High frequency of multiple paternity in the largest rookery of Mediterranean loggerhead sea turtles

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Abstract

Mating systems are a central component in the evolution of animal life histories and in conservation genetics. The patterns of male reproductive skew and of paternal shares in batches of offspring, for example, affect genetic effective population size. A prominent characteristic of mating systems of sea turtles seem to be a considerable intra- and interspecific variability in the degree of polyandry. Because of the difficulty of observing the mating behaviour of sea turtles directly in the open sea, genetic paternity analysis is particularly useful for gaining insights into this aspect of their reproductive behaviour. We investigated patterns of multiple paternity in clutches of loggerhead sea turtles in the largest Mediterranean rookery using four highly variable microsatellite loci. Furthermore, we tested for a relationship between the number of fathers detected in clutches and body size of females. More than one father was detected in the clutches of 14 out of 15 females, with two clutches revealing the contribution of at least five males. In more than half the cases, the contributions of different fathers to a clutch did not depart from equality. The number of detected fathers significantly increased with increasing female body size. This relationship indicates that males may prefer to mate with large, and therefore productive, females. Our results suggest that polyandry is likely to increase effective population size compared to a population in which females would mate with only one male; male reproductive contributions being equal.

Keywords: *Caretta caretta*, Mediterranean, microsatellites, paternal contributions, polyandry, Zakynthos

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Introduction

Mating systems and the extent of multiple matings by female and male individuals are an important component in the evolution of animal life-history traits. Since mating often poses disadvantages to females such as an increased risk of predation or disease transmission (e.g. Magnhagen 1991; Loehle 1997), females — if they are in control of mating — are expected to mate with several males only if this renders benefits. The acquisition of direct benefits (e.g. to insure fertilization if the quantity of sperm from one male is insufficient to fertilize all eggs, or to provide access to resources by additional males) is a plausible explanation for multiple mating by females. The concept, however, of genetic benefits (Birkhead & Møller 1993) to females mating with several males is a controversially debated field in evolutionary ecology. Few studies unequivocally demonstrate genetic benefits to females mating with several males in animal populations (e.g. Newcomer *et al.* 1999; Fedorka & Mousseau 2002; Tregenza & Wedell 2002). Indeed, it has been suggested that females may make the 'best of a bad job' without getting genetic benefits by mating multiply when costs of resistance to mating exceed costs of mating (Watson *et al.* 1998; Lee & Hays 2004). Apart from their topicality in behavioural ecology, male reproductive skew, female mating frequency and patterns of male contributions to offspring batches in animal populations are important parameters in conservation

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genetics. Genetic effective population size, for example, is partly dependent on female mating behaviour (Balloux & Lehmann 2003). Detailed knowledge on mating systems may thus be fundamental for the assessment of the conservation status of endangered species or populations.

Female sea turtles, which are thought to be in control of mating (Berry & Shine 1980), can obtain no apparent direct benefits from multiple mating. Males do not provide access to environmental resources and, although females lay several large clutches at intervals of about two weeks during one nesting season (Miller 1997), it has been established that mating with one male would be sufficient to fertilize all the eggs of a female turtle during an entire reproductive season (FitzSimmons 1998; Pearse & Avise 2001). Yet a number of studies of several sea turtle species have demonstrated multiple paternity in clutches and hence multiple mating (e.g. Hoekert *et al.* 2002; Moore & Ball 2002; Lee & Hays 2004; Jensen *et al.* 2006). Sea turtles are difficult organisms in which to experimentally investigate the potential effects of mating with several males on female fitness (i.e. the genetic benefits hypothesis). This is mainly due to their complex and elusive life history and large adult body size. But their conservation status (www.redlist.org) and complex life history, encompassing considerable intra- and interspecific variability in the degree of polyandry, certainly make investigations of their mating systems a worthwhile enterprise. Moreover, because of their temperature-dependent sex determination (Wibbels 2003), climate change may lead to perturbations in hatchlings (e.g. Hawkes *et al.* 2007) and eventually in the operational sex ratios, which in turn are likely to affect the frequency of multiple mating by males and females (Jensen *et al.* 2006).

