Insights into the mating systems of green turtle populations from molecular parentage analyses

Submitted by

Lucy Isabel Wright

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature: …………………………………………………………..

ABSTRACT

Gaining a good understanding of marine turtle mating systems is fundamental for their effective conservation, yet there are distinct gaps in our knowledge of their breeding ecology and life history, owing largely to the difficulty in observing these highly mobile animals at sea. Whilst multiple mating by females, or polyandry, has been documented in all marine turtle species, the fitness consequences of this behaviour have not been fully investigated. Furthermore, male mating patterns, operational sex ratios and the number of males contributing to breeding populations are poorly understood, impeding accurate assessments of population viability. In this thesis, I use molecular-based parentage analysis to study, in detail, the genetic mating system of two green turtle (*Chelonia mydas*) populations. In the focal population in northern Cyprus, I show that, despite exhibiting a strongly female-biased hatchling sex ratio and contrary to our expectations, there are at least 1.3 breeding males to every nesting female. I go on to assess the breeding frequency of male turtles in the population and determine that males do not breed annually at this site, demonstrating that the observed relatively equal sex ratio of breeders is not the result of a few males mating every year, but that the number of breeding males in the population is greater than expected. I show that 24% of nesting females in the population produce clutches with multiple paternity, but do not detect any fitness benefits to polyandrous females, and discuss the potential role of sexual conflict in influencing female mating decisions. Finally, I reveal a high frequency of multiple paternity in green turtle clutches on Ascension Island, one of the largest green turtle rookeries in the world, and discuss possible causes of variation in the level of polyandry among marine turtle populations. The results presented here shed new light on aspects of marine turtle mating systems that are challenging to study, and illustrate the value of molecular data, not only in describing mating patterns, but in elucidating aspects of life history and behaviour that would otherwise be very difficult to ascertain.

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AUTHOR'S DECLARATION

All chapters presented in this thesis were written by L.I. Wright under the guidance and supervision of A.C. Broderick, T. Tregenza and A. McGowan. All molecular analyses were conducted at the NERC Biomolecular Analysis Facility, University of Sheffield, under the supervision of T. Burke and D.A. Dawson, with guidance from A. Krupa and G.J. Horsburgh. Fieldwork in northern Cyprus was carried out as part of the Marine Turtle Conservation Project under the supervision of W.J. Fuller, with the assistance of R. Snape, K.L. Stokes, L Collyer and the dedicated support of numerous volunteers. Specific author contributions to chapters are detailed below:

Chapter 2: Turtle mating patterns buffer against disruptive effects of climate change.

Lucy I. Wright, Kimberley L. Stokes, Wayne J. Fuller, Brendan J. Godley, Andrew McGowan, Robin Snape, Tom Tregenza and Annette C. Broderick

I conducted fieldwork, collected all samples, carried out all genetic analyses, analysed the genetic data and was the lead author on the manuscript. KLS analysed the satellite tracking data, wrote the satellite tracking results section of the manuscript and produced Figure 2. RS supervised fieldwork, under the management of WF, BJG and ACB. RS additionally assisted with data and sample collection in the field and attached the satellite transmitter. AM, TT and ACB provided guidance on data analysis and writing, and all co-authors provided useful comments on the manuscript.

Chapter 3: Reconstruction of paternal genotypes over multiple breeding seasons reveals male green turtles do not breed annually.

Lucy I. Wright, Wayne J. Fuller, Brendan J. Godley, Andrew McGowan, Tom Tregenza and Annette C. Broderick

I conducted fieldwork, collected samples, carried out all genetic analyses, analysed the data and wrote the manuscript. WJF, BJG and ACB oversaw and managed fieldwork. AM, TT and ACB provided guidance on data analysis and writing, and all co-authors provided useful comments on the manuscript. In addition, R Snape, K.L. Stokes and L

Collyer assisted with data and sample collection in the field, particularly in 2009, and RS and KLS supervised fieldwork.

Chapter 4: No benefits of polyandry to female green turtles

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I conducted fieldwork, collected samples, carried out all genetic analyses, analysed the data and wrote the manuscript. WJF, BJG and ACB oversaw and managed fieldwork. AM, TT and ACB provided guidance on data analysis, presentation and writing, and all co-authors provided useful comments on the manuscript. In addition, R Snape, K.L. Stokes and L Collyer assisted with data and sample collection in the field, particularly in 2009, and RS and KLS supervised fieldwork.

Chapter 5: High level of multiple paternity in green turtle clutches at Ascension Island

Lucy I Wright, Kate E Plummer, Sam B Weber, Brendan J Godley, Andrew McGowan, Tom Tregenza, Annette C Broderick

I carried out all genetic analyses, analysed the data and wrote the manuscript. KEP and SBW carried out fieldwork and collected samples, under the management of BJG and ACB. KEP and AM extracted DNA from some of the samples. AM, TT and ACB provided guidance on data analysis, presentation and writing, and all co-authors provided useful comments on the manuscript.

LIST OF ABBREVIATIONS

CCL	Curved carapace length
CCW	Curved carapace width
GLMM	Generalised linear mixed model
HWE	Hardy-Weinberg equilibrium
mtDNA	Mitochondrial DNA
N_e	Effective population size
OSR	Operational sex ratio
PCR	Polymerase chain reaction
PrDM	Probability of detecting multiple paternity
REML	Restricted maximum likelihood
SCL	Straight carapace length
SCW	Straight carapace width
TSD	Temperature-dependent sex determination

CHAPTER 1

General introduction

The application of molecular genetics to the study of mating systems in natural populations is now commonplace and has provided considerable insights into aspects of reproductive biology that would otherwise be difficult to address. For example, molecular studies of parentage have commonly revealed extra-pair paternity (e.g. Kempenaers *et al.* 1992; reviewed in Hughes 1998) and shown multiple mating by both males and females to be widespread across taxa (reviewed in Simmons 2001; Avise *et al.* 2002; Griffith *et al.* 2002; Uller and Olsson 2008). They have provided information on individual variation in reproductive success within natural populations (Coltman *et al.* 1998; Pearse *et al.* 2002; Frasier *et al.* 2007), which, in the case of depleted populations, can shed light on mechanisms impacting population recovery (e.g. Frasier *et al.* 2007; Gottelli *et al.* 2007). Mating system and variation in male and female reproductive success can strongly influence genetic effective population size (N_e), inbreeding risk and levels of gene flow between populations (Frankham 1995), hence, understanding the mating systems of threatened species is key to their effective conservation (Anthony and Blumstein 2000). Molecular approaches are particularly valuable for studying elusive species that are difficult to capture or observe, and, in these situations, molecular data can greatly contribute to life history information. Indeed, Avise (1996) notes that one of the most important ways in which genetics can contribute to conservation is by increasing our understanding of particular species' biology.

The research presented in this thesis uses a combination of molecular techniques and direct field studies to investigate the mating system of the green turtle (*Chelonia mydas*). In the following pages I provide background information on the life history of the green turtle; summarize our current understanding of marine turtle breeding behaviour, highlighting major gaps in our knowledge; and describe how molecular parentage-based approaches have been previously used to study marine turtle mating systems.

Life history of the green turtle

Distribution and status

The green turtle belongs to the family Cheloniidae, which can be traced back to the Cretaceous era and today encompasses six of the seven extant species of marine turtle (Pritchard 1997). Green turtles are distributed circum-globally in tropical and sub-tropical waters and nest on mainland beaches, coral reef islands and remote oceanic islands in all major ocean basins. The largest nesting assemblages occur in the northern Great Barrier Reef, Australia (Raine Island; approximately 18,000 females nesting annually (Seminoff 2004)) and the Caribbean coast of Costa Rica (Tortuguero; approximately 17,000-37,000 females nesting annually (Troëng and Rankin 2005)), however, nesting colonies of varying magnitude are numerous and widely distributed, with nesting occurring in over 80 countries worldwide (Hirth 1997).

Green turtles have a long history of human exploitation and the intensive harvest of turtles and eggs has caused drastic populations declines across their global range (Parsons 1962; Frazier 2003; McClenachan *et al.* 2006). Most populations remain severely depleted relative to historical levels, although there are very few known examples of complete extirpation (McClenachan *et al.* 2006; Bell *et al*. 2010). Where populations have been afforded protection from exploitation and have been subject to effective monitoring and conservation efforts, significant population recoveries have taken place (Chaloupka *et al.* 2008) and some populations are increasing rapidly (e.g. Tortuguero, Costa Rica (Troëng and Rankin 2005), Ascension Island (Broderick *et al.* 2006) and the Archie Carr National Wildlife Reserve, Florida (Chaloupka *et al*. 2008)). Green turtles are protected by the Convention on International Trade in Endangered Species (CITES), the Convention on Migratory Species (CMS) and are currently listed as ‘Endangered’ by the World Conservation Union (IUCN), based on a reduction of >50% over the past 3 generations (www.redlist.org, accessed 26 April 2012). This global listing is controversial due to the favourable status of many green turtle populations (Broderick *et al*. 2006; Godfrey and Godley 2008; Seminoff and Shanker 2008), however, there is no doubt that some regional populations are at risk of extinction due to ongoing anthropogenic threats (McClenachan *et al.* 2006; Wallace *et al*. 2011). It is important to note that population censuses are typically based on annual numbers of nests or nesters, rather than absolute adult population size, which, although a reliable index of population trends when monitored over long time periods (Seminoff 2004), provide no information about the male or juvenile components of a population.

Life cycle

Green turtles are highly migratory and have a complex life history that involves large scale movements at both juvenile and adult life stages (reviewed in Musick and Limpus 1997; Plotkin 2003). Gaining a full picture of their ecology away from the nesting beach has, therefore, been challenging, but advances in the use of satellite telemetry (Godley *et al.* 2008) and molecular genetics (Bowen and Karl 2007), in addition to more traditional tagging studies (e.g. Balazs 1976; Limpus *et al.* 1992), have substantially contributed to our understanding of their life history. Upon emerging from their nest, hatchling green turtles enter the sea and swim actively offshore, eventually reaching the open ocean, where, although based on little direct data, it is thought that they remain in oceanic, pelagic habitats for a number of years (Musick and Limpus 1997; Bolten 2003). Hatchling and young juvenile green turtles may be carried passively in ocean currents, as has been demonstrated for loggerhead turtles (*Caretta caretta*) in the Atlantic (Musick and Limpus 1997), potentially crossing entire ocean basins. Once they reach 20-35cm carapace length, juveniles recruit to a series of neritic habitats (Bolten 2003), accompanied by a switch from epipelagic to benthic feeding and from omnivory to herbivory (Heppell *et al.* 2003). Adult green turtles generally reside in neritic foraging grounds (reviewed in Plotkin 2003; Godley *et al.* 2008), feeding largely on sea grasses and algae (Bjorndal 1997), however, recent satellite tracking studies of post-nesting females in Japan and the Galapagos Islands suggest that some individuals spend time foraging in oceanic habitats (Hatase *et al.* 2006; Seminoff *et al*. 2008), indicating a degree of plasticity in feeding behaviour.

Green turtles reach maturity between 26-40 years of age (Seminoff 2004), after which both males and females make cyclical reproductive migrations between fixed foraging and breeding grounds that can be hundreds or thousands of kilometres apart (reviewed in Godley *et al.* 2008). Females typically migrate to nesting grounds at intervals of 2-5 years and lay an average of 3 clutches per breeding attempt, each of around 112 eggs (reviewed in Miller 1997). Females show strong fidelity to nesting sites both within and between nesting seasons, and remain in near-shore waters during the inter-nesting period (Hays *et al.* 2000; Godley *et al.* 2008). The movements of adult male turtles are less well understood, due to the difficulty of locating and accessing them at sea. Observations of flipper-tagged males in Australia suggested that male green turtles migrate to breeding grounds more frequently than females (Limpus 1993), and satellite tracking of male hawksbill (*Eretmochelys imbricata)* (van Dam *et al.* 2008), leatherback (*Dermochelys coriacea*) (James *et al*. 2005) and loggerhead turtles (Hays *et al.* 2010) demonstrate that males can return to breeding sites in consecutive years. However, robust data on male breeding frequency are lacking. Genetic analyses have confirmed that both male and female green turtles return to nest in their natal regions (natal philopatry), contributing to strong maternal genetic structure between nesting colonies, despite populations overlapping in foraging sites (Bowen and Karl 2007), although there is evidence for male-mediated gene flow between rookeries (Karl *et al.* 1992; FitzSimmons *et al.* 1997a).

Temperature-dependent sex determination and sex ratios

Sex determination in all species of marine turtles (as well as many other reptiles) is sensitive to the temperature regime experienced in the incubation environment (Temperature-dependent sex determination, or TSD (Janzen and Paukstis 1988)) (reviewed in Wibbels 2003). In turtles, developing embryos are thermally sensitive during the middle third of the incubation period and, in marine turtles, high temperatures produce female embryos whilst lower temperatures produce males (Yntema and Mrosovsky 1982). The pivotal temperature, at which a 1:1 sex ratio is produced, lies between approximately 28°C and 31°C in all species (Wibbels 2003), and is estimated to be around 29°C in green turtles (reviewed in Hawkes *et al.* 2009). Mixed sex ratios are produced within a transitional range of temperatures (TRT), above which all offspring will be female and below which all will be male (Wibbels 2003). Female-biased hatchling sex ratios are typical in marine turtles, and in some cases this bias can be extreme (>90% female; e.g. green turtle nests in northern Cyprus (Broderick *et al.* 2000) and loggerhead turtle nests in Florida (Hanson *et al.* 1998)). Estimates of natural hatchling sex ratios in green turtle populations to date range from 67-100% female (Hawkes *et al.* 2009). Freedberg and Wade (2001) propose that natal philopatry of females promotes female-biased sex ratios in reptiles, since nest sites that produce an excess of females will expand as females originating from such sites return to nest there, and themselves produce predominately female offspring.

The dynamics of sex ratios within marine turtle populations and the consequences of female-biased offspring sex ratios for the reproductive ecology of marine turtle populations are poorly understood. Data assessing the sex ratios of juvenile and adult green turtle populations are scarce, owing to the difficulties in sampling turtles at sea, but generally demonstrate a moderate female bias (reviewed in Wibbels 2003), although a male biased adult sex ratio was reported in the Great barrier Reef (Chaloupka and Limpus 2001). The female-biased hatchling sex ratios observed in marine turtle populations are predicted to be exacerbated under future climate change scenarios (Fuentes *et al*. 2010; Witt *et al.* 2010), leading to concerns that the viability of some marine turtle populations may become threatened by a lack of males. With this in mind, it is of particular importance to know whether hatchling sex ratios persist to adult life stages, and to gain an understanding of operational sex ratios (the ratio of males to females that are ready to mate in any particular breeding season (Emlen and Oring 1977)) and their influence on mating systems and fertility of marine turtle populations.

Mating system of green turtles

Courtship and mating

Despite a wealth of literature relating to females and hatchlings on the nesting beach, there are limited data describing courtship and mating under natural conditions (e.g. Booth and Peters 1972; Limpus 1993). Courtship and mating generally occur in established areas that may be directly offshore from nesting beaches (Broderick *et al.* 2003), or in regional breeding grounds, from which females disperse to a number of different nesting sites (Limpus 1993). Males have also been observed intercepting receptive females as they migrate to nesting beaches (Meylan *et al.* 1992), and genetic evidence of male-mediated gene flow among regional populations that share common migration routes suggests that some mating occurs during the reproductive migration (FitzSimmons *et al*. 1997a). Males are highly active during the breeding season (Hays *et al*. 2001) and pursue females aggressively (Booth and Peters 1972). Females can refuse copulation with courting males and demonstrate a variety of avoidance behaviours including swimming away, folding their hind flippers, circling to face the male, biting, adopting a vertical ‘refusal’ position and even beaching themselves (Booth and Peters 1972). Mating pairs are often accompanied by ‘attendant’ males that may attempt to dislodge the mounted male, and both males and females often receive injuries (Booth and Peters 1972; Miller 1997).

Mating occurs approximately one month before nesting (Owens 1980; Wood and Wood 1980). Males appear to be sexually active for around a month (Limpus 1993) whilst females are probably receptive for just 2-4 days (Booth and Peters 1972; Owens 1980; Comuzzie and Owens 1990). The majority of mating activity takes place before the peak of nesting (Booth and Peters 1972; Broderick *et al.* 2003), but Godley *et al.* (2002a) observed low levels of mating at Ascension Island well after the peak of nesting activity, which they attribute to the continued arrival of receptive females at the breeding ground. Females are thought to store sperm to fertilize all clutches within a nesting season and there is no evidence that they re-mate during the inter-nesting interval between laying successive clutches (e.g. FitzSimmons 1998; Kichler *et al.* 1999). In freshwater turtles, females also utilize sperm stored from previous breeding seasons to fertilise clutches (Pearse *et al.* 2002), but it is not known whether female marine turtles, with longer breeding intervals, are able to store viable sperm over multiple years.

Mating system

Male green turtles exhibit scramble mating tactics (Jessop *et al.* 1999); they do not defend females or resources, but competitively search for and court females. Both males and females have been observed mating multiply within a breeding season (e.g. Limpus 1993; FitzSimmons 1998), and subsequent molecular studies have confirmed that multiple paternity of clutches is common in all seven marine turtle species (Table 1). The results of these genetic analyses have revealed wide variation in the proportion of females that produce multiple paternity clutches both within and between species (Table1). For example, within olive ridley turtle populations, estimates of multiple paternity range between 20-92%, whilst in green turtle rookeries as few as 15% and as many as 100% of females produced clutches with multiple sires.

Table 1. Studies of multiple paternity (MP) to date in all marine turtle species. Modified and updated from Bowen & Karl (2007).

Species	Region	No. females	No. loci	% MP (females)	Citation
Green (*Chelonia mydas*)	Costa Rica	8	2	63	(Peare *et al*. 1998)
	Great Barrier Reef	13	5	15	(FitzSimmons 1998)
	Ascension Island	3	2	100	(Ireland *et al.* 2003)
	Ascension Island	18	2-5	61	(Lee and Hays 2004)
Loggerhead (*Caretta caretta*)	Australia	24	allozymes	33	(Harry and Briscoe 1988)
	Florida	3	2	33	(Bollmer *et al*. 1999)
	Florida	70	2-4	31	(Moore and Ball 2002)
	Greece	15	4	93	(Zbinden *et al*. 2007)
Leatherback (*Dermochelys coriacea*)	Costa Rica	4	2	0	(Rieder *et al.* 1998)
	US Virgin Islands	4	6	0	(Dutton *et al.* 2000)
	Costa Rica	20	3	10	(Crim *et al.* 2002)
	US Virgin Islands	12	7	42	(Stewart and Dutton 2011)
Olive ridley (*Lepidochelys olivacea*)	Surinam	10	2	20	(Hoekert *et al.* 2002)
	Costa Rica	13	2	92*	(Jensen *et al.* 2006)
	Costa Rica	13	2	31	(Jensen *et al.* 2006)
Kemps ridley (*Lepidochelys kempi*)	Mexico	26	3	58	(Kichler *et al.* 1999)
Hawksbill (*Eretmochelys imbricata*)	Malaysia	10	5	20	(Joseph and Shaw 2010)
Flatback (*Natator depressus*)	Australia	9	4	67	(Theissinger *et al.* 2009)

* Arribada rookery

Within species, there appears to be a relationship between female breeding population size/ density and incidence of multiple paternity, with multiple paternity occurring at a greater frequency in higher density populations (Ireland *et al.* 2003; Jensen *et al.* 2006), although there are notable exceptions to this pattern (FitzSimmons 1998; Zbinden *et al.* 2007). However, there are many additional factors that are likely to influence the level of multiple mating in marine turtle breeding populations, including operational sex ratio, the costs of mating and of resisting mating, or potential benefits of multiple mating, which have not been fully investigated

There is evidence from diverse taxa that polyandrous females can gain fitness benefits (Jennions and Petrie 2000; Hosken and Stockley 2003; Simmons 2005). In theory, female green turtles could receive a number of direct or indirect benefits from mating with multiple males (reviewed in Uller and Olsson 2008), potentially explaining the high levels of multiple paternity observed in some populations. However, in spite of a growing number of paternity studies in all marine turtle species, very few have investigated the potential fitness consequences of polyandry and, to date, none have revealed any clear benefits to polyandrous females (Lee and Hays 2004; Zbinden *et al.* 2007). Studies focusing on male mating behaviour and male mating success in marine turtles are scarce (Limpus 1993), however, molecular parentage analyses have provided information on relative siring success of different males within clutches (e.g. Ireland *et al*. 2003; Lee and Hays 2004; Jensen *et al*. 2006; Stewart and Dutton 2011), and, in one case, revealed genetic evidence of polygyny (Crim *et al.* 2002) (i.e. the same male genotype appeared in the clutches of more than one female), which supports observations of males mating with multiple females in the wild (Booth and Peters 1972; Limpus 1993). No studies of genetic paternity in marine turtles have directly sampled males, so investigations into male mating success in relation to male traits (e.g. size) have not been possible, although studies have been carried out in freshwater species (Pearse *et al.* 2002).

