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Reconstruction of paternal genotypes over multiple breeding seasons reveals male green turtles do not breed annually

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Abstract

For species of conservation concern, knowledge of key life-history and demographic components, such as the number and sex ratio of breeding adults, is essential for accurate assessments of population viability. Species with temperature-dependent sex determination can produce heavily biased primary sex ratios, and there is concern that adult sex ratios may be similarly skewed or will become so as a result of climate warming. Prediction and mitigation of such impacts are difficult when life-history information is lacking. In marine turtles, owing to the difficultly in observing males at sea, the breeding interval of males is unknown. It has been suggested that male breeding periodicity may be shorter than that of females, which could help to compensate for generally female-biased sex ratios. Here we outline how the use of molecular-based paternity analysis has allowed us, for the first time, to assess the breeding interval of male marine turtles across multiple breeding seasons. In our study rookery of green turtles (*Chelonia mydas*), 97% of males were assigned offspring in only one breeding season within the 3-year study period, strongly suggesting that male breeding intervals are frequently longer than 1 year at this site. Our results also reveal a sex ratio of breeding adults of at least 1.3 males to each female. This study illustrates the utility of molecular-based parentage inference using reconstruction of parental genotypes as a method for monitoring the number and sex ratio of breeders in species where direct observations or capture are difficult.

Keywords: breeding frequency, *Chelonia mydas*, genotype reconstruction, marine turtle, sex ratio

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Introduction

Effective population size (N_e) (Wright 1931) is a central component of conservation biology that reflects the vulnerability of a population to inbreeding and random genetic drift. Decreasing values of N_e erode genetic variation and increase the risk of population extinction because of inbreeding, accumulation of deleterious alleles and the loss of evolutionary potential (Gilpin & Soulé 1986; Frankham *et al.* 2003; Frankham 2005). In natural populations, N_e is strongly influenced by variation in reproductive success and sex ratio (Frankham 1995). A good understanding of mating system as well as key life-history and demographic components, such as the number and sex ratio of breeding adults is, therefore, fundamental for the assessment and effective management of species and populations (Anthony & Blumstein 2000).

Species with temperature-dependent sex determination (TSD) can produce heavily biased primary sex ratios (Bull & Charnov 1989), and there is concern that climate warming will exacerbate this trend, ultimately threatening population persistence through the production of offspring of a single sex (Janzen 1994; Mitchell *et al.* 2008; Wapstra *et al.* 2009; Witt *et al.* 2010). In marine turtle populations, gaps in our knowledge of mating

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systems currently impede accurate evaluations of the potential impacts of climate change (Hawkes *et al.* 2009). Specifically, the breeding frequency of males is not known, and operational sex ratios (the ratio of fertilizable females to sexually active males at a given time (Emlen & Oring 1977)) are poorly understood, owing to the difficulty in observing or capturing male turtles at sea. Existing population censuses are typically based on estimated numbers of nesting females (e.g. Broderick *et al.* 2002; Dutton *et al.* 2005), meanwhile the number of males contributing to breeding populations is unknown.

Molecular parentage-based approaches to study mating systems are particularly appropriate in marine systems, where mating is rarely observed and high vagility of species limits access to them (e.g. Frasier *et al.* 2007; DiBattista *et al.* 2008). Sibship reconstruction from neutral genetic markers makes it possible to determine family structure even when it is not possible to sample candidate parents (e.g. Wang 2004; Wang & Santure 2009), and this approach has been used to infer mating system (Gottelli *et al.* 2007; DiBattista *et al.* 2008) and estimate effective population size (Liu & Ely 2009; Israel & May 2010; Kanno *et al.* 2010). The molecular ecology of marine turtles is an active area of research, and genetic methods have revealed that polyandry is widespread across marine turtle species (reviewed in Lee 2008; Uller & Olsson 2008). However, to date, molecular studies of mating systems in marine turtles have focused heavily on the question of female promiscuity, whilst many aspects of reproduction in male turtles have not been fully addressed.