We used microsatellites, highly variable DNA-markers, to investigate patterns of multiple paternity in loggerhead sea turtles (*Caretta caretta*) on the island of Zakynthos (Greece), the largest rookery of this species in the Mediterranean Sea (Margaritoulis *et al.* 2003). Casual observations of individual females mating with more than one male have been reported there (G. Schofield, unpublished). However, anecdotal observations on female mating behaviour seem not a reliable predictor of multiple paternity patterns in sea turtles (FitzSimmons 1998). Our first goal was therefore to determine—for the first time in a Mediterranean sea turtle rookery—the frequency of multiple paternity and the within-clutch skew in male contributions. In addition, we explored the relationship between the number of fathers detected in clutches and female body size as well as between hatching success of clutches and the number of fathers detected. The study of variation of such phenotypic and fitness traits in females having mated with different numbers of males may help to understand the causes and effects of variation in polyandry within animal populations.

Materials and methods

Study area

The nesting area of the Bay of Laganas on Zakynthos (hereafter referred to as Zakynthos) consists of six discrete but adjacent nesting beaches. These beaches are monitored comprehensively by ARCHELON (The Sea Turtle Protection Society of Greece). On average, nearly 1300 clutches are laid there annually (Margaritoulis 2005).

Field methods

We collected tissue samples of females and their offspring on Gerakas beach (37°43′N, 20°53′E), one of the nesting beaches of the Bay of Laganas. For two reasons, female mating strategies are most likely not connected to the beach within the Bay of Laganas where the females nest and hence results from clutches sampled on one beach can be viewed as valid for the entire rookery. First, individual females frequently alternate between the beaches of the bay for successive egg layings within a season (ARCHELON internal reports, unpublished data). Second, the whereabouts of females even in the period between successive nestings within a reproductive season seem not related to the specific beach of the Bay of Laganas where females nested or will nest (Zbinden *et al.* 2007). We sampled adult females at night during beach patrols conducted by ARCHELON collaborators. Samples consisting of skin plugs were taken from flipper-tagging with plastic rototags. Samples were stored in absolute ethanol. We measured female size (curved carapace length, notch to tip), marked clutch locations during night patrols and located egg chambers the following morning. We triangulated the position of clutches to marker poles located at the back of the beach. Clutches were either marked by a metal cage placed in the sand above the clutch (where this was required for nest protection) or with several labelled stones placed beneath the sand surface. We could identify study clutches during the entire incubation and unequivocally assign hatchlings to specific clutches. From 40 days of incubation onwards, a fence was built around study clutches during the night. We checked fences at intervals of < 30 min for emerged hatchlings. Non-destructive blood samples (10–50 µL) of hatchlings were taken from the dorsal cervical sinus according to Bennett (1986). We either pierced the blood vessel with a 27 g, 19 mm needle and collected the drop of blood with a micropipette, or used an insulin syringe with a 30 g, 8 mm needle. Blood samples were stored in lysis buffer (FitzSimmons *et al.* 1999) at room temperature for up to several weeks in the field and later frozen at –20 °C. All hatchlings were released immediately after sampling. If a large number of hatchlings emerged together, a random part of them was released without

being sampled, ensuring that no animal was trapped for longer than 1 h. In keeping with ARCHELON monitoring protocols, we excavated clutches 14 days after first emergence and categorized contents into hatched eggs, eggs with no visible embryos and dead embryos in various stages of development according to Miller (1999). We preserved tissue of recently dead embryos and hatchlings that were found during excavations in absolute ethanol. We collected samples from clutches laid from mid June to mid July in 2003 and 2004. In total, we collected offspring samples from 16 females comprising a total of 21 clutches and over 700 offspring samples. There are indications that first-time and experienced breeders differ in the seasonality of arrival in the nesting area (Hamann *et al.* 2003). Because we sampled neither at the very start nor at the very end of the nesting season (Margaritoulis 2005), we are confident to have obtained material from a random sample of breeding females.

Skin plugs from additional nesting females (not more than one sample per female, and from females not identical to the females sampled for paternity) and tissue samples of recently dead offspring found during excavations of clutches with known mothers (from which no sample was available) were collected in 2003–2005 on various beaches of the Bay of Laganas. They were used in addition to the genotypes of the mothers sampled for paternity analysis to determine population allele frequencies and genetic diversity measures and to perform tests on the suitability of the genetic markers for paternity analysis. This reference sample for the population comprised a total of 53 individuals.

