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Does polyandry really pay off?: The effects of multiple mating and number of fathers on morphological traits and survival in clutches of nesting green turtles at Tortuguero

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Despite the long debate of whether or not multiple mating benefits the offspring, studies still show contradicting results. Multiple mating takes time and energy. Thus, if females fertilize their eggs with a single mating, why to mate more than once? We investigated and inferred paternal identity and number of sires in 12 clutches (240 hatchlings) of green turtles (*Chelonia mydas*) nests at Tortuguero, Costa Rica. Paternal alleles were inferred through comparison of maternal and hatchling genotypes, and indicated multiple paternity in at least 11 of the clutches (92%). The inferred average number of fathers was three (ranging from 1 to 5). Moreover, regression analyses were used to investigate for correlation of inferred clutch paternity with morphological traits of hatchlings fitness (emergence success, length, weight and crawling speed); and size of the mother; and an environmental variable (incubation temperature). We suggest and propose two different comparative approaches for evaluating morphological traits and clutch paternity, in order to infer greater offspring survival. First, clutches coded by the exact number of fathers and second by the exact paternal contribution (fathers who gives greater proportion of the offspring per nest). We found significant differences ($P < 0.05$) in clutches coded by the exact number of fathers for all morphological traits. A general tendency of higher values in offspring sired by two to three fathers was observed for the length and weight traits. However, emergence success and crawling speed showed different trends which unable us to reach any further conclusion. The second approach analysing the paternal contribution showed no significant difference ($P > 0.05$) for any of the traits. We conclude that multiple paternity does not provide any extra benefit in the morphological fitness traits or the survival of the offspring, when analysed following the proposed comparative statistical methods.

2 **Title page**

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4 morphological traits and survival in clutches of nesting green turtles at Tortuguero

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17 **Abstract**

18 Despite the long debate of whether or not multiple mating benefits the offspring, studies still show
19 contradicting results. Multiple mating takes time and energy. Thus, if females fertilize their eggs with a
20 single mating, why to mate more than once? We investigated and inferred paternal identity and number
21 of sires in 12 clutches (240 hatchlings) of green turtles (*Chelonia mydas*) nests at Tortuguero, Costa
22 Rica. Paternal alleles were inferred through comparison of maternal and hatchling genotypes, and
23 indicated multiple paternity in at least 11 of the clutches (92%). The inferred average number of fathers

24 was three (ranging from 1 to 5). Moreover, regression analyses were used to investigate for correlation
25 of inferred clutch paternity with morphological traits of hatchlings fitness (emergence success, length,
26 weight and crawling speed); and size of the mother; and an environmental variable (incubation
27 temperature). We suggest and propose two different comparative approaches for evaluating
28 morphological traits and clutch paternity, in order to infer greater offspring survival. First, clutches
29 coded by the exact number of fathers and second by the exact paternal contribution (fathers who gives
30 greater proportion of the offspring per nest). We found significant differences ($P < 0.05$) in clutches
31 coded by the exact number of fathers for all morphological traits. A general tendency of higher values
32 in offspring sired by two to three fathers was observed for the length and weight traits. However,
33 emergence success and crawling speed showed different trends which unable us to reach any further
34 conclusion. The second approach analysing the paternal contribution showed no significant difference
35 ($P > 0.05$) for any of the traits. We conclude that multiple paternity does not provide any extra benefit
36 in the morphological fitness traits or the survival of the offspring, when analysed following the
37 proposed comparative statistical methods.

38 **Introduction**

39 Although the fertilization of eggs in many female animals is usually achieved through a single mating,
40 multiple mating is nevertheless a relatively common observation in natural populations. While some
41 authors have argued that its prevalence is intriguing, due to the energetic fitness costs that such
42 behaviour incurs (Lee & Hays, 2004; Bilde et al., 2009), others have demonstrated how females may
43 gain indirect genetic benefits to offset the cost by mating with multiple males (Byrne & Roberts, 2000;
44 LaBrecque et al., 2014). For example, polyandry may provide genetic benefits by improving the
45 chance that females will acquire “good” genes or by enhancing the genetic diversity of their progeny
46 (Yasui, 1997).

47 Traditionally behavioural observations have been used to determine the mating patterns in many
48 vertebrate and invertebrate species. However, the application of nuclear DNA markers as an alternate
49 tool has transformed such research, by providing direct insights into parentage in natural populations
50 (Packer et al., 1991; Craighead et al., 1995; Keane, Dittus & Melnick, 1997; FitzSimmons, 1998; Uller
51 & Olsson, 2008) and enabling investigation into the genetic consequences of multiple matings. For
52 example, genetic polyandry of surveyed birds species occurs regularly in 86% of the species (Michl et
53 al., 2002; Griffith, Owens & Thuman, 2002). As a result, sperm from different males may compete to
54 fertilize a single clutch of eggs. This is the case in many species of insects, gastropods, fish, amphibian,
55 birds and reptiles (FitzSimmons, 1998; Garcia-Vazquez, 2001; Jones & Clark, 2003; García-González
56 & Simmons, 2005; Chandler & Zamudio, 2008; Beese et al., 2008; Noble, Keogh & Whiting, 2013;
57 LaBrecque et al., 2014); or in progeny from the same brood in mammals (Clapham & Palsbøll, 1997;
58 Shurtliff, Pearse & Rogers, 2005). Thus, sperm competition may be an important factor in the
59 evolution of reproduction of many organisms, although the precise mechanism that determines sperm
60 success is not fully understood (Jones & Clark, 2003).

61 As with all six other sea turtle species, female green turtles have been observed to undergo polygamous
62 breeding (Pearse & Avise, 2001), that often result in multiple paternity (MP) among offspring from a
63 single clutch. Both short term sperm storage and sperm competition have been proven to be important
64 aspects of turtle mating system (FitzSimmons, 1998; Phillips et al., 2013), and as a result several
65 hypotheses have been proposed regarding the benefits of this behaviour. These include increased
66 offspring viability and genetic diversity, fertilization assurance and procurement of compatible gametes
67 (FitzSimmons, 1998; Jennions & Petrie, 2000; Uller & Olsson, 2008). The adaptive value of polyandry
68 as a mating strategy could be explained in terms of sperm competition, sperm storage and sperm
69 degradation during the mating process; which occurs within female oviducts and/or the egg

70 fertilization. As in many other reptiles species, sea turtles are capable of long-term sperm storage for
71 several years (Ewing, 1943). However, sperm viability does decline drastically after time (Pearse &
72 Avise, 2001). Most turtle species breed at intervals of two to five years (Galbraith, 1993), making any
73 stored sperm from previous seasons highly valuable – due to its genetic variability across seasons – but
74 most likely of low quality.

75 Previous studies have demonstrated that the frequency of MP varies both between (Moore & Ball,
76 2002; Hoekert et al., 2002; Crim et al., 2002; Theissinger et al., 2008; Phillips et al., 2013; Noble et al.,
77 2013), and within species (Galbraith, 1993; FitzSimmons, 1998; Ireland et al., 2003; Jensen et al.,
78 2006; LaBrecque et al., 2014). For green turtles, evidence of MP has been reported in several studies,
79 but the proportion of clutches with MP varies among populations. For example, 9% of the clutches
80 analysed in Australia (FitzSimmons, 1998), 50% of clutches in the Caribbean Tortuguero rookery
81 (Peare & Parker, 1996), 61% of clutches on Ascension Island (Lee & Hays, 2004), and the highest
82 documented frequency of MP at 75% in the Eastern Pacific (Pearse & Avise, 2001).

83 All this raises some important questions such as, why these large differences in MP occur among
84 populations of the same species? Are there any measurable benefits to the offspring from MP such as
85 increased hatching success, increased survival in the clutches and/or benefits in morphological traits
86 (e.g. length, weight and crawling speed) when compared by the exact number of inferred fathers in the
87 clutches? On the other hand, there may be other factors that might affect these morphological traits,
88 and may confound the results (e.g. incubation temperature, rainfall or the size of the parents).

89 Whether MP results in fitness benefits to the species remains unclear. In a previous study from
90 Ascension Island in the middle Atlantic, the fitness in clutches was found to be not correlated with the
91 paternity (Lee & Hays, 2004). The measurements of fitness used were the size of the female, clutch
92 size, proportion of eggs fertilized, proportion of eggs hatching and offspring survival. However, the

93 authors of this study did not measure direct physical or morphological traits in the hatchlings. In the
94 present study, most measures from the Lee & Hays (2004) study were included in the analysis.
95 Moreover, in addition we expanded by including measures of morphological traits of offspring survival
96 measured by the emergence success; weight, length and crawling speed in order to generate additional
97 parameters that may provide a more detail insight into the offspring fitness. Furthermore, we
98 incorporate and propose two different comparative approaches to evaluate clutches paternity effect in
99 the offspring fitness, which are in more detail described below.

