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

Alonzo Alfaro-Núñez, Michael P. Jensen, F. Alberto Abreu-Grobois

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 $MgCl_2$). 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*

Female population analysis. To assess the genetic diversity of the Tortuguero population the 41 female

turtles were genotyped at eight microsatellite loci. MICRO-CHECKER software version 2.2.3 (Van

Oosterhout et al., 2004) was used to check the microsatellite data for null alleles, stutter errors, short

allele dominance and allelic dropout. To estimate the allele frequencies and the frequency of null

alleles IDENTITY4.0 software (Wagner & Sefc) was used. To determine if the data fit Hardy-Weinberg

proportions, GENEPOP version 3.3 (Raymond & Rousset, 1995) was used as implemented online at

http://genepop.curtin.edu.au/genepop_op1.html. For this procedure, the Markov chain method with the

default parameters suggested online was used (Guo & Thompson, 1992) (5,000 dememorizations with

1,000 batches and 5,000 iterations per batch). Fischer's method for combining independent test results

across loci was used. GENEPOP was also used to test for genotypic linkage disequilibrium between loci

using the standard Markov chain parameters of 1,000 dememorizations with 100 batches and 1,000

iterations per batch. Fischer's method for combining independent test results across loci was used

throughout.

Paternity analysis. Two microsatellite primers, Or-4 and Or-7, did not yield sufficiently reproducible

PCR product in the hatchling's DNA and were therefore excluded from the paternity analysis. Though,

the mean number of hatchlings sampled per clutch at Tortuguero was 45, two clutches had a limited

number of offspring (>25). Therefore, to prevent statistical artefact for the analyses, the sample size

genotyped in this study was established to 20 offspring per clutch. Thus, twelve different nests and 20

random selected offspring from each nest were examined for paternity. Maternal and offspring

genotypes were determined directly from the sampled females and hatchlings. To calculate the

probability of detecting MP with one known parent, we used the PRDM software (Neff & Pitcher,

2002). To determine the actual number offspring that are required to detect multiple sired broods with

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 = length, x = MP). The model also allows the

213 use of multiple covariates, $y = \alpha + \beta_1 * x_1 + \beta_2 * x_2 + \beta_3 * x_3 + \beta_4 * x_4 + ... \beta_n * x_n$ (e.g. lme(length ~ MP,

214 random=~1|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=1father; 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-CHECKER 2.2.3, 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 IDENTITY 4.0

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 ($MP = 99.6 ± 18.8$ and

266    $SP = 82.0 ± 0$). However, emergence success percentage ($MP = 85.8\% ± 9.5$ and $SP = 85.4\% ± 0$)

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 (± 1.2°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

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

On the other hand, if sperm from four or more males interacted in the female oviducts, out-competition 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 mechanist 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 (± 1.2°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 me 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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Figures legends

Fig. 1 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.

Fig. 2 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.

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.

References

487 Aggarwal RK, Lalremruata A, Velavan TP, Pavani Sowjanya A, Singh L 2007. Development and characterization of ten
488   novel microsatellite markers from olive ridley sea turtle (Lepidochelys olivacea). *Conservation Genetics* 9:981–984.
489 Beese K, Armbruster GFJ, Beier K, Baur B 2008. Evolution of female sperm-storage organs in the carrefour of
490   stylommatophoran gastropods. *Journal of Zoological Systematics and Evolutionary Research* 46:49–60.
491 Bilde T, Foged A, Schilling N, Arnqvist G 2009. Postmating Sexual Selection Favors Males That Sire Offspring with Low
492   Fitness. *Science* 324:1705–1706.
493 Bjorndal KA, Bolten AB, Troëng S Population structure and genetic diversity in green turtles nesting at Tortuguero, Costa
494   Rica, based on mitochondrial DNA control region sequences. *Marine Biology* 147:1449–1457.
495 Broderick AC, Coyne MS, Fuller WJ, Glen F, Godley BJ 2007. Fidelity and over-wintering of sea turtles. *Proceedings of*
496   *the Royal Society B: Biological Sciences* 274:1533–1539.
497 Burgess EA, Booth DT, Lanyon JM Swimming performance of hatchling green turtles is affected by incubation
498   temperature. *Coral Reefs* 25:341–349.
499 Byrne PG, Roberts JD 2000. Does multiple paternity improve fitness of the frog Crinia georgiana? *Evolution; international*
500   *journal of organic evolution* 54:968–973.
501 Chaloupka M, Kamezaki N, Limpus C 2008. Is climate change affecting the population dynamics of the endangered Pacific
502   loggerhead sea turtle? *Journal of Experimental Marine Biology and Ecology* 356:136–143.
503 Chandler CH, Zamudio KR 2008. Reproductive success by large, closely related males facilitated by sperm storage in an
504   aggregate breeding amphibian. *Molecular Ecology* 17:1564–1576.
505 Clapham PJ, Palsbøll PJ 1997. Molecular analysis of paternity shows promiscuous mating in female humpback whales
506   (Megaptera novaeangliae, Borowski). *Proceedings. Biological sciences / The Royal Society* 264:95–98.
507 Clark AG 1999. Female x male interactions in Drosophila sperm competition. *Science* 283:217–220.
508 Craighead L, Paetkau D, Reynolds HV, Vyse ER, Strobeck C 1995. Microsatellite analysis of paternity and reproduction in
509   Arctic grizzly bears. :255–261.
510 Crim JL, Spotila LD, Spotila JR, O'Connor M, Reina R, Williams CJ, Paladino FV 2002. The leatherback turtle,
511   Dermochelys coriacea, exhibits both polyandry and polygyny. *Molecular Ecology* 11:2097–2106.
512 Debade X, del Aguila DN, Harrison E 2008. Report on the 2007 green turtle program at Tortuguero, Costa Rica. *Caribbean*