Microsatellite genotyping and characterization of microsatellite loci

DNA was extracted using magnetic beads (Promega, Wallisellen, Switzerland), after standard digestion and ethanol purification. All samples were typed at the four microsatellite loci Cc7 (FitzSimmons 1998), Cc117 (FitzSimmons *et al.* 1995), Cc141 (FitzSimmons *et al.* 1999) and CCM2 (Moore & Ball 2002). Primers were labelled with different fluorescent markers and all loci were amplified simultaneously with a Multiplex PCR kit (QIAGEN, Basel, Switzerland), according to the recommendations of the manufacturer. The amplified fragments were resolved on an automated DNA sequencer (ABI 3100). A negative control was included in every polymerase chain reaction (PCR). Alleles were scored with GENEMAPPER® version 3.0 (ABI, Rotkreuz, Switzerland) and checked by eye. Samples with unclear results were repeated, where necessary in single-locus reactions. Genotyping success was over 95% in female skin-plug and hatchling blood samples, but below 50% in tissue samples from dead embryos and hatchlings. Repeatability of genotyping, assessed from about 200

single-locus genotypes (spread equally across loci) was over 99%.

Deviations from Hardy–Weinberg equilibrium (HWE) for each locus and from linkage equilibrium between all pairs of loci were tested in the population reference sample with Fisher's exact tests based on the approach of Guo & Thompson (1992) using GENEPOP version 3.2 (Raymond & Rousset 1995) with 1 000 000 steps in the Markov chain (100 batches with 1000 iterations). Mean expected paternal exclusion probabilities (probabilities of excluding a single randomly chosen unrelated individual from parentage) were calculated across loci in the program GERUD version 1.0 (Jones 2001). Statistical power of detecting multiple paternity (probability of detecting multiple paternity) was assessed for each clutch based on the model of Neff & Pitcher (2002). This model applies a Monte Carlo simulation and takes into account the number of loci analyzed and their allele frequencies in the population, the number of offspring included, the genotype of the mother and the expected skews in the contribution of fathers. For the latter, we examined two situations: equal and skewed (10 : 90%) contributions by two fathers (the model determines the probability to detect multiple paternity, irrespective of the number of fathers involved in a clutch).

Paternity analysis

Maternal genotypes were determined directly from sampled females and observed in the offspring genotypes. Paternal alleles were inferred from offspring genotypes once maternal alleles were accounted for. If an extra paternal allele indicating an additional father appeared in only one offspring at one locus among the offspring of a mother, this allele was tentatively classified as a mutation. We assessed the minimum number of fathers by using the program GERUD version 1.0 (Jones 2001). This program reconstructs all possible multilocus genotypes of fathers and searches for the minimum number of males (due to technical limitations, up to five males) that can explain the progeny array. If multiple solutions of father genotypes are obtained for a given minimum number of fathers, GERUD can rank them based on the Mendelian segregation of alleles and the allele frequencies in the population. The contributions of individual fathers can likewise be calculated in GERUD. Since technical limitations prevent the resolution of five or more males, the last two procedures can only be conducted for clutches with up to four males. We tested for each clutch whether the male contributions departed from equality (χ^2-tests).

Correlates of paternity

We conducted an ordinal logistic regression analysis to assess whether the number of detected fathers depends on

the body size of the mothers, designating the number of detected fathers as the dependent variable. An ordinal scaling was chosen for the number of detected fathers since the paternity analysis did not allow differentiating between five or more fathering males (see above). To control for possible artefacts caused by differences between clutches in the probability to detect fathers, we included the number of offspring analyzed (which is the main factor determining the probability to detect fathers) and the interaction between female size and the number of offspring analyzed as additional factors in this regression model. The analysis was carried out with the program JMP IN © (version 4.04, SAS Institute). A second logistic regression was conducted with the program R (R Developmental Core Team 2005) to assess potential relationships between hatching success in clutches (defined as dependent variable) and the number of fathers detected (defined as independent variable). We included the number of offspring analyzed and the interaction between the number of offspring analyzed and the number of fathers detected as additional factors in this regression model.

Results

As assessed in the population reference sample of 53 individuals (including 16 mothers of the clutches used for paternity analyses), all four loci were variable with eight to 11 alleles per locus and observed heterozygosities of 0.64–0.93 (Cc117: eight alleles, observed heterozygosity (H_O) = 0.70, expected heterozygosity (H_E) = 0.72; Cc141: nine alleles, H_O = 0.64, H_E = 0.72; Cc7: 11 alleles, H_O = 0.93, H_E = 0.85; CCM2: eight alleles, H_O = 0.74, H_E = 0.70). Genotype frequencies at all loci were within expectations of HWE ($P > 0.05$), and no evidence of genotypic disequilibrium between pairs of loci was found ($P > 0.05$). Paternity exclusion probability for all four loci combined was 0.956. Probabilities of detecting multiple paternity were ≥98% for all clutches when assuming equal paternal contributions of two fathers, and ≥77% when assuming a considerable skew in paternal contributions (Table 1).