All species of marine turtles have TSD and a large number of studies across species, populations and geographic ranges indicate that hatchling sex ratios biased towards females (the sex produced at warmer temperatures) are the norm (reviewed in Hawkes *et al.* 2009) and are likely to increase under future climate change scenarios (Fuentes *et al.* 2010; Witt *et al.* 2010), causing concern that lack of males might threaten the persistence of depleted populations. Assessing the juvenile and adult sex ratios of marine turtle populations at sea is logistically challenging and is further complicated by their complex life history and migrations (Bowen & Karl 2007). Whether female-biased sex ratios remain at later life stages is not clear; a number of studies suggest that juvenile and adult sex ratios are moderately female-skewed (e.g. Stabenau *et al.* 1996; Casale *et al.* 2005; Braun-McNeill *et al.* 2007; Delgado *et al.* 2010); however, Chaloupka & Limpus (2001) report a male-biased adult sex ratio despite a female-biased juvenile sex ratio of green turtles in the southern Great Barrier Reef. Furthermore, relatively equal sex ratios of breeders have recently been reported in green and leatherback *(Dermochelys coriacea)* turtle populations, even though offspring sex ratios in these populations are female-skewed (Stewart & Dutton 2011; Wright *et al.* 2012). These results could suggest sex differences in adult dispersal or sex-biased mortality of juvenile or adult turtles. There is some evidence that hatchlings from cooler nests (and therefore likely to be male) are larger and show increased swimming ability than hatchlings from warmer nests (Booth & Evans 2011), which may reduce their vulnerability to predation (Janzen *et al.* 2000a,b). Alternatively, male turtles might breed more frequently than female turtles (Limpus 1993). If female-skewed hatchling sex ratios persist in adult populations, then a consequence of more frequent mating by males could be that operational sex ratios of breeding aggregations in a given year are more equal than expected. In other words, a higher reproductive rate for male turtles would mean that the ratio of males to females that are ready to mate in any one breeding season could be relatively equal even if the adult sex ratio is female biased.

Marine turtles are capital breeders (Bonnet *et al.* 1998), and females typically reproduce at intervals of several years (reviewed in Miller 1997; Hamann *et al.* 2003). Smaller energetic requirements for reproduction in males than females may allow them to mate more frequently, and there are reports of some male loggerhead *(Caretta caretta)*, leatherback and green turtles migrating to breeding grounds annually (Limpus 1993; James *et al.* 2005; Hays *et al.* 2010). Whilst a shorter reproductive interval in males might help to ensure mate availability even if adult populations are highly female biased (Hays *et al.* 2010), a small male population size will, nevertheless, result in inbreeding and loss of genetic variation, which will be exacerbated if there is a large skew among individuals in reproductive success (Frankham 1995; Karl 2008). Considering the potential of climate warming to further feminise marine turtle populations, it is critical that we have a better understanding of current sex ratios of older size classes. Because of the implications for N_e and population viability, it is important that we distinguish whether the observed relatively equal sex ratios of breeders in marine turtle populations are the result of a few males breeding annually or of larger adult male population sizes than predicted based on offspring sex ratios.

Here we use microsatellite analysis to specifically address the question of male mating periodicity in a green turtle rookery in northern Cyprus that exhibits a highly female-biased offspring sex ratio (86–96% female, Broderick *et al.* 2000; Wright *et al.* 2012). Although male marine turtles are difficult to locate and sample at sea, female turtles and their offspring are easily accessible on the nesting beach. We intensively sampled and genotyped mothers and offspring and used sibship reconstruction and paternal genotype reconstruction to identify males across three successive breeding seasons. By sampling a large proportion of the nesting female population at the study site (>90% of females that successfully nested at the site each year were included in the study), we were also able to estimate the sex ratio of breeding adults and the number of males successfully reproducing, with the aim of better understanding the male contribution to the gene pool and subsequent implications for N_e.

Materials and methods

Study site

The study was conducted in a wild population of green turtles at Alagadi Beach, northern Cyprus during three consecutive breeding seasons from 2008 to 2010. The Mediterranean green turtle population consists of approximately 300–400 females nesting annually (Broderick *et al.* 2002) and was recently ranked as the most endangered green turtle population in the world (Wallace *et al.* 2011). Alagadi Beach is among the most important nesting sites for green turtles in the Mediterranean (Kasparek *et al.* 2001), supporting a total breeding population of approximately 100 females (Broderick *et al.* 2002). The annual nesting population is highly variable, but averaged 30 females per year during the study period (range 23–40). Nesting is localized in the Mediterranean occurring mostly in Turkey, Cyprus and Syria and in much smaller numbers in Israel and Egypt (Broderick *et al.* 2002; Rees *et al.* 2008). The closest major breeding rookery to Alagadi is the Karpaz Peninsula of northern Cyprus, approximately 83 km away. Alagadi beach consists of two short coves (2 km total), on which the nesting population of green turtles has been monitored comprehensively since 1992. All nesting females are marked individuals, and all clutches are recorded and attributed to individual females.