The genetic structure of breeding assemblages is poorly understood in marine turtles. As noted above, natal philopatry has been documented in both genders based on maternally inherited mitochondrial DNA (mtDNA) haplotypes, however, in males this behaviour has only been investigated in a single population of green turtles (FitzSimmons *et al.* 1997b). Nuclear DNA analyses have revealed a contrasting pattern of male-mediated gene flow between nesting populations; it is assumed that nuclear gene flow is most likely occurring where adult populations overlap in feeding grounds or shared migratory corridors on route to natal breeding areas (FitzSimmons *et al.* 1997a; Bowen and Karl 2007), but natal homing behaviour in male turtles may also be less precise. Although there are reports of male turtles showing strong fidelity to breeding sites (Limpus 1993; James *et al.* 2005), when males return to small nesting colonies where mating opportunities may be scarce, male turtles might maximise their reproductive success by visiting multiple rookeries in search of additional receptive females, as suggested by a satellite tracking study of male leatherbacks migrating to breeding grounds in the Caribbean (James *et al.* 2005). It is also conceivable that males produced on cooler (potentially higher latitude) nesting beaches contribute to reproduction in non-natal rookeries that produce predominantly female offspring, and may be important to the fertility and persistence of such rookeries (Heppell *et al.* 2003).

Molecular ecology of marine turtles

Marine turtles are long-lived, highly migratory and spend the vast majority of their lives in the ocean, making direct field studies of many aspects of their behaviour and ecology very difficult. Molecular genetic studies have been employed to address some of the gaps in our knowledge and the genetic perspective has shed light on numerous features of their life history, behaviour and evolution that are essential to their effective conservation (reviewed in Avise 2007; Bowen and Karl 2007). For example, mtDNA analyses have revealed a complex population structure, whereby nesting populations show strong matrilineal spatial structure, but juveniles from distinct rookeries overlap extensively on ‘mixed-stock’ feeding grounds (Bowen and Karl 2007). Mixed-stock genetic analyses allow the natal origin of juveniles to be assessed, and can highlight feeding areas or migration routes along which juveniles from multiple rookeries may be vulnerable to exploitation or fisheries interactions. As outlined above, genetic studies of paternity have also provided substantial insights into marine turtle breeding behaviour that would be have been impossible to obtain through direct observations. The body of literature utilizing molecular data in marine turtle studies is rapidly expanding and there are several excellent, recent reviews of this work, addressing population genetics, phylogeography, hybridisation, mating behaviour and conservation genetics of marine turtles (Avise 2007; Bowen and Karl 2007), as well as the current molecular approaches relevant to marine turtle studies (Lee 2008). Below, I briefly describe the genetic methods that have been used to study paternity in marine turtle populations to date.

Microsatellite DNA markers

The most popular genetic markers for use in paternity analyses are microsatellites; short tandem repeats of 1-6 nucleotides (base pairs, or bp) found throughout the nuclear genomes of most taxa (Goldstein and Schlotterer 1999). The number of repeats of the core 1-6bp sequence is highly variable between individuals, due to a high mutation rate of microsatellite loci, which alters the number of repeats and, therefore, the length of the tandem sequence (Eisen 1999). A single microsatellite locus varies in length, typically between 5 and 40 or more repeats (Selkoe and Toonen 2006). The microsatellite locus is flanked at either end (flanking regions) by DNA sequences that are usually highly conserved within species and sometimes between closely related species (Selkoe and Toonen 2006). Primers (short sequences of single-copy DNA, or oligonucleotides) are designed to bind to the flanking region (and are generally species-specific), and amplify the microsatellite locus within a polymerase chain reaction (PCR). Alleles of differing lengths can then be resolved by gel electrophoresis or in an automated DNA sequencer.

The highly variable nature of microsatellites means that they are often informative even in small or recently bottlenecked populations. Provided that there is some polymorphism at the chosen microsatellite loci within the population, combining data from multiple loci can provide a unique multi-locus genotype for every individual analysed, thereby offering a robust and statistically powerful means of comparing individuals and populations (Selkoe and Toonen 2006). The number of microsatellite loci needed to resolve paternity of a group of offspring depends on the variability of each microsatellite locus within the population, the size of the offspring sample (Neff and Pitcher 2002; Wang and Santure 2009) and whether the aim of the study is to simply detect the occurrence of multiple paternity within a clutch, or to assign offspring to individual sires (for which greater power is required) (Lee 2008). To date, seven or fewer microsatellite loci have been used to examine genetic paternity in marine turtles (Table 1), however, the use of larger numbers of loci would increase confidence in paternity and sib-ship assignments, and allow more precise reconstruction of inferred paternal genotypes (see below) (Lee 2008). Until recently, paternity studies were limited by the small number of microsatellite loci available, however, in the past few years, a host of new markers have been developed for marine turtles (e.g. Miro-Herrans *et al.* 2008; Dutton and Frey 2009; Roden and Dutton 2010). A search of the European Molecular Biology Laboratory (EMBL) nucleotide sequence database (http://www.ebi.ac.uk/embl/ accessed April 2012) listed 138 published microsatellite loci across six species. Furthermore, the flanking regions of microsatellite loci are highly conserved among marine turtle species (FitzSimmons *et al.* 1995), hence, existing primers often successfully amplify the same microsatellite locus in all species.

Parentage analysis

In diploid organisms microsatellite alleles are inherited bi-parentally, one copy from each parent. In studies of paternity in marine turtles, blood or tissue samples are generally collected from mothers and offspring whilst on the nesting beach, and their genotypes at multiple microsatellite loci can be determined directly. Males are difficult to access and rarely sampled, but their genotypes can be inferred from the offspring genotypes; maternal genotypes are compared with offspring genotypes and, once the maternal alleles have been accounted for, paternal alleles can be identified at each locus (Avise 2007; Lee 2008). A crude estimate of minimum number of fathers contributing to a clutch of offspring can be estimated by counting the number of paternal alleles present; each father can potentially contribute two different alleles, so three or more paternal alleles at any one locus indicates multiple paternity of the clutch. In practice, information from multiple loci is used to infer multiple paternity, since genotyping error or mutation could produce an unexpected paternal allele at one locus (FitzSimmons 1998). The probability of detecting multiple paternity within a clutch increases with the number of offspring that are genotyped, as this increases the chance that all paternal alleles are represented (Wang 2004, Wang and Santure 2009). However, marine turtle clutches are large and it is not necessary to sample them to completion; the statistical power to detect multiple paternity can be assessed based on the number of offspring sampled and the number and variability of the loci used (Neff and Pitcher 2002).

In order to accurately determine the number of fathers contributing to clutches, to assign offspring to specific fathers, and to reconstruct paternal genotypes, multiple microsatellite loci are needed and a more sophisticated statistical approach is required to analyse the microsatellite data. Accompanying the rapid advances in the availability and application of genetic markers, there has been a proliferation in statistical methods to analyse genetic data for pedigree information, implemented in a variety of software packages (reviewed in Blouin 2003; Jones and Ardren 2003). Not all of these approaches are suitable for analysing the marker data generally available in marine turtle studies, where mothers and groups of siblings or half-sibs are sampled but there is no marker information for fathers. For example, the allocation approach employed in the program CERVUS (Marshall *et al*. 1998) determines the likelihood that a particular individual is the parent of a given offspring based on similarities of their multilocus genotypes, requiring genotypic information for at least some candidate fathers. The majority of paternity studies in marine turtles to date have used the program GERUD (Jones 2005), which determines the minimum number of fathers from a group of full and/or half-sibs and reconstructs the parental genotypes based on segregation of paternal alleles and their deviation from Mendelian expectations, and does not require prior information about the genotypes of either parent. However, the program is not able to assign offspring to specific fathers, cannot cope with missing data at any loci, and does not incorporate error. This final point is of importance because genotyping errors are common, even when steps have been taken to avoid or identify them (Bonin *et al*. 2004; Selkoe and Toonen 2006), and can strongly affect parentage inference; a single typing error in a multi-locus genotype can result in a correct relationship being rejected (Wang 2004). Furthermore, because GERUD is generally used to analyse a single clutch of offspring at a time, valuable information about other sibling relationships is lost, for example paternal half-sibs arising from a male mating with more than one female would not be identified.

An alternative approach to infer parentage is by grouping offspring into full-sib and half-sib families, as implemented using a maximum-likelihood method in the program COLONY (Wang 2004; Wang and Santure 2009). COLONY considers all sampled offspring together, partitions them simultaneously into full-sib and half-sib groups, assigns candidate parents to sibling families and infers the genotypes of unknown parents (fathers). The program is robust to missing data, allows for genotyping error, and has no limit (aside from computing power) to the number of offspring included or the amount of marker information (number of loci), resulting in greatly improved statistical power to infer paternity (Wang and Santure 2009). I have used COLONY version 2.0 (Wang and Santure 2009) to infer sibship and parentage relationships throughout this thesis, enabling me to simultaneously analyse large numbers of offspring samples collected over multiple breeding seasons and identify both maternal and paternal half-sibship, as well as full-sibship, both within and between years. This information has allowed me to comprehensively assess mating patterns within a focal green turtle population and has shed light on aspects of behaviour and life history that have not previously been adequately addressed.

Aims and objectives

The over-arching aim of this thesis was to combine molecular-based parentage analyses with long-term nesting data to improve our understanding of the mating system of green turtles. I aimed to capitalise on the recent increase in the number of polymorphic genetic markers available for use in marine turtles, and developments in parentage and sibship assignment methods, to provide robust data on paternity and sibling relationships. Using this information, I address some of the gaps in our knowledge of marine turtle breeding ecology. Specifically, male mating behaviour is poorly understood and the number of males contributing to breeding populations is generally unknown. I aim to provide data on male mating frequency and sex ratios of breeders, which will contribute to more accurate assessments of population viability. Additionally, although polyandry has been widely documented in marine turtles, few studies have attempted to assess the fitness consequences of polyandrous behaviour. I aim to assess potential fitness benefits that female marine turtles might receive from mating with multiple males.

Study sites

The majority of research included in this thesis was conducted at Alagadi beach, northern Cyprus, a major nesting site for green turtles in the Mediterranean. The green turtle population in the Mediterranean is considered to be at high risk of extinction (Wallace *et al.* 2011); there are approximately 900-1200 adult female green turtles remaining, of which around 100 nest at Alagadi beach (Broderick *et al.* 2002). The small size of the rookery enables comprehensive monitoring of nesting activity; every female is marked and sampled for genetic analysis, and every clutch is recorded and assigned to the relevant female. Females nest on average every three years at this site, so, by conducting fieldwork over three consecutive breeding seasons, I was able to capture and determine paternity of offspring for a large proportion of the nesting population.

Data collection for **chapter five** was conducted at Ascension Island. In contrast to Alagadi beach, the nesting population of green turtles at Ascension Island is large (11-15000 adults females) and expanding (Broderick *et al.* 2006). Consequently, only a small proportion of the nesting population was included in my analysis. Both populations have a female-biased sex ratio of hatchlings, although this is more pronounced at Alagadi beach (Ascension Island: 75% female (Godley *et al.* 2002b); Alagadi: >90% female (Broderick *et al.* 2000)), but the adult sex ratios are unknown. Both populations have been subject to historical harvest (Sella 1982; Broderick *et al.* 2006) but, whilst the Ascension Island population appears to be rapidly recovering, the Mediterranean green turtle population remains depleted.

Thesis format and structure

The remaining chapters presented in this thesis are written as discrete units of study, two of which have been published in peer-reviewed journals, and two have been submitted in their current form. As a result, there is some repetition between chapters, particularly in the methods sections and literature cited. The data chapters are presented in the format of manuscripts, as they were submitted for publication.

In **chapter two** I present the results of my first year of study at Alagadi beach, northern Cyprus. I use molecular-based paternity analysis, including near-saturation coverage of nesting turtles within a single breeding season, to assess the genetic mating system and determine the number of males contributing to reproduction at this site. The chapter is written within the framework of the predicted effects of climate warming in species with temperature-dependent sex determination systems, and argues that male mating patterns currently shown in our study population help to maintain relatively equal operational sex ratios despite highly female-biased hatchling sex ratios. In **chapter three** I explicitly aim to address the question of male mating periodicity, which has not previously been determined in any green turtle population. I use paternity analysis and paternal genotype reconstruction to identify males that sired offspring across three consecutive breeding seasons, and infer their breeding frequency at the study site. With this extended dataset I also build upon the results in **chapter two** and more accurately assess male mating patterns and the sex ratio of breeding individuals in the population.

In **chapters four** and **five** I turn my attention to female mating strategies. In **chapter four** I assess the frequency of multiple paternity in green turtle clutches laid over multiple breeding seasons at Alagadi beach, and explore the relationship between polyandry and components of female reproductive success and offspring fitness. The frequency with which multiple paternity occurs in nesting populations of marine turtles varies widely between species and populations. In order to provide comparative data from a different nesting population, in **chapter five**, I investigate levels of multiple paternity of clutches at the green turtle rookery on Ascension Island, which is an order of magnitude larger than the focal north Cyprus rookery. I discuss a number of factors that potentially influence levels of polyandry in marine turtle populations. Finally, I summarise my results from all four data chapters in **chapter six**, outline the contribution that they make to our knowledge of marine turtle mating systems, and discuss their potential relevance to conservation of marine turtle populations.

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CHAPTER 2

Turtle mating patterns buffer against disruptive effects of climate change

Lucy I Wright[a], Kimberley L Stokes[a], Wayne J Fuller[b], Brendan J Godley[a], Andrew McGowan[a], Robin Snape[a, c], Tom Tregenza[a], Annette C Broderick[a, 1]

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[a] Centre for Ecology and Conservation, University of Exeter, Cornwall Campus, Penryn, TR10 9EZ, UK.

[b] Marine and Fisheries Research Institute, European University of Lefke, Gemikonagi, Lefke, North Cyprus, Mersin 10, Turkey.

[c] North Cyprus Society for Protection of Turtles, PK65, Kyrenia, North Cyprus, Mersin 10, Turkey.

[1] Corresponding author: Dr Annette Broderick

E-mail: A.C.Broderick@exeter.ac.uk

SUMMARY

For organisms with temperature-dependent sex determination (TSD) skewed offspring sex ratios are common, however, climate warming poses the unique threat of producing extreme sex ratio biases that could ultimately lead to population extinctions. In marine turtles, highly female skewed hatchling sex ratios already occur and predicted increases in global temperatures are expected to exacerbate this trend, unless species can adapt. However, it is not known whether offspring sex ratios persist into adulthood, or whether variation in male mating success intensifies the impact of a shortage of males on effective population size. Here we use parentage analysis to show that in a rookery of the endangered green turtle (*Chelonia mydas*), despite an offspring sex ratio of 95% females, there were at least 1.4 reproductive males to every breeding female. Our results suggest that male reproductive intervals may be shorter than the 2-4 years typical for females, and/or that males move between aggregations of receptive females, an inference supported by our satellite tracking, which shows that male turtles may visit multiple rookeries. We suggest that male mating patterns have the potential to buffer the disruptive effects of climate change on marine turtle populations, many of which are already seriously threatened.

Key words

Marine turtle, temperature-dependent sex determination (TSD), climate change, sex ratio, mating patterns, *Chelonia mydas*.

INTRODUCTION

Understanding and predicting how climate change impacts species with temperature-dependent sex determination (TSD) is critical for their conservation. In these species, sex is determined by the temperature regime experienced during embryonic development and even small temperature changes can produce offspring sex ratios that are heavily biased [1, 2]. Many populations of species with TSD already exhibit offspring sex ratios skewed towards the sex produced at warmer temperatures e.g. males in tuatara [3] and females in marine and freshwater turtle populations [4, 5]. Future climate change scenarios are predicted to increase these sex ratio biases with implications for population viability [2, 3, 6, 7]. Potential consequences include a reduction in effective population size (N_e) that will exacerbate the negative effects of inbreeding and increase genetic drift in small populations [8], the inability to find mates leading to reduced fecundity or female infertility [9], and, under more extreme climate projections, the production of single sex cohorts [3, 7].

In principle, TSD species could adapt to a warming climate through various mechanisms including evolution of the pivotal temperature (at which 50% of either sex is produced) and/or transitional range of temperatures at which there is a mixed sex ratio, and behavioural change in nesting phenology or nest site selection [2, 10]. Although maternal nest site choice has been shown to compensate for geographical differences in nest temperature in a lizard [11], and marine turtles have shown climate-related phenological nesting responses [12] (but see [13]), evidence suggests that these mechanisms may not be adequate to compensate for climate effects on sex ratio, especially in long-lived and late-maturing reptiles [10]. For example, the evolution of nest site choice and threshold temperature (above which female offspring are produced) are predicted to be slow in response to climate warming in a freshwater turtle, and unlikely to effectively offset sex ratio bias resulting from rapid climate change [10]. Furthermore, earlier nesting by females, due to individual plasticity in the timing of first nesting, is predicted to have a modest compensatory effect on offspring sex ratios compared to the perturbing effect of even small increases in summer temperature [14]. Although extant species with TSD have clearly survived and responded to substantial historical temperature fluctuations [15], and have adapted to geographical temperature variation (e.g. [11, 16]), it is uncertain whether they will be able to keep pace with anticipated rates of future climate change.

All species of marine turtles have TSD, with females being produced at higher temperatures, males at lower temperatures and 50% of either sex at around 29°C (reviewed in [4]). Hatchling sex ratios biased towards females are typical and in all green turtle populations studied to date, estimates of hatchling sex ratios range from 67-100% female (reviewed in [4]). The few data available regarding juvenile and adult sex ratios in marine turtles suggest that the female biases seen at hatching are maintained at older life stages (e.g. [17, 18] but see [19]), hence, a climate induced increase in female biased primary sex ratios could threaten the viability of marine turtle populations through a reduction in N_e and associated genetic effects, and potential reproductive failure due to scarcity of males. These consequences are expected to be particularly deleterious in small populations, where a reduction in the number of males could conceivably drop below a critical minimum required to maintain a fertile population (e.g. [9]), and in areas where incubation temperatures already result in extremely female-biased offspring production [7]. Larger populations, and those that encompass rookeries at nesting range extremes where more males are produced, may be more robust to offspring sex ratio skews [4], except under the most extreme climate-change scenarios. Any reductions in N_e due to lack of males will be further intensified if, as seen in most animals, variation among individuals in reproductive success results in only a small proportion of the available males siring most of the offspring in subsequent generations.

There is currently a scarcity of information regarding mating behaviour in male turtles. Operational sex ratios are poorly understood [7] (but see [20] for information relating to OSR) and data on the reproductive success of individual males are lacking in marine turtle species, despite the influence of these parameters on population dynamics and N_e [8, 21]. These gaps in our knowledge currently prevent an accurate evaluation of the potential impacts of climate change on marine turtle populations [4]. To clarify whether the mating patterns of marine turtle populations increase or decrease their vulnerability to climate change and better understand the male contribution to the gene pool, we determined the number of males successfully breeding in a green turtle rookery in northern Cyprus, that consists of approximately 100 nesting females [22] and already exhibits a highly female-skewed hatchling sex ratio (86-96% female [23]). There are an estimated 300-400 female green turtles nesting annually in the Mediterranean, with nesting restricted to the eastern basin, occurring mostly in Cyprus and Turkey [22]. The proximity of nesting areas in Cyprus and Turkey likely results in similar incubation temperature regimes and indeed other major Mediterranean rookeries also produce highly female biased hatchling sex ratios (e.g. 92% female at Akyatan, Turkey [24]). We hypothesised that the sex ratio of breeding adults in our study rookery would, therefore, also be female biased, with fewer males than females contributing to reproduction.

Unlike female marine turtles, males rarely come ashore and the difficulty in catching them at sea limits access to them. We have overcome this problem by intensively sampling and genotyping mothers and offspring and employing sibship reconstruction and parentage inference methods to estimate the number of males successfully siring offspring and detect any skew in male reproductive success that might further reduce N_e. In addition, we report satellite tracking results from our study site that provide new insights into male mate searching behaviour and lend support to the inferences drawn from our parentage assignments.

MATERIALS AND METHODS

The study was conducted in a wild population of green turtles at Alagadi beach, northern Cyprus during the 2008 breeding season (May-October). Tissue samples were taken from 20 nesting females of known identity (representing 91% of females that successfully nested at this site in 2008) and up to 23 (mean ± sd = 21.9 ± 1.55, range 15-23) offspring from one or more clutches per female. The final dataset was comprised of 809 offspring from 37 clutches. Offspring sex was estimated for the 2008 nesting season from incubation durations (as previously described in [23]) and includes all clutches that successfully hatched at the site in 2008.

