are indicated. Observed heterozygosity ($H_{observed}$), expected heterozygosity ($H_{expected}$), probability of two individuals share the same genotype (q), and probability of detecting multiple paternity at one locus (d) were calculated as described in the Material and methods section.

| Locus | Primer Sequence (5′ → 3′) | AT [°C] | # of Alleles | Allele Length [bp] | | $H_{observed}$ | $H_{expected}$ | $q$ | $d$ |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Expected | Observed | | | | |
| **Dc99**‡ | CACCCATTTTTTCCCATTG<br>ATTTGAGCATAACTTTTCGTGG | 60 | 8 | 130–140 | 118–136 | 0.66 | 0.70 | 0.23 | 0.41 |
| **Cc117**† | TCTTTAACGTATCTCCTGTAGCTC<br>CAGTAGTGTCAGTTCATTGTTTCA | 55 | 11 | 224–252 | 224–244 | 0.83 | 0.85 | 0.05 | 0.69 |
| **Ei8**† | ATATGATTAGGCAAGGCTCTCAAC<br>AATCTTGAGATTGGCTTAGAAATC | 58 | 15 | 192–254 | 224–278 | 0.85 | 0.87 | 0.04 | 0.71 |
| **Cm3**† | AATACTACCATGAGATGGGATGTG<br>ATTCTTTTCTCCATAAACAAGGCC | 57 | 6 | 169–187 | 172–188 | 0.78 | 0.78 | 0.09 | 0.57 |
| **Cm58**† | GCCTGCAGTACACTCGGTATTTAT<br>TCAATGAAAGTGACAGGATGTACC | 60 | 2 | 119–125 | 118–120 | 0.09 | 0.11 | 1.76 | 0.04 |

†Fitzsimmons et al., 1995; ‡Dutton, 1995.

PCR products were diluted between 1:10 and 1:30 and analysed on a LICOR 4300 DNA Analyser (LiCOR Biosciences).

Mothers were run alongside their offspring. To reduce scoring errors all mothers were run again flanked by other females sampled in Playa Gandoca, and females from the East Pacific nesting population in Cost Rica, as a reference.

### 2.4. Data analyses

To establish a base-line, frequencies of allele distribution for each locus were calculated from 36 females ($n = 38$ for locus Dc99 and $n = 34$ for locus Ei8) of the population nesting in Gandoca. Allele frequencies among males were assumed to equal those of the females. Probabilities for the Hardy-Weinberg-Equilibrium were estimated with the Markov-Chain method using the software ARLEQUIN 3.01 (Excoffier et al., 2005). ARLEQUIN was also used to test loci used in the paternity analysis for pairwise linkage disequilibrium (P).

The observed heterozygosity (H) at a specific locus was calculated following the formula in Hanotte et al. (1991).

$$H = 1-\sum(\mathrm{p_i}^2),$$

where $p$ is the frequency of the $i$th allele for $n$ alleles. The observed heterozygosity was compared to an unbiased, expected heterozygosity by ARLEQUIN.

To estimate the probability that two unrelated individuals within the population share the same genotype at a single locus, the following unbiased formula published by Waits et al. (2001) was used, which corrects for differences in sample size.

$$q_{(\mathrm{unbiased})} = \left[\left(\mathrm{n^3(2a_2{}^2-a_4)-2n^2(a_3+2a_2)+n(9a_2+2)-6}\right)\right]/[\mathrm{(n-1)(n-2)(n-3)}]$$

where $n$ is the sample size, $a_i$ equals $\Sigma p_j^i$, and $p_j$ is the frequency of the $j$th allele.

To calculate the probability that two unrelated individuals share a common genotype across several loci (Q), the probabilities derived from each locus were multiplied (Hanotte et al., 1991; Waits et al., 2001). The probability of detecting multiple paternity at a single locus (d) and with a combination of loci (D) were calculated with formulae published by Westneat et al. (1987), which rely on the allele frequencies within the population and the genotype arrangements from all possible matings within a representative sample of the population following FitzSimmons, 1998. The probability of detecting multiple paternity with a given sample size was further assessed with regard to possible skewed father contributions for the loci combination that was used to score females and their offspring using the PrDM software. PrDM integrates the information on the number of loci, allele frequencies within population, sample size of offspring, and the mother's genotype (Neff and Pitcher, 2002).

Paternal alleles were inferred through simple autosomal Mendelian inheritance by comparing the known genotypes of the offspring to their mothers' genotype and excluding the alleles of the mother.

The program GERUD 2.0 (Jones, 2005) was used to reconstruct the genotypes of the alleged fathers from the observed paternal alleles. If several combinations were possible, results were ranked after allele frequencies observed within the studied population and their probability based on Mendelian segregation. The presence of three or more paternal alleles at more than one locus was assumed to have been derived from multiple paternity. The exception was one female where GERUD suggested a second father even though extra-paternal alleles were only detected at one locus. Consequently, it was concluded that these extra paternal alleles in female H15 also derived from multiple paternity and that undetected complementary paternal alleles on the other loci were due to the homozygosity of one of the fathers for a very common allele within population.