100 This study aims to determine the level of MP for the green turtle population nesting at Tortuguero,
101 Costa Rica. What's more, we aim to provide a relative measure of fitness in terms of morphological
102 traits of clutches and hatchlings into the wild, defined as emergence success, length, weight and
103 crawling speed. This combination of measurements may (i) support previous results in which MP was
104 found to provide NO benefits to offspring fitness, or (ii) contradict most studies by reaching new
105 conclusions where MP may be found to provide direct morphological benefits at the initial stage of the
106 sea turtles lifecycle.

107 **Materials and Methods**

108 *Field sampling*

109 Samples were collected in 2007 at Tortuguero (10°32'42.26"N, 83°30'11.35"W), on the Caribbean
110 coast of Costa Rica. This is the largest green turtle rookery in the world that comprises 25% of the
111 global abundance of nesting green females (Seminoff et al., 2014). Tortuguero is a 45 km long beach.
112 This study was concentrated on the northern 2 km of the beach. Tissue samples were taken from the
113 trailing edge of the left front flipper from 41 nesting females using a scalpel. Iodine was immediately
114 applied to the wound to avoid infection. If not previously tagged, adult turtles were tagged with Inconel
115 flipper tags in both front flippers to ensure identification. The minimum curved carapace length (CCL)

116 was recorded using a measuring tape. The location of the nests on the beach was recorded by
117 triangulation; information used 45 days later to track them down before hatching was expected.
118 Additionally, smart i-button data loggers (ACR Systems Inc. Vancouver, Canada) were placed in small
119 Ziploc plastic bags in the middle of the nest together with the eggs. 12 smart i-buttons were placed into
120 different nests to register the temperature changes during the incubation process (5 data loggers were
121 lost and never recovered). Temperature was recorded every hour in seven different nests during the
122 entire incubation period (approximately 60 days). Information from the smart i-button data loggers was
123 downloaded using the software ACR SMARTBUTTON READER 1.32. To protect nests from fly infestation
124 and restrain hatchlings after emergence, mesh cylinder cages covered with mosquito netting were
125 placed over each nest. Nests were observed every two hours; seven days at week during the emergence
126 phase to prevent hatchlings from overexposure to the sun. Tissue samples were taken from the
127 posterior edge of the carapace from 530 hatchlings (from 12 different nests, averaging 45 hatchlings
128 per nest). All tissue samples were preserved in a saturated NaCl with 20% DMSO (Dutton et al., 1999)
129 at ambient temperature in the field, and thereafter shipped to our labs in Denmark and preserved at -20
130 °C in the laboratory.

131 Immediately after tissue collection, length, weight and crawling speed were recorded from each
132 hatchling. Right after, the neonates were released. Offspring length and weight were measured using a
133 250 mm vernier calliper and a 50 g manual scale, respectively. Crawling speed was recorded by using a
134 one-meter long tube (15 cm diameter) placed on the sand. Hatchlings were released on one side and
135 allowed to crawl to the other end, and the time spent by hatchlings in this trajectory was used as a
136 measure of the crawling speed.

137 *Laboratory analysis*

138 *Microsatellites*. A total of 297 turtles (41 adult females and 256 hatchlings) were used and analysed for
139 variation at eight microsatellite loci in this study. Tissue samples were finely chopped and digested
140 overnight with proteinase-K at 52°C. Total genomic DNA was then extracted using Invisorb Spin
141 Tissue Kit (Invitek Inc. Berlin, Germany) following the manufacturer's instructions. The microsatellite
142 primers (Table 1) used in this study were developed for different species of sea turtles; Cm3, Cm58,
143 Cm72, Cm84, Cc117 (FitzSimmons, Moritz & Moore, 1995), Cc7 (FitzSimmons, 1998), Or-4 and Or-
144 7 (Aggarwal et al., 2007). Forward primers were end-labelled with fluorescent dye TaqMan® (Applied
145 Biosystems). Following the QIAGEN Multiplex PCR Handbook manufacturer instructions, the
146 microsatellites were multiplexed in the amplification stage and onwards. Each PCR was carried out in
147 a 10-µL reaction volume containing 1-µL of genomic DNA, 1-µL of primer mix (2 µM each primer),
148 3-µL RNase-free water and 5-µL of QIAGEN multiplex PCR master mix (provides a final
149 concentration of 3 mM MgCl₂). Amplification were carried out in a PXE 0.2 Thermal Cycler with an
150 enzyme activation step at 95°C for 15 minutes, followed by 30 cycles of denaturation at 94°C for 30°C
151 s, annealing at 57°C for 90 s and extension at 72°C for 60 s, and a final extension step at 60°C for 30
152 min. To check for contamination negative controls were included in all PCR runs.

153 After successful amplifications, 1-µL of each PCR product was mixed with LIZ 500 size standard and
154 HI-DI Formamide mixture, and denatured at 94°C for 4 min, snap-cooled on ice and loaded onto an
155 ABI 3130 DNA sequencer (Applied Biosystems). PCR fragment lengths were scored using
156 GENEMAPPER version 4.0 (Applied Biosystems). Using the DNA extracts of the 297 turtles, the
157 procedure of PCR amplification and genotyping was repeated and no genotypic inconsistencies were
158 found for any locus among replications (at least two replications were made for each sample).

159 *Data Analysis*

160 *Female population analysis.* To assess the genetic diversity of the Tortuguero population the 41 female
161 turtles were genotyped at eight microsatellite loci. MICRO-CHECKER software version 2.2.3 (Van
162 Oosterhout et al., 2004) was used to check the microsatellite data for null alleles, stutter errors, short
163 allele dominance and allelic dropout. To estimate the allele frequencies and the frequency of null
164 alleles IDENTITY4.0 software (Wagner & Sefc) was used. To determine if the data fit Hardy-Weinberg
165 proportions, GENEPOP version 3.3 (Raymond & Rousset, 1995) was used as implemented online at
166 http://genepop.curtin.edu.au/genepop_op1.html. For this procedure, the Markov chain method with the
167 default parameters suggested online was used (Guo & Thompson, 1992) (5,000 dememorizations with
168 1,000 batches and 5,000 iterations per batch). Fischer's method for combining independent test results
169 across loci was used. GENEPOP was also used to test for genotypic linkage disequilibrium between loci
170 using the standard Markov chain parameters of 1,000 dememorizations with 100 batches and 1,000
171 iterations per batch. Fischer's method for combining independent test results across loci was used
172 throughout.

173 *Paternity analysis.* Two microsatellite primers, Or-4 and Or-7, did not yield sufficiently reproducible
174 PCR product in the hatchling's DNA and were therefore excluded from the paternity analysis. Though,
175 the mean number of hatchlings sampled per clutch at Tortuguero was 45, two clutches had a limited
176 number of offspring (>25). Therefore, to prevent statistical artefact for the analyses, the sample size
177 genotyped in this study was established to 20 offspring per clutch. Thus, twelve different nests and 20
178 random selected offspring from each nest were examined for paternity. Maternal and offspring
179 genotypes were determined directly from the sampled females and hatchlings. To calculate the
180 probability of detecting MP with one known parent, we used the PRDM software (Neff & Pitcher,
181 2002). To determine the actual number offspring that are required to detect multiple sired broods with
182 high probability (80 and 95%) the software takes into account: (i) number of loci; (ii) frequencies and

183 number of alleles; and (iii) number of sires and reproductive skew. To test the power of detecting MP
184 under different scenarios paternal contributions and number of fathers, different simulations using three
185 different combinations of loci (Table 2) were carried out.

186 To determine and to reconstruct the genotypes of the unknown fathers, we used software GERUD2.0
187 (Jones, 2005). Paternal alleles were inferred from offspring genotypes once maternal alleles were
188 determined. Alleles present in hatchlings that were different from the maternal alleles and, in addition,
189 alleles that were homozygous in some hatchlings were considered to be paternal. In a diploid organism,
190 any instance of more than two paternal alleles is an indication of multiple paternity. The inferred
191 paternal alleles in a clutch were then tested in combination to determine which set of potential paternal
192 genotypes could have produced the entire array. This approach produces multiple minimum-father
193 solutions consistent with a given progeny array (parameters were setup to the maximum: 500,000
194 MaxNumSols; 2,000,000 MaxPPgens; 2,000,000 MaxGPgens and 200,000 MaxMaternalgens;
195 Personal communication Adam G. Jones). GERUD2.0 calculates relative likelihoods for each solution
196 and picks the solution with the highest likelihood. The most likely minimum number of fathers for each
197 clutch was calculated. The simulation package GERUDSIM2.0 (Jones, 2005) was used to test the
198 reliability of GERUD2.0 to correctly determine the number of sires and to correctly reconstruct their
199 genotypes. Based on the allele frequencies of the markers being used the program simulated progeny
200 arrays. This approach allows a simple assessment of confidence in the performance of GERUD2.0.