(a) Genotyping

Genomic DNA was extracted from tissue samples using a standard ammonium acetate precipitation method [25]. Samples were genotyped at 14 polymorphic microsatellite loci designed for use in sea turtles (Table 1). Primers were labelled with fluorescent dyes (6-FAM, HEX or NED). PCR amplification was carried out in an MJ Research model PTC DNA Engine Tetrad thermal cycler according to the following schedule: 95°C for 15min followed by 35 cycles of 94°C for 30s, 58°C for 90s and 72°C for 60s, and finally one cycle of 60°C for 30min. 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). Samples that failed to amplify at all loci, or which displayed unexpected alleles were re-amplified and re-scored. Any samples that still failed to amplify at a minimum of 8 loci were excluded from further analyses, however, multilocus genotypes were complete at all 14 loci for >90% of individuals.

(b) Characterisation of microsatellite loci

Samples from 60 adult turtles (all collected from females that nested at the study site between 2007 and 2009) 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 [26]. Linkage disequilibrium between all pairs of loci was tested using a Markov chain method in GENEPOP version 4.0.10 [27]. Probability of detecting multiple paternity was assessed using PrDM software [28] (downloaded 2009), assuming both equal and skewed (10:90%) paternal contributions to clutches.

(c) Paternity analysis

Sibship and parentage inference were carried out in COLONY version 2.0 [29] assuming an error rate of 0.01 for allelic dropout and 0.02 for genotyping error. COLONY uses a maximum likelihood model to assign sibship and parentage relationships. Individuals are clustered based on full-sib, half-sib (maternal and paternal) and parent-offspring relationships, candidate parents are assigned to full-sibling groups and genotypes of unknown parents are inferred [29, 30]. Ten replicate runs of ‘medium’ length were conducted on the same dataset. Each of the 10 replicate runs used different random number seeds to initiate the simulated annealing process. Maternity of all offspring was known. All genotyped offspring were analysed together in order to identify any paternal half-sibs, which would indicate males that sired offspring with more than one female.

(d) Satellite tracking

In 2009 a satellite transmitter (KiwiSat 101, Sirtrack, New Zealand) was attached to a male turtle from the same study site according to previously described protocol [31]. The male was tracked via satellite for 81 days. Locations were obtained using the Argos system and Satellite Tracking and Analysis Tool (STAT) [32], and mapped in ArcGIS 9.3.1. (for full details see electronic supplementary material).

RESULTS

(a) Offspring sex ratio

Incubation durations of clutches in 2008 ranged from 43-59 days (mean 48.3 ± 3.48 (sd), n=57) with only 3 clutches exceeding the pivotal incubation duration (at which 50:50 sex ratio is found) of 56 days [23]. Based on incubation durations, using the methodology of Broderick *et al*. [23], we estimate that the overall offspring sex ratio at this site in 2008 was 95% female.

(b) Paternity analysis

Parentage analysis of >800 offspring revealed that a minimum of 28 unique males sired offspring from 20 nesting females, demonstrating an unexpected sex ratio of breeders of at least 1.4 males to each female (Fig. 1). There was a high degree of convergence between the ten COLONY runs (Supplementary Table 1 – see electronic supplementary material). All runs identified 20 family clusters, all of which contained a single mother and all her offspring with one or multiple fathers (i.e. all offspring in the clusters were full sibs or maternal half-sibs). Because the number of clusters was equal to the number of females included in the analysis, and no paternal half-sibs were identified, the results indicate that no male sired offspring across females. The analysis revealed 28-30 full sibling families and the total number of unique fathers contributing offspring to these full-sibling groups also ranged from 28 -30 across the ten runs, with 28 fathers being most likely in five runs.

30% (6 out of 20) of females in this study produced clutches with multiple paternity (Supplementary Table 2 - see electronic supplementary material), but interestingly, we found no evidence that any single male sired offspring with more than one female at this rookery. This is evidenced by the lack of paternal half-sibs in the COLONY analysis, despite near complete sampling (>90% of females that successfully nested at Alagadi in 2008 were included in the study).

(c) Characterisation of microsatellite loci

All loci conformed to expectations of Hardy-Weinberg equilibrium ($P>0.05$), showed low probability of null alleles and showed no evidence of genotypic linkage disequilibrium after correction for multiple tests [33]. Combined exclusion probability (second parent) for all 14 loci was >0.99, and probability of detecting multiple paternity, assuming 2 fathers with skewed paternal contributions (10:90%) and 20 offspring sampled per clutch, was 0.876.

(d) Satellite tracking

The male turtle tracked from the study site travelled in proximity to (< 20 km) multiple nesting beaches in Cyprus and Turkey before travelling to North Africa (Fig. 2), in a pattern consistent with mate searching behaviour (see electronic supplementary material). The breeding sites along the route account for 58% of green turtle nesting in the Mediterranean according to maximum nest numbers taken from the literature [22, 34-36].

DISCUSSION

Our finding that more males than females contributed to reproduction in this study was contrary to our expectations, considering the extremely female-skewed hatchling sex ratio reported at this rookery (this study, [23]).Operational sex ratios (OSRs) [37] depend not only on adult sex ratio but also the potential reproductive rate of each sex

[38]. Our results might reflect more frequent breeding periodicity in males than females, resulting in sex ratios of adults on breeding grounds that are much less female biased than would be expected based on offspring sex ratios alone, as recently demonstrated in loggerhead turtles (*Caretta caretta*) [39]. A higher breeding frequency of males compared to females would help to explain the persistence of female-biased populations, by ensuring mate finding and the maintenance of marine turtle fertility even at low population size [40]. More frequent breeding by males will not, however, ameliorate the effects of low $N_{e,}$ and populations with few males will still suffer negative effects of inbreeding and loss of genetic variation. Alternative explanations for our results may be that females are able to store sperm from previous breeding seasons to produce viable offspring, as has been recorded in freshwater turtles that breed annually [41], or that there are sex differences in mortality rates leading to an adult sex ratio that does not reflect that seen at primary life stages.

Additionally, some of the males breeding at our study site may originate from (thus far unidentified) rookeries elsewhere in the Mediterranean that produce more balanced offspring sex ratios, although nesting in this population is limited to the eastern Mediterranean where nest incubation temperatures are likely similar to or higher than those in Cyprus. Natal philopatry is a central life history component in marine turtles and has been documented in both males and females, although the precision with which either sex returns to natal sites is not clear [42]. Lower levels of genetic divergence at nuclear compared to mitochondrial DNA (mtDNA) markers is consistent with male-mediated gene flow between rookeries and suggests that males are more plastic in their philopatric behaviour [43]. Additionally, although courtship and mating is thought to occur close to the nesting beach at this colony [44], in some populations mating occurs at regional courtship areas from which females disperse to nesting rookeries that can be distant from the mating site, hence males are associated with regional nesting populations rather than specific rookeries [20].

Our satellite tracking of a post-breeding male turtle from our study site in 2009 supports the conjecture that males might mate at multiple breeding grounds. In contrast to post-breeding female turtles, which travel from this site directly to foraging grounds in Turkey, Syria or North Africa [45], the male took a 348km diversion to Turkey before travelling to the North African coast, passing in proximity to multiple green turtle nesting beaches [22, 34-36]. The exceptional navigational abilities of marine turtles are well documented [46] and tentatively suggest that the observed detour was strategic. Mating activity in marine turtles can overlap significantly with the nesting season [47] and males are typically sexually active for a period of around one month [20]. Given the seasonality of nesting in the Mediterranean [22], it is possible that the male tracked in this study was seeking receptive females both in Cyprus and Turkey. It is not known whether there is maternal population structure among the major nesting sites for this species in the Mediterranean. In addition to ensuring mate finding and maintaining equal OSRs on mating grounds, if aggregations of nesting females are distinct (in terms of mtDNA), then the movement of males between breeding grounds will contribute to nuclear gene flow between rookeries and reduce inbreeding and loss of genetic variation that would occur if very small numbers of males were reproducing at each breeding site.

Although polyandry has been documented across marine turtle species (reviewed in [48]), and was recorded in 30% of females in this study, the observation that males only sired offspring with a single female at the Alagadi rookery (Fig. 1) is surprising and is in contrast to observations of polygynous behaviour at other green turtle breeding grounds [20]. In aggregate breeding systems with no parental care, regardless of whether or not males defend females or resources, multiple mating opportunities for both males and females are expected [37]. It is clear that males may have mated with additional females at other breeding sites, however, the genetic mating pattern observed at this rookery allows more males to participate in reproduction compared to a strictly monogamous system [21, 49], further contributing to the maintenance of genetic variation in this population.

Whilst previous work has focused on the potential of plasticity in maternal nesting behaviour to counter the sex ratio biases induced by climate warming [10, 11] this study highlights the role of mating behaviour in maintaining relatively equal operational sex ratios despite highly female biased hatchling sex ratios. Whether the breeding pattern observed at this nesting site results from males breeding more frequently than females, males moving between aggregations of receptive females, or from other processes leading to a more equal OSR than expected based on offspring sex ratios, warrants further investigation due to the potential implications for $N_{e.}$ It is important to note that the sex ratio of breeders observed in this study reflects hatchling sex ratios approximately 30 years ago (due to late age at maturity in green turtles, reviewed in [50]), however, sea surface temperatures at this site have risen by, on average, less than 1°C over the past 50 years suggesting a female bias in offspring produced at that time [7]. Nonetheless, future adult sex ratios could be much more female biased than at present. Current mating patterns will help to preserve genetic variation that may be critical if marine turtles are to adapt behaviourally or physiologically to a warming climate and have, no doubt, contributed to their persistence through historical climatic upheaval.

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

locus	original source for locus development	n	no. alleles	H_O	H_E
A6	[51]	60	5	0.733	0.711
B103	[51]	60	5	0.617	0.702
B123	[51]	59	5	0.627	0.636
C102	[51]	60	5	0.550	0.550
D105	[51]	60	8	0.683	0.784
D2	[51]	59	10	0.797	0.733
Cm3	[52]	60	7	0.550	0.523
Cm58	[52]	60	7	0.833	0.806
Klk314	[53]	60	4	0.433	0.491
Or7	[54]	60	5	0.717	0.656
Cc2	[55]	60	8	0.717	0.759
Cc28	[55]	60	4	0.650	0.716
CcP7D04	[56]	59	8	0.847	0.794
CcP7E11	[57]	60	4	0.550	0.496

(H_O, observed heterozygosity; $H_{E,}$ expected heterozygosity)

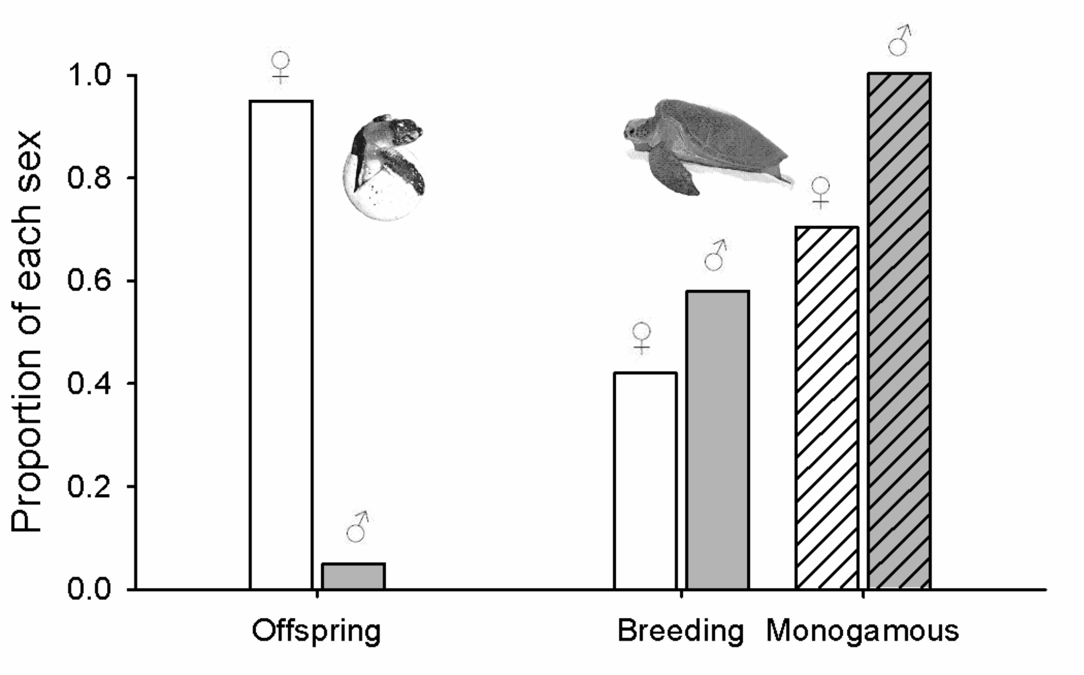

Figure 1. Sex ratios and monogamy of green turtles. Proportion of female (un-shaded) and male (shaded) offspring and breeding adults for the 2008 nesting season at our study site. Also shown (hashed) are the proportion of breeding adults that were monogamous at the study site, i.e. the proportion of females that had a single sire for their offspring and the proportion of males that only sired offspring from one female at this rookery (Note: use of the term monogamy in this figure relates only to the Alagadi rookery; males may have mated with additional females at other nesting sites within the wider Mediterranean population). Offspring sex is estimated from incubation durations [23] and includes all clutches that hatched successfully at this site in 2008.

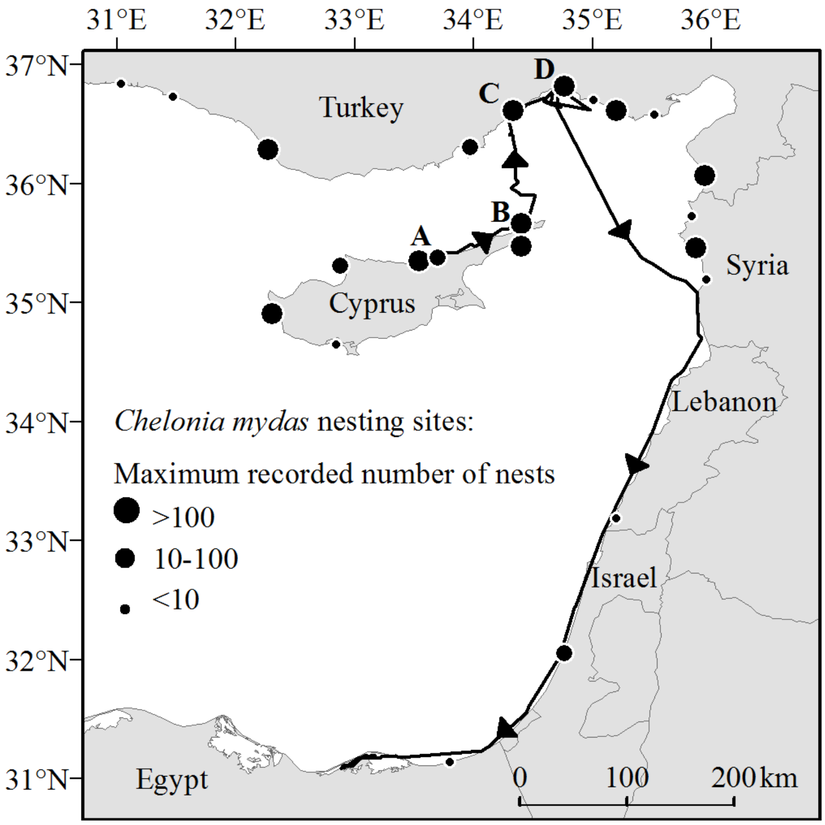

Figure 2. Route of adult male green turtle from Cyprus. Route of an adult male turtle that was released post-breeding at Alagadi Beach, Cyprus and tracked to Egypt via the Turkish coast. Major green turtle nesting beaches along the route are labelled (A-D): A; Alagadi and the Cyprus North beaches 8-9th June. B; North Karpaz beaches 10-11th June. C; Alata 14th June. D; Kazanlı 15-26th June. Data for nesting numbers are taken from the literature [22, 34-36].

ELECTRONIC SUPPLEMENTARY MATERIAL

Satellite tracking methods and results

In 2009 a satellite transmitter was attached to a male turtle from the same study site and the male was tracked via satellite for 81 days. The route was reconstructed using data of Argos location classes 3, 2 and 1 (estimated errors of less than 1km). A standard filter was applied to exclude locations inferring implausible speeds (>5 km h^{-1}) and turning angles (<25^o) (Witt *et al*., 2010). Minimum speeds were calculated assuming straight line travel between transmissions. From Alagadi beach the turtle travelled east along the north coast of Cyprus, passing a smaller nesting rookery to another major nesting site. Although minimum speeds averaged 1.5 km h^{-1} along the coast, there was a demonstrable slowing to 0.7 km h^{-1} at this second major rookery. The turtle undertook his first pelagic crossing to reach the Turkish coast where he spent 12 days travelling at low speeds (average 0.6 km h^{-1}) within Mersin Bay in proximity to (< 20 km) the nesting sites of Alata, Kazanlı and Akyatan, the latter being the single most important rookery for the Mediterranean population of green turtles, in a pattern consistent with mate searching behaviour. The turtle embarked on a second pelagic crossing to reach Syria, then followed the Eastern Basin coastline to the Bay of Tinah, Egypt, where he remained for a further 43 days of transmission at low speeds (minimum average 0.1 km h^{-1}). The diversion to Turkey took a total of 19 days, adding 348 km to the journey, and increased the total journey length by 44% when compared to a beeline route (taken from the last location at the north eastern tip of Cyprus to the point at which the turtle met the Syrian coast).

Supplementary References

Witt, MJ, Åkesson, S, Broderick, AC, Coyne, MS, Ellick, J, Formia, A, Hays, GC, Luschi, P, Stroud, Stedson, Godley, BJ, 2010 Assessing accuracy and utility of satellite-tracking data using Argos-linked Fastloc-GPS. *Anim. Behav*. **80**, 571-581

Supplementary Table 1: Summary of results from ten COLONY analyses. (Family clusters contain individuals that are related directly or indirectly via sibship or shared parentage. Individuals between clusters are unrelated [29]. In our analysis all clusters contained a single mother and all her offspring from one or multiple inferred fathers. See Results).