Since females deposit on average five to seven clutches within one nesting season on the same beach, one to three clutches of each female were compared with each other, to see if females re-mated during intervals between nestings, and if these matings were successful. To express the statistical similarity in father compositions between different clutches the Sørensen Index.

$$(\mathrm{QS} = 2\mathrm{C}/(\mathrm{A}+\mathrm{B}))$$

(Sørensen, 1957)

was used, where A and B are the number of fathers in clutch A and B, and C is the number of fathers shared by clutch A and B.

The total number of observed fathers across all analysed clutches of one female was considered the observed mating frequency. As a quantity, that takes into account the actual paternities of contributing males, the effective number of mates ($m_e$) was determined for each female using the formula.

$$\mathrm{m_e} = 1/\sum \mathrm{p_i}^2-1$$

(Starr, 1984)

where $p_i$ is the proportion of offspring in a clutch sired by male $i$, with following correction for sampling error by Pamilo (1993):

$$\sum \mathrm{p_i}^2 = \left(\mathrm{N}\sum \mathrm{y_i}^2-1\right)/(\mathrm{N}-1),$$

where $y_i$ are the observed contributions by each sire, and $N$ is the number of sampled offspring.

## 3. Results

### 3.1. Marker analyses

36 females of the nesting population in Playa Gandoca were genotyped at microsatellite loci Cc117, Cm3, and Cm58, and $n = 38$ and $n = 34$ females, respectively, at loci Dc99 and Ei8 to determine a base-line for allele frequencies in the nesting population (Fig. 2). It was possible to score these females without problems at all five loci manually using the *SAGA$^{GT}$ GENERATION2* software (version 3.2.1, Personal Edition).

No locus showed significant deviation from Hardy-Weinberg equilibrium and expected and observed heterozygosity levels ranged from 0.11 to 0.87 and 0.09 to 0.85 respectively. Per locus two to 15 alleles were detected (Table 1).

Values for linkeage-disequilibrium (P) of loci combinations suggest a random association of chosen loci (range 0.07–0.86) (Table 2). The combination of microsatellite loci Ei8-Cc117- Dc99 was used in paternity assay based on their probability to detect multiple paternity, the level of heterozygosity and polymorphism they showed in leatherbacks (Dutton, 1995; FitzSimmons et al., 1995; Dutton et al., 1999; FitzSimmons et al., 1999), and on how well they amplified.

The probability for sharing the same genotype (Q) across the loci Ei8-Cc117-Dc99 was $5 \times 10^{-3}$. The probability for detecting multiple paternity at a single locus (d) ranged from 0.04 to 0.71, but probability of detection (D) rose to 0.95, when loci Ei8, Cc117 and Dc99 were combined (Table 2). The probability of detection (D) across all five loci initially used was also 0.95 (Table 2), but Cm58 showed a very shallow allelic diversity (a total of two alleles, Fig. 2) and Cm3 did not amplify well in hatchlings.

To assess the accuracy of detecting multiple paternity with a given sample size in the case of a skewed contribution by sires, the probability to detect multiple paternity was calculated with our chosen loci combination Dc99-Cc117-Ei8 based on our allele frequency data for the whole population for different paternal skews with the program PrDM.