We excluded some of the total of 629 offspring samples that were successfully genotyped at all four loci from the paternity analyses for one of the following reasons: in one clutch (from which 37 offspring were sampled), none of the alleles of the mother was present in most offspring at one locus (Cc7) and therefore this clutch was excluded from all further analyses (data not shown). Only one potential mutation at a maternal allele was detected in one offspring and the respective multilocus genotype excluded. We further excluded 20 offspring from 11 mothers because of potential mutations in paternal alleles (see methods). There was no obvious bias in the number of putative mutations across loci.

After excluding the previously mentioned samples, microsatellite data for all four loci were considered for 571

offspring from 15 females (Table 1; the dataset is available upon request). Proportions of offspring included in the paternity analyses ranged from 16% to 44% of total clutch size (average 27%, from Table 1). From five females, we had sampled two clutches (designated as A and B in Table 1). There is some evidence that all clutches of a female sea turtle within one season are fertilized by the same male(s) (Miller 1997; FitzSimmons 1998; Kichler *et al.* 1999). Our data seems to support this notion in that paternal alleles in the sets of two clutches from identical females were very similar. In one case (Female F1), one additional paternal allele was detected at one locus in both the first and second clutch sampled for the female. Four additional paternal alleles (of which three occurred in only one offspring) were observed in the second clutch sampled of Female F3 compared to its first clutch sampled, and one additional paternal allele occurred in its first clutch sampled. In the clutches sampled of Female F5, four paternal alleles were observed in the first, but not the second clutch sampled (two of which found in only one offspring) and no additional paternal alleles were found in the second compared to the first clutch. However, given the very small sample sizes (Table 1), this result seems statistically not meaningful. In the second clutch of Female F6 sampled, three additional paternal alleles were discovered and no additional alleles in its first clutch. However, it should be noted that the sample size in the first clutch was very small (Table 1). For Female F12, the exact same paternal alleles were detected in both of her clutches sampled. However, because one cannot fully exclude that females mated between clutches, we considered only one clutch per female (the one with the higher number of offspring sampled, with a sample size of > 10 offspring) throughout this study for further analysis if not stated otherwise. This conservative dataset contains 481 offspring from 14 mothers (in bold in Table 1).

Multiple paternity was found in all but one clutch and two clutches had a minimum of five different fathers (Table 1). In most cases we could not confidently assign specific sires to individual offspring due mainly to extensive allele sharing. In particular, we noted that in cases where some of the offspring shared the heterozygous genotype with the mother at one or several loci (i.e. when the inference of the paternal allele was ambiguous), many different solutions of male genotypes with similar relative contributions to the progeny array were determined by GERUD for that particular clutch. The confidence of ranking different solutions of male genotypes was therefore in most cases low, as predicted by Jones (2001) when more than two males are represented in a clutch: there were many solutions of allocating paternal genotypes to specific offspring in a clutch and these had similar likelihoods based on Mendelian segregation of alleles and allele frequencies of the loci under consideration. Therefore, for

Table 1 Sampling design and number of inferred fathers. Data are shown for individual clutches as well as pooled clutches for cases where two clutches of the same female were analyzed. Of these, the clutch with the higher number of offspring sampled (in bold) is considered for further analysis and interpretation. The probabilities of detecting multiple paternity (PrDM) with respect to the number of offspring included (*N*) were estimated for equal and skewed (10 : 90%) paternal contributions