Sample collection

Tissue biopsies were taken from nesting female turtles of known identity from the trailing edge of the foreflipper. Clutches were marked, and upon hatching, tissue biopsies were taken from a sample of hatchlings in each clutch from the trailing edge of the carapace. Live and dead hatchlings as well as dead embryos from unhatched eggs were sampled. In 2008, tissue samples were stored in Queen's lysis buffer (Seutin *et al.* 1991), and in subsequent years, tissue samples were stored in 96% alcohol. In 2008, two clutches were sampled from a subset (16 of 20) of females. Inferred paternity was highly correlated in the successive clutches of these females, so in further years, we reduced our sampling effort to a single clutch per female. In total, 94 clutches from 78 females were sampled, representing 92% of females that successfully produced offspring at the site during the study period.

Genotyping

Genomic DNA was extracted from tissue samples using a standard ammonium acetate precipitation method (Nicholls *et al.* 2000). Samples were genotyped at 14 polymorphic microsatellite loci designed for use in sea turtles and previously shown to amplify and be polymorphic in the green turtle (Table 1). However, one marker, D105, was later removed from analysis because of evidence of null alleles. Primers were labelled with fluorescent dyes (6-FAM, HEX or NED) and the loci

Table 1 Characterization of microsatellite loci in green turtles (*Chelonia mydas*) at Alagadi, northern Cyprus

Locus	Reference	*n*	No. alleles	H_O	H_E	PIC
A6	Dutton & Frey (2009)	88	6	0.750	0.734	0.687
B103	Dutton & Frey (2009)	88	5	0.636	0.673	0.619
B123	Dutton & Frey (2009)	87	5	0.655	0.631	0.562
C102	Dutton & Frey (2009)	88	5	0.614	0.625	0.563
D105*	Dutton & Frey (2009)	88	8	0.648	0.787	0.754
D2	Dutton & Frey (2009)	87	10	0.828	0.760	0.726
Cm3	FitzSimmons *et al.* (1995)	88	8	0.568	0.546	0.517
Cm58	FitzSimmons *et al.* (1995)	88	7	0.807	0.808	0.776
Klk314	Kichler *et al.* (1999)	88	4	0.477	0.507	0.408
Or7	Aggarwal *et al.* (2004)	88	5	0.716	0.660	0.614
Cc2	Monzon-Arguello *et al.* (2008)	88	8	0.727	0.766	0.730
Cc28	Monzon-Arguello *et al.* (2008)	88	4	0.716	0.728	0.672
CcP7D04	Shamblin *et al.* (2009)	87	8	0.851	0.791	0.757
CcP7E11	Shamblin *et al.* (2007)	88	4	0.500	0.484	0.443

H_O, observed heterozygosity; H_E, expected heterozygosity; PIC, polymorphic information content.

*Removed prior to parentage analyses.

amplified in two multiplex PCR sets. PCR amplification was carried out in an MJ Research model PTC DNA Engine Tetrad thermal cycler according to the following schedule: 95 °C for 15 min followed by 35 cycles of 94 °C for 30 s, 58 °C for 90 s and 72 °C for 60 s and finally one cycle of 60 °C for 30 min. Allele sizes were assigned using an internal size standard (Genescan-500-ROX; Applied Biosystems), an ABI 3730 DNA Analyser and ABI GeneMapper 3.7 software (Applied Biosystems). All PCRs were run with positive and negative controls.

A minimum of 20 offspring from each clutch were analysed. Samples that failed to amplify at all loci were re-amplified and re-scored. Any samples that still failed to amplify at a minimum of 10 loci were excluded from further analyses. Multilocus genotypes were complete at all of the 13 final loci for >97% of individuals. In total 2042 offspring (mean ± SD = 21.7 ± 1.2 hatchlings per clutch, range = 18–23, Table 2) and 78 mothers were included in the final parentage analysis.

To assess genotyping error rate, approximately 5% of samples were re-extracted, re-amplified and re-scored in a blind fashion and compared with original allele calls. Differences in the repeated genotypes were observed at seven allele calls of 3273 (0.21% error), and the highest error at any particular locus was 0.8% at locus B103. We also assessed the entire data set for null alleles, allelic dropout and genotyping error using the program MicroErrorAnalyzer (Wang 2010), which compares genotypes of parent–offspring dyads. Maximum likelihood estimates of error rates were 0.004 for null alleles (although all of these were at locus D105, which was removed from the analysis), <0.0001 for allelic dropout and 0.0003 for genotyping error.