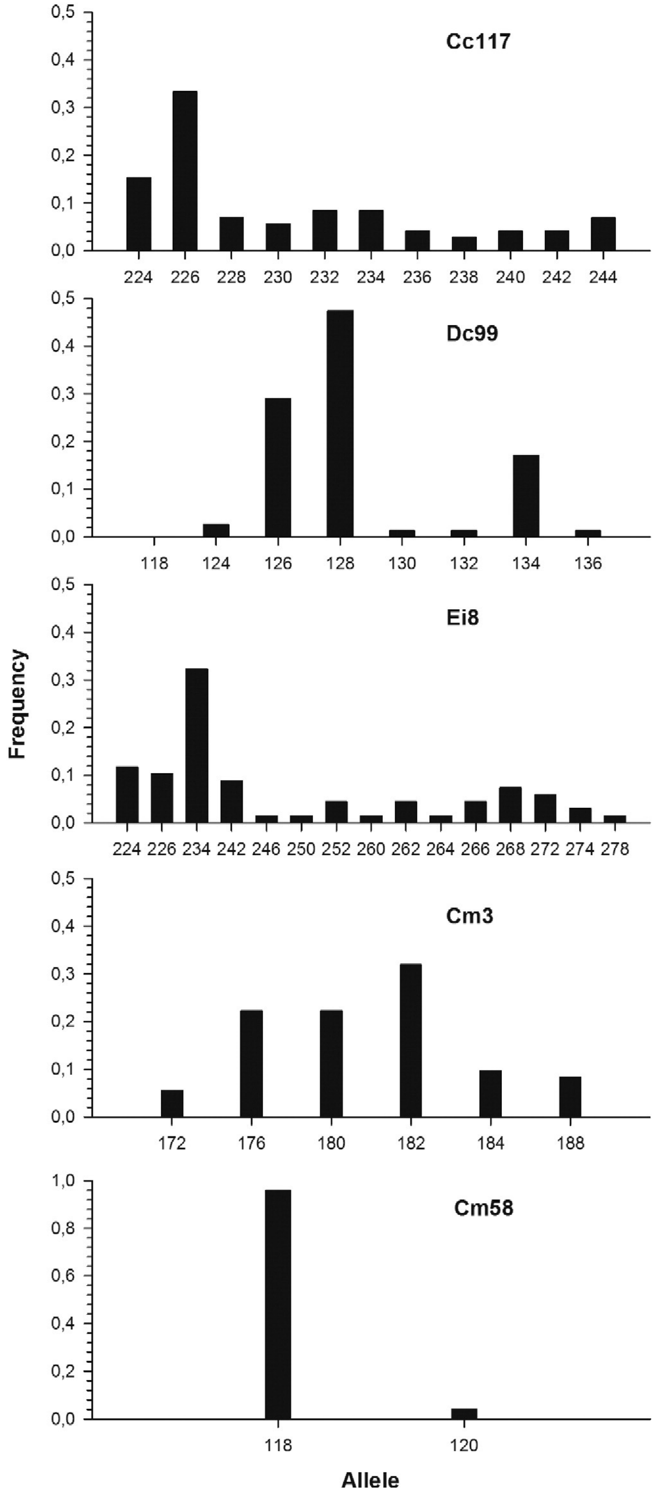

**Fig. 2.** Allele frequencies for five microsatellite loci observed in 36 (38 for Dc99, and 34 for Ei8) adult female leatherbacks nesting in Playa Gandoca, Costa Rica during the nesting season 2008.

**Table 2**
Probability of two random individual leatherback turtles at Playa Gandoca, Costa Rica sharing a genotype (*Q*) and of detecting multiple paternity (*D*) using various combinations of loci.

| Loci combination | *D* | Q | Linkage disequilibrium (*p* value)[a] |
|---|---|---|---|
| Ei8 — Cc117 | 0.91 | 0.0021 | 0.86376 |
| Ei8 — Dc99 | 0.83 | 0.0093 | 0.06941 |
| Cc117 — Dc99 | 0.81 | 0.0113 | 0.59188 |
| Ei8 v Cc117 — Dc99 | 0.95 | 0.0005 | |
| Across all loci | 0.95 | $1.36346 \times 10^{-05}$ | |

[a] Significant linkage disequilibrium indicated by $p < 0.05$.

To obtain a PrDM value of 98% in clutches sired by two males with an even contribution, sampling 15 offspring is sufficient, and to detect multiple paternity with a probability of 99% in clutches sired by three fathers, sampling 10 offspring is sufficient if paternal contributions are even. The highest observed paternity skew in the Gandoca nesting population was 77% between three clutches of one particular female, of which 29 offspring were sampled in total from the different nests. This yielded in a 99% confidence that all contributing males were detected. If the paternity skew is 90%, which only appears in a single clutch in this study, up to 50 offspring would have been necessary to detect a second male with a confidence of 97. Detection power was lacking in this particular case, but it could have only raised the level of multiple paternity detected, and thus does not alter the general findings.

Fewer than 10 offspring per clutch were sampled, mainly due to permit limitations, but also because of the number of offspring produced in different clutches. Given the PrDM values, the average possibility of detecting multiple paternity was on average 93% for each clutch, indicating that the chance of non-detection was approximately <7% in clutches of females with multiple sires.

### 3.2. Paternity assay

373 hatchlings of one to three clutches per female from 18 different females were genotyped with an average of 21.91% (range 8–80%) hatchlings per clutch (Table 3).

**Table 3**
Number of clutches per female and offspring per clutch sampled, as well as the sampling effort per clutch (%) for the paternity assay in the leatherback population nesting in Gandoca, Costa Rica. N = number of hatched offspring per clutch, n = number of offspring sampled.

| Female ID | No. of clutches analysed | Hatchlings per clutch analysed [N/n] | Sampling effort per clutch [%] |
|---|---|---|---|
| A2 | 1 | 1 [53/10] | 19 |
| A3 | 2 | 1 [41/8] | 20 |
| | | 2 [65/15] | 23 |
| B16 | 3 | 1 [57/10] | 18 |
| | | 2 [45/15] | 33 |
| | | 3 [10/8] | 80 |
| C5 | 2 | 1 [63/8] | 13 |
| | | 2 [67/15] | 22 |
| C21 | 2 | 1 [39/8] | 21 |
| | | 2 [10/3] | 30 |
| D6 | 3 | 1 [26/6] | 23 |
| | | 2 [46/15] | 33 |
| | | 3 [40/15] | 38 |
| E7 | 2 | 1 [74/8] | 11 |
| | | 2 [63/10] | 16 |
| E13* | 2 | 1 [51/9] | 18 |
| | | 2 [53/10] | 19 |
| F9 | 3 | 1 [50/5] | 10 |
| | | 2 [33/7] | 21 |
| | | 3 [44/16] | 36 |
| F20 | 1 | 1 [72/11] | 15 |
| G8 | 2 | 1 [87/15] | 17 |
| | | 2 [73/6] | 08 |
| G22 | 1 | 1 [95/15] | 16 |
| H15* | 3 | 1 [69/6] | 09 |
| | | 2 [77/14] | 18 |
| | | 3 [46/9] | 20 |
| I11 | 1 | 1 [42/11] | 26 |
| I19* | 2 | 1 [61/10] | 16 |
| | | 2 [52/15] | 29 |
| J12 | 1 | 1 [93/10] | 11 |
| J18* | 2 | 1 [68/10] | 15 |
| | | 2 [86/15] | 17 |
| K14 | 2 | 1 [30/6] | 20 |
| | | 2 [57/15] | 26 |