201 Using the paternal genotypes inferred in GERUD2.0, the probability that two fathers sharing a common
202 genotype was calculated using the software GENALEX6.0 (Peakall & Smouse, 2006).

203 *Statistical analysis.* Once maternal, offspring and paternal genotypes for each nest had been inferred,
204 paternal identity was assigned manually to each offspring. Thus, data was grouped by families

205 containing mother and fatherhoods and morphological traits of hatchlings fitness (emergence success,
206 length, weight and crawling speed).

207 The software R version 3.1.2 (Team, 2008) was used to calculate the statistical regression analyses with
208 random effects. The Pearson's method was used for the correlation matrices. Results are presented for
209 the additive mixed model as this model has one degree of freedom. ANOVA test and *F*-statistic were
210 used to determine the significant effect between the inferred paternity and the morphological traits. The
211 basic model used for the analysis was defined with the formula, $y = \alpha + \beta * x_1$; where α is the intercept,
212 and β is the fixed effect size for the covariate x_1 (e.g. $y = \text{length}$, $x = \text{MP}$). The model also allows the
213 use of multiple covariates, $y = \alpha + \beta_1 * x_1 + \beta_2 * x_2 + \beta_3 * x_3 + \beta_4 * x_4 + \dots + \beta_n * x_n$ (e.g. *lme*(length ~ MP,
214 random=~I|mother, data=data)); where length is notated as the dependent variable and MP as the
215 independent variable. All models had the random family error effect for the mother, which must be
216 considered due to the multiple nests. In this model there is always an assumption of causality, in the
217 sense that we assumed that β will affect the variable y (e.g. MP should affect the offspring length). Any
218 possible interactions between the variables were also checked. Two main comparative approaches were
219 evaluated for the clutches paternity; where the one factor ANOVA and *F*-statistic were tested to
220 determine the level of variation between the observations. The first approach evaluated clutches coded
221 as the exact number of fathers; where nests were analysed assuming an additive effect by their number
222 of inferred fathers on each nest (e.g. SP=1 father; MP=2fathers, 3fathers, 4fathers and 5fathers). Using
223 the paternal genotypes inferred in GERUD2.0, the second approach analysed the paternal contribution
224 (fathers who gives greater proportion of the offspring), where offspring within each family is giving a
225 value according to the paternal ranking of the proportion contributed (e.g. offspring of father who
226 contributed the most within a nest is giving a value of 1, offspring of father who contributed the second
227 highest within a nest is giving a value of 2, etc).

228 Regression analyses were also performed by correlating the morphological traits (length, weight and
229 crawling speed) with each other. Moreover, two other analyses were also made: (i) effect of the
230 environmental variable (incubation temperature mean values) on morphological traits; and (ii) effect of
231 mother size (CCL) on incubation temperature and on morphological traits.

232 **Results**

233 *Female population analysis.* The eight microsatellite loci varied in allele number (6 – 24) and in
234 observed heterozygosity (0.61 – 0.90) (Table 1). The mean overall deviation from the Hardy-Weinberg
235 proportions, F_{IS} was 0.062. However, for two loci, Cc117 and Cm84 were relatively high ($F_{IS} = 0.200$
236 and $F_{IS} = 0.144$, respectively). All loci were in Hardy-Weinberg proportion ($P > 0.05$). Based on results
237 from MICRO-CHECKER2.2.3, the same two loci (Cc117 and Cm84) indicated homozygote excess, which
238 suggests that null alleles may be present. In the remaining loci, there was no evidence for scoring error
239 due to stuttering or allelic dropout. Estimated frequency of null alleles calculated in IDENTITY4.0
240 showed also a relative high percentage for the same two loci (Cc117 : 8.7% and Cm84 : 6.2%). No
241 genotypic linkage disequilibrium was detected between any loci ($P > 0.05$).

242 *Paternity analysis.* The paternity analysis was performed using the four microsatellite loci (Cc7, Cm3,
243 Cm58 and Cm72) that amplified consistently and appeared most reliable (no evidence of null alleles).
244 Assuming equal paternal contributions, the probability of detecting MP using PRDM software with four
245 loci was very high when sampling 20 offspring (PrDM = 0.999). The number of fathers and number of
246 loci did not significantly affect this probability. A 66 : 33 skew or even 33 : 33 : 33 of paternal
247 contribution had little apparent affect on PrDM (Table 2). However, a highly skewed paternal
248 contribution of 80 : 10 : 10 and up to 90 : 10 did affect the PrDM relatively slight when 20 offspring
249 were sampled. Furthermore, an estimate of paternal contribution showed that the average proportion of
250 offspring sired by the “primary” male (male who gives greater proportion of the offspring) was higher

251 than 50% across all MP clutches (see Fig. 1 labelled as father 1). In five nests, the primary father
252 proportion of hatchlings was > 80%, but only one was inferred to have just a single father (N07). The
253 probability of detecting MP at the most skewed paternal contribution (90 : 10; two fathers) with four
254 loci was PrDM = 0.868, so we assumed that the observation of SP in this nest was most likely correct.
255 Thus, the null hypotheses of single paternity could not be rejected in only one nest (8%) and MP was
256 found for the remaining 11 clutches (92%). The number of paternal alleles across all loci varied (from
257 one up to seven alleles per locus) for the MP clutches. Between two to five fathers were inferred in the
258 multiply sired clutches (Table 3) and each father contributed from one to 19 offspring (see Fig. 1).
259 There was no evidence of fathers sharing the same genotype as no match was found across all loci nor
260 at one locus. A total number of 35 different fathers were found to contribute to the paternity of the 12
261 different clutches. The allele frequencies obtained from IDENTITY4.0 were used in the simulation
262 package GERUDSIM2.0.

263 *Statistical analysis.*

264 There was a difference in the mean clutch size between multiply sired (114 ± 18.8) and singly sired (96
265 ± 0) clutch. The same was evident for the mean number of emerged hatchlings ($MP = 99.6 \pm 18.8$ and
266 $SP = 82.0 \pm 0$). However, emergence success percentage ($MP = 85.8\% \pm 9.5$ and $SP = 85.4\% \pm 0$)
267 showed to be almost identical.

268 Using the additive mixed regression model, the first comparative approach where clutches were
269 grouped by the exact number of fathers, provided a significant difference ($P < 0.05$) for all
270 morphological traits. Offspring's weight and more so the length showed a tendency of comprising most
271 of the higher values when sired by two and three fathers (see Fig. 2). However, the lowest values for
272 the emergence success were recorded in nests sired by two fathers (Table 3). Box plot diagrams were
273 made to graphically describe the distribution groups of the raw data through their five-father number

274 summary (see Fig. 3). The diagrams reveal the tendency of higher values within the groups of two and
275 three fathers for the length and weight traits; and so lower values in the groups of four and five fathers
276 to be independently of the mother size. However, an exception to this pattern was observed for the
277 crawling speed trait, which showed its highest values for the five fathers group (see Fig. 2-3). The
278 number of observations (or hatchlings) per number of fathers was determined; 1 father = 20 (8.3%), 2
279 fathers = 80 (33.3%), 3 fathers = 80 (33.3%), 4 fathers = 20 (8.3%) and 5 fathers = 40 (16.6%). 66.6%
280 of the observations were contained within the two and three fathers sub-groups.

281 The paternal contribution analysis where the association between proportion of clutch paternity and the
282 morphological traits was assessed showed no significant difference ($P > 0.05$) for none of the
283 parameters. Hence, primary fathers (1 single father) appeared not to confer fitness advantages to their
284 specific offspring compared with secondary fathers (see Fig. 3).

285 The morphological traits of hatchlings fitness (length, weight and crawling speed) were plotted into
286 linear regressions to determine the correlation between the three parameters per nest (see Fig. 4). Not
287 surprisingly, there was a highly significant correlation between length and weight of ($R^2=0.97\%$; $P <$
288 0.001). However, non-significant correlation ($P > 0.05$) was found between the parameter crawling
289 speed correlated with length and weight. These correlation analyses were in addition performed using
290 all the 530 hatchlings (including all those that were not genotyped and hence not used to infer
291 paternity), and the same pattern was found for this combined data set overall.

292 Incubation temperature was recorded for a random seven of the 12 clutches, all of which showed MP;
293 consequently it was not possible to test for differences against the unique SP nest. The highest
294 temperature recorded was 37.0°C in nest N12 during the last third time period of incubation. The
295 lowest temperature was 26.0°C recorded in both nests N04 and N11 in the beginning of the first third
296 of incubation. The mean incubation temperature recorded for all nests was 31.8°C ($\pm 1.2^\circ\text{C}$).