Samples from 88 adult turtles (78 mothers that were included in the study plus nine additional females and one male sampled from the same site between 2007 and 2010) were used to determine population allele frequencies and assess the suitability of the microsatellite markers for paternity analysis. Allele frequency analysis and tests of deviation from Hardy–Weinberg equilibrium (HWE) were performed in the program CERVUS version 2.0 (Marshall *et al.* 1998). Linkage disequilibrium between all pairs of loci was tested using a Markov chain method in GENEPOP version 4.0.10 (Raymond & Rousset 1995).

Paternity analysis

Sibship and parentage inference were carried out in COLONY version 2.0 (Wang & Santure 2009). COLONY uses a maximum likelihood model to assign sibship and parentage relationships. Offspring are clustered into full- and half-sib families, candidate parents are assigned to clusters and genotypes of unknown parents are inferred. Three replicate runs of 'long' length were conducted on the same data set assuming an error rate of 0.004 for allelic dropout and 0.008 for genotyping error (based on the highest error rate per locus observed in our error tests). Each of the replicate runs used different random number seeds to initiate the simulated annealing processes. Maternity of all offspring was known. All genotyped offspring were analysed together to detect any paternal half-sibs, which would identify males that sired offspring in more than 1 year or with more than one female within a year.

Results

Over the 3-year study period, our parentage analysis detected at least 98 different males that sired offspring from the 78 study females, indicating a sex ratio of breeding adult turtles of at least 1.3 males (M):1 female (F) across the 3 years, with a minimum of 1.1 M:1 F (2009) and maximum of 1.5 M:1 F (2008) in any particular breeding season (Fig. 1). There was a high degree of convergence between the three COLONY runs. Two of the three runs found the most likely number of unique males that sired offspring at this site across the study period to be 98, whilst one run found 100 unique sires to be most likely. We only concluded that a particular male had sired offspring with multiple females or that a female had mated with more than one male, when all three runs gave consistent results. Using these conservative estimates of the number of mates, for males and females, both within and between seasons, we infer 99 unique sires (Table 2).

Only three (3%) inferred males were assigned offspring in more than one breeding season (two males: 2008 and 2009; one male: 2008 and 2010), whilst 97% of males were assigned offspring in only one breeding season within the study period. The males that sired offspring only in 2008 or 2010 (59 of 99; 60%) had a minimum breeding interval of 3 years with females at the Alagadi rookery within the study period, whilst the males that sired offspring only in 2009 (37 of 99; 37%) had a minimum breeding interval of 2 years. No males were assigned offspring in all 3 years, suggesting that male turtles do not generally breed annually at this site. No females reproduced more than once at this site during the 3-year study. There was no consistent evidence from all three COLONY runs that any single male sired offspring with more than one female within a breeding season (i.e. COLONY did not consistently identify any paternal half-sibs within a breeding season), providing evidence that no particular males were able to dominate reproduction. However, the analysis did identify multiple paternity in the clutches of 19 (24%) females, inferring a maximum of four fathers in any single clutch (Table 2).

Table 2 Inferred paternity and number of fathers per clutch. For some clutches, the number of inferred fathers differed across the three COLONY runs; we show minimum and maximum numbers of inferred fathers per clutch, but only conclude multiple paternity when all runs detect at least two fathers (shown in bold, column 5). No candidate males were sampled; all inferred father IDs (column 7) refer to unsampled putative males and are inferred from the most conservative estimates across all three COLONY runs. Numbers of offspring per clutch that were consistently assigned to putative sires are shown in brackets. Males assigned offspring in more than 1 year are shown in bold