In 14 of 18 families no evidence for multiple mating was found and inference of paternal alleles was unambiguous.

Extra paternal alleles were detected in four out of 18 (22.22%) females analysed (Table 4). Two of these females (E13 and H15) were first-time nesters (neophytes), while two females (I19 and J18) had been nesting for at least two and eight years, respectively. Three of the other 14 females were also neophytes, two had been nesting less than five years, seven had been nesting less than 10 years, and two had been nesting more than ten years.

Offspring genotypes from 14 of the analysed females were consistent with only one father, while offspring from the other four females showed two to three different sires (Table 4, Fig. 3). The program GERUD 2.0 confirmed the suggested number of mates per female and their genotypes in three of these females, which were first manually assigned by comparing genotypes of mothers and their offspring on three loci. GERUD suggested a second contributing father for female H15, based on allele frequencies within the population, and female H15 was also included in the group of females with evidence of multiple paternity. Multiple fathers were consistently evident in all clutches analysed of females with multiple paternity, except in the first clutch of female E13. A missing maternal allele in four hatchlings in the second clutch of female E13 at locus Cc117 led to exclusion of these hatchlings from the paternity assay.

No inferred paternal genotype was observed twice, which indicates that 25 different males contributed to the clutches of the 18 females analysed (Table 4).

### 3.3. Contribution of males

In all clutches of females, where more than one patriline was detected among their offspring, the contribution of each sire was uneven within and between clutches (Fig. 3). Between the clutches laid by the same female, the composition and frequency of contributing fathers also differed. In some clutches of multiply mated females a majority of offspring was sired by a single male. Subsequent clutches, however, differed in paternity, with another male siring most offspring (Fig. 3). In subsequent clutches of two females a "new" male not detected in previous clutches sired a high percentage (Fig. 3) of offspring (female E13 and I19). In female E15 a male detected in the first clutch was not detected in the second clutch but again in the third (Fig. 3).

Similarities between clutches of females were compared using the Sørensen Similarity Index (QS), where 1 is 100% similar and 0 is completely different. Values for QS ranged from 0.5 to 1, showing relatively high similarities between subsequent clutches of females. Clutches of female J18 had identical composition of fathers in each clutch. Composition of fathers in other females showed similarities between different clutches of the same female, but were not identical (Fig. 3). Lowest similarity between clutches had female H15 with 0.5 between clutch 1 and 3 (Fig. 3).

Effective mating frequencies ($m_e$) ranged from 1 to 3.18 in single clutches for females where multiple paternity was detected and from 1.63 to 3.13 over the sequences of clutches laid by the same female and were in general lower than the observed number of males contributing to clutches (Fig. 3).

## 4. Discussion

### 4.1. Paternity assay

Here, it is demonstrated that at least 22.22% of female leatherbacks nesting in Gandoca exhibited multiple paternity in their clutches. This level of multiple paternity is more than twice as high as previously found in the same nesting population (Curtis, 1998). Another paternity study of leatherback turtles conducted in Playa Grande, Costa Rica (Pacific coast), showed a level of 10% multiple paternity (Crim et al., 2002), and a study conducted, contemporaneously to this study, in a different Caribbean nesting population in St. Croix (US Virgin Islands) (Stewart and Dutton, 2011) found 41.7%, even though a prior conducted study found no multiple paternity at all (Dutton et al., 1998).

The higher levels of multiple paternity detected suggest that natural mating systems of leatherback turtles might be more polyandrous than previously observed (Curtis, 1998; Dutton et al., 1998; Crim et al., 2002). Long-term conservation efforts of declining populations (Dutton et al., 2005; Chacón-Chaverri and Eckert, 2007) were only recently established when first paternity studies were conducted. Natural mating patterns could have been altered by small population size. An

**Table 4**
Depicted are the genotype of each female and inferred paternal genotypes among her offspring for the loci combination Dc99-Cc117-Ei8 of the paternity assay, the year in which each female was tagged the first time, and the number of males found among offspring of each female of the leatherback nesting population in Gandoca, Costa Rica in 2008.