297 The incubation temperature was found correlated with the emergence success, crawling speed and
298 mother size ($P < 0.005$). Further, a highly significant correlation ($R^2 = 0.81$; $P < 0.001$) was found
299 between incubation temperature and mother size, while other morphological traits showed no
300 correlation ($P > 0.05$). Mother size (as measured by the CCL) showed a highly significant correlation
301 with the emergence success and crawling speed ($P < 0.001$). No significant correlation was found
302 between mother size and any of the other morphological traits ($P > 0.05$).

303 **Discussion**

304 *Multiple mating*

305 The observation of a MP frequency of at least 92% in this study is the highest ever recorded for green
306 turtles. There might be true differences in MP between green turtle populations, but methodological
307 artefacts may also cause the discrepancy. For example, MP was previously reported for green turtles at
308 Tortuguero with a frequency of 50% (Bjorndal, Bolten & Troëng). However, the previous study used
309 only two microsatellites in two different nests with less than 15 offspring each and also used a different
310 approach (UPGMA method). The power of inferring unknown parentage is based on number of
311 markers, number of offspring and the allele frequencies of the population. Such power differences
312 between studies only affect the probability of type II errors (the probability of false negatives), so we
313 conclude that our very high frequency of MP is unlikely to be upwards biased. However, we are fully
314 aware that the final use of only four microsatellite loci was low and it can potentially has its limitations
315 and therefore we caution the general interpretation of the conclusion drawn in this study. From this and
316 other studies, we can conclude that MP is not a rare or occasional event; it is in fact a general and
317 widespread mating strategy for the green turtle with a variation in frequency amongst breeding
318 population sizes. At present, no long-term study has been conducted to measure the frequency of MP

319 within and across seasons of the same population, so it is hard to say whether this is a characteristic
320 attribute for a given population.

321 This study examined a limited number of turtle clutches; 12 out of more than 170,000 estimated nests
322 in Tortuguero in 2007 (Debade, del Aguila & Harrison, 2008). Moreover, the sampling of females was
323 limited to the northern 2 km out of 45 km nesting beach. Green turtles have shown strong nesting site
324 fidelity (Broderick et al., 2007) and females nesting in close proximity to each other at Tortuguero
325 have been proven to be genetically closely related ($R^2 = 0.27\%$; $P < 0.001$) (Peare & Parker, 1996).
326 However, our results from the genetic diversity analysis did not indicate a close relation between the
327 females. It would be interesting to investigate further (within a coherent methodological framework)
328 how the frequency of MP varies on a spatial and temporal scale incorporating further measurements of
329 the mating process from also male turtles.

330 Most of our knowledge about female selecting between multiple males comes from experiments where
331 two or more males are presented simultaneously to a female. Under natural conditions, however,
332 females of many species rarely encounter potential mates simultaneously. This is due to mate choice
333 often being constrained by time, mobility, predation pressure and multiple males fighting over a single
334 female (commonly observed in green turtles). Thus the costs of comparing several potential mates can
335 be considerably high (Klemme, Eccard & Ylönen, 2006; LaBrecque et al., 2014). We find it more
336 likely that sea turtle female under breeding conditions might mate with the first male they encounter
337 and they will probably mate again if another male appears and so on. Thus, we suggest multiple mating
338 to be a common and general mating strategy in green turtles only limited by potential mate encounters
339 and effective sex ratios at the breeding grounds.

340 The question here is which males sperm will fertilize most of the offspring. Several different theories
341 have been proposed in this regard. Laboratory experiments in *Drosophila* have shown that later-mating

342 males tend to father a greater proportion of the offspring and that there is a great variability among
343 genotypes of males and of females in the magnitude of this later-male advantage (Clark, 1999; Stewart
344 & Dutton, 2014). Similar analysis in spiders have also suggested that fertilization success should be
345 biased towards later mates (Watson, 1991). FitzSimmons (1998) suggested that male green turtles that
346 have successfully inseminated females with sufficient sperm might be out-competed by previous
347 mates. If fertilization from previous season's mating occurred, there is a possible loss of sperm through
348 time in storage tubules, or older sperm may be less viable (Ewing, 1943; FitzSimmons, 1998). Equal
349 male contribution by random sperm mixture could also occur. Nonetheless, non- equal paternal
350 contribution was observed in this analysis (see Fig. 1) and there appears to be a primary father siring
351 ranging from 40% to more than 90% of the offspring in a clutch. This could be caused by sperm
352 competition or simply by the fact that male turtle that had mated either first or last to fertilize the
353 majority of the eggs but without providing any fitness improvement. Therefore, if there were no
354 competition and just random mixing of sperm one would expect an even contribution of fathers.

355 *Morphological fitness traits*

356 Despite contradictory results from previous work (Jennions & Petrie, 2000; Fisher, Double & Moore,
357 2006; Jennions et al., 2007), the main conclusion in this study is that polyandry does not have a clear
358 influence in the morphological fitness traits measured in green turtle offspring. The inferred average
359 number of fathers was three (ranging from 1 to 5). This in returns suggests that most females
360 successfully got fecundated by at least three males. Regardless of this tendency observed for clutches
361 fathered by two to three different males which grouped the largest and heaviest offspring, we cannot
362 ignore the fact that this may be entirely random as the same pattern was NOT followed by the other
363 traits (e.g. emergence success and crawling speed). This is, in fact, the first time that effect of exact
364 number of fathers has been assessed for a turtle population into the wild. In a manipulative mating

365 experiment in small marsupials, offspring of polyandrous females (mated with exactly three males)
366 were measured to grow faster than offspring of monogamous females (Fisher et al., 2006); supporting
367 the potential relevance of evaluating multiple mating on fitness by the exact number of fathers for other
368 organisms as well. It has been suggested that even though multiple mating includes males that are of
369 poorer quality and thus potentially decreasing the fitness of offspring, most of a female's offspring
370 would be sired by dominant high quality males (Klemme et al., 2006). In theory, paternity should
371 consistently be biased towards males with high fitness values. In other words, paternity should be
372 biased towards males that elevate offspring performance ('intrinsic male quality' hypothesis).
373 However, there could also be selection against males where paternal-maternal genome interactions
374 will, in fact, lower the offspring performance ('genetic compatibility' hypothesis) (Jennions et al.,
375 2007). These two hypotheses correspond to the contrast between additive (e.g. 'good gene') and
376 nonadditive (e.g. dominance) genetics effects (Leal, 2001). To what extent do males vary predictably
377 in their effect on offspring fitness? One male should satisfy the basic fertilization needs but without
378 necessarily granting higher fitness. Thus, having multiple fathers might increase the chance of some
379 fathers to produce stronger (fitter) offspring so that on average MP nests are more successful. In our
380 study, the observed tendency of most of the nests to be fathered by two to three males and within those
381 two father clusters offspring morphological traits (weight and length) were in average significant
382 higher. This may suggest that there is a natural maximum size that may be explained in terms of sperm
383 competition between males, which might ensure that a higher proportion of bigger offspring are
384 produced. Nevertheless, this theory can be immediately overruled as the lowest emergence success
385 values were recorded in nests sired by two fathers, bigger does not necessary provide fitter.

386 On the other hand, if sperm from four or more males interacted in the female oviducts, out-competition
387 might occur resulting in a lower fitness (Pearse & Avise, 2001). We suggest that "best quality" sperm

388 interaction may come out of the combination of two to three males to provide the “optimal” fitness in
389 the offspring. Nonetheless, how precisely does this mechanism occur and work in sea turtles is still not
390 known.

391 The paternal contribution analysis showed no correlations between none of the traits. Hence, we found
392 no evidence of improved fitness in the offspring when sired by a primary father (male who gives
393 greater proportion of the offspring) compared to any other secondary fathers. Similar results were
394 concluded in Lee and Hays (2004) study.

395 In this study, measurements of morphological traits (crawling speed, length and weight) were taken
396 following the same procedure. However, field and random error effects should be expected.

397 Measurements between nests or families took place at different locations during different hours and
398 even with different environmental conditions (e.g. rain, wet or dry sand, time of the day temperature,
399 etc.) within those 2 km of the beach. Hence, we should expect variation and differences in offspring
400 performance in terms of the measurement of fitness between nests defined for this study. For instances,
401 it was observed a large variation in the crawling speed measures, the longer the offspring were
402 retained, the slower they crawled probably because they become exhausted. The sand temperature and
403 moisture, as well as the sampling time hour, seemed also to affect the offspring performance. All these
404 may explain the great bias observed for the crawling speed, while length and weight showed not to be
405 affected by retention time.