Mother ID—Clutch	Year	No. of offspring sampled	Clutch size	Min. inferred fathers	Max. inferred fathers	Inferred father ID
G001-1	2008	23	111	1	1	F1(23)
G001-3	2008	22	138	1	1	F1(22)
G008-1	2008	22	89	**2**	2	F6(21), F7(1)
G008-3	2008	23	107	1	1	F6(23)
G048-1	2008	22	119	**3**	3	F14(5), **F15(11)**, F16(6)
G048-2	2008	22	140	**3**	4	F14(4), **F15(15)**, F16(3)
G056-1	2008	22	76	**2**	2	F17, F18
G056-3	2008	22	98	1	2	F18
G057-1	2008	21	40	**2**	2	F19(6), F20(15)
G057-3	2008	23	121	1	1	F19(23)
G152-1	2008	21	69	**2**	2	F29(20), F30(1)
G155-2	2008	23	144	**2**	2	**F31(20)**, F32(3)
G155-4	2008	20	117	**2**	2	**F31(16)**, F32(4)
G158-1	2008	22	119	1	1	F36(22)
G158-2	2008	22	136	1	1	F36(22)
G168-1	2008	23	111	1	1	F40(23)
G168-3	2008	22	123	1	1	F40(22)
G177-1	2008	19	158	1	1	F42(19)
G182-1	2008	23	123	1	1	F43(23)
G195-1	2008	22	105	1	1	F51(22)
G195-4	2008	23	117	1	1	F51(23)
G197-2	2008	21	138	1	1	**F52(21)**
G197-3	2008	22	110	1	1	**F52(22)**
G198-1	2008	22	99	1	1	F53(22)
G198-4	2008	21	105	1	1	F53(21)
G199-1	2008	22	76	1	1	F54(22)
G199-3	2008	18	96	1	1	F54(18)
G200-1	2008	21	111	1	1	F55(21)
G200-2	2008	20	101	1	1	F55(20)
G201-1	2008	22	95	1	1	F56(22)
G202-2	2008	22	115	1	1	F57(22)
G202-4	2008	22	116	1	1	F57(22)
G203-1	2008	23	95	1	1	F58(23)
G203-3	2008	23	100	1	1	F58(23)
G204-1	2008	22	95	**2**	2	F59(22), F60(2)
G204-3	2008	19	122	**2**	2	F59(15), F60(4)
G015-1	2009	21	145	**2**	2	F10(16), F11(5)
G020-2	2009	21	88	1	1	F12(21)
G071-1	2009	21	131	1	1	F22(21)
G156-2	2009	21	97	**2**	2	F33(15), F34(6)
G157-3	2009	21	137	1	1	F35(21)
G166-1	2009	21	75	**2**	2	F38(14), F39(7)
G185-2	2009	19	102	1	1	F44(19)
G187-1	2009	21	99	1	1	F45(21)
G189-2	2009	21	95	**2**	2	F46(18), F47(3)
G190-1	2009	21	93	1	1	F48(21)
G191-1	2009	21	109	1	1	F49(21)
G192-1	2009	21	126	1	1	F50(21)
G205-1	2009	20	88	1	2	F61(20)
G206-1	2009	21	81	1	1	F62(21)

Table 2 Continued.

Mother ID—Clutch	Year	No. of offspring sampled	Clutch size	Min. inferred fathers	Max. inferred fathers	Inferred father ID
G207-3	2009	23	123	1	1	F63(23)
G208-1	2009	19	57	1	1	F64(19)
G209-1	2009	21	95	1	1	F65(21)
G210-1	2009	21	69	1	1	F66(21)
G211-1	2009	21	95	1	2	F67(21)
G212-1	2009	21	104	1	1	F68(21)
G213-1	2009	21	111	1	1	F69(21)
G214-1	2009	21	104	**2**	2	F70(14), F71(7)
G215-1	2009	21	106	1	1	**F52(21)**
G216-3	2009	21	94	1	1	F72(21)
G218-1	2009	21	99	1	1	F73(21)
G219-1	2009	21	98	1	1	**F31(21)**
G220-1	2009	21	116	1	1	F74(21)
G221-1	2009	21	108	1	1	F75(21)
G222-1	2009	21	123	1	1	F76(21)
G223-1	2009	21	83	1	1	F77(21)
G224-1	2009	21	123	1	1	F78(21)
G225-1	2009	21	77	1	1	F79(21)
G226-1	2009	21	79	1	1	F80(21)
G227-1	2009	22	86	1	1	F81(22)
G002-2	2010	23	149	**3**	3	F2(18), F3(4), F4(1)
G006-2	2010	23	162	1	1	F5(23)
G009-2	2010	23	112	**2**	2	F8(22), F9(1)
G044-2	2010	23	119	1	1	F13(23)
G055-2	2010	22	126	1	1	**F15(22)**
G058-2	2010	21	138	1	1	F21(21)
G080-2	2010	23	182	1	1	F23(23)
G086-2	2010	23	115	**3**	3	F24(16), F25(6), F26(1)
G087-2	2010	23	150	**2**	2	F27(21), F28(2)
G163-2	2010	23	129	1	1	F37(23)
G172-2	2010	22	126	1	1	F41(22)
G229-2	2010	23	117	1	1	F82(23)
G230-2	2010	23	88	1	1	F83(23)
G231-2	2010	23	75	**2**	3	F84(16), F85
G232-2	2010	23	115	1	1	F86(23)
G233-2	2010	23	100	**3**	3	F87(8), F88(11), F89(4)
G235-2	2010	23	117	**3**	3	F90(21), F91(1), F92(1)
G236-1	2010	23	112	1	1	F93(23)
G237-2	2010	23	94	1	1	F94(23)
G238-1	2010	23	119	1	1	F95(23)
G239-2	2010	23	160	1	1	F96(23)
G240-2	2010	23	107	1	1	F97(23)
G241-1	2010	23	154	1	1	F98(23)
G242-1	2010	23	106	1	2	F99(23)