| Female ID | Year tagged | Maternal genotypes | | | Inferred paternal genotypes | | | Total no. of males |
|---|---|---|---|---|---|---|---|---|
| | | Dc99 | Cc117 | Ei8 | Dc99 | Cc117 | Ei8 | |
| A2 | 1999 | 126/128 | 226/232 | 234/278 | 126/126 | 230/250 | 234/252 | 1 |
| A3 | 2000 | 126/126 | 226/226 | 234/252 | 126/134 | 224/226 | 226/234 | 1 |
| B16 | 2008 | 128/134 | 234/236 | 234/242 | 126/128 | 226/228 | 264/272 | 1 |
| C5 | 2002 | 126/126 | 226/230 | 224/234 | 126/132 | 224/234 | 224/262 | 1 |
| C21 | 2008 | 126/126 | 242/244 | 272/272 | 126/134 | 224/226 | 262/282 | 1 |
| D6 | 2003 | 128/134 | 226/234 | 268/272 | 128/128 | 226/226 | 234/262 | 1 |
| E7 | 2002 | 126/128 | 226/228 | 234/268 | 126/128 | 228/234 | 224/234 | 1 |
| E13* | 2008 | 130/134 | 226/238 | 242/274 | 128/128 | 228/230 | 266/242 | 2 |
| | | | | | 126/128 | 228/230 | 234/264 | |
| F9 | 2005 | 126/128 | 232/244 | 234/260 | 126/132 | 226/230 | 224/242 | 1 |
| F20 | 1998 | 126/126 | 230/232 | 224/266 | 128/128 | 226/234 | 224/234 | 1 |
| G8 | 2005 | 126/128 | 226/228 | 242/268 | 126/126 | 226/238 | 224/234 | 1 |
| G22 | 1994 | 126/128 | 224/240 | 224/266 | 128/136 | 224/234 | 234/276 | 1 |
| H15* | 2008 | 126/128 | 226/228 | 226/234 | 126/134 | 228/240 | 234/266 | 3 |
| | | | | | 128/134 | 228/228 | 256/268 | |
| | | | | | 134/134 | 228/228 | 226/264 | |
| I11 | 2001 | 128/136 | 228/234 | 226/252 | 128/134 | 228/244 | 234/242 | 1 |
| I19* | 2006 | 126/134 | 226/236 | 224/234 | 118/118 | 236/244 | 226/234 | 3 |
| | | | | | 118/134 | 224/244 | 224/266 | |
| | | | | | 134/134 | 226/232 | 224/266 | |
| J12 | 2008 | 128/134 | 224/226 | 234/268 | 126/128 | 232/248 | 234/256 | 1 |
| J18* | 2000 | 126/132 | 224/236 | 224/234 | 124/126 | 248/252 | 224/260 | 3 |
| | | | | | 124/126 | 226/226 | 250/250 | |
| | | | | | 124/126 | 226/232 | 242/264 | |
| K14 | 2002 | 128/134 | 228/234 | 226/234 | 128/128 | 224/226 | 250/260 | 1 |