406 *Environmental effect*

407 The positive correlation found between mean incubation temperature and emergence success allows us
408 to suspect that, within a certain range the higher incubation temperature increase the hatchling success
409 as previous has already been proposed for green turtles and other reptile species (Lin et al., 2005;
410 Burgess, Booth & Lanyon). Sexual determination in sea turtles is influenced by the temperature of the

411 sand in which the eggs develop and sex is determined in the middle first third of the incubation
412 (Wyneken, Godfrey & Bels, 2007; Stewart & Dutton, 2014). The incubation temperature that results in
413 50% of each sex is termed the pivotal temperature. For the green sea turtle the mean pivotal
414 temperature is 28.8°C (Mrosovsky, 1994). Nests with lower incubation temperatures will produce more
415 males, whereas nests with higher temperatures will produce more females. Pivotal temperature is
416 expected to differ between populations of the same species (Standora & Spotila, 1985; Mrosovsky,
417 1994; Stewart & Dutton, 2014; Godfrey & Mrosovsky). No study has reported the pivotal temperature
418 for the Tortuguero population. Therefore, for this study 28.8°C was considered the pivotal temperature
419 for the green turtle species as this is the documented value for another relative close geographical site
420 in Suriname (Godfrey & Mrosovsky; Kaska et al.). In this study, the mean temperature recorded in the
421 middle first third period of the incubation was 30.2°C ($\pm 1.2^\circ\text{C}$). This suggests that there was a strong
422 female-biased sex ratio of hatchling at the Tortuguero green turtle population during the 2007 season.
423 The same result has been reported for other species of sea turtles in different nesting population, which
424 has prompted concerns that global warming might be expected to skew the sex ratio towards females
425 (Mrosovsky, 1994; Hays et al., 2003; Chaloupka, Kamezaki & Limpus, 2008). Under this scenario, if
426 this leads to a very low proportion of males at the breeding grounds we could also suggest that MP, as
427 a natural process, will be reduced in frequency causing a decline in hatchling's emergence success.
428 Recent evidence suggests that the interval between breeding seasons is less in male turtles than females
429 (Hays, Mazaris & Schofield, 2014) and hence when female hatchlings dominate, operational (adult
430 breeding) sex ratios are likely to be more balanced (Laloë et al., 2014). Hence MP may continue even
431 when hatchling sex ratios are heavily female skewed.

432 The mean incubation temperature also showed a negative correlation with the size of the female turtle.
433 This suggests that the larger females dig cooler nests. This can possibly be explained by the fact that

434 larger females having longer hind flippers allowing the animal to dig deeper in the sand. Nevertheless,
435 contradictory to our hypothesis that the deeper the eggs are laid the lower the mean temperature, it has
436 been reported that nest depth has little influence on nest temperature (van de Merwe, Ibrahim &
437 Whittier, 2006; The Chu, Booth & Limpus, 2008).

438 In summary, all these lead us to conclude that in fact the incubation temperature factor has a great
439 influence on the offspring morphological traits. If so, incubation temperature together with 2 to 3
440 fathers siring clutches may be important factors defining and perhaps shaping the morphological fitness
441 traits of green turtle hatchlings in Tortuguero.

442 **Competing interests**

443 The authors declare that they have no competing interests.

444 **Author's contributions**

445 AAN conceived of the study, carried out the statistical data analysis, study design and drafted the
446 manuscript; MPJ and FAAG participated in the design of the study, data analysis and coordination and
447 helped to draft the manuscript. All authors read and approved the final manuscript.

448 **Animal research ethics statement**

449 All tissue skin samples were collected in strict accordance with the recommendations in the Guide for
450 the Care and Use of Laboratory Animals of the National Institutes of Health (Eight Edition, 2011); and
451 exported under relevant CITES permits (host institute permit DK03). Moreover, samples were
452 originated from live animals following a protocol made specially and approved by Universidad de
453 Costa Rica (UCR) in cooperation with the Costa Rican Ministry of Environment and Energy (MINAE).

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460 Greame Hays and one anonymous reviewer for the comments and improvements made to an earlier
461 version of this manuscript.

462 **Figures legends**

463 **Fig. 1** Paternal contribution for all nests having multiple paternity (MP) and single paternity. The
464 different colours represent the proportion of offspring that each father has contributed per nest. The
465 first colour in the bottom of each column represents the primary father or the father that contributed to
466 most offspring until the last colour in the top representing the father with the small offspring
467 contribution.

468 **Fig. 2** Distributions of length (**A**), weight (**B**) and speed (**C**) of green turtle hatchlings under categories
469 specifying the exact number of inferred fathers on each nest (e.g. SP=1 father; MP=2fathers, 3fathers,
470 4fathers and 5fathers). The red lines indicate the 95% confidence interval. The diagrams revealed the
471 tendency of higher values within the groups of two and three fathers for the length and weight traits.
472 However, an exception to pattern was measured for the crawling speed (**C**) trait, which showed its
473 highest values for the five fathers group. These results suggest a significant difference in fitness (as
474 measured by our criteria) between hatchlings resulting from clutches fathered by one or more fathers.

475 **Fig. 3** Box plot describing the raw data of length (A), weight (B) and crawling speed (C) by number of
476 father groups observations through their five-number summaries, the smallest observation represented
477 by the lowest line (sample minimum), lower quartile (Q1) 25% ≤ the lower line of the box, median
478 (Q2) 50% of the observations ≤ the bold line into the box, upper quartile (Q3) 75% of the observations
479 ≤ the upper line of the box, and largest observation (sample maximum) upper highest line.

480 **Fig. 4** Linear regressions between offspring morphological traits in the next order: weight and length
481 (A); crawling speed and length (B); and crawling speed and weight (C). Regressions were plotted for
482 each nest, and *P*-values estimated in an overall. Regressions between weight (g) and length (mm)
483 showed a high significant correlation ($P < 0.001$). The regressions between crawling speed and length
484 (B) showed however in an overall non-significant correlation ($P > 0.05$). Furthermore, non-significant
485 correlation ($P > 0.05$) was neither observed globally between crawling speed and weight (C) traits.

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616

Figure 1 (on next page)

Paternal contribution

Paternal contribution for all nests having multiple paternity (MP) and single paternity. The different colours represent the proportion of offspring that each father has contributed per nest. The first colour in the bottom of each column represents the primary father or the father that contributed to most offspring until the last colour in the top representing the father with the small offspring contribution.