The 14 microsatellite markers used in the analysis were all variable in our green turtle population (Table 1). All loci conformed to expectations of HWE ($P > 0.05$) and showed low frequency of null alleles, except for D105 where CERVUS estimated a frequency of >0.9. Marker D105 was therefore excluded from the analysis. No pair of loci showed evidence of genotypic linkage disequilibrium (after correction for multiple tests, Verhoeven *et al.* 2005). Combined exclusion probability (second parent) for the remaining 13 loci was >0.999.

Discussion

Our results demonstrate a sex ratio of 1.3 males to each female over the 3-year study period and indicate that, contrary to expectations, there is currently no shortage

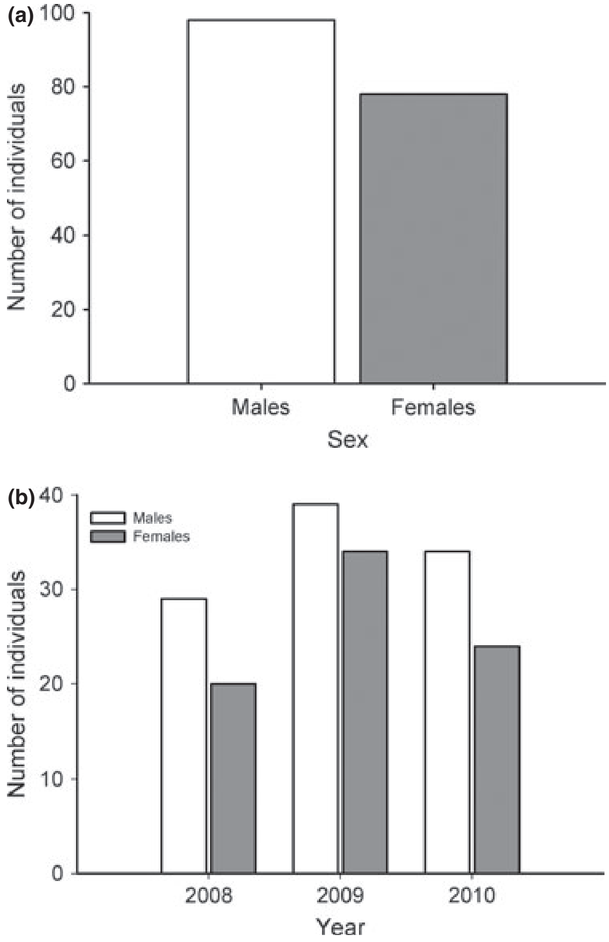

Fig. 1 Number of nesting females (shaded) that were included in the study and inferred number of unique males that sired offspring from those females (a) across the 3-year study period and (b) within each breeding season at Alagadi, northern Cyprus.

of adult male turtles at this rookery, despite a highly female-biased hatchling sex ratio (Broderick *et al.* 2000; Wright *et al.* 2012). We show that the observed sex ratio of breeders is not because of a large proportion of males mating annually, as has been suggested in some other marine turtle populations (Limpus 1993; Hays *et al.* 2010), but reflects a sex ratio of breeding adults that is actually close to even.

Only three of 99 (3%) males returned to breed more than once during the study period, suggesting that male breeding frequencies may be similar to those in females, although further breeding seasons would need to be studied to confirm this pattern. Female green turtles in this population reproduce on average every 3 years (Broderick *et al.* 2003), and no female nested in more than 1 year during the 3-year study period. Other aspects of breeding behaviour are similar in both sexes; like females, males can undertake long migrations between feeding and breeding grounds, show fidelity to breeding areas (Limpus 1993; James *et al.* 2005) and are philopatric to breeding grounds in natal regions (FitzSimmons *et al.* 1997a).

