

# Turtle mating patterns buffer against disruptive effects of climate change

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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 per cent 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.

**Keywords:** marine turtle; temperature-dependent sex determination; climate change; sex ratio; mating patterns; *Chelonia mydas*

## 1. INTRODUCTION

Understanding and predicting how climate change impacts species with temperature-dependent sex determination (TSD) are 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 the 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, owing to individual plasticity in the timing of first nesting, is predicted to have a modest compensatory effect on offspring sex ratios compared with 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 [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 per cent of either sex at around 29°C (reviewed in Hawkes *et al.* [4]). Hatchling sex ratios

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biased towards females are typical and in all green turtle populations studied to date, estimates of hatchling sex ratios range from 67 to 100 per cent female (reviewed in Hawkes *et al.* [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 ([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 owing to scarcity of males. These consequences are expected to be particularly deleterious in small populations, where the number of males could conceivably be reduced to below a critical minimum required to maintain a fertile population [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  $N_e$  owing 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 (OSRs) 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 probably 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 hypothesized 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

Table 1. Characterization of microsatellite loci in green turtles (*Chelonia mydas*) at Alagadi, northern Cyprus.  $H_O$ , observed heterozygosity;  $H_E$ , expected heterozygosity.

locus	original source for locus development	no.		$H_O$	$H_E$
		$n$	alleles		
A6	[26]	60	5	0.733	0.711
B103	[26]	60	5	0.617	0.702
B123	[26]	59	5	0.627	0.636
C102	[26]	60	5	0.550	0.550
D105	[26]	60	8	0.683	0.784
D2	[26]	59	10	0.797	0.733
Cm3	[27]	60	7	0.550	0.523
Cm58	[27]	60	7	0.833	0.806
Klk314	[28]	60	4	0.433	0.491
Or7	[29]	60	5	0.717	0.656
Cc2	[30]	60	8	0.717	0.759
Cc28	[30]	60	4	0.650	0.716
CcP7D04	[31]	59	8	0.847	0.794
CcP7E11	[32]	60	4	0.550	0.496

provide new insights into male mate-searching behaviour and lend support to the inferences drawn from our parentage assignments.

## 2. MATERIAL 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  $\pm$  s.d. =  $21.9 \pm 1.55$ , range 15–23) offspring from one or more clutches per female. The final dataset comprised 809 offspring from 37 clutches. Offspring sex was estimated for the 2008 nesting season from incubation durations (as previously described in Broderick *et al.* [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 15 min followed by 35 cycles of 94°C for 30 s, 58°C for 90 s and 72°C for 60 s, and finally one cycle of 60°C for 30 min. Allele sizes were assigned using an internal size standard (Genescan-500-ROX, Applied Biosystems), an ABI 3730 DNA Analyser and ABI GeneMapper 3.7 software (Applied Biosystems). Samples that failed to amplify at all loci, or that displayed unexpected alleles, were re-amplified and re-scored. Any samples that still failed to amplify at a minimum of eight loci were excluded from further analyses. However, multi-locus genotypes were complete at all 14 loci for more than 90 per cent of individuals.

### (b) Characterization 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 v. 2.0 [33]. Linkage disequilibrium between all pairs of loci was tested using a Markov chain method in GENEPOP v. 4.0.10 [34]. Probability of detecting multiple paternity was assessed using PRDM software [35] (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 v. 2.0 [36] 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-sib groups and genotypes of unknown parents are inferred [36,37]. 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 a previously described protocol [38]. The male was tracked via satellite for 81 days. Locations were obtained using the Argos system and Satellite Tracking and Analysis Tool (STAT) [39], and mapped in ARCGIS v. 9.3.1. (for full details, see the electronic supplementary material).

## 3. RESULTS

### (a) Offspring sex ratio

Incubation durations of clutches in 2008 ranged from 43 to 59 days (mean  $48.3 \pm 3.48$  (s.d.),  $n = 57$ ), with only three 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 per cent female.