Paternal contribution in single and multiple father clutches

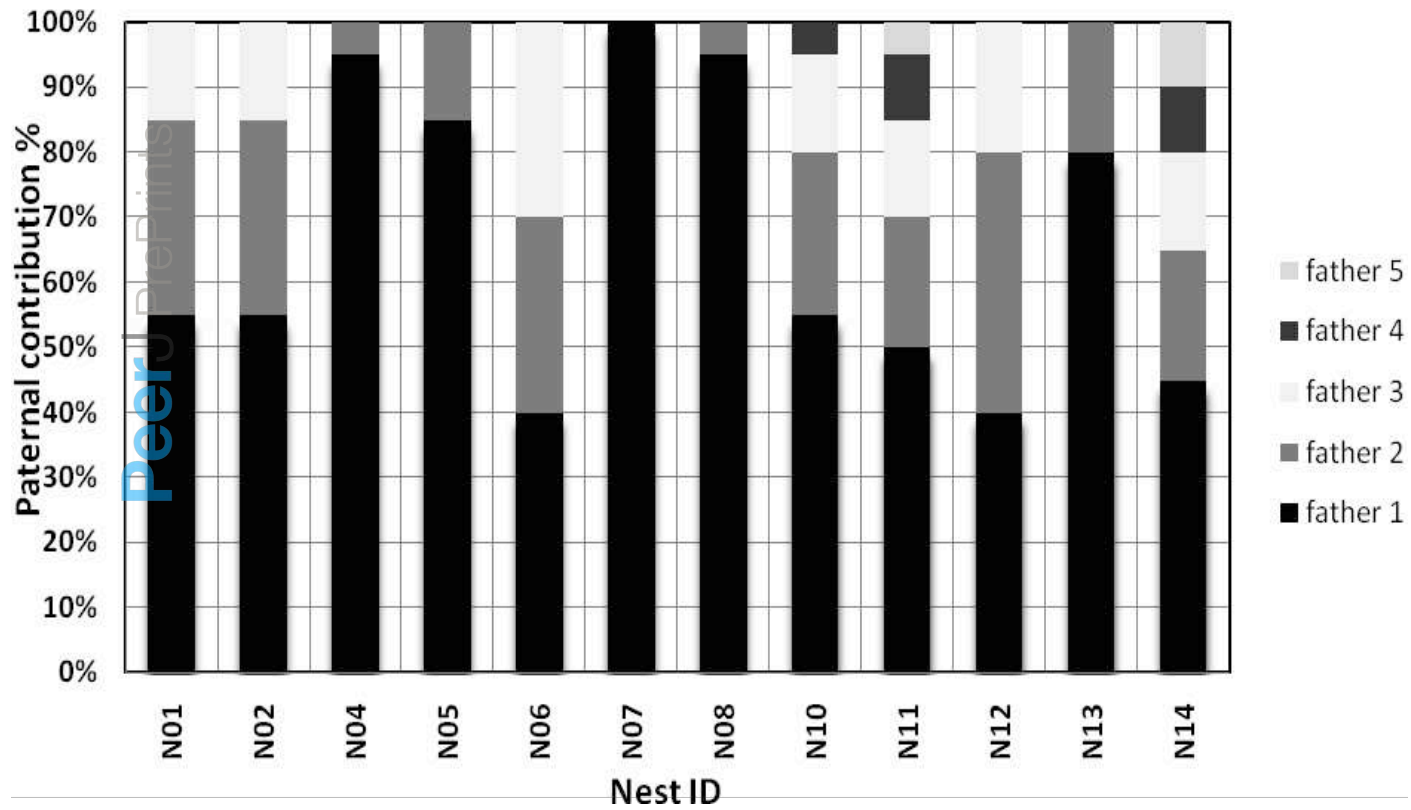


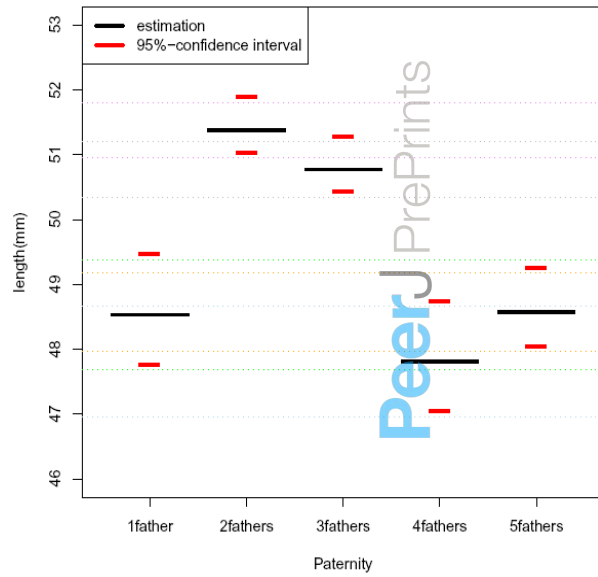
Figure 2 (on next page)

Distributions of correlated morphological traits with 95% confidence intervals

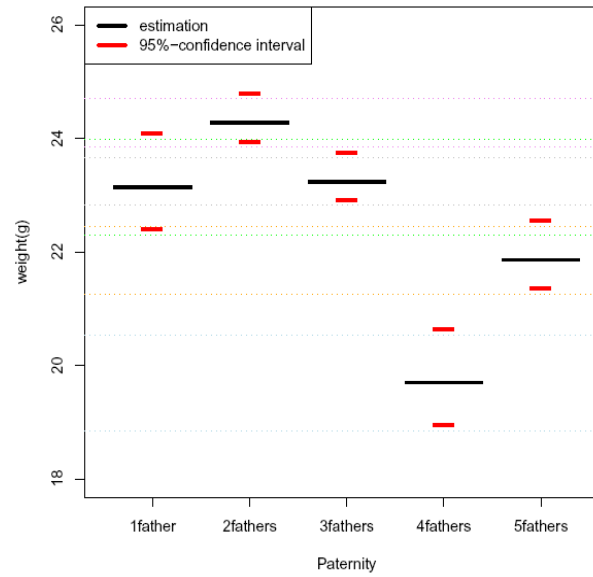
Distributions of length (**A**), weight (**B**) and speed (**C**) of green turtle hatchlings under categories specifying the exact number of inferred fathers on each nest (e.g. SP=1father; MP=2fathers, 3fathers, 4fathers and 5fathers). The red lines indicate the 95% confidence interval. The diagrams revealed the tendency of higher values within the groups of two and three fathers for the length and weight traits. However, an exception to pattern was measured for the crawling speed (**C**) trait, which showed its highest values for the five fathers group. These results suggest a significant difference in fitness (as measured by our criteria) between hatchlings resulting from clutches fathered by one or more fathers.

A

Distribution of hatchlings length on number of fathers

**B**

Distribution of hatchlings weight on number of fathers

**C**

Distribution of hatchlings crawling speed on number of fathers

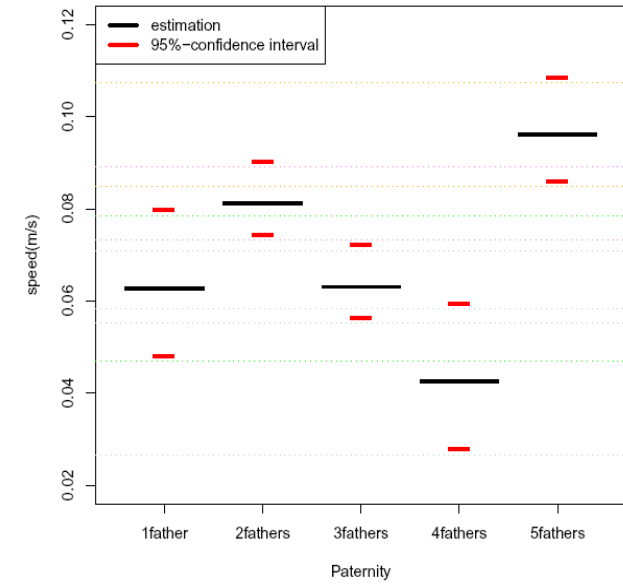


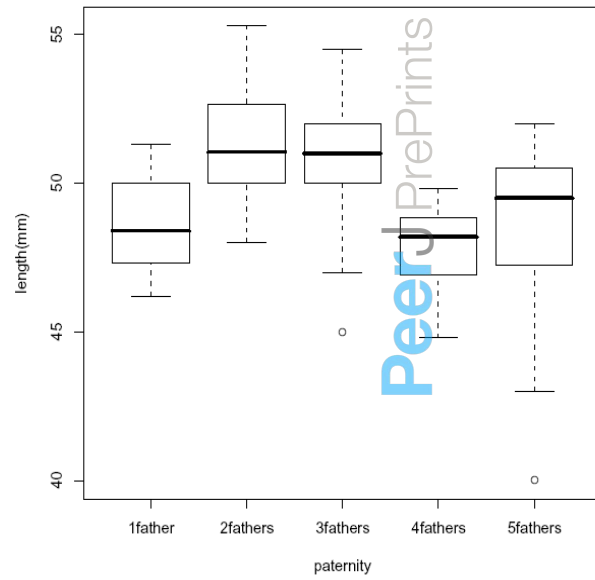
Figure 3 (on next page)

Box plot of morphological traits correlations

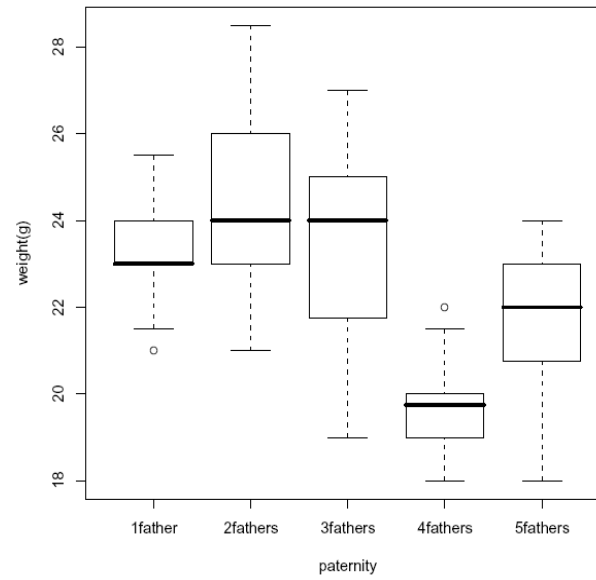
Box plot describing the raw data of length (**A**), weight (**B**) and crawling speed (**C**) by number of father groups observations through their five-number summaries, the smallest observation represented by the lowest line (sample minimum), lower quartile (Q1) 25% \leq the lower line of the box, median (Q2) 50% of the observations \leq the bold line into the box, upper quartile (Q3) 75% of the observations \leq the upper line of the box, and largest observation (sample maximum) upper highest line.