Other studies report that males migrate to breeding grounds on an annual basis. Hays *et al.* (2010) tracked three male loggerhead turtles in the Mediterranean for up to 12 months, in which time all of them migrated back to breeding grounds. Female loggerhead turtles in the Mediterranean reproduce on average every 2 years, with a significant number breeding annually (Broderick *et al.* 2003; Hays *et al.* 2010), so perhaps accumulating the energy stores required for reproduction in this omnivorous species is more easily achieved than in the herbivorous green turtle (Broderick *et al.* 2001). Likewise, two male leatherback turtles tracked in the western Atlantic re-migrated to the same breeding grounds in two consecutive years, but two other male turtles remained in feeding grounds throughout the breeding season, suggesting that re-migration interval varies among males in the population (James *et al.* 2005). Critically, neither of these studies demonstrated that the males tracked to breeding grounds actually mated in either year, and the observed migrations could be resource related. Data from tag returns and observations of green turtles in Australia provide evidence that males reproduce every 1–2 years compared with an average reproductive interval of 4.7 years for females in the same population (Limpus 1993), indicating that reproductive interval in male turtles varies between species and populations and may depend upon quality of foraging grounds and migratory distance (Hamann *et al.* 2003). It is possible that a large proportion of the male green turtles in our study rookery migrated to the breeding grounds annually, but did not successfully reproduce, although this seems unlikely. It is also possible that males mated annually with females at other rookeries and therefore were not detected in this study, despite evidence that male turtles show fidelity to courtship areas in successive breeding migrations (Limpus 1993). Sampling offspring from multiple rookeries throughout the Mediterranean would be the logical next step to address this question.

The disparity between the observed approximately equal sex ratio of breeders and the highly female-skewed sex ratio of hatchlings at our study site could be explained by a number of causes. First, age at maturity in green turtles is estimated to be 30–40 years (reviewed in Heppell *et al.* 2003); hence, current adult sex ratios reflect hatchling sex ratios several decades ago and may become more female biased in the future, although Witt *et al.* (2010) report only small average increases in sea surface temperatures at this site (<1 °C) over the past 50 years. Sex differences in age at maturity could also cause disagreement between primary and breeding sex ratios. Although there is limited evidence of males reaching maturity earlier than females in the freshwater painted turtle (*Chrysemys picta*; Freedberg & Bowne 2006), there is no direct evidence that this is the case in marine turtles, and the difference would have to be large to offset the highly female-skewed offspring sex ratio that we observe in northern Cyprus.

Second, our results could indicate strongly female-biased mortality in early life stages, but owing to a dearth of information relating to the pelagic juvenile and subadult phases in marine turtle life cycles, we can only speculate whether this is the case (although the few data available suggest the female bias is maintained in juvenile populations, for example, Chaloupka & Limpus 2001; Braun-McNeill *et al.* 2007; Delgado *et al.* 2010), and if so, what the potential causes of a sex difference in mortality could be. Hatchlings are extremely vulnerable to predation whilst entering the ocean and in near-shore waters; hence, more rapid dispersal to the open ocean is likely to enhance survival (Gyuris 1994; Booth & Evans 2011). Cooler incubation temperatures produce larger hatchlings (Booth & Evans 2011), but there is conflicting evidence regarding the effect of incubation temperature on swimming ability. Recently Booth & Evans (2011) showed that hatchlings from cooler nests (incubation temperatures below 30 °C, that is, those that produce a proportion of males) showed greater swimming ability than those incubated at warmer temperatures (above 30 °C, that is, producing females); however, an earlier study showed the opposite effect (Burgess *et al.* 2006). Nonetheless, larger green turtle hatchlings experienced significantly lower mortality rates during early offshore migration across a reef at Heron Island, Australia (Gyuris 2000), possibly due to gape limitation of predatory fish. In northern Cyprus, and other nesting regions where female-producing incubation temperatures are the norm, the only nests likely to produce any male hatchlings are those laid very early or late in the nesting season when ambient temperatures are cooler. It is feasible that predation at these times is reduced because of seasonal fluctuations in predator abundance, resulting in higher survival of male hatchlings (but see Whelen & Wyneken (2007) for an example of predation increasing towards the end of the hatching season). Directly assessing early mortality in hatchling and juvenile marine turtles would be logistically extremely difficult, but studies on freshwater turtles suggest no significant sex differences in early mortality between the sexes (Janzen & Morjan 2002). Furthermore, there are no reported sex differences in subadult or adult distribution or migration routes that might make one sex more vulnerable to interactions with fisheries or other causes of mortality at subadult life stages, although comparatively little is known about the movement of male turtles, placing particular importance on studies of their spatial and breeding ecology.