COLONY run	number of mothers	number of family clusters	number of full-sib families	total number of unique fathers
1	20	20	28	28
2	20	20	28	28
3	20	20	28	28
4	20	20	28	28
5	20	20	28	28
6	20	20	29	29
7	20	20	29	29
8	20	20	29	29
9	20	20	29	29
10	20	20	30	30

Supplementary Table 2: The number of paternal alleles at each locus and number of inferred fathers per clutch. (Multiple paternity is inferred when there are more than 3 paternal alleles at a locus (shown in bold). For some clutches the number of inferred fathers differed across the ten COLONY runs; we show minimum and maximum numbers of inferred fathers per clutch, but only conclude multiple paternity when all runs detect at least 2 fathers).

mother id	**clutch id**	**n[1]**	**number of paternal alleles at each locus**														**min. inferred fathers**	**max. inferred fathers**
			A6	**CC28**	**CCP7-D04**	**CCP7-E11**	**CM3**	**D105**	**D2**	**OR7**	**B103**	**B123**	**C102**	**CC2**	**CM58**	**KLK-314**		
G001	G001.1	23	2	2	1	1	2	2	2	1	2	2	2	2	1	2	1	1
	G001.3	22	2	2	1	1	2	2	2	1	2	2	2	2	1	2	1	1
G008	**G008.1**	**23**	**2**	**3**	**3**	**2**	**1**	**2**	**3**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**
	G008.3	23	1	2	2	2	1	2	2	1	2	2	2	2	2	2	1	1
G048	**G048.1**	**22**	**4**	**3**	**5**	**3**	**2**	**4**	**5**	**3**	**2**	**3**	**3**	**3**	**3**	**2**	**3**	**3**
	G048.2	**23**	**4**	**3**	**4**	**3**	**1**	**4**	**5**	**3**	**2**	**3**	**3**	**3**	**3**	**2**	**3**	**4**
G056	G056.1	22	2	2	2	2	2	2	1	1	2	2	2	2	1	1	1	2
	G056.3	23	2	2	2	2	2	2	1	1	2	2	2	2	1	1	1	2
G057	**G057.1**	**21**	**3**	**2**	**2**	**2**	**2**	**3**	**2**	**2**	**3**	**3**	**3**	**2**	**2**	**2**	**2**	**3**
	G057.3	23	2	2	2	1	2	2	2	2	2	1	2	2	1	2	1	3
	G057.4	**15**	**3**	**2**	**2**	**2**	**2**	**3**	**3**	**2**	**3**	**2**	**3**	**2**	**2**	**2**	**2**	**3**
G152	**G152.1**	**21**	**2**	**3**	**2**	**2**	**3**	**2**	**3**	**2**	**2**	**3**	**1**	**2**	**2**	**2**	**2**	**2**
G155	**G155.2**	**23**	**2**	**2**	**1**	**2**	**3**	**2**	**3**	**2**	**2**	**2**	**1**	**2**	**3**	**2**	**2**	**3**
	G155.4	**21**	**2**	**1**	**1**	**2**	**3**	**2**	**3**	**2**	**3**	**3**	**2**	**3**	**3**	**2**	**2**	**3**
G158	G158.1	23	1	2	1	1	2	1	1	2	1	2	2	2	1	1	1	1
	G158.2	22	1	2	1	1	2	1	1	2	1	2	2	2	1	1	1	1

mother id	**clutch id**	**n[1]**	**number of paternal alleles at each locus**														**min. inferred fathers**	**max. inferred fathers**
			A6	**CC28**	**CCP7-D04**	**CCP7-E11**	**CM3**	**D105**	**D2**	**OR7**	**B103**	**B123**	**C102**	**CC2**	**CM58**	**KLK-314**		
G168	G168.1	23	1	2	2	1	1	2	2	2	2	1	1	2	1	1	1	1
	G168.3	22	1	2	2	1	1	2	2	2	2	1	1	2	1	1	1	1
G177	G177.1	20	1	2	2	1	2	1	2	2	2	2	1	2	2	1	1	1
G182	G182.1	23	2	1	2	2	1	1	2	1	2	1	1	1	2	1	1	1
G195	G195.1	23	2	1	2	2	2	1	2	2	1	2	2	2	2	1	1	1
	G195.4	23	2	1	2	2	2	1	2	2	1	2	2	2	2	1	1	1
G197	G197.2	23	1	2	2	2	1	2	1	1	1	1	2	2	1	1	1	1
	G197.3	22	1	2	2	2	1	2	1	1	1	1	2	2	1	1	1	1
G198	G198.1	22	1	2	2	1	1	2	2	2	2	2	2	1	2	1	1	1
	G198.4	21	1	2	2	1	1	2	2	2	2	2	2	1	2	1	1	1
G199	G199.1	22	2	1	2	1	2	2	2	2	2	1	1	1	2	1	1	1
	G199.3	19	2	1	2	1	2	2	2	2	2	1	1	1	2	1	1	1
G200	G200.1	21	2	2	2	1	1	2	1	2	2	1	2	2	2	1	1	1
	G200.2	21	2	2	2	1	1	2	1	2	2	1	2	2	2	1	1	1
G201	G201.1	22	1	2	2	2	1	2	2	1	2	1	1	1	2	2	1	1
G202	G202.2	22	2	2	2	1	1	1	2	1	2	2	2	2	2	2	1	1
	G202.4	22	2	2	2	1	1	1	2	1	2	2	2	2	2	2	1	1
G203	G203.1	23	1	1	2	2	1	1	2	1	2	2	2	2	2	2	1	1
	G203.2	23	1	1	2	2	1	1	2	1	2	2	2	2	2	2	1	1
G204	**G204.1**	**22**	**2**	**2**	**4**	**2**	**2**	**4**	**2**	**3**	**2**	**3**	**1**	**2**	**4**	**2**	**2**	**2**
	G204.3	**20**	**3**	**2**	**4**	**3**	**2**	**4**	**2**	**3**	**2**	**3**	**1**	**3**	**4**	**2**	**2**	**2**

[1] n refers to number of offspring genotyped per clutch

CHAPTER 3

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

Lucy I Wright[1], Wayne J. Fuller[2], Brendan J Godley[1], Andrew McGowan[1], Tom Tregenza[1], Annette C Broderick [1a]

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[1]Centre for Ecology and Conservation, University of Exeter, Cornwall Campus, Penryn, TR10 9EZ, UK.

[2]European University of Lefke, Marine and Fisheries Research Institute, Lefke , Mersin 10, North Cyprus.

[a] Corresponding author: Dr Annette Broderick

E-mail: A.C.Broderick@exeter.ac.uk

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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 is 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 three year study period, strongly suggesting that male breeding intervals are frequently longer than one 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.

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 due to 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 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 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, leatherback and green turtles migrating to breeding grounds annually (Limpus 1993; James *et al.* 2005; Hays *et al.* 2010). While 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. Due to 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 (*Chelonia mydas*) 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 3 consecutive breeding seasons from 2008-2010. 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 localised in this population, 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 83km away. The beach consists of two short coves (2km 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 un-hatched eggs were sampled. In 2008 tissue samples were stored in Queen's lysis buffer (Seutin *et al.* 1991), in subsequent years tissue samples were stored in 96% alcohol. In 2008 two clutches were sampled from a subset (16 out 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 due to evidence of null alleles. Primers were labelled with fluorescent dyes (6-FAM, HEX or NED) and the loci amplified in 2 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 15min followed by 35 cycles of 94°C for 30s, 58°C for 90s and 72°C for 60s, and finally one cycle of 60°C for 30min. 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 to original allele calls. Differences in the repeated genotypes were observed at 7 allele calls out of 3273 (0.21% error), and the highest error at any particular locus was 0.8% at locus B103. We also assessed the entire dataset 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 9 additional females and 1 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 dataset 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 in order to detect any paternal half-sibs, which would identify males that sired offspring in more than one year or with more than one female within a year.

RESULTS

Over the three 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 three years, with a minimum of 1.1M:1F (2009) and maximum of 1.5M:1F (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 3 (3%) inferred males were assigned offspring in more than one breeding season (2 males: 2008 and 2009; 1 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 out of 99; 60%) had a minimum breeding interval of three years with females at the Alagadi rookery within the study period, whilst the males that sired offspring only in 2009 (37 out of 99; 37%) had a minimum breeding interval of two years. No males were assigned offspring in all three years, suggesting that male turtles do not generally breed annually at this site. No females reproduced more than once at this site during the three 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 4 fathers in any single clutch (Table 2).

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 three year study period, and indicate that, contrary to expectations, there is currently no shortage 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 due to 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 3 out 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 three years (Broderick *et al.* 2003), and no female nested in more than one year during the three 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 3 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 two 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, 2 male leatherback turtles tracked in the western Atlantic re-migrated to the same breeding grounds in two consecutive years, but 2 other 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 to 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 due to a number of causes. Firstly, 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.

Secondly, our results could indicate strongly female-biased mortality in early life stages, but, due to a dearth of information relating to the pelagic juvenile and sub-adult 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, e.g. 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, i.e. those that produce a proportion of males) showed greater swimming ability than those incubated at warmer temperatures (above 30°C, i.e. 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 due to 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 sub-adult or adult distribution or migration routes that might make one sex more vulnerable to interactions with fisheries or other causes of mortality at sub-adult life stages, although comparatively little is known about the movement of male turtles, placing particular importance on studies of their spatial and breeding ecology.

Thirdly, 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 4 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, since 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 (Pritchard *et al.* 2000); the analysis suggested that a single population (K=1) was most likely (Figure S1, Supporting Information), although we consider this result to be conservative due to the uncertainty associated with our inferred multilocus genotypes (see 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 non-overlapping 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

Table 1. **Characterisation 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

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 2 fathers (shown in bold, column 5). No candidate males were sampled; all inferred father IDs (column 7) refer to un-sampled 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 one 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)

Mother ID - Clutch	Year	No. of offspring sampled	Clutch size	Min inferred fathers	Max inferred fathers	Inferred father ID
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)
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)

Mother ID - Clutch	Year	No. of offspring sampled	Clutch size	Min inferred fathers	Max inferred fathers	Inferred father ID
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)

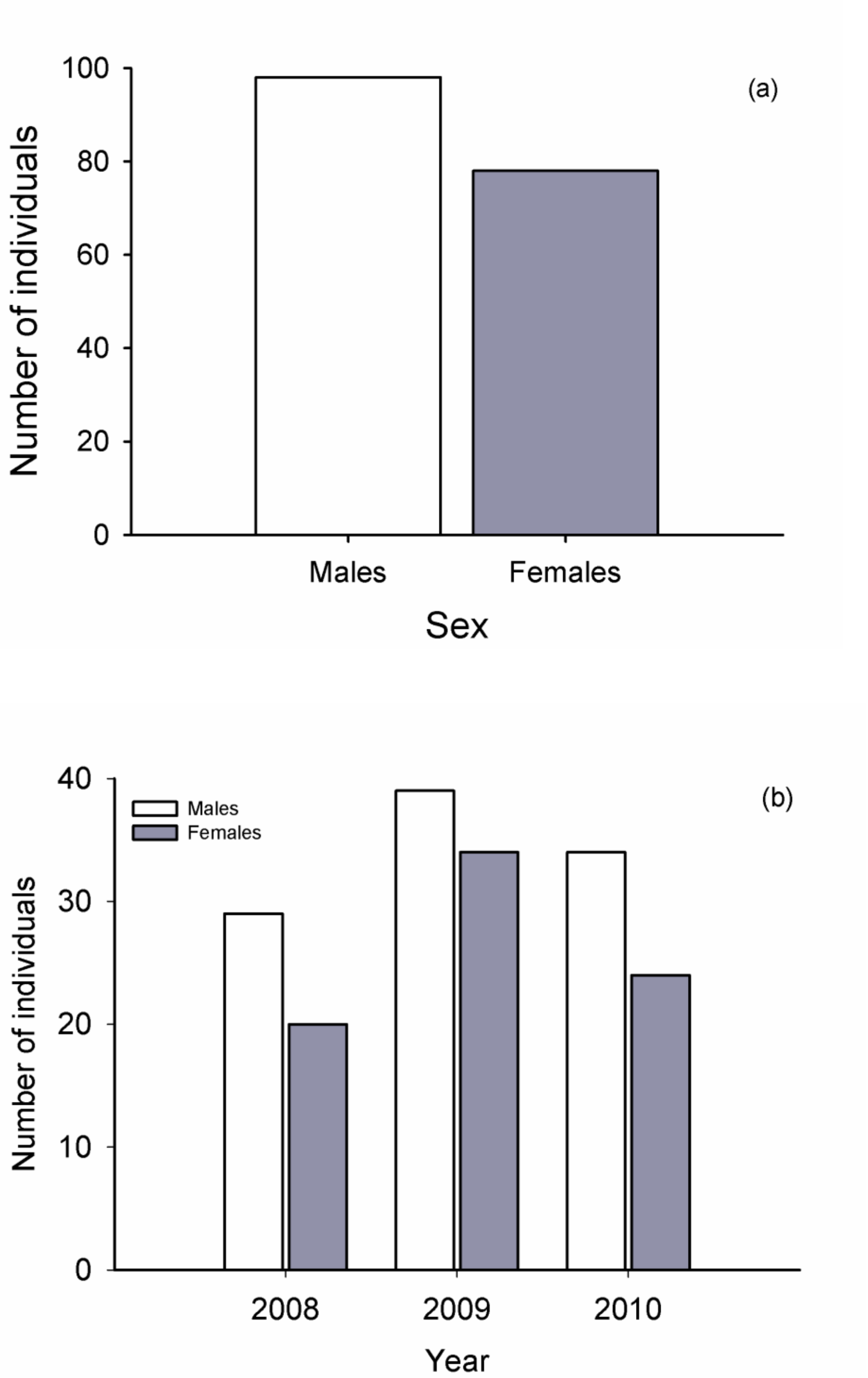


Figure 1. Number of nesting females (shaded) that were included in the study and inferred number of unique males that sired offspring from those females a) across the 3 year study period, and b) within each breeding season at Alagadi, northern Cyprus.

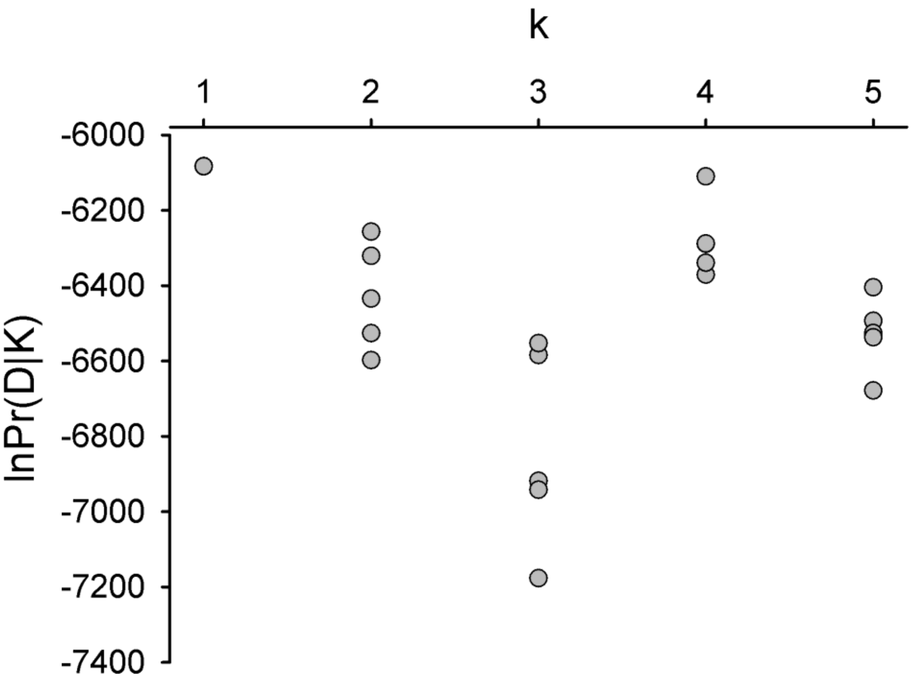
SUPPORTING INFORMATION

Genetic structure methods

We used the program Structure v.2.3.3 (Pritchard *et al.* 2000) to investigate possible genetic differentiation among females and inferred males breeding at our study site. We used genotypes at 13 loci from 87 adult female turtles that nested at the site between 2007 and 2010 and 87 male genotypes that were inferred using reconstructed single-locus paternal genotypes from the Colony (Wang 2004, Wang & Santure 2009) analysis. Colony only reconstructs parental genotypes on a single locus basis and makes no attempt to infer multilocus parental genotypes. Inferring multilocus genotypes from single locus genotypes is difficult to do accurately because, with increasing numbers of loci, it quickly becomes highly likely that the inferred multilocus genotype is incorrect at at least one locus (Colony user guide, version 2.0). We only used single locus genotypes that were inferred with a probability of >0.75 and only included inferred males with genotypes reconstructed at a minimum of 10 loci. Nonetheless, we urge that the results of the Structure analysis are considered conservative. We ran the analysis assuming a K (the number of potential distinct genetic clusters) of 1 to 5, with an admixture model, correlated allele frequencies and no prior location information. We conducted five iterations for each K with a run length of 200 000 iterations after a burn-in of 100 000 iterations.

Genetic structure results

Results of the Structure analyses suggest that there is no genetic differentiation within the female and inferred male turtles breeding at our study site. The mean log-likelihood was highest when assuming one cluster (K = 1), compared to values assuming a K of 2–5 (fig S1). Furthermore, when assuming a K of 2-5 all individuals in the sample were highly admixed and none were strongly assigned to one cluster or another, suggesting a single population.



Supplementary Figure S1. Output from a Structure analysis of 87 adult female turtles that nested at the study site from 2007 - 2010 and 87 inferred male genotypes based on reconstructed single-locus paternal genotypes from a Colony analysis. We ran five iterations of the analysis for each value of 'K' (the number of clusters) to determine the likelihood that the genetic structure of the data is explained by 'K' clusters.

CHAPTER 4

No benefits of polyandry to female green turtles

Lucy I Wright[1], Wayne J. Fuller[2], Brendan J Godley[1], Andrew McGowan[1], Tom Tregenza[1], Annette C Broderick [1a]

This chapter is *in review* at *Behavioral Ecology* in its present form

[1]Centre for Ecology and Conservation, University of Exeter, Cornwall Campus, Penryn, TR10 9EZ, U.K.

[2]European University of Lefke, Marine and Fisheries Research Institute, Lefke , North Cyprus, Mersin 10, Turkey

[a] Corresponding author: Dr Annette Broderick

E-mail: A.C.Broderick@exeter.ac.uk

Short Title: No benefits of polyandry in turtles

ABSTRACT

Multiple paternity is extremely common in natural populations of almost all reptiles studied to date, suggesting that pay-offs from polyandrous mating systems are important in these taxonomic groups. However, strong evidence in support of direct or indirect benefits to females is scarce. We examined the relationship between polyandry and components of female reproductive success and offspring fitness in the promiscuous green turtle (*Chelonia mydas*), a species that exhibits highly variable levels of multiple paternity. We did not detect any clear fitness benefits to polyandrous females in this study and we discuss the potential of sexual conflict to influence female mating patterns in marine turtles. We show that polyandrous females produce significantly smaller clutches than monandrous females, highlighting a potential cost to polyandry in green turtles. Furthermore, multiple paternity was more common in returning females (recorded breeding in a previous season) than females nesting for the first time at our study site, possibly reflecting increased encounter rates with males or sperm storage across breeding seasons. Our results reveal potentially complex influences of female traits, environment and mating strategy on components of reproductive success and we discuss the challenges associated with unraveling the costs and benefits of multiple mating in natural populations.

Key words: Polyandry, multiple paternity, Mediterranean, microsatellites, *Chelonia mydas*

INTRODUCTION

Female promiscuity is common in natural populations across almost all taxa studied (Birkhead and Møller 1998) but, despite substantial evidence that polyandry can increase female fitness (Arnqvist and Nilsson 2000; Jennions and Petrie 2000; Simmons 2005), its adaptive significance in the wild remains controversial (Jennions and Petrie 2000; Arnqvist and Kirkpatrick 2005; Akçay and Roughgarden 2007; Uller and Olsson 2008). Mating can carry significant costs to females, such as exposure to disease (Thrall *et al*. 2000), increased predation risk (Rowe 1994), time and energy costs (Watson *et al.* 1998), as well as the risk of physical harm (Crudgington and Siva-Jothy 2000), hence, the persistence of this behavior in natural populations suggests that these costs should be offset by net benefits. In many cases females can receive direct benefits from mating with multiple partners, which include all material benefits ranging from fertilization assurance to provision of or access to resources and parental care of offspring (reviewed in Arnqvist and Nilsson 2000; Hosken and Stockley 2003). Polyandrous females may also gain indirect genetic benefits, via the processes of sperm competition and/or cryptic female choice, if these processes result in the production of more viable offspring (reviewed in Jennions and Petrie 2000; Hosken and Stockley 2003; Simmons 2005). Indirect benefits can potentially arise through the increased genetic quality (Yasui, 1997; Fisher *et al.* 2006), sperm competitive ability (Keller and Reeve 1995), genetic compatibility (Zeh and Zeh 1996; Zeh and Zeh 1997; Tregenza and Wedell 2000), or genetic diversity (Yasui 1998) of offspring. However, unequivocally demonstrating that polyandrous females gain indirect benefits in the wild is challenging and evidence in support of these hypotheses is largely restricted to laboratory experiments (Simmons 2005) (but see Foerster *et al*. 2003; Garant *et al.* 2004; Fisher *et al*. 2006)). Multiple mating by females may also arise in the absence of benefits to females, as a consequence of male coercion (Clutton-Brock and Parker 1995) or to avoid the costs of male harassment (‘convenience polyandry’) (Thornhill and Alcock 1983). In this situation, multiple mating is driven by strong selection on males to increase their reproductive success and multiple paternity arises as a result of conflict between the sexes over mating rate, with females potentially paying a net cost (Arnqvist and Rowe 2005; Hosken and Stockley 2005; Maklakov *et al.* 2005).

In the vast majority of reptiles, parental care of offspring is absent and there is no evidence that males provide any resources to females other than sperm, yet multiple paternity of clutches is widespread in reptiles, occurring in all species that have been studied to date (Uller and Olsson 2008). Furthermore, it can be extremely common within natural populations; high levels of multiple paternity have been reported in numerous species of snakes and lizards as well as some marine turtles (Jensen *et al*. 2006; Zbinden *et al.* 2007; Uller and Olsson 2008). There is limited evidence from reptiles that multiple paternity is associated with indirect genetic benefits. Early work in adders (*Vipera bergus*) and sand lizards (*Lacerta agilis*) suggested that polyandrous females suffered lower offspring mortality (Madsen *et al.* 1992; Olsson *et al.* 1994), possibly through female selection of more compatible sperm (Olsson *et al.* 1996), and subsequent studies have found correlations between multiple paternity of clutches and increased clutch size, hatching success or offspring viability (e.g. Pearse *et al.* 2002; Blouin-Demers *et al*. 2005; Madsen *et al.* 2005; Uller and Olsson 2005; Eizaguirre *et al.* 2007; Zbinden *et al*. 2007; Olsson *et al.* 2010). Whether the widespread occurrence of multiple paternity in reptiles can be explained by indirect benefits, however, has been questioned (Lee and Hays 2004; Uller and Olsson 2008; but see Madsen 2008).