A

Boxplot of hatchlings length by number of fathers

**B**

Boxplot of hatchlings weight by number of fathers

**C**

Boxplot of hatchlings speed by number of fathers

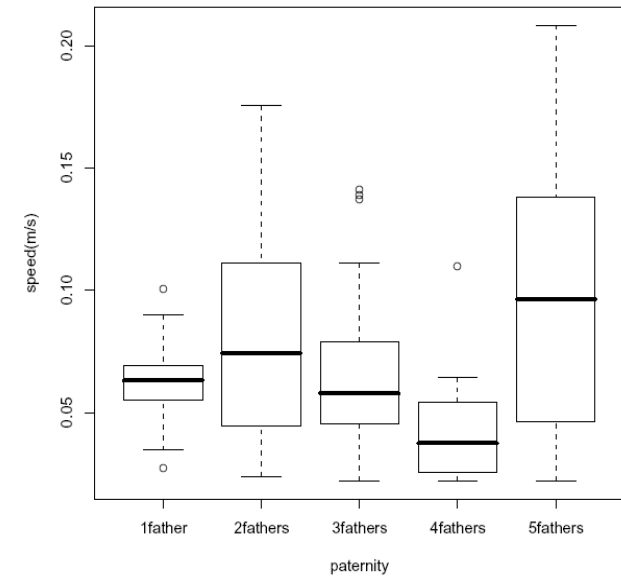


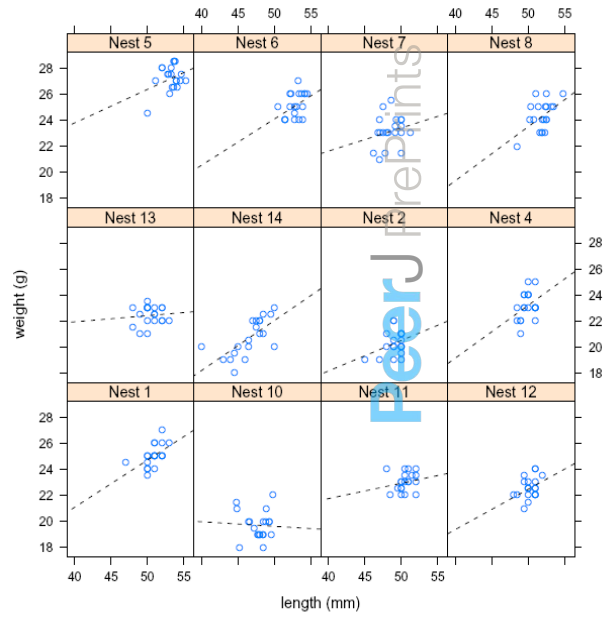
Figure 4 (on next page)

Linear regressions of morphological traits

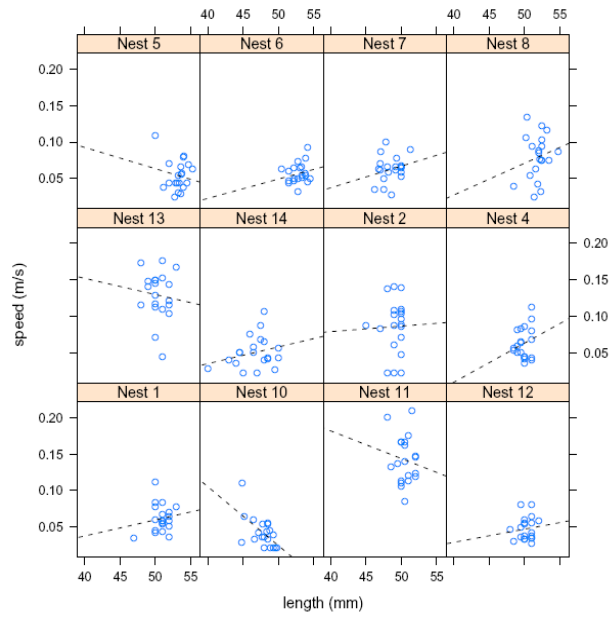
Linear regressions between offspring morphological traits in the next order: weight and length (**A**); crawling speed and length (**B**); and crawling speed and weight (**C**). Regressions were plotted for each nest, and *P*-values estimated in an overall. Regressions between weight (g) and length (mm) showed a high significant correlation ($P < 0.001$). The regressions between crawling speed and length (**B**) showed however in an overall non-significant correlation ($P > 0.05$). Furthermore, non-significant correlation ($P > 0.05$) was neither observed globally between crawling speed and weight (**C**) traits.

A

Linear regressions between weight and length by nests

**B**

Linear regressions between speed and length by nests

**C**

Linear regressions between speed and weight by nests

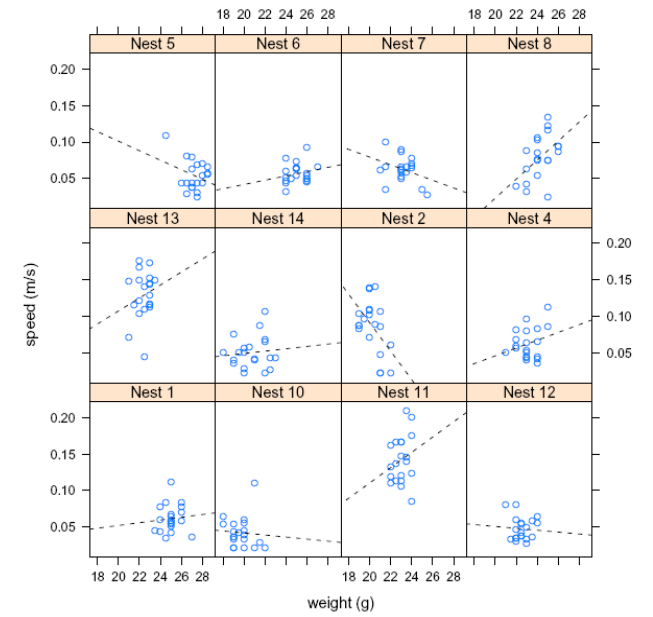


Table 1 (on next page)

Table1

Eight different microsatellite loci, primer sequences where the forward primers were end-labelled with fluorescent dye TaqMan®, sea turtle species from which the primers were designed, annealing temperature, allele length, number of alleles (NA), expected heterozygosity (H_E) and observed heterozygosity (H_O) for 41 adult females sample size.

2 **Table 1** Eight different microsatellite loci, primer sequences where the forward primers were end-
 3 labelled with fluorescent dye TaqMan®, sea turtle species from which the primers were designed,
 4 annealing temperature, allele length, number of alleles (NA), expected heterozygosity (H_E) and
 5 observed heterozygosity (H_O) for 41 adult females sample size.

Loc us	Primer sequence (5' → 3')	Species	Annealing temperature (°C)	Allele length (bp)	N A	H_E	H_O
Cc1 17	TCTTTAACGTATCTCCTG TAGCTC	<i>Caretta caretta</i>	57	230-260	1 1	0. 87	0. 71
	CAGTAGTGTCAGTTCATT GTTCA						
Cc7	TGCATTGCTTGACCAATT AGTGAG	<i>Caretta caretta</i>	57	160-220	1 7	0. 92	0. 93
	ACATGTATAGTTGAGGA GCAAGTG						
Cm 3	AATACTACCATGAGATG GGATGTG	<i>Chelonia mydas</i>	57	154-198	1 0	0. 75	0. 63
	ATTCTTTTCTCCATAAAC AAGGCC						
Cm 58	GCCTGCAGTACACTCGG TATTTAT	<i>Chelonia mydas</i>	57	124-156	8	0. 63	0. 61
	TCAATGAAAGTGACAGG ATGTACC						
Cm 72	CTATAAGGAGAAAGCGT TAAGACA	<i>Chelonia mydas</i>	57	228-298	2 4	0. 90	0. 90
	CCAAATTAGGATTACAC AGCCAAC						
Cm 84	TGTTTTGACATTAGTCCA GGATTG	<i>Chelonia mydas</i>	57	316-356	1 5	0. 90	0. 78
	ATTGTTATAGCCTATTGT TCAGGA						
Or- 4	AGGCACACTAACAGAGA ACTTGG	<i>several species</i>	52	81-125	1 3	0. 88	0. 88
	GGGACCCTAAAATACCA CAAGACA						
Or- 7	GGGTTAGATATAGGAGG TGCTTGATGT	<i>several species</i>	52	210-240	6	0. 64	0. 71
	TCAGGATTAGCCAACAA GAGCAAAA						

6

Table 1 The table shows the 8 different microsatellite loci, primer sequences where the forward primers were end-labelled with fluorescent dye TaqMan®, sea turtle species from which the primers were designed, annealing temperature, allele length, number of alleles (NA), expected heterozygosity (H_e) and observed heterozygosity (H_o) for 41 adult females sample size.