513   *Conservation*.

514 Dutton PH, Bowen BW, Owens DW, Barragan A, Davis SK 1999. Global phylogeography of the leatherback turtle
515   (Dermochelys coriacea). *Journal of Zoology* 248:397–409.

516 Ewing HE 1943. Continued fertility in female box turtles following mating. *Copeia*:112–114.

517 Fisher DO, Double MC, Moore BD 2006. Number of mates and timing of mating affect offspring growth in the small
518   marsupial Antechinus agilis. *Animal Behaviour* 71:289–297.

519 FitzSimmons NN 1998. Single paternity of clutches and sperm storage in the promiscuous green turtle (Chelonia mydas).
520   *Molecular Ecology* 7:575–584.

521 FitzSimmons NN, Moritz C, Moore SS 1995. Conservation and Dynamics of Microsatellite Loci Over 300-Million Years of
522   Marine Turtle Evolution. *Molecular Biology and Evolution* 12:432–440.

523 Galbraith DA 1993. Multiple paternity and sperm storage in turtles. *Herpetological journal* 3:117–123.

524 Garcia-Vazquez E 2001. Alternative Mating Strategies in Atlantic Salmon and Brown Trout. *Journal of Heredity* 92:146–
525   149.

526 García-González F, Simmons LW 2005. Sperm Viability Matters in Insect Sperm Competition. *Current Biology* 15:271–
527   275.

528 Godfrey MH, Mrosovsky N Pivotal temperature for green sea turtles, Chelonia mydas, nesting in Suriname.
529   *ingentaconnect.com*.

530 Griffith SC, Owens IPF, Thuman KA 2002. Extra pair paternity in birds: a review of interspecific variation and adaptive
531   function. *Molecular Ecology* 11:2195–2212.

532 Guo SW, Thompson EA 1992. Performing the Exact Test of Hardy-Weinberg Proportion for Multiple Alleles. *Biometrics*
533   48:361.

534 Hays GC, Broderick AC, Glen F, Godley BJ 2003. Climate change and sea turtles: a 150-year reconstruction of incubation
535   temperatures at a major marine turtle rookery. *Global Change Biology* 9:642–646.

536 Hays GC, Mazaris AD, Schofield G 2014. Different male vs. female breeding periodicity helps mitigate offspring sex ratio
537   skews in sea turtles. *Frontiers in Marine Science* 1.

538 Hoekert WEJ, Neuféglise H, Schouten AD, Menken SBJ 2002. Multiple paternity and female-biased mutation at a
539   microsatellite locus in the olive ridley sea turtle (Lepidochelys olivacea). *Heredity* 89:107–113.

540 Ireland JS, Broderick AC, Glen F, Godley BJ, Hays GC, Lee P, Skibinski D 2003. Multiple paternity assessed using
541   microsatellite markers, in green turtles Chelonia mydas (Linnaeus, 1758) of Ascension Island, South Atlantic. *Journal*
542   *of Experimental Marine Biology and Ecology* 291:149–160.