Multiple paternity (and hence polyandry) has been documented in all seven marine turtle species (reviewed in Bowen and Karl 2007; Joseph and Shaw 2010). Considerable variation in the level of multiple paternity, both between and within species, is an interesting aspect of their reproductive biology and is possibly influenced by the abundance and sex ratio of breeding individuals (Jensen *et al*. 2006). Although many studies have addressed the question of female promiscuity in marine turtles, few have attempted to assess whether females gain benefits from mating with multiple males (Lee and Hays 2004; Zbinden *et al.* 2007) and, to date, no benefits have been clearly demonstrated. This has led some authors to conclude that female turtles ‘make the best of a bad job’ and mate multiply in response to male harassment (Lee and Hays 2004). Male marine turtles are aggressive in their pursuit of receptive females (Booth and Peters 1972; Miller 1997; Bowen and Karl 2007) and females undoubtedly pay energetic costs in order to avoid their advances. However, mating is also likely to incur substantial costs to female turtles (e.g. physical injury; Miller 1997), hence, the causes and fitness consequences of multiple paternity in turtles may be complex and difficult to unravel. Furthermore, indirect benefits of polyandry on offspring fitness may be subtle in comparison to environmental effects on incubating clutches (Godley *et al.* 2001; Glen *et al.* 2003; Lee and Hays 2004; Foley *et al*. 2006) and measures of offspring fitness are difficult to assess in such long-lived species, so genetic benefits may have gone undetected in previous studies.

We have used microsatellite analysis to study in detail the genetic mating system of a green turtle (*Chelonia mydas*) population in northern Cyprus, with the aim of increasing our understanding of mating strategies in marine turtles. We previously reported that 24% (19 out of 78) of females at this site produced clutches with multiple paternity (Wright *et al.* 2012). Here we extend our analysis to investigate whether females that were polyandrous gained fitness benefits by examining components of reproductive success and offspring fitness.

MATERIALS AND METHODS

Field methods and sample collection

Samples were collected from nesting female turtles and their offspring at Alagadi beach, northern Cyprus, during three breeding seasons; May to October, 2008 - 2010. Tissue samples were taken from nesting females of known identity from the trailing edge of the foreflipper. Female size was recorded (curved carapace length (CCL) and width (CCW)) and nest locations were marked. Upon hatching, tissue biopsies were taken from a sample of hatchlings in each clutch from the trailing edge of the carapace. Hatchlings were measured (straight carapace length (SCL), straight carapace width (SCW) and weight) before being released. Nests were allowed to hatch naturally and were excavated when no further hatchlings had emerged for 48 hours, or after 5 days since the first hatchling emerged, whichever was sooner. Live and dead hatchlings found inside the nest, as well as dead embryos from un-hatched eggs were also sampled. In 2008, tissue samples were stored in Queen’s lysis buffer (Seutin *et al.* 1991), in subsequent years tissue samples were stored in 96% alcohol. Clutch size, fertilization success, hatching success and the number of hatchlings surviving to leave the nest were recorded for each clutch, through excavation of clutch contents once hatching was complete. Un-hatched eggs were classified as unfertilized if there were no gross signs of embryo presence. In 2008, two clutches were sampled from a subset (16 out of 20) of females. Inferred paternity was highly correlated in the successive clutches of these females (see Results), so in further years we reduced our sampling effort to a single clutch per female. In total, 94 clutches from 78 females were sampled for microsatellite analysis of paternity, representing 87% of females that nested at the site during the study period.

Female green turtles at Alagadi beach lay on average three clutches of eggs within a breeding season (Broderick *et al.* 2003) and our study females laid between one and six clutches (mean = 3). We collected samples for paternity analysis from a maximum of two clutches per study female and additionally collected data on clutch size, fertilization success, hatching success, the number of hatchlings surviving to leave the nest (subsequently referred to in the text as ‘emergence success’) and hatchling size for all clutches that the study females laid throughout the season. If there was evidence of multiple sires in at least one genotyped clutch from a particular female, then that female was considered to be polyandrous and all of her subsequent un-genotyped clutches were also classified as polyandrous clutches. The full dataset consisted of 219 clutches from 78 females (13 clutches failed due to inundation so were excluded from the analysis), however, we conducted some statistical analyses (those requiring assignment of individual offspring to specific sires, see below) on the subset of 94 genotyped clutches.

Genotyping and parentage analysis

Mothers and offspring were genotyped at 13 microsatellite loci, as previously described (Wright *et al.* 2012). All loci were designed for use in marine turtles, were variable in our population, conformed to expectations of Hardy Weinberg equilibrium, showed low probability of null alleles and showed no evidence of linkage disequilibrium (Wright *et al.* 2012). Between 18 and 23 offspring were genotyped per clutch (mean ± sd, 21.7 ± 1.2) representing 12-52% of the total clutch size (mean 21%). In total 2042 offspring and 78 mothers were successfully genotyped at a minimum of 10 loci, but genotypes were complete at all 13 loci for 97% of individuals. Sibship reconstruction and parentage inference were carried out using the program COLONY v2.0 (Wang 2004; Wang and Santure 2009) as previously described (Wright *et al.* 2012). In brief, three replicate runs of ‘long’ length were conducted on the same dataset assuming an error rate of 0.004 for allelic dropout and 0.008 for genotyping error. Each of the replicate runs used different random number seeds to initiate the simulated annealing processes. We only concluded that a female had mated with more than one male when all three runs gave consistent results. Probability of detecting multiple paternity (assessed using PrDM software (Neff and Pitcher 2002; downloaded November 2011) with 20 offspring sampled per clutch was 0.874 when assuming 2 fathers and skewed paternal contributions (10:90%) and 0.999 when assuming 3 fathers and skewed paternal contributions (10:20:70%).

Statistical analyses

All statistical analyses were run in GenStat release 13.2 (GenStat 2010) using Restricted Maximum Likelihood (REML) and Generalized Linear Mixed Models (GLMM) with a binomial error structure and a logit link function. REMLs and GLMMs allow both fixed and random factors as well as covariates to be fitted and the random terms control for the use of repeated measurements (Schall 1991). The significance of fixed terms in linear mixed models is calculated using maximum likelihoods and is assessed by their Wald statistics, which are distributed as χ^2 for each term fitted last in the model. Full models contained higher order interactions and final models were selected using stepwise elimination of non-significant terms ($P>0.05$) (for discussion of the use of stepwise modeling see (Whittingham *et al.* 2006; Murtaugh 2009)). Residuals were checked for normality and homoscedasticity after each deletion step.

We firstly investigated the effect of polyandry on estimators of female reproductive success, including clutch size (total number of eggs including unfertilized eggs), fertilization success (the proportion of a clutch that showed signs of embryo development; numerator, number of fertilized eggs; denominator, clutch size), hatching success (numerator, number of hatched eggs; denominator, clutch size) and emergence success (the proportion of a clutch that survived to leave the nest; numerator, number of eggs that hatched minus number of hatchlings found dead in the nest; denominator, clutch size). We also examined the effect of polyandry on mean hatchling size (SCL x SCW). Polyandry (binary variable: polyandrous versus monandrous clutches) was included in all models as a fixed factor and female ID was included as a random term to control for the inclusion of repeat clutches from the same females. Covariates included female size (CCL), lay date (number of days since 1 January; both linear and quadratic effects), year, incubation duration and clutch size.

Hypotheses relating to indirect benefits from good genes and cryptic female choice predict that males achieving a high paternity share in multiply sired clutches should produce 'better' offspring (Yasui 1997; Zeh and Zeh 1997). Therefore, using just the 94 genotyped clutches in which we assigned paternity to specific offspring, we also investigated whether males that achieved a high paternity share produced higher quality offspring than males that sired smaller proportions of a clutch, and whether these preferred males sired better quality offspring than sires of monandrous clutches. Within polyandrous clutches, males that sired the highest number of genotyped offspring were classified as primary fathers, whilst additional males that sired smaller proportions of the clutch were classified as secondary fathers. Considering only hatched offspring from polyandrous clutches that were consistently assigned to fathers (n = 452), we investigated whether primary fathers produced larger offspring than secondary fathers, using the same covariates as previous models, with primary or secondary father included as a fixed factor and female ID and clutch ID included as random terms. Considering only hatched offspring sired by primary fathers in polyandrous clutches and hatched offspring from monandrous clutches (n = 1687), we investigated whether primary fathers from polyandrous clutches produced larger offspring than fathers from monandrous clutches, using the same covariates as previous models, with polyandry included as a fixed factor and female ID and clutch ID included as random terms. Results are presented as means ± standard error unless otherwise stated.

RESULTS

Paternal contributions to clutches and patterns of paternity in successive clutches

Parentage analysis was successfully carried out on 94 clutches from 78 females. Multiple paternity was detected in 23 clutches from 19 (24%) females (subsequently referred as 'polyandrous clutches') and the minimum number of inferred fathers per clutch ranged from 1-3 (Wright *et al.* 2012). The proportion of females that were polyandrous did not differ significantly across the three breeding seasons (Goodness-of-fit test; $\chi^2 = 3.35$, df = 2, p = 0.187). Within polyandrous clutches, primary males (i.e. males that sired the highest proportion of genotyped offspring) gained between 48% and 96% of the paternity share (mean 77%). Secondary males gained between 4% and 35%

(mean 19%) and, when a third male was detected (n = 6 clutches), they gained between 4% and 23% (mean 11%) of the paternity share. In 17 out of 21 polyandrous clutches, paternal contributions to clutches were significantly skewed from equal contributions (χ^2 all $P < 0.05$) (two clutches from one female were not analyzed because offspring could not be consistently assigned to specific sires, due to extensive allele sharing).

Identical paternal alleles were found in the successive clutches of 13 out of 16 females, suggesting that females did not generally re-mate between clutches. Paternal contributions to successive clutches were very similar and in polyandrous clutches the primary male in the first clutch was also the primary male in the second clutch in all cases. For two females, the COLONY analyses identified an additional father in the first clutch compared to the second clutch, but in both cases the additional male sired only a small proportion of the clutch, so we may have failed to detect these additional fathers in successive clutches due to their small paternal contributions. For one female, two of the three COLONY runs found that the same two fathers had contributed to both clutches, but one run found two fathers in the first clutch but only the primary father in the second clutch.

Polyandry and female size / status

Polyandry was more common in returning females (females that had nested in previous years at the study site) compared to females that nested for the first time at Alagadi beach (Goodness of fit test, $\chi^2 = 9.32$, df = 1, $p = 0.002$, Figure 1), but, although returning females were larger than first time nesters in this study (t-test, $t = 6.53$, df = 75, $p < 0.001$), female size (CCL) did not significantly differ between polyandrous and monandrous females (t-test; $t = 1.50$, df = 89, $p = 0.137$). This was true even when considering returning females and first time nesters separately (returning females; t-test, $t = 0.94$, df = 32, $p = 0.354$; first-time nesters; Mann-Whitney U test, $U = 77.0$, $n_1 = 38$, $n_2 = 5$, $p = 0.519$).

Effects of polyandry on fitness related traits

Total clutch size increased significantly with female size (REML; $W = 74.51$, $p < 0.001$) and lay date (REML; $W = 17.61$, $p < 0.001$) as expected based on previous studies (e.g. Broderick *et al.* 2003), but was negatively affected by polyandry (REML; $W = 7.20$, p

<0.01) with clutches from polyandrous females being significantly smaller than clutches from monandrous females (107.17 ± 2.55 and 112.75 ± 1.42 respectively, Figure 2). There was no effect of polyandry on fertilization success (GLMM; W = 2.15, p = 0.146), hatching success (GLMM; W = 0.09, p = 0.763), or emergence success (GLMM; W = 1.71, p = 0.195). Fertilization success was significantly positively affected by lay date (GLMM; W = 12.73, p <0.001). Both hatching success and emergence success were significantly affected by an interaction between female size and lay date (GLMM; hatching success; W = 5.55, p = 0.019; emergence success; W = 5.89, p = 0.016), and an interaction between female size and incubation duration (GLMM; hatching success; W = 7.69, p <0.01; emergence success; W = 6.32, p = 0.013); smaller females benefited from higher emergence success than larger females and this effect was stronger when incubation duration was long and when females nested later in the season (Figure 3). Hatching success was also affected by an interaction between incubation duration and year (GLMM; W = 4.03, p = 0.046). There was no effect of polyandry on mean hatchling size (REML; W = 1.33, p = 0.254). Mean hatchling size was affected by a complex interaction between female size, clutch size and lay date (REML; W = 5.94, p = 0.016) and an interaction between female size and incubation duration (REML; W = 6.79, p = 0.010).

Relationship between relative male siring success and offspring quality

Within polyandrous clutches, hatchling size was not influenced by whether the sire was a primary or secondary father (REML; W = 2.07, p = 0.151), but was positively affected by incubation duration (REML; W = 15.18, p <0.01), as expected due to lower incubation temperature (Glen *et al*. 2003), and by an interaction between clutch size and lay date (REML; W = 5.59, p = 0.041). Furthermore, primary fathers did not have a higher proportion of offspring surviving to leave the nest (Fishers exact test; p = 0.143). Offspring sired by primary fathers in polyandrous clutches were also not different in size than offspring from monandrous females (REML; W = 0.64, p = 0.426), but hatchling size (considering offspring from primary fathers in polyandrous clutches and monandrous clutches) was significantly affected by incubation duration (REML; W = 47.42, p <0.001), year (REML; W = 7.40, p = 0.009) and lay date (REML; W = 4.00, p = 0.049).

DISCUSSION

We found no evidence in this study to suggest that polyandry confers direct or indirect benefits to female green turtles. Our analysis revealed no differences in fertilization success, hatching success, emergence success (the number of hatchlings surviving to leave the nest) or mean hatchling size between polyandrous and monandrous females. Furthermore, there was no evidence that males that sired a large proportion of polyandrous clutches produced better quality offspring than males that achieved a smaller paternity share, or than sires of monandrous clutches. In contrast, we show that polyandrous females produce significantly smaller clutches than monandrous females, highlighting a potential cost to polyandry in green turtles. Other factors that had a strong influence on components of female reproductive success were lay date, incubation duration (related to temperature) and female size, with large female size seeming to have an unexpected negative influence on emergence success (Figure 3).

Potential benefits of polyandry in marine turtles

Many of the proposed material benefits of mating with multiple partners, such as nuptial gifts and parental care, are unlikely to be relevant to marine turtles, and indirect genetic effects, or benefits relating to cost minimization have been given more attention as potential explanations for multiple paternity in these species (Pearse and Avise 2001; Lee and Hays 2004). However, direct benefits include acquiring an adequate supply of sperm and hedging against male infertility, and these factors may play an important role in species that lay large numbers of eggs, including marine turtles. Although sperm limitation in many taxa seems unlikely considering the large numbers of sperm generally transferred in a normal ejaculate, fertility benefits are thought to play an important role in the maintenance of polyandry in insects (Arnqvist and Nilsson 2000) and may function via replenishing depleted sperm supplies or by guarding against sperm degradation or male sterility. Additionally, Uller and Olsson (2005) reported a positive correlation between number of copulations (whether these were with the one or multiple males was not known) and the proportion of fertile eggs in common lizards (*Lacerta vivipara*) and suggested that infertility risk could be a major driver of multiple mating in some reptiles, although it has been argued that fertility assurance is less likely to explain polyandry in birds (Birkhead and Møller 1998). However, we found no evidence that polyandry increased fertilization success of female turtles in this study.

There is considerable evidence that polyandry affords indirect genetic benefits to females across a range of taxa (Jennions and Petrie 2000; Hosken and Stockley 2003). Our results do not provide any support for indirect benefits of polyandry in green turtles, however, in more than 80% of polyandrous clutches in this study, paternal contributions were significantly skewed towards one or more males, raising questions about potential post-copulatory selection processes. The intrinsic male quality hypothesis suggests that females may gain benefits from polyandry if males that are successful in sperm competition also produce higher quality offspring (e.g. Hosken *et al.* 2003; Fisher *et al.* 2006) or if females are able to cryptically select sperm from high quality males. Furthermore, by biasing paternity towards genetically more compatible males, polyandrous females could benefit from increased offspring viability, potentially reducing the effects of inbreeding or increasing disease resistance of their young (e.g. Olsson *et al.* 1996; Tregenza and Wedell 2002). Both theories suggest that paternity should be biased towards particular males, and that those males should produce more viable progeny. Positive relationships between multiple male partners and offspring viability or hatching success have been demonstrated in squamate reptiles (Madsen *et al.* 1992; Olsson *et al.* 1994; Madsen *et al.* 2005; Eizaguirre *et al.* 2007; Olsson *et al.* 2010) and are consistent with both hypotheses, although the results of the early studies (Madsen *et al.* 1992; Olsson *et al.* 1994) were attributed to reduced production of inbred young. However, Uller and Olsson (2008) argue that the high degree of within-clutch multiple paternity in reptile populations itself casts doubt over the ability of females to control fertilization (but see (Calsbeek and Sinervo 2004) for an example of post-copulatory sperm selection in a lizard).

We did not find any evidence that primary males (that achieved a high paternity share in polyandrous clutches) sired ‘better’ offspring than secondary fathers or sires of monandrous clutches; within polyandrous clutches primary males did not sire larger or more viable offspring than secondary males. Nevertheless, the small size of our study population of green turtles (the Mediterranean green turtle population consists of approximately 300-400 females nesting annually (Broderick *et al.* 2002)), coupled with the fact that both male and female marine turtles exhibit natal philopatry (Bowen and Karl 2007) and high nest site fidelity (Limpus 1993; Broderick *et al.* 2002) make it plausible that inbreeding avoidance has played a role in the evolution or maintenance of polyandry in this population. An alternative explanation for the skewed paternal contributions to clutches in our study is that they reflect sperm precedence rather than any female-driven process. It has been hypothesized that first-male sperm precedence is likely to influence paternity patterns in marine turtles, due to the location of sperm storage tubules high in the female reproductive tract, and the behavior of male turtles, which leave breeding grounds early in the nesting season rather than continuing to mate with females as they ovulate new eggs (FitzSimmons 1998).

Gaining meaningful measures of female and offspring fitness is difficult in marine turtles due to their long-lifespan and complex life-history, and our components of fitness were limited to the short-term measures of fertilization success, hatching success and emergence success of offspring, and offspring size. Benefits of polyandry in this species may only become evident if the reproductive success of females and their offspring are estimated over much longer timescales. Nevertheless, hatching success and offspring size are strongly associated with post-hatching offspring survival in pythons and freshwater turtles (Madsen and Shine 1998; Janzen, Tucker, and Paukstis 2000) and are likely to be important fitness components. Direct or indirect fitness benefits associated with polyandry may also vary between individuals depending, for example, on female condition (Eizaguirre *et al.* 2007) or experience (Whittingham and Dunn 2010) and between environments, potentially impacting our ability to detect them.

The potential roles of sexual conflict and convenience polyandry

Recent reviews of multiple paternity in birds and reptiles have questioned the importance of indirect benefits to females, and instead highlighted the roles of sexual conflict and ecological factors, such as population density and sex ratio, in driving multiple mating by females (Westneat and Stewart 2003; Arnqvist and Kirkpatrick 2005; Akçay and Roughgarden 2007; Uller and Olsson 2008). In the absence of positive fitness effects, costs of mating and of resistance to mating may be of central importance to female mating strategies and levels of polyandry are likely to be influenced by mate encounter rates and levels of competition for access to mates. Sexual harassment by courting males can be extremely costly to females (Clutton-Brock and Parker 1995; Arnqvist and Rowe 2005; Sakurai and Kasuya 2008; Gay *et al.* 2009) and may be an expensive drain on energy resources, especially for capital breeders such as marine turtles (Bonnet *et al.* 1998), which rely largely on stored energy during the breeding period. Cost minimization could explain polyandry in this scenario, if the cost of resistance to mating exceeds that of accepting additional mates. Lee and Hays (2004) suggested that such ‘convenience polyandry’ (Thornhill and Alcock 1983) was the most likely explanation for multiple paternity in a population of green turtles, where no fitness benefits were detected. This idea is supported by work that demonstrates higher levels of multiple paternity in high density marine turtle populations, presumably due to increased encounter rates with males (Jensen *et al*. 2006). Male turtles exhibit very aggressive mating behavior (see Miller 1997; Bowen and Karl 2007; and references therein); females are often pursued by multiple males simultaneously and mating pairs can be harassed and bitten by attendant males (Booth and Peters 1972; Miller 1997). There is evidence from a number of species that females will accept copulations from harassing males if they cannot avoid them (Clutton-Brock and Parker 1995), particularly when females are persistently courted by more than one male concurrently, but experimental support for convenience polyandry is sparse, particularly in vertebrates (Huchard *et al*. 2012) (but see (Rowe 1992; Cordero and Andrés 2002; Arnqvist and Rowe 2005) for examples in invertebrates).