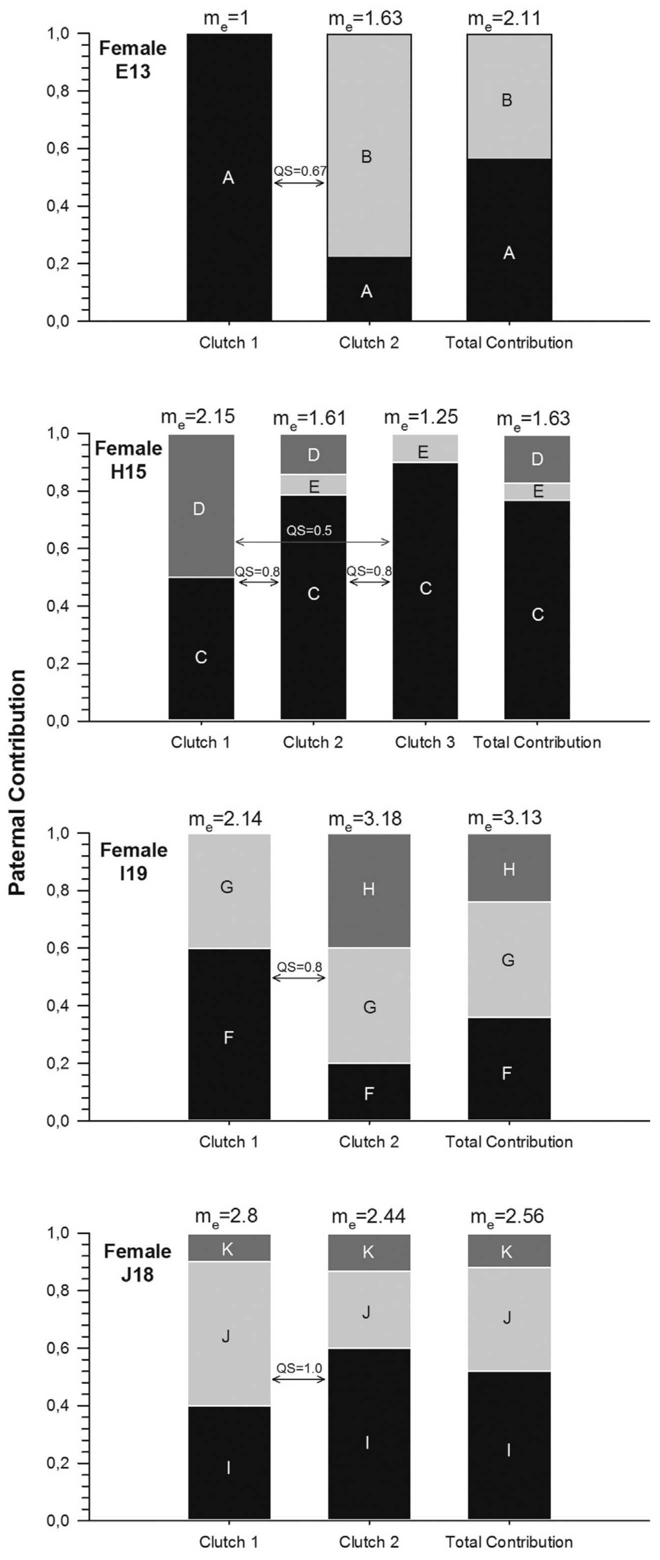

**Fig. 3.** The relative contribution from observed fathers for each clutch and across all clutches (n) laid by each of the four nesting females that exhibited multiple paternity at Playa Gandoca, Costa Rica. The Sørensen Similarity Index (QS) indicates similarities of father composition between clutches (where 1 is 100% similar and 0 is completely different), and the effective mating frequency ($m_e$) accounts for the actual paternities of contributing males based on the frequency of their contribution.

Allee effect and a resulting patchy and pelagic distribution of potential mates could have caused the low levels of multiple paternity previously detected (Courchamp et al., 1999). More than a decade later, the Northwest Atlantic ocean subpopulation of leatherbacks turtles, which the ACR is part of, is thought to be stable or has even slightly increased in size in some nesting populations, and the IUCN has changed its status from previously critically endangered to least concern in 2013 (Spotila et al., 1996; Troeng et al., 2004; Dutton et al., 2005; Girondot et al., 2007; Eckert et al., 2012; Tiwari and Wallace, 2013).

Still, the majority of analysed clutches in this study showed only single paternity. In general the density of breeding turtles (males and females) seems to be positively correlated with levels of multiple paternity, as Jensen et al. (2006) discovered in olive ridley turtles when comparing turtles nesting either in *arribada*-congregations (high density) or solitarily (low density). They found lower levels of MP (30%) in solitarily nesting turtles, whereas among *arribada*-nesting females the level of MP was as high as 92% with also a high number of contributing fathers per clutch. Hence, the pelagic life-history of leatherbacks and the general scattered distribution of individuals might be another reason for low levels of multiple paternity.

Temperature-dependent sex determination (TDSD) in sea turtles and rising temperatures on nesting beaches, due to climate change and beach development (Hawkes et al., 2009), are thought to produce a surplus in female leatherback hatchlings (range 53.4%-100% females) (Eckert et al., 2012) and it has been proposed that this could lead to a skewed OSR among adults with a strong female bias (Eckert et al., 2012). If the alleged feminisation of leatherback populations is true, it could emphasise the already mentioned potential reasons for low levels of multiple paternity, because it would mean an even greater scarcity of breeding males. The feminisation of the sexual mature population, however, was not yet observed by studies. Operational sex ratios among adult leatherback turtles showed only a slight female bias (51.9–66.7%) (Eckert et al., 2012) so far, and the results of this study show that each female analysed mated with a different male. Males might also balance out possible female biased sex-ratios with shorter reproductive and migratory cycles of one year instead of every second or third year, returning to breed more frequently than females (James et al., 2005; Hays et al., 2010; Stewart and Dutton, 2011; Eckert et al., 2012). Hays et al. (2014) provide evidence that shorter breeding periodicities in males are likely to occur broadly across all sea turtle populations. Therefore, multiple paternity could indicate a balanced operational sex ratio.

### *4.2. Sperm storage*

Evidence exists that sea turtles are able to store sperm in their oviduct for at least the duration of a nesting season, and even for several years (Gist and Jones, 1989; Gist and Congdon, 1998; Pearse and Avise, 2001). The consistent paternity in the majority of females (15 out of 18, including Family J18) supports the hypothesis in so far that sperm is stored from mating at the beginning of the nesting season and is used in all consecutive clutches in the given nesting season. The question is if leatherback turtles mate only one time successfully prior to the nesting season, or if they mate with several mates in a short time. If leatherbacks mate only once per nesting season the observed multiple paternity in a few females could be the result of residual sperm left over and stored from a previous nesting season mixed with sperm received in the current nesting season. Residual sperm from previous nesting seasons as a reason for found multiple paternity does not seem likely, though, for this study, since two of the four females with multiple paternity were neophytes, who are assumed to not have mated in previous nesting seasons. Furthermore, contributions of secondary males do not decrease from the first to subsequent clutches as would be expected from residual sperm, which should be less viable or only present in small quantities. Moreover, if residual sperm would be the reason for detected multiple paternity, why do still so many females (more than 75%) show only single paternity? If sperm is stored for several nesting seasons, a majority of clutches should be expected to show secondary males, at least at low frequencies. Unless the reason for the single paternity is that females cannot find a mate every season, due to a lack of males, and are using the same stored sperm to fertilise their clutches throughout more than one nesting season.