543 Jennions MD, Petrie M 2000. Why do females mate multiply? A review of the genetic benefits. *Biological Reviews* 75:21–
544   64.

545 Jennions MD, DRAYTON JM, BROOKS R, HUNT J 2007. Do female black field crickets Teleogryllus commodus benefit
546   from polyandry? *Journal of Evolutionary Biology* 20:1469–1477.

547 Jensen MP, Abreu-Grobois FA, Frydenberg J, Loeschcke V 2006. Microsatellites provide insight into contrasting mating
548   patterns in arribada vs. non-arribada olive ridley sea turtle rookeries. *Molecular Ecology* 15:2567–2575.

549 Jones AG 2005. Gerud 2.0: a computer program for the reconstruction of parental genotypes from half-sib progeny arrays
550   with known or unknown parents. *Molecular Ecology Notes* 5:708–711.

551 Jones B, Clark AG 2003. Bayesian sperm competition estimates. *Genetics* 163:1193–1199.

552 Kaska Y, Downie R, Tippett R, Furness RW Natural temperature regimes for loggerhead and green turtle nests in the
553   eastern Mediterranean. *nrcresearchpress.com*.

554 Keane B, Dittus WPJ, Melnick DJ 1997. Paternity assessment in wild groups of toque macaques Macaca sinica at
555   Polonnaruwa, Sri Lanka using molecular markers. *Molecular Ecology* 6:267–282.

556 Klemme I, Eccard JA, Ylönen H 2006. Do female bank voles (Clethrionomys glareolus) mate multiply to improve on
557   previous mates? *Behavioral Ecology and Sociobiology* 60:415–421.

558 LaBrecque JR, Alva-Campbell YR, Archambeault S, Crow KD 2014. Multiple paternity is a shared reproductive strategy in
559   the live-bearing surfperches (Embiotocidae) that may be associated with female fitness. *Ecology and Evolution*
560   4:2316–2329.

561 Laloë J-O, Cozens J, Renom B, Taxonera A, Hays GC 2014. Effects of rising temperature on the viability of an important
562   sea turtle rookery. *Nature Climate Change* 4:513–518.

563 Leal SM 2001. Genetics and Analysis of Quantitative Traits. *American Journal of Human Genetics* 68:548.

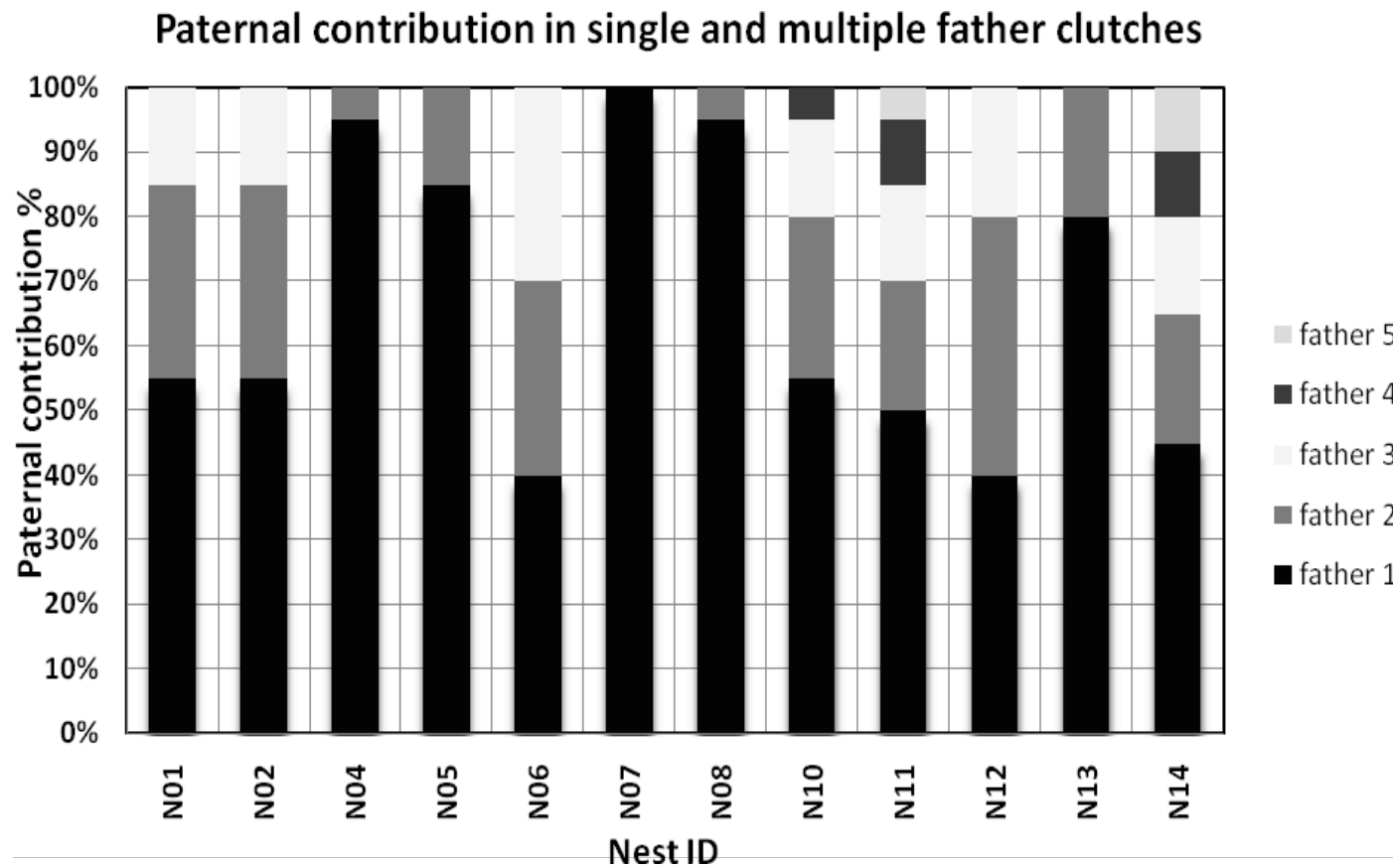
564 Lee PLM, Hays GC 2004. Polyandry in a marine turtle: females make the best of a bad job. *PNAS* 101:6530–6535.