The costs of mating and of resistance to mating are difficult to quantify in natural populations (but see Maklakov *et al.* 2005), particularly for long-lived organisms and no data exist from marine turtles. In semi-natural populations of common lizards where the adult sex ratios were manipulated, female survival, fecundity and lifetime reproductive success were reduced in male-biased populations (Fitze *et al.* 2005; Le Galliard *et al.* 2005, 2008). These results were attributed to increased male harassment, although it was not clear whether the costs were associated with multiple mating or with resistance to mating. Despite the costs, females were not more likely to mate multiply in male-biased compared to female-biased populations, opposing a theory of convenience polyandry. However, within polyandrous females, those in the male-biased populations had more fathers per clutch, suggesting that increased mating pressure on these females influenced the degree of polyandry (Fitze *et al.* 2005). Polyandrous female common lizards also harbor more diverse cloacal bacteria than monandrous females, presumably due to sexual transmission from multiple mates, with potential fitness consequences (White *et al.* 2011). Whether or not the apparent costs of multiple mating are mitigated by benefits in common lizards is not clear; Fitze *et al.* (2005) reported that polyandrous females produced larger clutches than monandrous females regardless of adult sex ratio, but Le Galliard *et al*. (2008) showed that the lifetime costs of being in a male biased population were not offset by indirect benefits via offspring.

We showed that polyandrous green turtles produced significantly smaller clutches than monandrous females. This result is in contrast to evidence from a freshwater turtle that clutches with multiple paternity are larger than clutches with only one sire, possibly due to male preference for larger (and more fecund) females (Pearse *et al.* 2002) (see also Zbinden *et al.* 2007). Whether the smaller clutch size in our study represents a cost to females of polyandry is unclear. Uller and Olsson (2005) also found that promiscuous female common lizards produced significantly smaller clutches, but heavier offspring, and speculated that this could be explained by more efficient yolk utilization. We found no effect of polyandry on mean hatchling size in this study, and, although demonstrated in other taxa (Sheldon 2000), there is no evidence that female marine turtles adjust nutrient allocation to eggs in response to polyandry or to male quality.

We showed that polyandry is significantly more common in returning females (that have previously nested at our study site) compared to first-time nesters (potential new recruits, although it is possible that they have nested previously elsewhere). Returning females were larger than first time nesters in this study and are most likely older and, therefore, more experienced females. However, we found no difference in size between polyandrous and monandrous females, in contrast to studies that have found multiple paternity to be positively correlated with female size in turtles (Pearse *et al*. 2002; Zbinden *et al.* 2007) and other reptiles (Eizaguirre *et al*. 2007; Lance *et al.* 2009). Our results could reflect a male preference for (and more intense courting of) older or more experienced females, or perhaps returning females arrive at mating grounds earlier and encounter more males than first-time breeders. Alternatively, this result may reflect sperm storage across breeding seasons, with returning females utilizing residual sperm from a previous breeding season. Long-term sperm storage has been reported in freshwater turtles (up to 4 years (Ewing 1943)), as well as multiple paternity resulting from the use of sperm stored from previous seasons (Pearse *et al.* 2002). Whether sperm stored over several years is of any value in marine turtles is not clear, and will depend on breeding intervals of particular females.

Due to the difficulty of reliably observing mating behavior of marine turtles at sea, molecular parentage analysis has been fruitfully employed to gain insights into their mating strategies (reviewed in Bowen and Karl 2007; Lee 2008). Here, we used microsatellites to detect multiple paternity of clutches and infer polyandry, as do many other studies of mating systems in natural populations (e.g. Lee & Hays, 2004; Eizaguirre *et al.* 2007; DiBattista *et al.* 2008). However, using this approach to examine potential benefits of polyandry is not straightforward because, under scenarios of cryptic female choice or strong sperm competition, multiple mating might not result in multiple paternity (Birkhead and Møller 1998). For example, Fitzsimmons (1998) reported a low level of multiple paternity in green turtles in the southern Great Barrier Reef despite observations of multiple mating by females in the population, and Moore *et al.* (2009) report that some socially polyandrous female tuatara (*Sphenodon punctatus*) produce single paternity clutches, highlighting the value of combining genetic and behavioral data. If female turtles are using polyandry to enable them to choose among several males, then some of the females that produced single paternity clutches in this study might have mated multiply but been very good at selecting sperm from one particular male, or one male may have been highly successful under sperm competition, possibly compromising our ability to reveal benefits of polyandry. Additionally, multiple paternity in turtles can result from sperm storage across multiple breeding seasons rather than multiple mating within one reproductive attempt (Pearse *et al.* 2002), which could bias estimates of within year polyandry. Behavioral observations or experimental manipulations are required in conjunction with the use of molecular techniques, in order to gain a more complete understanding of the fitness consequences of multiple mating, although either approach would be challenging for marine turtles, except in captivity.

Conclusions

The ubiquity of multiple paternity in marine turtle populations, albeit at widely varying levels, suggests that female promiscuity is common in these species and that pay-offs from polyandrous behavior are important. We were unable to demonstrate any direct or indirect benefits to polyandrous females in this study, suggesting that alternative explanations for the high frequency of multiple paternity in marine turtles should be explored. We show that polyandry is more common in returning females compared to first time breeders at our study site, which could potentially reflect increased encounter rates with males or sperm storage. The costs of mating and of resistance to mating, possibly influenced by population density and sex ratio, may well be key determinates of polyandry in marine turtles, although quantifying these costs in natural populations would be difficult. Our results highlight the apparently complex influences of female traits, environment and mating strategy on reproductive success in natural populations and the challenges associated with unraveling them.

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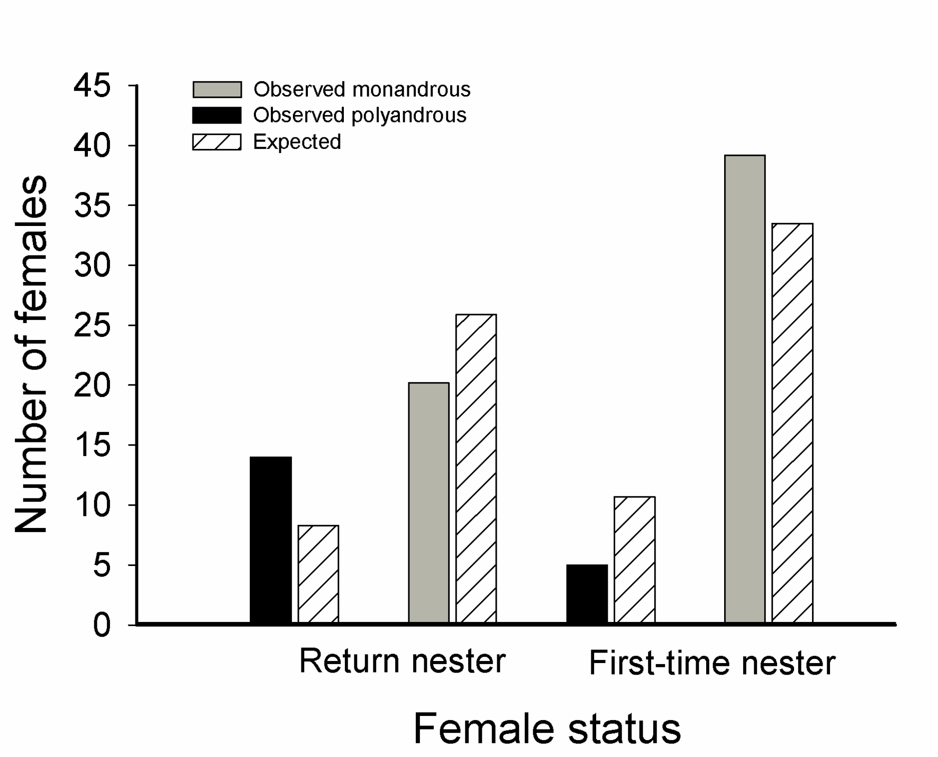


Figure 1. Numbers of polyandrous females (black bars) and monandrous females (grey bars) that were returning females (had previously nested at our study site) compared to first-time nesters. Solid bars show observed values, hashed bars show expected values.

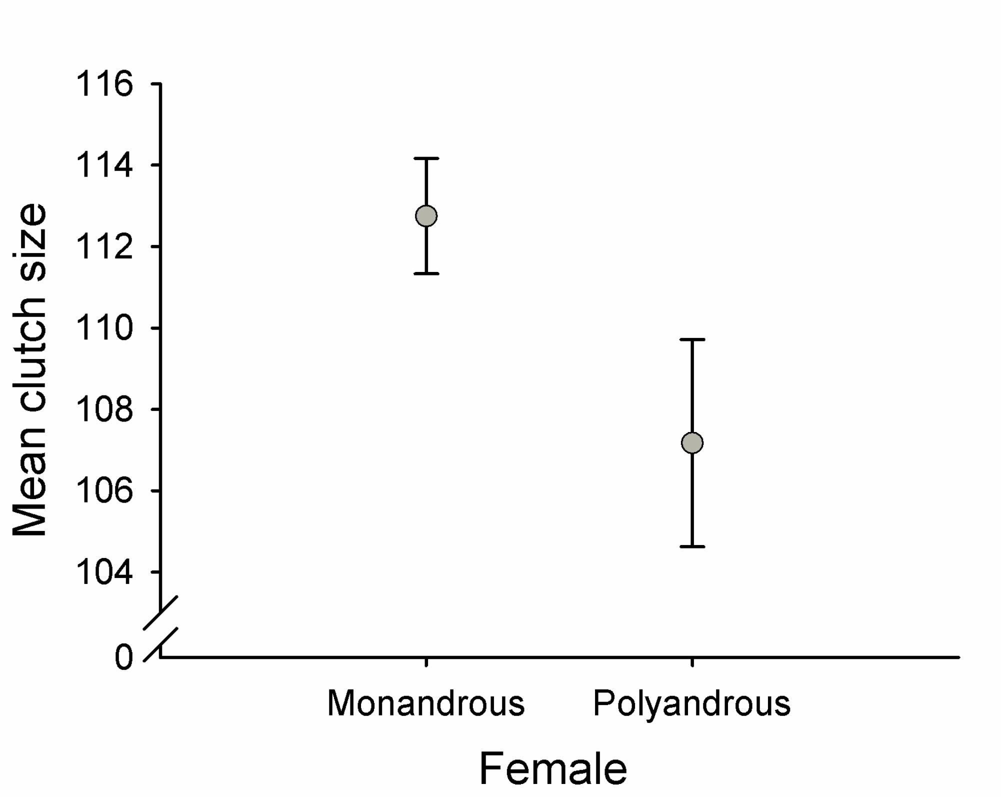
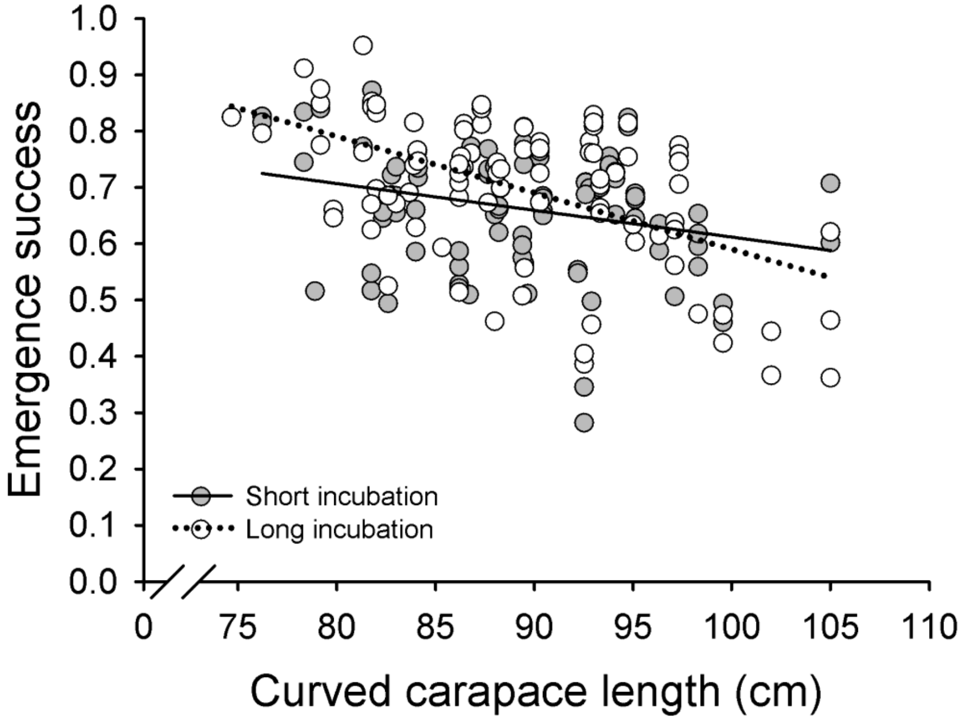


Figure 2. Effect of polyandry on total clutch size. Data points show fitted values of the models, which incorporate all predictor variables. Values are means ± 1 standard error.

a)


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Emergence success
Short incubation
Long incubation
0
75
80
85
90
95
100
105
110
Curved carapace length (cm)

b)

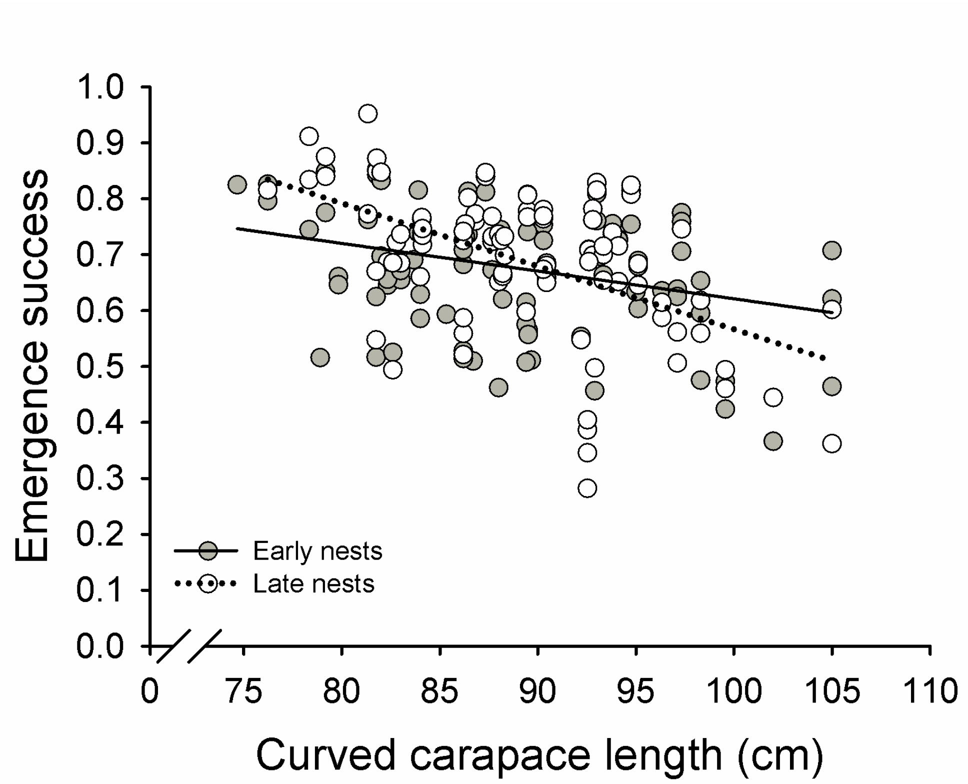

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Emergence success
Early nests
Late nests
0
75
80
85
90
95
100
105
110
Curved carapace length (cm)

Figure 3. Effect of female size (curved carapace length) and; a) incubation duration, and; b) lay date on emergence success of clutches. Data points show fitted values of the models, which incorporate all predictor variables. Nests were categorised as; a) having short or long incubation durations based on whether incubation duration was less than or equal to (short), or exceeded (long) the median value of 48 days incubation duration; b) early or late nests based on whether they were laid on or before (early), or after (late) the median lay date (182 Julian days).

CHAPTER 5

High level of multiple paternity in green turtle clutches at Ascension Island

Lucy I Wright, Kate E Plummer, Sam B Weber, Brendan J Godley, Andrew McGowan, Tom Tregenza, Annette C Broderick[a]

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Centre for Ecology and Conservation, University of Exeter, Cornwall Campus, Penryn, TR10 9EZ, UK.

[a] Corresponding author: Dr Annette Broderick

E-mail: A.C.Broderick@exeter.ac.uk

ABSRACT

Multiple paternity has been recorded in all seven species of marine turtle, but the frequency with which it occurs varies substantially both within and among species, and the factors influencing female remating rate remain poorly understood. In this study, we examined paternity of green turtle (*Chelonia mydas*) clutches laid at Ascension Island, one of the largest green turtle rookeries in the world. Our results reveal multiple sires in the clutches of 91% (10 out of 11) of study females, with up to four fathers contributing to a single clutch. This is amongst the highest incidence of multiple paternity recorded in any marine turtle population. In contrast to many other nesting populations, where a single male tends to dominate paternity within a clutch, the relative siring success of different fathers was similar in over half of the clutches analysed. Additionally, the same males sired offspring in the successive clutches of all study females, supporting the hypothesis that females do not mate between nesting events, but use stored sperm to fertilize clutches throughout the nesting season. We show that offspring size varies among fathers, however, within multiply sired clutches we found no evidence that males that achieved a high paternity share produced larger offspring. We compare our results to documented levels of multiple paternity in other marine turtle populations and discuss the potential influences on female remating rate in these species.

INTRODUCTION

Animal mating strategies, such as female remating rate and the conflicting interests of males and females, are key areas of research in evolutionary biology, but also have important consequences for population dynamics and the conservation of threatened species. For example, operational sex ratios and reproductive skew strongly influence genetic effective population size (Frankham 1995; Anthony and Blumstein 2000; Hare *et al.* 2011), and male harassment can increase female mortality rate, with consequences for population growth rate and persistence (Le Galliard *et al*. 2005; Johanos *et al*. 2010). Over the past two decades, the use of molecular techniques has substantially advanced our understanding of mating systems in wild populations, often revealing unexpected paternity distributions (e.g. Hughes 1998; Griffith *et al*. 2002; Gottelli *et al*. 2007) and showing multiple mating by females, or polyandry, to be common across taxa (reviewed in Avise *et al*. 2002; Griffith *et al.* 2002; Uller and Olsson 2008). In marine turtles, due to the difficulties in reliably observing mating pairs at sea, the application of molecular methods to the study of mating behaviour has proven to be particularly valuable. Molecular parentage analyses have documented multiple paternity in all seven species of marine turtles (reviewed in Bowen and Karl 2007; Joseph and Shaw 2010), demonstrating widespread polyandry in this animal group. Molecular techniques have also provided information on the number and sex ratio of breeding individuals (Stewart and Dutton 2011; Wright *et al*. 2012a), the mating frequency of male turtles (Wright *et al.* 2012b), and have revealed substantial variation in the rates of multiple paternity among species and nesting populations (Jensen *et al.* 2006; Bowen and Karl 2007; Lee 2008), raising interesting questions about the factors that influence the prevalence of polyandry in marine turtles.

Marine turtles are promiscuous breeders with both males and females mating with multiple partners (Hamann *et al.* 2003). The mating system has been described as scramble polygamy (Jessop *et al.* 1999), characterized by males competitively searching for and courting females, with intermittent inter-male aggression. Mating takes place either close to nesting beaches or in regional breeding grounds from which females disperse to nesting rookeries (Limpus 1993). Females are then thought to use stored sperm to produce multiple clutches of eggs at approximately 2 week intervals throughout the nesting season (Miller 1997): a hypothesis which is supported by genetic data showing consistent paternity across successive clutches of individual females in some populations (e.g. FitzSimmons 1998; Kichler *et al.* 1999; Joseph and Shaw 2010; Stewart and Dutton 2011). Marine turtles do not defend territories, form pair bonds or care for their young beyond nesting (Pearse and Avise 2001), hence, there is likely to be strong selection on males to increase their reproductive success by mating with as many females as possible. Females are able to refuse copulations from courting males (Booth and Peters 1972), suggesting that multiple paternity is a consequence of females choosing to mate multiply, rather than from males forcing copulations. Female turtles could potentially receive direct benefits from multiple mating, such as fertility assurance (Uller and Olsson 2005), or gain indirect benefits through increased fitness of their offspring (Jennions and Petrie 2000; Hosken and Stockley 2003; Uller and Olsson 2008). However, while numerous studies have documented multiple paternity in marine turtles, no fitness benefits of polyandry have yet been clearly demonstrated, leading to the suggestion that multiple paternity in these species may be driven by sexual conflict, with females mating multiply due to high costs of resisting male harassment (convenience polyandry (Thornhill and Alcock 1983)) (Lee and Hays 2004).