Third, many of the females at our study site could have mated with males hatched on distant nesting beaches elsewhere in the Mediterranean. It has been shown that both male and female marine turtles are philopatric to mating grounds in their natal regions, resulting in genetic structuring among nesting colonies, as revealed by mitochondrial DNA sequences (reviewed in Bowen & Karl 2007). Comparisons between genetic divergence at nuclear and mitochondrial DNA markers, however, have revealed evidence for male-mediated gene flow between rookeries and between some regional populations with shared migratory pathways (FitzSimmons *et al.* 1997b; Bowen & Karl 2007). In contrast to females that typically show strong fidelity to specific nesting sites (Broderick *et al.* 2002), when mating takes place in regional courtship grounds that can be distant from the nesting beaches, males can mate with females from multiple rookeries (Limpus 1993). Although no regional breeding grounds have been identified yet for green turtles in the Mediterranean, and mating is thought to occur close to the nesting beaches (Broderick & Godley 1997), males might move around aggregations of receptive females throughout the region. A single adult male green turtle was satellite-tracked from our study site in northern Cyprus in 2009, immediately following mating (Wright *et al.* 2012), and travelled through or close to multiple nesting sites in Cyprus and Turkey within the breeding season, before moving to feeding grounds off the coast of North Africa. Similarly, James *et al.* (2005) reported that four male leatherback turtles tracked to breeding grounds ranged widely during the breeding season in the vicinity of multiple small nesting colonies, possibly seeking mating opportunities with females at each site. The extent of male movements during the breeding season may depend on the number of females available at the natal breeding ground, as males returning to small nesting colonies ranged more widely than those returning to larger nesting colonies (James *et al.* 2005). If the males breeding at our study site originate from distant nesting beaches, and if gene flow has been sufficiently limited to maintain genetic differentiation between nesting populations, then genetic assignment of the females and inferred males could provide evidence to support this conjecture. However, we found no evidence of genetic differentiation among females and inferred males breeding at our study site using the program STRUCTURE (version 2.3.3) (Pritchard *et al.* 2000); the analysis suggested that a single population ($K = 1$) was most likely (Fig. S1, Supporting Information), although we consider this result to be conservative because of the uncertainty associated with our inferred multilocus genotypes (see Appendix S1, Supporting Information for details of the methods and results of this analysis).

Few estimates of effective population size (N_e) exist for marine turtles (Rivalan *et al.* 2006; Theissinger *et al.* 2009), and those that do all relate to long-term (inbreeding) N_e. Methods of estimating contemporary N_e make assumptions that are not met in long-lived marine turtles (such as nonoverlapping generations, temporally distant samples in terms of generations or unbiased primary sex ratios) or require information on key demographic parameters that are unknown (Wang *et al.* 2010) (for reviews see Palstra & Ruzzante 2008; Hare *et al.* 2011). N_e is strongly affected by sex ratio and variance in reproductive success among individuals (Frankham 1995). The current close to even sex ratio of breeding adults and the relatively large number of males contributing to reproduction in our study rookery will help to maintain N_e and, therefore, genetic variation, at least on a local scale. Expanding this study to incorporate other green turtle rookeries throughout the Mediterranean would provide a more robust estimate of the adult sex ratio of the wider population and allow a more accurate viability assessment.

To our knowledge, this study represents the most comprehensive assessment of paternity in any marine turtle species. We have validated the use of molecular-based sibship analysis as a method to census successfully breeding males in a marine turtle population (Pearse *et al.* 2001; Schwartz *et al.* 2007; Lee 2008), as well as addressing aspects of male mating behaviour, such as breeding interval, which have previously been logistically very difficult to study. Our approach offers a means of monitoring breeding sex ratios and identifying any future changes that may result from current climate warming.

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Data accessibility

Microsatellite genotypes: DRYAD entry doi: 10.5061/dryad.7dk0m36r.

Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1 Genetic structure methods and results.

Fig. S1 Output from a Structure analysis of 87 adult female turtles that nested at the study site from 2007 to 2010 and 87 inferred male genotypes based on reconstructed single-locus paternal genotypes from a Colony analysis.