| Year	Female (F) and clutch (A/B)	Mother carapace length (cm)	Hatching success (%)	*N*	Clutch size	PrDM Equal contribution	PrDM Skewed contribution	Minimum number of fathers
2003	F1 A	81	73	25	123	0.98	0.87	3
2003	**F1 B**	"	**97**	**38**	**127**	**0.99**	**0.94**	**2**
2003	F1 A&B	"	85	63	na	0.99	0.98	4
2003	**F2 A**	**90**	**91**	**32**	**137**	**0.99**	**0.91**	**4**
2003	F3 A	82	95	19	109	0.98	0.80	3
2003	**F3 B**	"	**73**	**52**	**142**	**0.99**	**0.97**	**4**
2003	F3 A&B	"	84	71	na	0.99	0.98	5
2003	**F4 A**	**75**	**65**	**18**	**89**	**0.98**	**0.78**	**1**
2003	F5 A	91	87	10	148	*na	*na	*na
2003	F5 B	"	94	7	126	*na	*na	*na
2003	F5 A&B	"	91	17	na	0.99	0.77	4
2004	F6 A	91	88	10	143	*na	*na	*na
2004	**F6 B**	"	**79**	**45**	**133**	**0.99**	**0.96**	**3**
2004	F6 A&B	"	84	55	na	0.99	0.97	5
2004	**F7 A**	**87.5**	**66**	**31**	**71**	**0.99**	**0.91**	**2**
2004	**F8 A**	**84**	**68**	**21**	**108**	**0.98**	**0.83**	**4**
2004	**F9 A**	**89**	**73**	**36**	**159**	**0.99**	**0.93**	**4**
2004	**F10 A**	**91**	**74**	**38**	**126**	**0.99**	**0.95**	**4**
2004	**F11 A**	**84**	**76**	**40**	**92**	**0.99**	**0.94**	**5**
2004	**F12 A**	**86**	**81**	**43**	**115**	**0.99**	**0.95**	**5**
2004	F12 B	"	94	19	117	0.98	0.80	4
2004	F12 A&B	"	88	62	na	0.99	0.98	5
2004	**F13 A**	**87**	**85**	**22**	**115**	**0.99**	**0.84**	**3**
2004	**F14 A**	**68**	**59**	**41**	**143**	**0.99**	**0.95**	**2**
2004	**F15 A**	**82**	**64**	**24**	**100**	**0.99**	**0.87**	**2**
Total				571				

*Parameters not indicated due to individual clutch offspring array containing not more than 10 samples.

the evaluation of paternal contributions, we considered average values of the rendered solution. A qualitative comparison between average values and the most likely solution revealed that these were nearly identical (data not shown). The primary male sired on average 59% of offspring (51–68%, $n = 4$) in clutches where two males were detected; on average 51% (44–57%) in the two clutches where three males were found; and on average 45% (41–54%, $n = 5$) in cases where four males were discovered (Fig. 1). In five out of these 11 clutches (and in the same cases when considering the most likely solution), parental contributions deviated significantly from equality (goodness-of-fit χ^2-tests, $P < 0.05$; Fig. 1).

The ordinal logistic regression analysis revealed that in the full regression model (whole model test: $\chi^2 = 10.41$; d.f. = 3; $P = 0.015$), mother body size was the only factor with a significant effect ($\chi^2 = 4.84$; d.f. = 1; $P = 0.028$) on number of fathers detected in a clutch (number of offspring
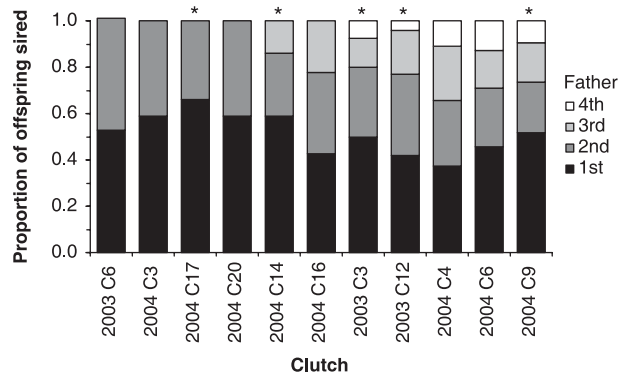
Fig. 1 Relative contribution of fathers to clutches being sired by two to four males. Shown are average values over multiple solutions. From mothers where two clutches were sampled, only the clutch with the higher number of offspring sampled is included. Asterisks above columns designate clutches with skewed paternal contributions.
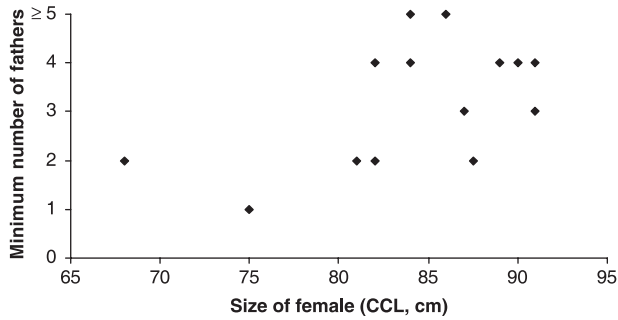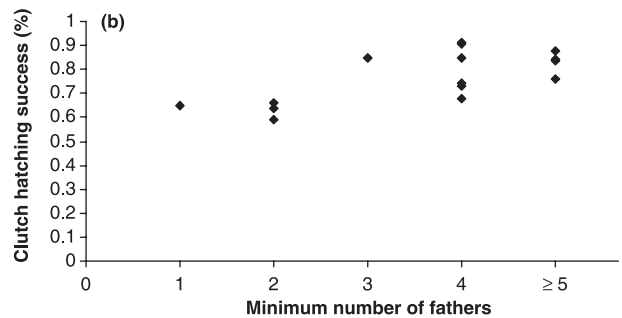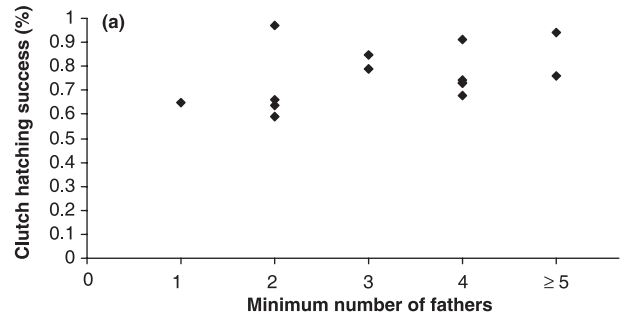
Fig. 2 Number of fathers detected in 14 clutches plotted against female body size (CCL: curved carapace length). From mothers where two clutches were sampled, only the clutch with the higher number of offspring sampled is included.