Although multiple paternity has been described in all species of marine turtles, its occurrence varies widely among nesting populations from almost none (FitzSimmons 1998) to virtually 100% of clutches (Ireland *et al.* 2003; Jensen *et al.* 2006; Zbinden *et al.* 2007). There are likely to be numerous population-specific influences on female remating rates that have contributed to the observed variation in frequency of multiple paternity in marine turtle populations. Population density and operational sex ratio will influence encounter rates of the same and the opposite sex and can have strong effects on mating tactics and mate choice (Emlen and Oring 1977; Kokko and Rankin 2006), with potential consequences for the level of multiple mating. Furthermore, the length and synchrony of female receptivity, the degree of aggregation of females (Emlen and Oring 1977), and the ability of males to coerce females into mating (Clutton-Brock and Parker 1995) may all influence the frequency of multiple mating, whilst sperm storage may influence multiple paternity of clutches (Uller and Olsson 2008). Jensen *et al.* (2006) compared multiple paternity in neighbouring rookeries of olive ridley turtles (*Lepidochelys olivacea*), one of which hosts a very high density mass-nesting (arribada) population, and the other a low density population of solitary nesting females. Multiple paternity was significantly more prevalent in the arribada rookery, which the authors attributed to the very high density of individuals in the breeding ground, and this result has been interpreted as further support for convenience polyandry in marine turtles (Bowen and Karl 2007; Lee 2008). However, theoretical and empirical work has revealed variable effects of density on female remating rate (Härdling and Kaitala 2005; Kokko and Rankin 2006). More studies of multiple paternity in marine turtles over a range of population densities are needed to better understand their mating strategies and the possible causes of variation in levels of polyandry.

In this study, we use microsatellites to examine multiple paternity in green turtles clutches at Ascension Island, one of the largest green turtle rookeries in the world (Broderick *et al.* 2006). We compare paternal contributions to multiple clutches from known females to assess patterns of paternity across the nesting season, and explore the relationship between relative siring success of males within multiple paternity clutches and offspring size. We discuss our results in relation to published levels of multiple paternity in other marine turtle populations.

METHODS

Study site

The study was conducted in a nesting population of green turtles at Long Beach, Ascension Island during the 2007 nesting season (January – May). Ascension Island is an isolated volcanic peak in the South Atlantic Ocean that hosts the second largest breeding population of green turtles in the Atlantic, estimated to contain 11,000 – 15,000 adult females (Broderick *et al.* 2006). There are 5809m of nesting beach around the island, but Long beach is the primary nesting area (Godley *et al.* 2001).

Field methods and sample collection

Tissue samples were taken from marked females (n = 11) immediately following nesting, from the trailing edge of the foreflipper. Female size was recorded (curved carapace length (CCL) and width (CCW)) and radio transmitters (Biotrack Ltd., Wareham, U.K.) were attached to the females' carapace, following previously described protocol (Godley *et al.* 2002a), to enable relocation of individuals during subsequent nesting events. Nests were marked and, upon hatching, either a blood sample (maximum 0.1 ml taken from the dorsal cervical sinus) or a small tissue sample (from the trailing edge of the carapace) was taken from a proportion of offspring from each clutch. Hatchlings were weighed and measured (straight carapace length (SCL), straight carapace width (SCW)) before being released. Nests were excavated 48 hours after the last emergence of hatchlings and tissue samples were taken from any live or dead hatchlings inside the nest, as well as from dead embryos from un-hatched eggs. Hatching success was determined by counting the number of un-hatched eggs and egg fragments remaining in the egg chamber. All blood and tissue samples were stored in Queen's lysis buffer (Seutin *et al.* 1991). Two clutches were sampled for each female to enable us to investigate across-season patterns of paternity in the clutches of specific individuals. Blood samples were taken from a further 34 nesting females in 2008 for use in characterizing microsatellite loci in green turtles at this site.

Genotyping and paternity analysis

We extracted genomic DNA from blood and tissue samples using a standard ammonium acetate precipitation method (Nicholls *et al.* 2000) and genotyped samples at 12 polymorphic microsatellite loci designed for use in marine turtles (Table 1). Primers were labelled with fluorescent dyes (6-FAM, HEX or NED) and the loci were amplified in 2 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 15min followed by 35 cycles of 94°C for 30s, 58°C for 90s and 72°C for 60s, and finally one cycle of 60°C for 30min. 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. We aimed to genotype a minimum of 20 offspring from each clutch. Samples that failed to amplify at all loci were re-amplified and re-scored up to three times and any samples that persistently failed were excluded from further analyses. Genotypes were resolved at a minimum of 8 loci for 502 offspring (mean ± sd = 22.8 ± 2.15 hatchlings per clutch, range = 19-28, Table 2) from 11 mothers. Genotypes were fully resolved at all 12 loci for 88% of individuals.

We assessed the repeatability of genotyping by re-extracting, re-amplifying and re-scoring approximately 8% of samples in a blind fashion and comparing them to original allele calls. We observed differences in the repeated genotypes at 5 allele calls out of 978 (0.5% error), however, at one locus (B123) the error rate reached 3.5%. We also used the program MicroErrorAnalyzer (Wang 2010) to analyse the entire dataset for null alleles, allelic dropout and genotyping error from known parent-offspring dyads. Maximum likelihood estimates of error rates using this software were <0.001 for null alleles and allelic dropout, and 0.001 for genotyping error. Samples from 45 adult turtles (11 mothers that were included in the study plus 34 additional females from the same site) were used to calculate population allele frequencies and test the markers for deviation from Hardy-Weinberg equilibrium (HWE), using 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 and Rousset 1995). Probability of detecting multiple paternity (PrDM) was assessed using PrDM software (downloaded November 2011) (Neff and Pitcher 2002).

Sibship and parentage analysis were carried out using the program COLONY version 2.0 (Wang and Santure 2009). Three runs of ‘long’ length were conducted on the same dataset assuming conservative error rates of 0.01 for allelic dropout and genotyping error for all loci except B123, for which the genotyping error rate was set to 0.035. Maternity of all offspring was known and all genotyped offspring were analysed together in order to detect full-sibs and both maternal and paternal half-sibs.

Statistical analysis

We firstly tested the prediction that there is an association between particular males and the size of their offspring, using Restricted Maximum Likelihood (REML) with hatchling size (SLC x SCW) as the response term and male identity (ID) included as a fixed factor. Female size (CCL), lay date (number of days since 1 January), incubation duration and total clutch size were included as covariates. Within clutches demonstrating multiple paternity, we went on to rank fathers based on the absolute number of offspring that were assigned to them, regardless of whether the relative male contributions to the clutch were significantly skewed from equal contributions or not. We then explored the relationship between male rank and offspring size using the same modelling approach. Hatchling size (SLC x SCW) was used as the response term and male rank was included as a fixed factor, with the same covariates as the previous model. Female ID, clutch ID and male ID (second model only) were included as random factors to control for the inclusion of multiple individuals from the same clutch (and sire) and multiple clutches from the same females. All statistical analyses were conducted in the program GenStat release 13.2 (GenStat 2010). Second order interactions were included in the full models; final models were selected using stepwise elimination of non-significant terms (P>0.05) (for discussion of the use of stepwise modelling see (Whittingham *et al.* 2006; Murtaugh 2009)); and residuals were checked for normality and homoscedasticity after each deletion step.

RESULTS

Patterns of multiple paternity

Multiple paternity was detected in 20 of 22 study clutches from 10 of 11 (91%) females in this study. For all females, COLONY assigned the same putative fathers to both of the clutches that we sampled, supporting the notion that females do not remate between successive clutches, but instead use stored sperm to fertilize clutches throughout the nesting season. We found no evidence that any males sired offspring with more than one female in this study, demonstrated by the lack of paternal half-sibs in the COLONY analysis. Thus, our analysis revealed that 24 unique males sired offspring from the 11 study females. If this small sample is representative of the entire population then it would imply a breeding sex ratio of 2.2 males to each female. Only one female produced clutches with a single sire, suggesting that the majority of females at this site are polyandrous. Of the remaining females, we detected two sires in the clutches of eight females, three sires for one female and four sires for the final female (Table 2, Figure 1). In 8 out of 20 clutches with multiple paternity, paternal contributions to clutches significantly deviated from equal contributions (χ^2, all P< 0.05, Figure 1). Primary males, which were assigned the most offspring in the clutch, sired between 48% and 90% of the clutch. Although the primary male in the first clutch was also the primary male in the second clutch for the majority of females (9 out of 11), relative paternal contributions did vary across clutches. For example, for four females, paternal contributions were significantly skewed from equal contributions in one of their clutches but not in the other (Figure 1).

Influence of male ID and male rank on offspring size

Hatchling size was significantly affected by male ID (REML; W = 35.43, p = 0.047) suggesting that different males give rise to different sized offspring. However, males that achieved a high paternity share within multiply sired clutches did not also produce larger offspring; i.e. hatchling size was not influenced by male rank (based on the absolute number of offspring assigned to each male within a clutch) (REML; W = 1.88, p = 0.179). Hatchling size in this study was positively affected by incubation duration (REML; W = 11.56, p < 0.01, Figure 2), and by lay date (REML; W = 7.61, p = 0.022).

Microsatellite markers

All 12 microsatellite markers were polymorphic in our population; the number of alleles per locus ranged from 3 to 11 (Table 1). All markers conformed to expectations of HWE (P>0.05) and showed low frequency of null alleles. 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 12 loci was >0.999. Probability of detecting multiple paternity (PrDM) assuming 2 fathers with equal paternal contributions and 23 offspring sampled per clutch (the mean number genotyped per clutch in this study) was >0.99; assuming 2 fathers with skewed paternal contributions (10:90%) PrDM was 0.91; and assuming 3 fathers and skewed paternal contributions (10:20:70%) PrDM was >0.99. When we reduced the number of offspring sampled per clutch to 19 (the lowest number sampled from any of our clutches) PrDM assuming 2 fathers with skewed paternal contributions (10:90%) dropped to 0.86 but exceeded 0.99 for the other scenarios.

DISCUSSION

The frequency of multiple paternity in marine turtle clutches has previously been shown to vary dramatically among populations. We found multiple paternity in the clutches of 91% of females (10 out of 11) included in this study, which, taken together with previous work at this site (Ireland *et al.* 2003; Lee and Hays 2004), suggests a very high level of polyandry among female green turtles nesting on Ascension Island. Previous studies of paternity in green turtles at this nesting site have documented multiple fathers in the clutches of 61% (n =18; Lee and Hays 2004) and 100% (n =3; Ireland *et al.* 2003) of females, suggesting that the frequency of multiple paternity may vary among breeding seasons within the same nesting population, potentially influenced by annual fluctuations in the numbers of breeding individuals (Heppell *et al.* 2003). The frequency of multiple paternity detected in this study is much higher than that reported in green turtle nesting populations in Australia (15%, n = 13, (FitzSimmons 1998)) , Cyprus (24%, n = 78, (Wright *et al.* 2012b)), and is similar to observed levels of multiple paternity in a loggerhead turtle rookery in Greece (93%, n =15, (Zbinden *et al.* 2007)) and a mass nesting olive ridley turtle rookery in Costa Rica (92%, n =13, (Jensen *et al.* 2006)). To what extent the observed levels of multiple paternity reflect actual levels of multiple mating is not clear; FitzSimmons (1998) reported a very low incidence of multiple paternity despite observations of females mating with multiple males in the population. Polyandrous females may produce single paternity clutches as a result of strong sperm competition or sperm selection (Uller and Olsson 2008). Alternatively, multiple paternity of clutches can arise via the use of stored sperm across multiple breeding seasons, as has been reported in freshwater turtles (Pearse *et al.* 2002), which would represent long-term polyandry rather than multiple mating within a breeding season. Nonetheless, it is clear that there is a high degree of variation in the level of multiple paternity between species, populations and in different breeding seasons.

We observed identical paternal alleles in the successive clutches of all females in the study, supporting the hypothesis that females do not mate between nesting events (or that subsequent copulations do not result in successful fertilisation of eggs), but mate prior to the onset of nesting and use stored sperm to fertilize clutches throughout the nesting season (Owens 1980). In previous paternity studies, identical fathers have been assigned to successive clutches of green (FitzSimmons 1998), loggerhead (*Caretta caretta*) (Zbinden *et al.* 2007), leatherback (*Dermochelys coriacea*) (Crim *et al.* 2002; Stewart and Dutton 2011), hawksbill (*Eretmochelys imbricata*) (Joseph and Shaw 2010) and Kemp’s ridley (*Lepidochelys Kempi*) (Kichler *et al.* 1999) turtles. Furthermore, mating activity of green turtles at Ascension Island appears to decline to a low level before the peak of nesting (Godley *et al.* 2002b) and male turtles have been observed leaving breeding grounds soon after nesting begins (Limpus 1993; Hays *et al.* 2010), suggesting that mating late in the nesting season is uncommon. However, for some females in this study, the relative contributions from sperm of multiple fathers altered between successive clutches, as has been observed in successive clutches of leatherback turtles (Crim *et al.* 2002), suggesting differential use of stored sperm or the depletion or degradation of sperm from particular males across the nesting season.

In more than half of the clutches with multiple paternity in this study, the relative contributions of different fathers were not different from equal paternal contributions, indicating similar siring success among fathers that contributed to these clutches. This result is in contrast to a previous study of paternity in this population where 9 out of 10 multiple paternity clutches were significantly skewed from equal paternal contributions (Lee and Hays 2004), and to green turtle clutches in Cyprus, where 81% of clutches showed skewed paternal contributions (Wright *et al.* in review), and Australia, where the primary males sired on average 96% of the clutch (FitzSimmons 1998). Relatively equal paternal contributions to multiple paternity clutches have also been reported in leatherback turtles (Crim *et al.* 2002) and, interestingly, in the loggerhead (Zbinden *et al.* 2007) and olive ridley (Jensen *et al.* 2006) turtle rookeries that showed similar high levels of multiple paternity to the green turtle rookery in this study. Perhaps in populations where females are under intense mating pressure, mating with multiple males in rapid succession could reduce sperm precedence effects. Alternatively, if numerous males of similar quality are available, then they may be equally successful under sperm competition (Birkhead and Møller 1998), or females may exercise less post-copulatory mate choice (Jennions and Petrie 2000) (although there is currently no evidence for cryptic female choice in marine turtles).

It is possible that female turtles benefit directly or indirectly from polyandry (Hosken and Stockley 2003; Uller and Olsson 2008), and that females in different populations benefit to a greater or lesser extent, influencing female remating rate and leading to the variable levels of multiple paternity across marine turtle populations. However, despite an increasing number of studies documenting multiple paternity in all species of marine turtles (reviewed in Bowen and Karl 2007), there is currently no clear evidence that females benefit from polyandrous mating behaviour (Lee and Hays 2004; Wright *et al.* in review) (but see Zbinden *et al.* 2007). Several hypotheses relating polyandry to indirect genetic benefits, including ‘trading up’, ‘good genes’ and cryptic female choice (reviewed in Jennions and Petrie 2000; Uller and Olsson 2008), predict that paternity should be skewed towards particular ‘high quality’ males and that those males should sire superior young. We show that offspring size varies among fathers; large hatchling size has previously been linked to increased post-hatching survival in green turtles (Gyuris 2000) and freshwater turtles (Janzen *et al.* 2000), hence, male traits could potentially influence female mate choice. However, we found no evidence in this study that males that achieve a high paternity share in multiple paternity clutches sire bigger offspring, although our relatively small sample size prevented a more thorough analysis of the potential beneficial effects of polyandry on female fitness.

In both the mass-nesting olive ridley rookery investigated by Jensen *et al.* (2006) and the loggerhead rookery studied by Zbinden *et al.* (2007), the observed high levels of multiple paternity were attributed, at least in part, to the very high density of breeding individuals expected to occur on the mating grounds. The olive ridley rookery hosts an average of 130,000 nesting females per 5-7 day nesting event and mating takes place offshore of the nesting beach in an extremely high density courtship patch (Jensen *et al.* 2006). In comparison, the loggerhead rookery hosts a small nesting population of no more than 500 nesting females, however, mating is thought to be confined to one small bay, hence, density of mating individuals could reach intense levels and lead to high mate encounter rates (Zbinden *et al.* 2007). The nesting population of green turtles on Ascension Island is large (approximately 3800 females nesting annually) and expanding (Broderick *et al.* 2006), the mating areas are localised, close to the nesting beaches (Godley *et al.* 2001; Godley *et al.* 2002b) and the density of breeding individuals is also likely to be high at this rookery.

Support for a relationship between population density and multiple paternity is provided by an apparent trend of increasing multiple paternity with nesting population size across marine turtle species (Ireland *et al.* 2003; Jensen *et al.* 2006), and a significant positive correlation between incidence of multiple paternity and breeding population size within the genus *Lepidochelys* (Jensen *et al.* 2006). The results of this study seem to be in broad agreement with this notion. However, there are notable exceptions to this pattern (e.g. FitzSimmons 1998; Zbinden *et al*. 2007), suggesting that population size is not always a good predictor of local density in mating areas (Kokko and Rankin 2006; Lee 2008), or that other factors may have an important influence on female remating rate. An effect of breeding population density on the frequency of multiple paternity is consistent with the theory that polyandry in marine turtles is a consequence of male harassment; empirical studies show that sexual conflict (Martin and Hosken 2003) and convenience polyandry (Lauer *et al*. 1996; Arnqvist and Rowe 2005) are elevated in high density populations, and aggregative behaviour is thought to facilitate male coercion and lead to increased rates of multiple paternity in sharks (Daly-Engel *et al.* 2010).

The operational sex ratio of the breeding population (OSR; the ratio of males to females that are ready to mate at any one time (Emlen and Oring 1977)) is also likely to influence the frequency of multiple mating. A biased OSR will increase the mate encounter rate for the limiting sex, whilst increasing competition for the limited sex and can intensify the effects of density (Grant *et al.* 1995; Kokko and Rankin 2006). A male-biased OSR might, therefore, be expected to increase the levels of multiple paternity, particularly if males are able to coerce females into accepting additional copulations, although empirical work has produced contradictory results regarding the effect of sex ratio bias on multiple paternity in reptiles. For example, in common lizards (*Lacerta vivipara*), the number of fathers contributing to multiple paternity clutches was greater in male-biased compared to female-biased enclosures, presumably due to increased male harassment, but overall, the frequency of multiple paternity was not more common in the male-biased environment (Fitze *et al*. 2005). Furthermore, in the mass-nesting olive ridley turtle rookery, where the level of multiple paternity was very high (Jensen *et al*. 2006), the OSR was apparently highly female biased. The OSR of the population at Ascension Island is not known; we found that 24 unique males had mated with our 11 study females, implying a male biased OSR. Frequent observations of attendant males harassing mating pairs (B. J. Godley, personal communication) supports this possibility, and the adult sex ratio could still be suffering the impacts of historical harvest of female green turtles at this site, which did not cease until the 1940s (Huxley 1999). The offspring sex ratio at Ascension Island is estimated to be approximately 75% female (3:1 females to males) (Godley *et al.* 2002c), but male-biased sex ratios of breeders have previously been reported in marine turtle populations that exhibit a highly female-biased offspring sex ratio (Stewart and Dutton 2011; Wright *et al.* 2012a,b).

The length and degree of synchrony of female receptivity will influence the OSR (Emlen and Oring 1977). Female green turtles are apparently receptive for 2-4 days

(Owens 1980; Comuzzie and Owens 1990), whilst males are thought to be sexually active for around a month (Limpus 1993). Although reproduction is highly seasonal, the arrival of females at breeding grounds may be spread over several months (Limpus 1993; Godley *et al*. 2002b; Hamann *et al.* 2003), hence, males most likely have the opportunity to mate with numerous females, and competition among males for mates may be high. Little is known about the spatial behaviour of males or females on mating grounds; males appear to be highly active (Hays *et al.* 2001), consistent with scramble mating tactics, and can travel large distances within the mating season (Hamann *et al.* 2003; James *et al.* 2005; Wright *et al*. 2012a) which will increase encounter rates with females and could also influence female remating rates.