565 Lin ZH, Ji X, Luo LG, Ma XM 2005. Incubation temperature affects hatching success, embryonic expenditure of energy
566   and hatchling phenotypes of a prolonged egg-retaining snake, Deinagkistrodon acutus (Viperidae). *Journal of Thermal*
567   *Biology* 30:289–297.

568 Michl G, Török J, Griffith SC, Ben C Sheldon 2002. Experimental analysis of sperm competition mechanisms in a wild bird

569   population. *PNAS* 99:5466–5470.
570   Moore MK, Ball RM 2002. Multiple paternity in loggerhead turtle (Caretta caretta) nests on Melbourne Beach, Florida: a
571   microsatellite analysis. *Molecular Ecology* 11:281–288.
572   Mrosovsky N 1994. Sex-Ratios of Sea-Turtles. *Journal of Experimental Zoology* 270:16–27.
573   Neff BD, Pitcher TE 2002. Assessing the statistical power of genetic analyses to detect multiple mating in fishes. *Journal of
574   Fish Biology* 61:739–750.
575   Noble DWA, Keogh JS, Whiting MJ 2013. Multiple mating in a lizard increases fecundity but provides no evidence for
576   genetic benefits. *Behavioral Ecology* 24:1128–1137.
577   Packer C, Gilbert DA, Pusey AE, O'Brieni SJ 1991. A molecular genetic analysis of kinship and cooperation in African
578   lions. *Nature* 351:562–565.
579   Peakall R, Smouse PE 2006. genalex 6: genetic analysis in Excel. Population genetic software for teaching and research.
580   *Molecular Ecology Notes* 6:288–295.
581   Peare T, Parker PG 1996. Local genetic structure within two rookeries of Chelonia mydas (the green turtle). *Heredity* 77 (
582   Pt 6):619–628.
583   Pearse DE, Avise JC 2001. Turtle mating systems: Behavior, sperm storage, and genetic paternity. *Journal of Heredity*
584   92:206–211.
585   Phillips KP, Jorgensen TH, Jolliffe KG, Jolliffe S-M, Henwood J, Richardson DS 2013. Reconstructing paternal genotypes
586   to infer patterns of sperm storage and sexual selection in the hawksbill turtle. *Molecular Ecology* 22:2301–2312.
587   Raymond M, Rousset F 1995. Genepop (Version-1.2) - Population-Genetics Software for Exact Tests and Ecumenicism.
588   *Journal of Heredity* 86:248–249.
589   Seminoff JA, Balazs GH, Dutton PH, Eguchi T, Haas HL, Hargrove SA, Klemm DL, Lauritsen AM, MacPherson SL, Opay
590   P et al. 2014. Green turtle (Chelonia mydas) Status Review under the U.S. Endangered Species Act. *NMFS, Silver
591   Spring, MD*.
592   Shurtliff QR, Pearse DE, Rogers DS 2005. Parentage analysis of the canyon mouse (Peromyscus crinitus): Evidence for
593   multiple paternity. *Journal of Mammalogy* 86:531–540.
594   Standora EA, Spotila JR 1985. Temperature Dependent Sex Determination in Sea Turtles. *Copeia* 1985:711.
595   Stewart KR, Dutton PH 2014. Breeding sex ratios in adult leatherback turtles (Dermochelys coriacea) may compensate for
596   female-biased hatchling sex ratios. *PLoS ONE* 9:e88138.
597   Team RDC 2008. Team, R Development Core (2008). R: A language and environment for statistical computing. R
598   Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0. *r-project.org*.
599   The Chu C, Booth DT, Limpus CJ 2008. Estimating the sex ratio of loggerhead turtle hatchlings at Mon Repos rookery
600   (Australia) from nest temperatures. *Australian Journal of Zoology* 56:57.
601   Theissinger K, FitzSimmons NN, Limpus CJ, Parmenter CJ, Phillott AD 2008. Mating system, multiple paternity and
602   effective population size in the endemic flatback turtle (Natator depressus) in Australia. *Conservation Genetics*
603   10:329–346.
604   Uller T, Olsson M 2008. Multiple paternity in reptiles: patterns and processes. *Molecular Ecology* 17:2566–2580.
605   van de Merwe J, Ibrahim K, Whittier J 2006. Effects of Nest Depth, Shading, and Metabolic Heating on Nest Temperatures
606   in Sea Turtle Hatcheries. *Chelonian Conservation and Biology* 5:210–215.
607   Van Oosterhout C, Hutchinson WF, Wills D, Shipley P 2004. MICRO-CHECKER: software for identifying and correcting
608   genotyping errors in microsatellite data. *Molecular Ecology Notes* 4:535–538.
609   Wagner H, Sefc K IDENTITY 1.0: Freeware program for the analysis of microsatellite data - Google Scholar.
610   *scholar.google.dk*.
611   Watson P 1991. Multiple paternity as genetic bet-hedging in female sierra dome spiders, Linyphia litigiosa (Linyphiidae).
612   *Animal Behaviour* 41:343–360.
613   Wyneken J, Godfrey MH, Bels V 2007. *Biology of Turtles*. CRC Press.
614   Yasui Y 1997. A "Good-Sperm" Model Can Explain the Evolution of Costly Multiple Mating by Females. *The American
615   Naturalist* 149:573.
616