Fig. 3 Hatching success (%) in relation to the number of fathers detected. In part a, only one clutch per female is included; part b shows the data from pooled clutches in cases where two clutches from a female were sampled.

included: $\chi^2 = 2.03$; d.f. = 1; $P = 0.15$; interaction: $\chi^2 = 2.20$; d.f. = 1; $P = 0.14$), indicating a positive correlation between the number of fathering males and body size of the mother (Fig. 2). When the same analysis was carried out with the pooled data for females for which two clutches had been sampled, both body size of the mother ($\chi^2 = 5.10$; d.f. = 1; $P = 0.024$) and the number of offspring included ($\chi^2 = 7.76$; d.f. = 1; $P = 0.005$) had a significant additive effect on number of fathers detected, while the interaction between these two factors had no significant effect ($\chi^2 = 0.32$; d.f. = 1; $P = 0.57$) in the full regression model (whole model test: $\chi^2 = 17.57$; d.f. = 3; $P < 0.001$), indicating independent positive relationships between the two factors and the number of fathers detected.

In the logistic regression analysis, the number of fathers detected was the only factor that showed a significant additive effect on hatching success ($\chi^2 = 15.62$; d.f. = 4; $P = 0.004$; number of offspring included: $\chi^2 = 0.70$; d.f. = 1; $P = 0.40$; interaction: $\chi^2 = 1.86$; d.f. = 2; $P = 0.39$). The scatter plot of the raw data (Fig. 3a), however, indicated a rather weak positive correlation between hatching success and the number of fathering males. The same analysis, carried out with the pooled data for females for which two clutches had been sampled, yielded qualitatively identical results but with a more pronounced effect of the number of fathers detected on hatching success (number of fathers detected: $\chi^2 = 62.67$; d.f. = 4; $P < 0.001$; number of offspring included: $\chi^2 = 0.32$; d.f. = 1; $P = 0.57$; interaction: $\chi^2 = 3.70$; d.f. = 2; $P = 0.17$; Fig. 3b).

Discussion

This first thorough paternity study in Mediterranean sea turtles revealed multiple paternity in clutches of 14 out of 15 females (93%). This is, together with the multiple paternity rate of 92% (12 out of 13 females analyzed) observed in a rookery of olive ridley sea turtle (*Lepidochelys olivacea*) on the Pacific coast of Costa Rica (Jensen *et al.*

2006), the highest rate of multiple paternity observed in a marine turtle so far. Given that we found no deviations from HWE and a very low apparent mutation rate (one mutation of a maternal allele in 571 offspring), the finding that multiple paternity is widespread in the loggerhead sea turtle rookery of Zakynthos is apparent. Moreover, some of the alleles considered to be mutations from paternal alleles might actually stem from extra fathers and their exclusion might have led to underestimating the minimum number of fathers. More importantly, the fact that the probability of detecting multiple paternity (skewed contributions) varies considerably among our study clutches indicates that a number of male contributions might have gone undetected mainly due to the low numbers of offspring sampled in some clutches.