In summary, we demonstrate an extremely high frequency of multiple paternity in green turtle clutches at Ascension Island, further highlighting the remarkably variable levels of multiple paternity both within and between marine turtle populations. Our results broadly fit into the postulated pattern of increasing multiple paternity with breeding population density (Ireland *et al*. 2003; Jensen *et al*. 2006; Lee 2008), but further studies are needed to fully understand the ecological and demographic influences on marine turtle mating strategies. Despite the high incidence of multiple paternity in the Ascension Island population, as in previous studies, we found no evidence that polyandrous females gain benefits from biasing paternity towards particular males, at least in terms of offspring size. Whilst there could be many other benefits of polyandry to female reproductive success or offspring survival that were not tested, our results are more consistent with the hypothesis that multiple paternity is driven by male harassment and is, thus, more prevalent in high density nesting populations where courtship encounters are more frequent.

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Table 1. Characterization of microsatellite loci in green turtles (*Chelonia mydas*) at Ascension Island.

Locus	**Reference**	**N**	**No. alleles**	**H_O**	**H_E**	**PIC**
A6	(Dutton & Frey 2009)	45	6	0.689	0.663	0.598
B103	(Dutton & Frey 2009)	45	6	0.444	0.468	0.430
B123	(Dutton & Frey 2009)	45	4	0.533	0.536	0.483
C102	(Dutton & Frey 2009)	45	3	0.556	0.497	0.391
Cm3	(Fitzsimmons *et al.* 1995)	44	9	0.591	0.577	0.551
Cm58	(Fitzsimmons *et al.* 1995)	45	9	0.756	0.807	0.773
Klk314	(Kichler *et al.*1999)	45	3	0.644	0.631	0.545
Or7	(Aggarwal *et al.* 2004)	45	6	0.733	0.755	0.703
Cc2	(Monzon-Arguello *et al.* 2008)	44	11	0.795	0.803	0.765
Cc28	(Monzon-Arguello *et al.* 2008)	45	4	0.622	0.609	0.538
CcP7D04	(Shamblin *et al.* 2009)	45	10	0.889	0.877	0.854
CcP7E11	(Shamblin *et al.* 2007)	45	7	0.689	0.655	0.601

H_O, observed heterozygosity; $H_{E,}$ expected heterozygosity; PIC, Polymorphic information content;

Table 2. Sample sizes for genotyped offspring and number of inferred fathers per clutch. Column 6 gives the most likely paternity and sibship inferences across the 3 COLONY runs. No candidate males were sampled; father IDs refer to un-sampled putative males. Numbers of offspring per clutch that were assigned to inferred sires are shown in brackets.

Mother ID – Clutch	**No. of offspring sampled**	**Clutch size**	**Hatching success %**	**Inferred no. of fathers**	**Inferred father ID (no. offspring sired)**
1-3	20	132	67	2	F1 (11), F2 (9)
1-4	23	124	74	2	F1 (11), F2 (12)
2-2	24	104	95	3	F3 (12), F4 (5), F5 (7)
2-4	24	111	90	3	F3 (7), F4 (14), F5 (3)
3-2	28	156	96	2	F6 (15), F7 (13)
3-4	23	159	93	2	F6 (13), F7 (10)
4-3	21	143	84	2	F8 (15), F9 (6)
4-5	23	144	88	2	F8 (19), F9 (4)
5-2	24	134	75	2	F10 (19), F11 (5)
5-3	24	146	92	2	F10 (21), F11 (3)
6-2	19	144	82	1	F12 (19)
6-4	23	170	85	1	F12 (23)
7-2	22	103	98	2	F13 (18), F14 (4)
7-4	20	82	98	2	F13 (18), F14 (2)
8-2	24	145	98	2	F15 (9), F16 (15)
8-5	22	146	90	2	F15 (8), F16 (14)
9-3	21	143	98	2	F17 (16), F18 (5)
9-5	22	162	90	2	F17 (12), F18 (10)
10-2	26	151	78	2	F19 (11), F20 (15)
10-4	20	138	65	2	F19 (7), F20 (13)
11-2	23	136	96	4	F21 (4), F22 (16), F23 (2), F24 (1)
11-3	25	146	96	4	F21 (5), F22 (12), F23 (4), F24 (4)

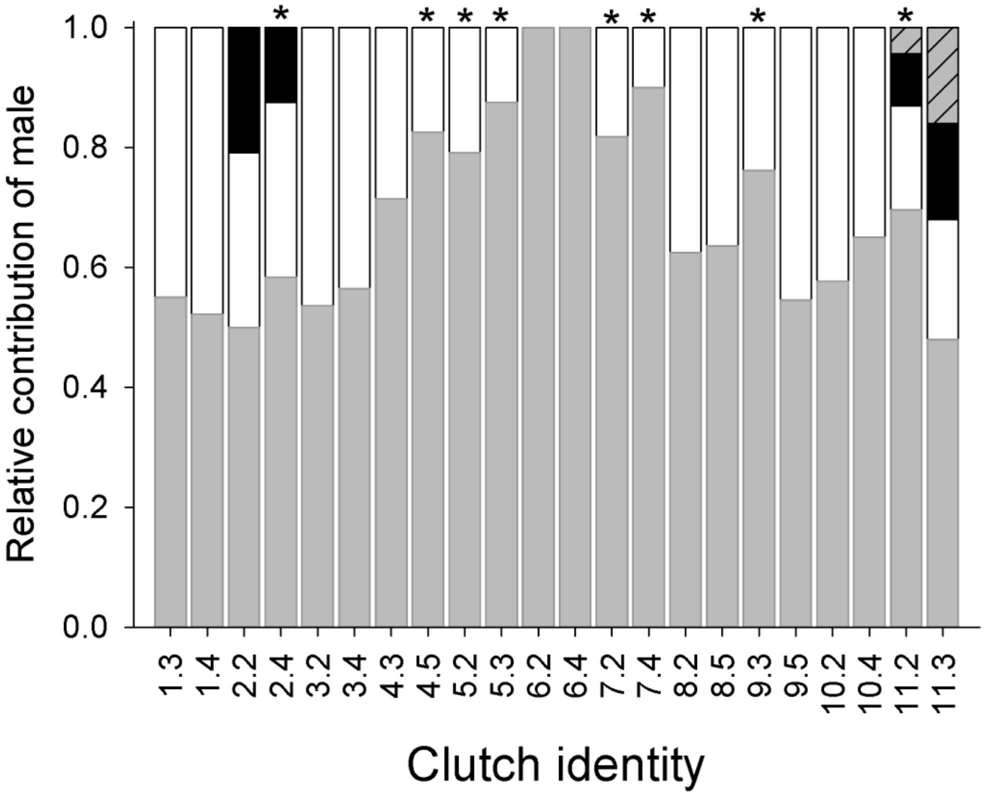

Figure 1. Relative siring success of males in each clutch (father 1 shown in grey, father 2 in white, father 3 in black and father 4 in grey hatched). Clutches in which the paternal contributions are significantly different from equal contributions are indicated with an asterisk.

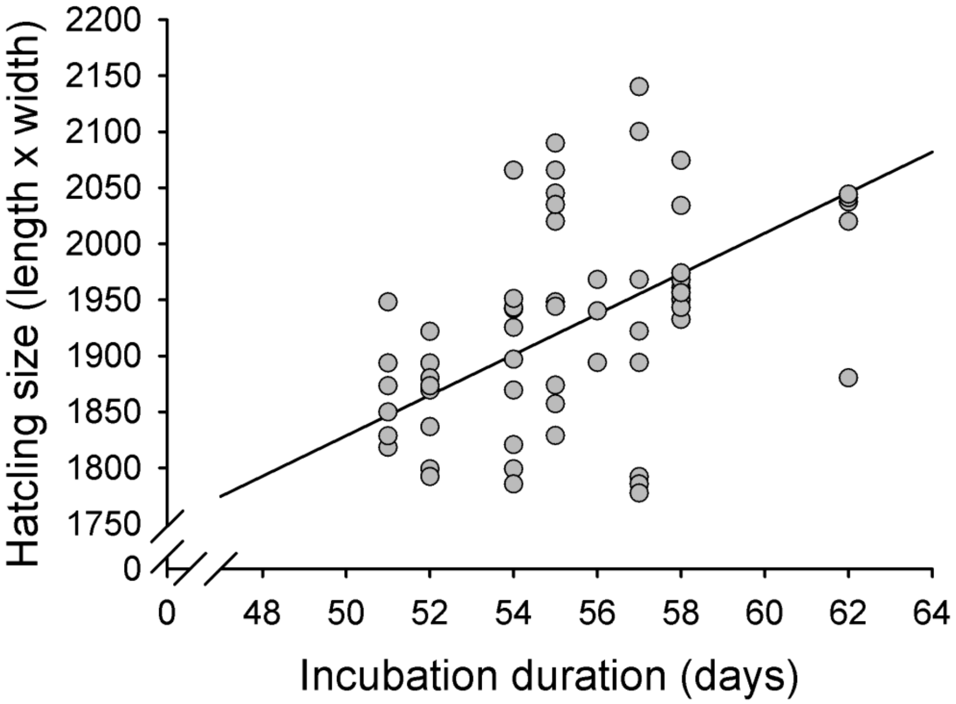

Figure 2. Effect of incubation duration on hatchling size. Data points show fitted values of the models, which incorporate all predictor variables.

CHAPTER 6

General discussion

The study of mating systems involves determining the number of mates that males and females obtain, the circumstances under which individuals acquire mates, the type of pair bonds they form, and often focus on how particular mating strategies affect reproductive success (Emlen and Oring 1977). It is now well known that the genetic mating system, based on the number of individuals that successfully reproduce and with whom, can differ substantially from the social mating system, based on observations of individuals associating with each other (Hughes 1998), hence, molecular techniques are now routinely employed in mating system studies and can reveal mating behaviour that would otherwise be hidden from observers, particularly in elusive species, including marine turtles (Avise *et al.* 2002). Multilocus genotypic data allow accurate parentage assignments, which, when accomplished for large numbers of individuals, can uncover the genetic mating system and enable the assessment of pay-offs from particular mating strategies (Avise *et al.* 2002). However, understanding genetic mating systems is also highly relevant to conservation; the loss of genetic variation in small populations is largely dependent on the genetic effective population size (N_e) (Wright 1931), which itself is strongly influenced by skewed sex ratios of breeders and variance in reproductive success (Frankham 1995). Furthermore, mating systems can influence mortality and population growth rates (reviewed in Anthony and Blumstein 2000) and can impact upon a populations recovery from exploitation (Greene *et al.* 1998), thus, the study of mating systems has clear significance for the conservation of threatened species (Caro 1998).

Studying marine turtle mating systems is complicated by their complex, highly mobile lives and the difficulty in locating and observing them at sea, resulting in the current scarcity of information relating to many aspects of their breeding behaviour and impeding accurate assessments of N_e in marine turtle populations. In this thesis I used microsatellite markers to assign parentage and determine the genetic mating system of two green turtle populations. I combined this data with field studies to assess the influence of female mating strategies on aspects of reproductive success, but also use this genetic data to infer sex ratios of breeding individuals and the breeding frequency of male turtles, which will contribute to future assessments of population viability. In the following pages I summarise the key findings of my research, highlight the contribution that my results make to the existing body of work, but also discuss the limitations of the work and make suggestions for future avenues of research.

Male mating patterns and operational sex ratios

The operational sex ratio (OSR; the ratio of males to females that are ready to mate at any one time) (Emlen and Oring 1977) is a key component of mating systems that influences intra-specific competition for mates, mate choice, sexual conflict and mating tactics. Related to the OSR, and of relevance to conservation due to its influence on N_e, is the sex ratio of individuals that successfully breed; a highly skewed sex ratio of breeders will reduce N_e and lead to a reduction in genetic diversity (Frankham 1995). Because offspring sex ratios are commonly highly female-skewed in marine turtle populations (Hawkes *et al.* 2009), and may become more so as a result of predicted climate change (Witt *et al.* 2010), there is concern that a lack of males will further endanger populations through reduced mate availability and/or the negative genetic effects of a decline in N_e. The primary sex ratio of our study population in Cyprus is extremely female-skewed (86%-96% female, (Broderick *et al.* 2000; Wright *et al.* 2012)) and I anticipated that the sex ratio of breeders would also be female-biased, however, in **chapters two** and **three** I show that it is actually male-biased, with at least 1.3 reproductive males to every nesting female. Furthermore, I show that no particular males were able to dominate reproduction, since there was no evidence of males that had mated with more than one female within a breeding season. The near saturation coverage of a single nesting colony enabled me to assess the sex ratio of breeders and the number of males contributing to reproduction much more accurately than had been achieved previously in any marine turtle population (e.g. Stewart and Dutton 2011).

It is often suggested in the literature that male turtles may breed annually, or certainly more frequently than females (Hamann *et al.* 2003; Hays *et al.* 2010), possibly due to lower costs of reproduction in males, but there is little unequivocal evidence to support this belief (Limpus 1993). It has also been proposed that more frequent mating by males than females could result in a relatively equal OSR even if the adult sex ratio is female-biased (Hays *et al.* 2010). I show in **chapter three** that male green turtles at my study site do not breed annually; in fact, 97% of males that contributed to reproduction at the study site over a three year period were assigned offspring in just one breeding season. This result demonstrates that the relatively equal sex ratio of breeders in a given year is not a result of the same males mating on an annual basis, but that different males successfully reproduced in all 3 breeding seasons, suggesting that the population of adult males at this site is larger than expected based on offspring sex ratios alone. The results from **chapters two** and **three** are encouraging in terms of the current genetic health of the Alagadi population, and its potential resilience to future climate change, but highlights a large disparity between the primary and adult sex ratios of green turtles at this site. In **chapter two**, the route taken by a single satellite tracked male turtle from the study site indicated that males may visit breeding grounds of different nesting populations within a breeding season, which could go some way to explaining our results, but further tracking studies of male turtles are clearly needed to confirm this pattern and would be an obvious focus for future work.

The movements and mating patterns of male marine turtles remain poorly understood, and the conclusions that can be drawn from my results are limited by this lack of knowledge. For example, although I show that male green turtles do not breed annually at Alagadi beach (**chapter three**), the caveat remains that these males could be mating at other nesting sites in subsequent years. Future satellite tracking studies of male green turtles in the Mediterranean between foraging and mating grounds, ideally over multiple breeding seasons, would shed light on this problem, although would clearly be dependent on adequate longevity and retention of satellite tags. Traditional mark and recapture methods could also offer a useful approach (Godley *et al*. 2008), although the lack of known specific breeding sites in the Mediterranean where males may aggregate currently make this approach difficult. If molecular-based paternity studies on the scale achieved in this thesis for Alagadi beach could be repeated at multiple rookeries throughout the Mediterranean, this would also offer a method of identifying males that successfully reproduce at multiple nesting sites.

Future research should also focus on the disparity between the observed relatively equal sex ratio of breeders and the highly female-biased hatchling sex ratio at our study site. If the males breeding at our study site originate from cooler nesting beaches elsewhere in (or outside of) the Mediterranean, then molecular methods may be able to reveal their natal origins. Maternally inherited mtDNA haplotypes have been commonly used in marine turtle studies to assign individuals to their natal populations, however, the segment of the mtDNA that is traditionally sequenced in marine turtles shows almost no variation in all Mediterranean green turtles sampled to date. This lack of variation currently prevents assignment of green turtles in the Mediterranean based on mtDNA. However, recent work sequencing a different portion of the mtDNA genome from green turtles in Israel demonstrates a promising amount of variation (Tikochinski *et al.* 2012) and may allow such assignments in the future, although this will require capturing and sampling males at sea. My results could also indicate a sex difference in mortality of green turtles at juvenile or adult life stages. Another interesting route for future work would be, firstly, to attempt to assess sex ratios of juvenile and adult green turtle populations in the Mediterranean (although there are difficulties associated with determining sex in juvenile turtles and in avoiding sampling bias; (Freedberg and Bowne 2006; Braun-McNeill *et al.* 2007)) and, secondly, to identify potential sources of mortality that could impact males and females differently. Tracking juvenile and adult turtles from foraging grounds could highlight sex differences in migration routes or dispersal that might make one sex more vulnerable to human-induced mortality. Finally, it is possible that OSRs of marine turtle populations will become more female-biased in the future, due to climate driven increases in female-skewed offspring production. Although the observation in **chapters two** and **three** that no males sired offspring with more than one female within a breeding season should currently help to maintain N_e, it implies that a relatively equal OSR may be important to maintain fertile populations of marine turtles and that males could become limiting if future OSRs become female biased. Furthermore, our finding that males do not reproduce more frequently than females at our study site suggests that mating periodicity in males will probably not be able to compensate for a female biased adult sex ratio and maintain an equal OSR, as has been previously suggested (Hays *et al.* 2010). Large-scale paternity analysis, as implemented in this thesis, offers a means of monitoring the sex ratio of breeding individuals, in order to identify and mitigate any future changes.

Male and female mating strategies

Polyandry has been demonstrated in almost all marine turtle populations that have been investigated, suggesting that pay-offs from this mating strategy may be important to female turtles, but very few studies have gone beyond simply documenting multiple paternity of clutches and investigated the potential benefits. In **chapter four** I examined the influence of polyandry on components of female reproductive success. Despite achieving a robust sample size, measuring multiple components of female and offspring fitness, and assigning offspring to specific sires in order to better test hypotheses relating to 'good genes' and cryptic female choice, I did not detect any clear fitness benefits to polyandrous females. This result is in line with the only previous study that explored fitness pay-offs to polyandrous females in green turtles, which also failed to detect any benefits (Lee and Hays 2004), and with a recent review of multiple paternity in reptiles (Uller and Olsson 2008), which concluded that polyandry in this taxonomic group is more likely to be driven by strong selection on males to mate with multiple females.

It has been suggested that multiple paternity in marine turtles arises as a result of convenience polyandry, with females 'giving in' to costly male harassment (Lee and Hays 2004), and the apparent trend of increasing occurrence of multiple paternity with increasing population size has been interpreted as support for this hypothesis (Bowen and Karl 2007; Lee 2008). In **chapter five**, I determined the frequency of multiple paternity in the large green turtle rookery on Ascension Island, and I reveal an extremely high level of multiple paternity, in broad agreement with this pattern. Although I was unable to test every potential benefit of polyandry to females in this study, overall, my results from **chapters four** and **five** are more consistent with the idea that multiple paternity in marine turtles is driven by male harassment and convenience polyandry rather than positive selection for direct or indirect benefits to females.

Surprisingly, I found no evidence that any males mated with more than one female within a breeding season (polygyny). Demonstrating polygyny using molecular markers is more difficult than detecting polyandry, because it requires sampling clutches from a large number of females in order to 'capture' multiple clutches sired by a particular male, as well as adequate marker power to discriminate between putative sires (Pearse *et al.* 2001; Avise 2007). At Alagadi beach I sampled clutches from approximately 90% of reproductive females each breeding season, and my set of molecular markers had very high exclusion probability, hence, I expected to detect polygyny if males were indeed mating with multiple females at this site. When nesting populations are small, as they are in the Mediterranean, limited mate availability or high synchrony of female receptivity may limit the ability of males to access multiple females. Perhaps under these conditions males maximise their reproductive success by mating with females at multiple rookeries, as suggested in **chapter two**, and conducting paternity analyses at additional nearby rookeries would be an informative next step in assessing male reproductive success.

Chapters four and **five** highlight the difficulties in assessing the fitness consequences of particular mating strategies in un-manipulated natural populations, particularly when behavioural observations are also difficult. As discussed in **chapter four**, experimental studies in captive populations would be necessary to gain a fuller understanding of the costs and benefits of polyandry in marine turtles, and to address questions relating to male mating behaviours, such as the influence of sperm precedence on paternity share. Nonetheless, as shown here, molecular markers can provide valuable information on the genetic mating system of elusive species, and further paternity studies in marine turtle populations that differ in density or sex ratio, for example, will help to shed light on factors that influence mating behaviour in these species.

In conclusion, this thesis has provided novel information relating to sex ratios, male periodicity of breeding, and mating strategies of male and females green turtles, but has also highlighted large gaps that remain in our knowledge of marine turtle natural history. Despite being among the most intensively studied of all reptiles, our understanding of many aspects of marine turtle mating systems, movements and life history are extremely limited. This thesis has provided data to suggest that adult sex ratios may not reflect those seen at hatching, but the mechanisms impacting the dynamics of sex ratios in marine turtle populations are unknown. My results challenge the long-assumed belief that male turtles mate more frequently than females, and also suggest that males may visit multiple rookeries within a breeding season, but a better understanding of male movements are needed before we can draw strong conclusions about male mating patterns. Finally, my results highlight variation in female remating rates among populations, but the factors affecting female mating decisions remain obscure. Molecular markers, when employed in conjunction with field observations and other technologies, can help us to address some of these challenging questions.

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