The limited sample size of hatchlings per clutch (in one case only 8%) could have led to an incidental non-detection of secondary fathers, and also to a skewed attribution of contribution as a result of random sampling noise. It does not seem likely, however, since secondary males fathered a substantial part of the offspring (7–79% per nest) (Fig. 3).

As a reason for single paternity in the majority of clutches Stewart and Dutton (2011) suggested that females mate with the same male in consecutive nesting seasons. This means that either leatherbacks would have to be able to recognise each other even after two to three years (female remigration intervals) (Eckert et al., 2012) from last copulation, and would have to meet again frequently or that there is such a small amount of breeding males present, that females re-mate with the same male by chance. Up-to-date no evidence exists that sea turtles are able to recognise their own kin let alone specific individuals (Waldmann and McKinnon, 1993). In small nesting populations such as St. Croix (Troeng et al., 2004; Dutton et al., 2005; Dow et al., 2007), fewer males should be present and chances might be higher to encounter the same male more than once. James et al. (2005), though, observed that males travelling to smaller nesting populations had a broader range, even swimming between several nesting beaches probably to find females, than males that travelled to bigger nesting aggregations, where they stayed close to its epicentre. The Atlantic Costa Rican rookery is a large nesting aggregation and the chances to meet the same male twice in one nesting season or even between seasons seems fairly unlikely, unless the operational sex ratio (OSR) in this aggregation is highly skewed and males are present in only very small quantities. Our results do not suggest a highly female biased OSR or substantial lack of mates. It can also be assumed that males engage in competitive behaviour and are actively courting females, possibly in groups, and hindering or dislodging other males from mating with the female (Carr and Carr, 1986; Eckert et al., 2012). Therefore, mating with the same male and only him in consecutive nesting season would happen only by chance and seems unlikely.

### 4.3. Sperm competition and timing of mating

It is also debated that either sperm competition within the female occurs or that the timing, when copulation is taking place, plays a crucial part in successful fertilisation. E.g. if copulation takes place after ovulation and egg production, sperm would not be able to fertilise eggs, and might not even reach the storage tubuli in the oviduct (Valverde, 1996; FitzSimmons, 1998). There might also be mechanisms of sperm competition, not yet identified, enabling males to influence the outcome of fertilisation, dominating over other males' sperm and siring the biggest portion of offspring in a given clutch or among all clutches during one nesting season (FitzSimmons, 1998; Chevalier et al., 1999). This study detected uneven contribution of fathers within one clutch and in following clutches. If sperm competition takes place, each clutch should show one primary male, siring the majority of the offspring, and secondary males in low frequencies, which sire only small portions of a clutch. A single dominant male was found in some clutches in this study, but not in all of them, and one primary male throughout all clutches of a female was found only in female H15. Effective mating frequencies ($m_e$) were close to the observed mating frequencies in single nests, except in the second and third nest of female H15, which indicates that the contribution of fathers was not strongly skewed, and suggests that sperm competition does not play a strong role. Females E13, I19 and J18 showed one dominant male in a one or two of their clutches, but not the same male each time. It is unknown how sperm mixes in storage tubuli, if it consists of a homogenous mixture which gives each male an equal probability to fertilise a certain proportion of eggs, or if the time of arrival in storage tubuli determines the frequency of sired eggs in a given clutch (e.g. last in, first out) (Owens, 1980; FitzSimmons, 1998).

The sperm quality or quantity might also have an effect on the levels of multiple paternity. In green sea turtles it has been observed that not every copulation is successful (FitzSimmons, 1998). Even though female green turtles in Australia have been seen to copulate frequently and with several males prior to nesting, levels of multiple paternity still remained low (0.3%) (FitzSimmons, 1998). This raised the question on how successful the average copulation is. It is very likely that the duration of copulation determines the quantity of sperm that gets transferred and is directly correlated to the probability of fertilising a certain proportion of eggs. Unlike the Cheloniidae, leatherback males are not able to clasp onto the female carapace due to the lack of a claw on their front flipper and might be easily dislodged by competing males or even by an unwilling female, and therefore, only insignificant quantities of sperm might reach oviduct of female during the majority of copulations.