Not only was the rate of multiple paternity very high in the Zakynthos rookery, but contributions of the different males were similar and departed from equality in less than half the clutches. In comparison, paternal contributions in a study of green sea turtles (*Chelonia mydas*) deviated from equal contributions in nine out of 10 multiply sired clutches (Lee & Hays 2004). Over a range of vertebrate taxa, paternal skews within batches of offspring are generally more pronounced than those found in Zakynthian loggerhead sea turtles (e.g. Largiadèr *et al.* 2001; Walker *et al.* 2002). The generally similar contributions of males within clutches in this study implies that polyandry is likely to

increase genetic effective population size (Balloux & Lehmann 2003). Polyandry in the present case thus may decrease the level of genetic drift in the population compared to a population where each female only mates with one male. Having said that, the effect of within-season polyandry on effective population size is reduced for species like sea turtles with a very long reproductive life compared to species with few lifetime reproductive events. Moore & Ball (2002) have pointed out the significance of polyandry for the colonization of new nesting beaches by only a few gravid females. The similar contribution of different males to clutches in the Zakynthos rookery indicates that a single clutch carries a high genetic diversity, which may reduce founder effects when a new nesting habitat is colonized by few females.

The Zakynthos rookery is to our knowledge the second loggerhead sea turtle rookery with reliable estimates of multiple paternity. Moore & Ball (2002) found evidence of multiple paternity in nearly a third of 70 *Caretta caretta* clutches from Florida. Taking into account that they genotyped only around 10 offspring per clutch, the frequency of multiply fathered clutches in this rookery is likely to be higher than what their data suggest. Considering that in another sea turtle species, the green turtle, striking differences in the rate of multiple paternity between rookeries seem to exist (FitzSimmons 1998; Lee & Hays 2004), it would be premature to generalize about multiple paternity in loggerhead sea turtles.

Density and abundance of individuals are likely to be key determinants of the frequency of multiple paternity. In individual bird species, for example, the rate of extra-pair copulations appears to increase with density (Westneat & Sherman 1997). Jensen *et al.* (2006) found a significantly higher incidence of multiple paternity in a solitary nesting rookery olive ridley sea turtles compared to a neighbouring mass-nesting one. The notion of an effect of density and abundance on the rate of multiple paternity is supported by a significant positive correlation between the rate of multiple paternity and the logarithm of population size in the marine turtle genus *Lepidochelys* (Jensen *et al.* 2006) as well as a trend in increasing incidence of multiple paternity with increasing population size across populations of different sea turtle species (Ireland *et al.* 2003; Jensen *et al.* 2006). However, the very high rate of multiple paternity found in the Zakynthos rookery with a comparably low breeding population size (estimated as no more than 500 females by the methods applied by Jensen *et al.* 2006 and hence placing it at the very low end of the spectrum of sea turtle rookeries for which multiple paternity data is available; Fig. 2; Jensen *et al.* 2006) does not fit into the postulated pattern. While the very high rate of multiple paternity in the Zakynthos rookery does not challenge the general hypothesis of the rate of multiple paternity being affected by population size, it emphasizes that other

factors (including species–specific differences in mating behaviour) are likely to play a considerable role in determining the rate of multiple paternity. Densities in the mating area for example are likely to be extraordinarily high in both the mass-nesting rookery investigated by Jensen *et al.* (2006) and the Zakynthos rookery, where the mating area seems confined to the Bay of Laganas. Spatial behaviour in the breeding area, mating opportunities along migration paths and operational sex ratio (the ratio of reproductively active males to females at any one time) might also affect the rate of multiple paternity. First, females in the study rookery seem to move little once they have laid their first clutch (Zbinden *et al.* 2007), but spatial behaviour during the mating season as well as that of males remains unknown. Second, very little is known about migratory behaviour of male sea turtles in the Mediterranean sea and hence the frequency of mating opportunities along migratory paths. Third, operational sex ratio is unknown in the study rookery. It should, however, be considered that operational sex ratio may vary from year to year due to the fact that the number of reproducing females is subject to high year-to-year fluctuations (Margaritoulis 2005), whilst males are thought to migrate each year (Miller 1997). Clutch numbers in the two seasons during which we collected samples were average (ARCHELON internal reports, unpublished data), and therefore the estimated frequency of multiple paternity is probably representative for the rookery.