### (b) Paternity analysis

Parentage analysis of more than 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 (figure 1). There was a high degree of convergence between the 10 COLONY runs (see electronic supplementary material, table S1). 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-sib families and the total number of unique fathers contributing offspring to these full-sibling

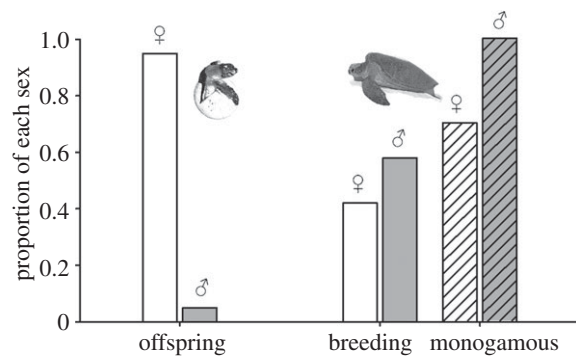


Figure 1. Sex ratios and monogamy of green turtles. The figure shows the proportions of female (un-shaded) and male (shaded) offspring and breeding adults for the 2008 nesting season at our study site. Also shown (hashed) are the proportions of breeding adults that were monogamous at the study site, 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.

groups also ranged from 28 to 30 across the 10 runs, with 28 fathers being most probable in five runs.

Thirty per cent (six out of 20) of females in this study produced clutches with multiple paternity (see electronic supplementary material, table S2), 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 (more than 90% of females that successfully nested at Alagadi in 2008 were included in the study).

### (c) Characterization of microsatellite loci

All loci conformed to expectations of HWE ( $p > 0.05$ ), showed low probability of null alleles and showed no evidence of genotypic linkage disequilibrium after correction for multiple tests [40]. Combined exclusion probability (second parent) for all 14 loci was greater than 0.99, and the probability of detecting multiple paternity, assuming two 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 multiple nesting beaches in Cyprus and Turkey before travelling to North Africa (figure 2), in a pattern consistent with mate-searching behaviour (see the electronic supplementary material). The breeding sites within 20 km of the route account for 58 per cent of green turtle nesting in the Mediterranean according to maximum nest numbers taken from the literature [22,41–43].

## 4. DISCUSSION

Our finding that more males than females contributed to reproduction in this study was contrary to our expectations, considering the extremely female-skewed



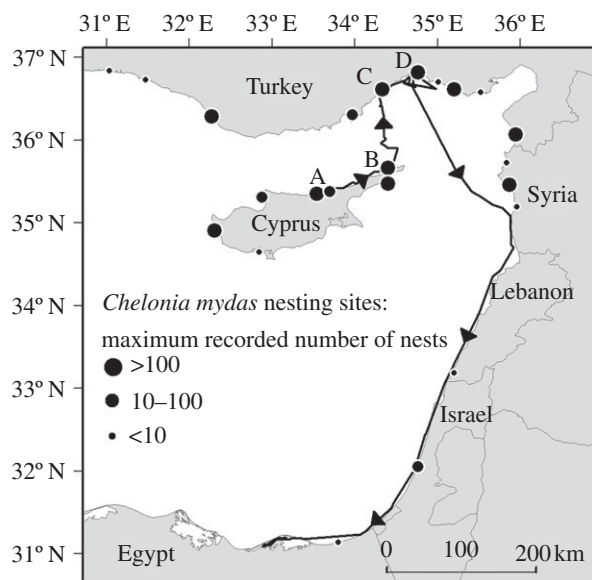


Figure 2. Route of an adult male green turtle that was released post-breeding at Alagadi Beach, Cyprus and tracked to Egypt via the Turkish coast. Major green turtle nesting beaches along the route are labelled (A–D): (A) Alagadi and the Cyprus North beaches, 8–9th June. (B) North Karpaz beaches, 10–11th June. (C) Alata, 14th June. (D) Kazanlı, 15–26th June. Data for nesting numbers are taken from the literature [22,41–43].

hatchling sex ratio reported at this rookery (this study, [23]). OSRs [44] depend not only on adult sex ratio but also on the potential reproductive rate of each sex [45]. 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*) [46]. A higher breeding frequency of males compared with 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 [47]. 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 [48], 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 probably 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 [49]. Lower levels of genetic divergence at

nuclear compared with mitochondrial DNA (mtDNA) markers is consistent with male-mediated gene flow between rookeries and suggests that males are more plastic in their philopatric behaviour [50]. Additionally, although courtship and mating are thought to occur close to the nesting beach at this colony [51], 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 [52], the male took a 348 km diversion to Turkey before travelling to the North African coast, passing in proximity to multiple green turtle nesting beaches [22,41–43]. The exceptional navigational abilities of marine turtles are well documented [53] and tentatively suggest that the observed detour was strategic. Mating activity in marine turtles can overlap significantly with the nesting season [54] 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 Lee [55]), and was recorded in 30 per cent of females in this study, the observation that males only sired offspring with a single female at the Alagadi rookery (figure 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 [44]. 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 with a strictly monogamous system [21,56], further contributing to the maintenance of genetic variation in this population.

While 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 owing 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 (owing to late age at maturity in green turtles, reviewed in Heppell *et al.* [57]). 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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