### 4.4. Benefits of multiple paternity

For males the benefits of polygyny seem to be obvious as an increase in sired offspring with little costs involved should result in a higher fitness (Lee and Hays, 2004). For Females, on the other hand, the benefits of multiple mating are not as palpable. Sea turtles are solitary animals that do not engage in parental care or complex social behaviour and a single mating is usually sufficient to fertilise all eggs of a female in a given nesting season. Thus, there are no apparent direct benefits (parental care, nuptial gifts) of polyandry for females. On the contrary, multiple mating seems very costly since it increases the risk of mortality through exposure to predation, transmitted diseases, and loss of energy through intense male harassment (Jennions and Petrie, 2000; Lee and Hays, 2004). It has been suggested that females only make the best of a bad job, since male harassment can be intense and energy consuming, leading to wounds which require weeks to heal, and at one point the benefits of giving-in to male harassment and to re-mate could probably exceed the costs involved of resisting aggressive male courtship behaviour (Jennions and Petrie, 2000). Indirect benefits could be the assurance of fertilisation, increased offspring viability, enhanced genetic diversity and sperm competition (Jennions and Petrie, 2000; Lee and Hays, 2004; Jensen et al., 2006), even though in the case of sea turtles probably only by chance. So far no mechanisms for sexual selection and female cryptic choice in sea turtles have been described (Lee and Hays, 2004). It can also reduce inbreeding in an environment where it might be impossible to identify close relatives, since sea turtles are thought to be not able to recognise their own kin (Waldmann and McKinnon, 1993).

The dependence of multiple paternity on age or experience of a female is possible. In our study, two of the four females (50%) showing multiple sires to their clutches were neophytes. The total proportion of neophytes within the group of sampled females was 27.78% (five of 18 females). A Fisher's Exact Test showed no significant differences ($p = 0.5646$). The paternity study conducted by Stewart and Dutton (2011) found two of the five females, which showed multiple paternity, to be neophytes and three females nested already for more than ten years. Hence, age dependence does not seem likely.

### 4.5. Inter-nesting mating

The appearance of a previously not detected male in succeeding clutches of three of the females (E13, H15, and I19) with multiple paternity has given rise to the assumption that inter-nesting mating might be responsible for these results. The values for the Sørensen Similarity Index support these findings, with indices considerably lower than 1.0 in the three families, which indicate that the different clutches within one family do not share the same composition of males in all nests. "New" fathers were present in such high frequencies (up to 0.79 in female E13) in some of the females' succeeding clutches that a sole non-detection in previous clutches seems unlikely. Males stay in waters close to nesting beaches for up to three month during nesting seasons (James et al., 2005) and could mate with females that have already mated earlier in the season. The physiological prerequisites for successful inter-nesting mating still need to be discussed. FitzSimmons (1998) argued that sperm might be unable to travel up the oviduct once egg production has started, since eggs are moving down and might flush out sperm travelling up the oviduct (first-male preference). Valverde (1996) though, was able to show that ovulation and egg production starts only a few hours after the last oviposition and lasts on average 80 h. He found shelled eggs within 51 h after last oviposition. This could mean that eggs stay in recess for several days unmoving. For leatherbacks, with inter-nesting intervals of about nine to ten days, it would be a recess for about a week, which would allow new sperm to reach the storage area above the albumin gland (Owens, 1980; Gist and Jones, 1989; Gist and Congdon, 1998) and which could be used in succeeding egg production.

### 4.6. Conclusions

Up till today gaps exist concerning leatherback mating systems, especially from the perspective of male leatherbacks. But also such basic information concerning mating areas and mating and courtship behaviour is still scarce. This study suggests that small population size could have an impact on the natural mating system of leatherback turtles. Levels of multiple paternity seem to be density dependent and, therefore, could be used as indicator for a stable or increasing population. The density dependency of multiple paternity in leatherback turtles and also the success-rate of matings should be tested in big leatherback nesting aggregations, such as Gabon or French Guiana/Suriname (Fossette et al., 2008).

Successful inter-nesting mating has not previously been observed in any other sea turtle paternity study yet. A reason could be either because no subsequent clutches of studied females were sampled (Kichler et al., 1999; Hoekert et al., 2002; Moore and Ball, 2002; Jensen et al., 2006), or only low levels of multiple paternity were observed (FitzSimmons, 1998; Crim et al., 2002) or a successful mating between nesting events could be an exception to the rule.

These new insights into mating patterns could help to manage and assess the genetic health of leatherback stocks world-wide and can assist in adjusting the focus of conservation efforts since genetic diversity plays a major part in the future survival of this species and its ability to adapt to changes.

## Compliance with ethical standards

Informed Consent: For this type of study formal consent is not required.

Ethical approval: All sampling procedures were in accordance with international and Costa Rican ethical standards and laws for the study and sampling of endangered animals and were conducted with official governmental permits.

Disclosure of potential conflicts of interest: The authors declare that they have no conflict of interest.

## Contributions

Christine Figgener: Research Idea, Sample Collection, Laboratory Work, Data Analysis, Manuscript.

Didiher Chacón-Chaverri: Permit Holder, Technical Assistance in the Field.

Michael P. Jensen: Data Analysis, Manuscript.

Heike Feldhaar: Research Idea, Funding, Data Analysis, Manuscript.

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