Locus	Primer sequence (5' → 3')	Species	Annealing temperature (°C)	Allele length (bp)	NA	H_e	H_o
Cc117	TCTTTAAGGTATCTCCTGTAGCTC CAGTAGTGTGTCAGTTTCATTGTTCA	<i>Caretta caretta</i>	57	230-260	11	0.87	0.71
Cc7	TGCATTGCTTGACCAATTAGTGAG ACATGTATAGTTGAGGAGCAAGTG	<i>Caretta caretta</i>	57	160-220	17	0.92	0.93
Cm3	AATACTACCATGAGATGGGATGTG ATTCCTTTCCATAAACAAGGCC	<i>Chelonia mydas</i>	57	154-198	10	0.75	0.63
Cm58	GCCTGCAGTACACTCGGTATTTAT TCAATGAAAGTGACAGGATGTACC	<i>Chelonia mydas</i>	57	124-156	8	0.63	0.61
Cm72	CTATAAGGAGAAAGCGTTAAGACA CCAAATTAGGATTACACAGCCAAC	<i>Chelonia mydas</i>	57	228-298	24	0.90	0.90
Cm84	TGTTTTGACATTAGTCCAGGATTG ATTGTTATAGCCTATTGTTCAAGGA	<i>Chelonia mydas</i>	57	316-356	15	0.90	0.78
Or-4	AGGCACACTAACAGAGAAGCTGG GGGACCTAAATACCAAGACA	several species	52	81-125	13	0.88	0.88
Or-7	GGGTTAGATATAGGAGGTGCTTGATGT TCAGGATTAGCCAACAGAGCAAAA	several species	52	210-240	6	0.64	0.71

Table 2 (on next page)

Table 2

Probability of detecting multiple paternity by using PrDM software (Neff & Pitcher 2002). Based on our baseline population frequencies, the model is used to determine the actual number of loci and offspring that are required to detect multiply mated broods with high probability (80 and 95%) and takes into account: (i) different number of loci; (ii) frequencies and number of alleles; and (iii) number of sires and reproductive skew. The three different combination of loci were, **8 loci** = Cc117, Cc7, Cm3, Cm58, Cm72, Cm84, Or4 and Or7; **6 loci** = Cc117, Cc7, Cm3, Cm58, Cm72 and Cm84; and finally **4 loci** = Cc7, Cm3, Cm58 and Cm72.

2 **Table 2** Probability of detecting multiple paternity by using PrDM software (Neff & Pitcher 2002).
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 8 finally **4 loci** = Cc7, Cm3, Cm58 and Cm72.

Number of fathers	Combinations of loci	Paternal contribution	Number of offspring sampled		
			10	20	30
2	8 loci	50:50	0.998	1.000	1.000
	6 loci		0.998	1.000	1.000
	4 loci		0.994	0.999	0.999
	8 loci	66:33	0.982	1.000	1.000
	6 loci		0.982	1.000	1.000
	4 loci		0.976	0.998	0.999
	8 loci	90:10	0.648	0.878	0.959
	6 loci		0.653	0.878	0.957
	4 loci		0.636	0.868	0.949
3	8 loci	33:33:33	1.000	1.000	1.000
	6 loci		1.000	1.000	1.000
	4 loci		1.000	1.000	1.000
	8 loci	50:25:25	0.999	1.000	1.000
	6 loci		0.999	1.000	1.000
	4 loci		0.998	1.000	1.000
	8 loci	80:10:10	0.891	0.988	0.999
	6 loci		0.890	0.989	0.999
	4 loci		0.882	0.986	0.998
4	8 loci	25:25:25:25	1.000	1.000	1.000
	6 loci		1.000	1.000	1.000
	4 loci		1.000	1.000	1.000
	8 loci	40:20:20:20	1.000	1.000	1.000
	6 loci		1.000	1.000	1.000
	4 loci		1.000	1.000	1.000
	8 loci	70:10:10:10	0.973	0.999	1.000
	6 loci		0.971	0.999	1.000
	4 loci		0.966	0.999	1.000
5	8 loci	20:20:20:20:20	1.000	1.000	1.000

6 loci		1.000	1.000	1.000
4 loci		1.000	1.000	1.000
8 loci		1.000	1.000	1.000
6 loci	40:15:15:15:15	1.000	1.000	1.000
4 loci		1.000	1.000	1.000
8 loci		0.893	0.989	0.999
6 loci	80:5:5:5:5	0.889	0.988	0.999
4 loci		0.881	0.985	0.999

9

10

Table 2 The table shows the probability of detecting multiple paternity by using PrDM software (Neff & Pitcher 2002). Based on our baseline population frequencies, the model is used to determine the actual number of loci and offspring that are required to detect multiply mated broods with high probability (80 and 95%) and takes into account: (i) different number of loci; (ii) frequencies and number of alleles; and (iii) number of sires and reproductive skew. The three different combination of loci were, **8 loci** = Cc117, Cc7, Cm3, Cm58, Cm72, Cm84, Or4 and Or7; **6 loci** = Cc117, Cc7, Cm3, Cm58, Cm72 and Cm84; and finally **4 loci** = Cc7, Cm3, Cm58 and Cm72.

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	8 loci	40:20:20:20	1.000	1.000	1.000
	6 loci		1.000	1.000	1.000
	4 loci		1.000	1.000	1.000
	8 loci	70:10:10:10	0.973	0.999	1.000
	6 loci		0.971	0.999	1.000
	4 loci		0.966	0.999	1.000
5	8 loci	20:20:20:20:20	1.000	1.000	1.000
	6 loci		1.000	1.000	1.000
	4 loci		1.000	1.000	1.000
	8 loci	40:15:15:15:15	1.000	1.000	1.000
	6 loci		1.000	1.000	1.000
	4 loci		1.000	1.000	1.000
	8 loci	80:5:5:5:5	0.893	0.989	0.999
	6 loci		0.889	0.988	0.999
	4 loci		0.881	0.985	0.999

Table 3 (on next page)

Table 3

Dataset of each nest analysed by mother length size (CCL), clutch size measured by the number of eggs, the number of hatchlings that emerged, the emergence success percentage, the incubation mean temperature registered in 7 different nests, the number of hatchlings genotyped. The number of alleles and number of non-maternal alleles = paternal alleles (*) at the microsatellite loci Cc7, Cm3, Cm58 and Cm72. The evidence of multiple paternity and the minimum number of fathers inferred by the program GERUD2.0.

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 6 evidence of multiple paternity and the minimum number of fathers inferred by the program GERUD2.0.

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Nest	Mother size CCL (cm)	Clutch size	Emergent hatchlings	Emergence success %	Incubation temperature (C°)	hatchlings genotyped	Cc7 (*)	Cm3 (*)	Cm58 (*)	Cm72 (*)	MP evidence	Minimum number of fathers
N01	105.07	92	78	84.78 %	-	20	3 (1)	3 (2)	2 (1)	4 (2)	yes	3
N02	104.13	97	91	93.81 %	-	20	6 (4)	4 (3)	3 (2)	6 (4)	yes	3
N04	116.33	138	125	90.58 %	30.91	20	4 (2)	2 (1)	3 (1)	4 (2)	yes	2
N05	105.20	128	114	89.06 %	-	20	4 (2)	3 (2)	3 (2)	4 (2)	yes	2
N06	108.47	94	86	91.49 %	31.77	20	4 (2)	3 (1)	3 (2)	6 (4)	yes	3
N07	107.37	96	82	85.42 %	-	20	4 (2)	3 (1)	3 (1)	4 (2)	no	1
N08	109.73	119	83	69.75 %	-	20	3 (1)	3 (1)	4 (2)	4 (2)	yes	2
N10	106.63	115	106	92.17 %	33.17	20	7 (5)	5 (3)	4 (2)	4 (2)	yes	4
N11	111.38	147	134	91.16 %	29.73	20	5 (3)	3 (2)	4 (2)	9 (7)	yes	5
N12	114.77	104	92	88.46 %	33.32	20	2 (1)	3 (2)	3 (2)	5 (3)	yes	3

N							3	3					
1				63.71			(1	(1	3	4			
3	105.10	124	79	%	32.01	20))	(2)	(2)	yes	2	
N							4	3					
1				88.66			(3	(1	7	6			
4	110.47	97	86	%	31.66	20))	(5)	(4)	yes	5	

8

Table 3 The table shows every nest analyzed defined by mother length size (CCL), clutch size measured by the number of eggs, the number of hatchlings that emerged, the emergence success percentage, the incubation mean temperature registered in 7 different nests, the number of hatchlings genotyped. The number of alleles and number of non-maternal alleles = paternal alleles (*) at the microsatellite loci Cc7, Cm3, Cm58 and Cm72. The evidence of multiple paternity and the minimum number of fathers inferred by the program GERUD2.0.

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N13	105.10	124	79	63.71%	32.01	20	3 (1)	3 (1)	3 (2)	4 (2)	yes	2
N14	110.47	97	86	88.66%	31.66	20	4 (3)	3 (1)	7 (5)	6 (4)	yes	5

9