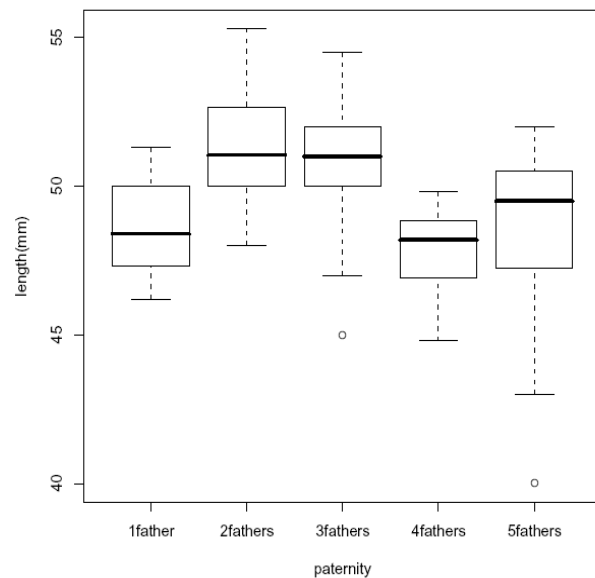
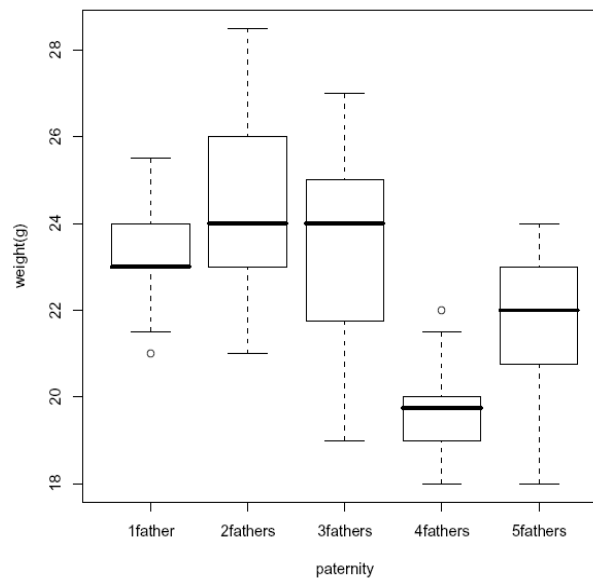
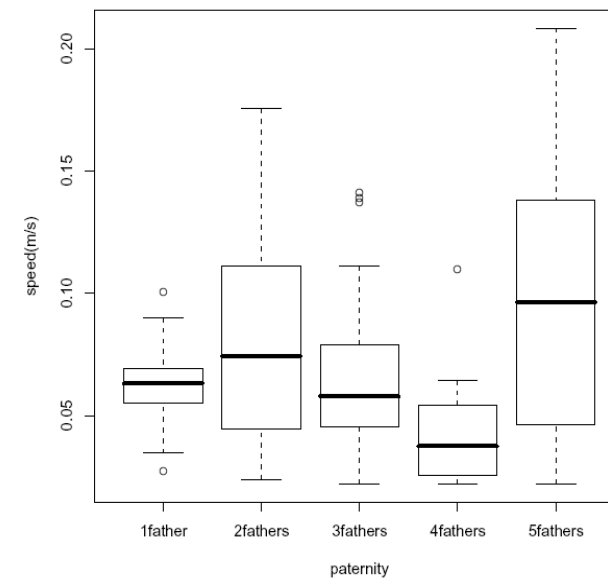
Paternal contribution

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.

Distributions of correlated morphological traits with 95% confidence intervals

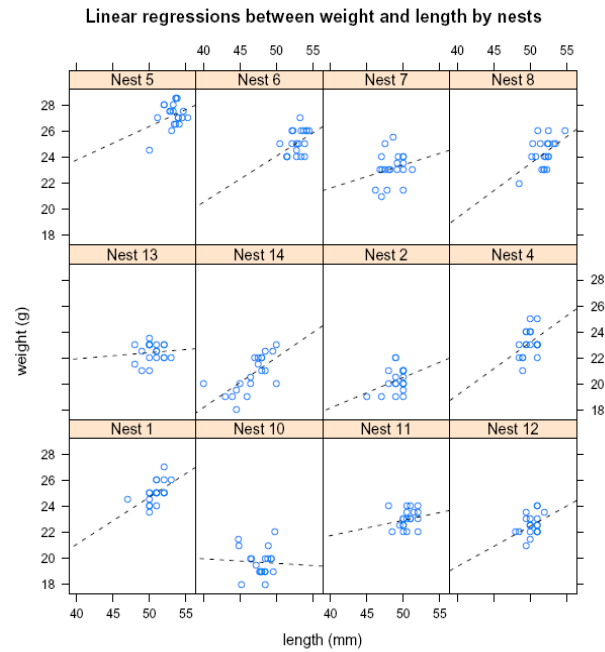
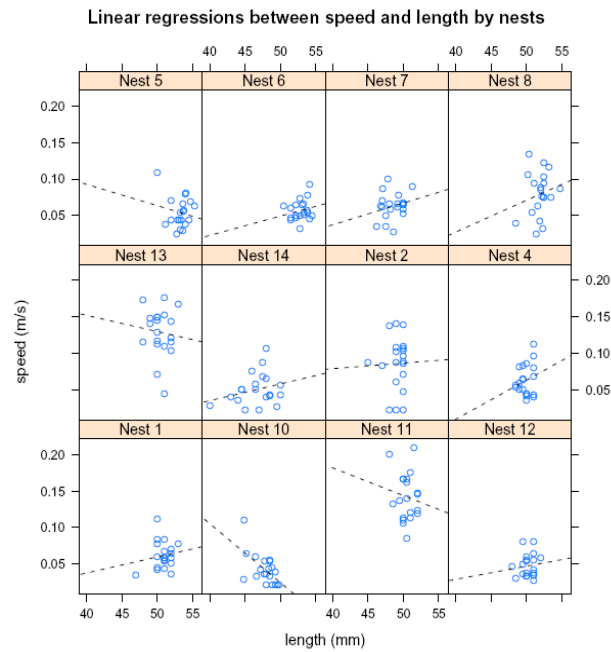
Distributions of length (**A**), weight (**B**) and speed (**C**) of green turtle hatchlings under categories specifying the exact number of inferred fathers on each nest (e.g. SP=1father; MP=2fathers, 3fathers, 4fathers and 5fathers). The red lines indicate the 95% confidence interval. The diagrams 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) betweenhatchlings resulting from clutches fathered by one or more fathers.