We found a positive correlation between the number of sires and female body size. While other publications on multiple paternity in marine turtles do not address this issue, Lee & Hays (2004) found mothers of multiply sired clutches of green sea turtles of Ascension Island to be, on average, larger than those laying clutches with only one father detected, although the result was not statistically significant. In a terrapin (*Chrysemys picta*) population, Pearse *et al.* (2002) found that clutch sizes in multiply sired clutches were significantly larger than in clutches in which only one father was detected. They suggest a male preference for large females, which are generally more fecund in reptiles. A relationship between paternity patterns and female body size may thus be a common trait in turtle populations, although we can only speculate on its biological significance. The trade-off between benefits and costs of female multiple matings may be size-dependent. In situations where variation in female fecundity is high and assessable by males, as is the case in most reptile species including sea turtles (Broderick *et al.* 2003; the relationship is not significant in our data set, most likely due to the relatively small sample size), male mate choice may be adaptive (Olsson 1993). This could lead to more intense male courting of large females and hence increased cost of resistance to mating. Alternatively and/or additionally, it is conceivable at least for sea turtles that larger females encounter more males than do smaller females, independent of male behaviour.

Firstly, larger females are likely to swim faster than smaller ones, and may thus arrive in the reproductive area earlier and hence spend more time mating. Secondly, average size at maturity might differ between turtles using disjoint foraging areas and mating opportunities along migratory routes might differ between migration paths.

For taxa with temperature-dependent sex determination such as crocodilian species, many turtles (including *Caretta caretta*) and some lizard species (Janzen & Paukstis 1991), climate change is expected to lead to systematic shifts in hatchling sex ratios under the assumption that populations will not be able to react fast enough to increasing temperatures. These expected shifts in hatchling (and eventually adult and operational) sex ratios are likely to affect the frequency of multiple paternity in populations with polyandrous mating systems. Intuitively, one might assume that a relatively low number of males in the mating area leads to a low frequency of multiple paternity. Jensen *et al.* (2006), however, hypothesized for sea turtles that a more female-biased sex ratio may generate higher rates of multiple paternity than a less female-biased ratio, due to decreased male–male competition. More research on the effect of operational sex ratio on both male and female mating frequency is clearly needed to anticipate one of the effects of climate change on population genetics parameters of species with temperature-dependent sex determination.

Whether or not shifts in the rate of multiple paternity will induce shifts in individual female reproductive output depends on whether females gain genetic benefits from mating with several males. Our results showed an increase in hatching success with increasing number of fathers siring a clutch, although the effect was not very high: especially when considering only individual clutches. This result — although its interpretation with regard to potential genetic benefits is inconclusive — is remarkable, given the scarcity of reported results on this issue among paternity studies in turtles (but see Pearse *et al.* 2002; Lee & Hays 2004). The positive correlation between hatching success and the number of sires could be explained by hatching success being related to size-dependent traits rather than frequency of multiple matings. For example, it may be speculated that mating frequency is dependent on the size of females, and that larger females produce more viable eggs and/or choose better nest sites — and that this, and not the frequency of mating, leads to higher hatching success in clutches. The paternity of unsuccessfully developing offspring (from tissue of embryos found dead at clutch excavation) could likewise be informative in assessing potential genetic benefits of female multiple matings (FitzSimmons 1998). If genetic compatibility or genetic quality differs amongst males to a degree that offspring from low quality males have an elevated chance of unsuccessful embryonic development, one would expect differences in

the frequency of paternal alleles between samples from hatchlings and those of embryos found dead at clutch excavation. However, due to in part the low genotyping success of this kind of tissue (see Materials and methods), our data set only contained seven samples of unsuccessfully developing offspring, whose genotypes did not contain exceptional paternal alleles.

In sea turtles, more males are produced at lower incubation temperatures and more females at higher temperatures (Wibbels 2003). In species with this sex-determining mode, global warming may potentially lead to a decrease in the proportion of males. Regardless of current speculations on the relationships between operational sex ratio, female multiple mating frequency, and individual female fitness, a lack of males will eventually lead to a reduction of effective population size and finally to reduced population reproductive output when not all eggs can be fertilized anymore. Long-term conservation of marine turtle populations should bear these aspects in mind.

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