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% ≤ the lower line of the box, median (Q2) 50% of the observations ≤ the bold line into the box, upper quartile (Q3) 75% of the observations ≤ 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

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

B

C

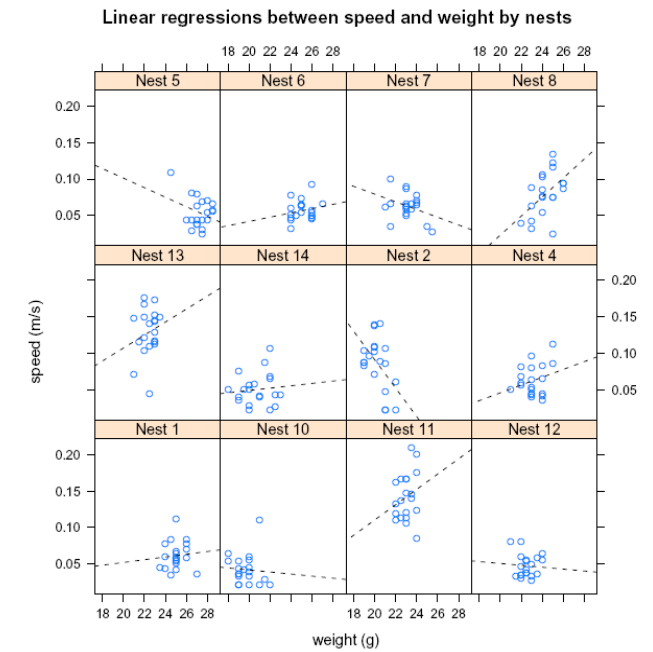



Table1

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.

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Locus	Primer sequence (5′ → 3′)	Species	Annealing temperature (°C)	Allele length (bp)	NA	H_E	H_O
Cc117	TCTTTAACGTATCTCCTGTAGCTC	*Caretta caretta*	57	230-260	11	0.87	0.71
	CAGTAGTGTCAGTTCATTGTTTCA						
Cc7	TGCATTGCTTGACCAATTAGTGAG	*Caretta caretta*	57	160-220	17	0.92	0.93
	ACATGTATAGTTGAGGAGCAAGTG						
Cm3	AATACTACCATGAGATGGGATGTG	*Chelonia mydas*	57	154-198	10	0.75	0.63
	ATTCTTTTCTCCATAAACAAGGCC						
Cm58	GCCTGCAGTACACTCGGTATTTAT	*Chelonia mydas*	57	124-156	8	0.63	0.61
	TCAATGAAAGTGACAGGATGTACC						
Cm72	CTATAAGGAGAAAGCGTTAAGACA	*Chelonia mydas*	57	228-298	24	0.90	0.90
	CCAAATTAGGATTACACAGCCAAC						
Cm84	TGTTTTGACATTAGTCCAGGATTG	*Chelonia mydas*	57	316-356	15	0.90	0.78
	ATTGTTATAGCCTATTGTTCAGGA						
Or-4	AGGCACACTAACAGAGAACTTGG	*several species*	52	81-125	13	0.88	0.88
	GGGACCCTAAAATACCACAAGACA						
Or-7	GGGTTAGATATAGGAGGTGCTTGATGT	*several species*	52	210-240	6	0.64	0.71
	TCAGGATTAGCCAACAAGAGCAAAA						

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Table2

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 finally **4 loci** = Cc7, Cm3, Cm58 and Cm72.

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

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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

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	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

Table3

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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3 eggs, the number of hatchlings that emerged, the emergence success percentage, the incubation mean

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

Nt	Mother size CCL (cm)	Clutch size	Emerged 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

| 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 |

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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