Investigating variation in the life-history strategy of marine turtles

Submitted by

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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: ……..

Dedication

This work is dedicated to Sue and Paul Bradshaw who are no longer with us, but without their love and support this work would not have been possible.

Abstract

Understanding the degree of connectivity among populations, forming migratory links and evaluating factors that influence reproductive fitness is fundamental for the successful management of migratory species of conservation concern. In this thesis I focus on a regionally important rookery of green turtles (*Chelonia* mydas) at Alagadi beach in northern Cyprus that has been intensively studied since 1992. I employ high resolution genetic markers with different modes of inheritance to reveal fine scale population structuring among four rookeries within a 200 km stretch of shoreline. The relative importance of four foraging areas and the annual contribution from each site to the breeding cohort are evaluated using the stable isotope ratios of the turtles calibrated by satellite telemetry. The stable isotope ratios of the turtles reveal that a previous undisclosed foraging site at Lake Bardawil in Egypt is critical for the population recovery of the Alagadi rookery. The temporal consistency of stable isotope ratios reaffirm that green turtles exhibit high fidelity to foraging sites allowing the evaluation of foraging area effects on reproductive traits to be evaluated over multiple seasons. I investigate the population sub-structuring of the Alagadi nesting aggregation grouped by the foraging area used. The absence of genetic structure supported the Learning Migration Goal Theory and provided evidence that the significant among site phenotypic variability in the body size of recruits, the length of interbreeding intervals and the date that the first nest of the season was made is a result of foraging areas effects. No significant among site phenotypic variability was found for the size or number of clutches laid. Among site variability in interbreeding intervals resulted in substantial variation in the reproductive potential of individuals due to assumed differences in resource availability and environmental factors. The multilocus genotypes generated for 243 nesting females were employed to reconstruct the first wild marine turtle pedigree using a full-likelihood sib-ship reconstruction approach. This revealed that the effective contribution to the next generation was unequal and allowed a minimum age to maturity to be estimated from parent – offspring assignations. However, sample size constraints prevented accurate estimates for the narrow-sense heritability of the five morphological and life-history traits from the quantitative genetic analysis. The multifaceted approach taken here to unravel the cryptic life-history of marine turtles emphasises the importance of long-term individual-based monitoring and the data generated can be employed to advise conservation strategy for this critically endangered regional management unit.

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The molecular work was undertaken at the NERC Biomolecular Analysis Facility in Sheffield and I would like to thank Terry Burke, Deborah Dawson, Gavin Horsburgh, Andy Krupa and Helen Hipperson for their assistance in the genotyping, sequencing and genetic analyses.

Finally, I would like to thank my brother Mark and all of my friends for their support throughout this academic journey, it has been emotional and I am truly grateful.

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Author's declaration

This work was funded by a NERC studentship grant 1353865 with additional funding awarded (NBAF869) for the molecular genetics work to be conducted at the Biomolecular Analysis Facility in Sheffield. All chapters within this thesis were written by P. J. Bradshaw under the supervision of B. J. Godley, A.C. Broderick and C. Carreras. 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 G.J. Horsburgh, A. Krupa and H. Hipperson. All stable isotope samples were prepared in the Daphne du Maurier research laboratories with samples analysed for carbon and nitrogen at the Stable Isotope Facility in the Environment and Sustainability Institute, both of which are located at the Penryn Campus of the University of Exeter. Stable isotope analysis of sulphur was conducted at Elemtex, Launceston. Platform terminal transmitters (PTTs) attached in 2015 were Spot-293A tags purchased from Wildlife Computers with data processing conducting in the satellite Tracking and Analysis Tool (STAT) facilitated by seaturtle.org. Fieldwork in northern Cyprus was conducted as part of the Marine Turtle Conservation Project under the supervision of W. Fuller with further assistance by R.T.E Snape, E. Duncan and L. Omeyer in addition to the numerous volunteers who collected tissue samples and nesting data. Specific author contributions are detailed below:

Chapter 1 Enhanced molecular tools reveal fine scale structuring among Mediterranean green turtle rookeries

Authors: Phil J. Bradshaw, Annette C. Broderick, Carlos Carreras, Wayne Fuller, Robin T.E. Snape, Lucy I. Wright and Brendan J. Godley.

I conducted fieldwork, collected genetic samples, genotyped, sequenced and analysed the genetic data and was the lead author on the manuscript. LIW conducted fieldwork, collected genetic samples, optimised the microsatellite array and provided genotype data from previously analysed samples. RTES conducted and supervised fieldwork, collected genetic samples and created the map. Fieldwork was managed by WF, ACB

and BJG. CC supervised analysis of the genetic data in addition to guidance on the genetic analysis and writing of the manuscript. All co-authors provided insightful comments which significantly improved the final manuscript.

Chapter 2 Satellite tracking and stable isotope analysis highlight differential recruitment among foraging areas in green turtles

Authors: Phil J. Bradshaw, Annette C. Broderick, Carlos Carreras, Rich Inger, Wayne Fuller, Robin T.E. Snape, Kimberley L. Stokes and Brendan J. Godley.

I conducted fieldwork, collected and prepared tissue samples for stable isotope analysis, analysed the data, and was the lead author on the manuscript. KLS processed the satellite telemetry tracks for turtles equipped with PTTs previous to 2015 and created the maps within the manuscript. RI was instrumental in organising the analyses of the sulphur isotopes. RTES supervised fieldwork and the attachment of the five PTTs to the selected green turtles in 2015. WF, ACB and BJG managed fieldwork in northern Cyprus and ACB and BJG provided significant guidance in writing the manuscript with all co-authors providing useful comments.

Chapter 3 Carry-over effects influence the reproductive potential of green turtles

Authors: Phil J. Bradshaw, Brendan J. Godley, Carlos Carreras, Wayne Fuller, Robin T.E. Snape, Kimberley L. Stokes, Lucy I. Wright and Annette C. Broderick.

I conducted fieldwork, collected and prepared tissue samples for stable isotope and genetic analysis, analysed the data and was the lead author on the manuscript. KLS conducted field work, collected tissue samples, processed satellite telemetry tracks and created the maps within the manuscript. LIW conducted fieldwork, collected genetic samples, optimised the microsatellite array and provided genotype data from previously analysed samples. RTES supervised fieldwork under the management of WF, ACB and BJG. BJG, CC and ACB provided significant guidance in writing the manuscript and all co-authors provided useful comments.

Chapter 4 A wild marine turtle pedigree reveals unequal effective contribution to the next generation

Authors: Phil J. Bradshaw, Annette C. Broderick, Carlos Carreras, Wayne J. Fuller, Helen Hipperson, Robin T.E. Snape, Stephen J. White, Lucy I. Wright and Brendan J. Godley.

I conducted fieldwork, collected genetic samples, sequenced and analysed the genetic data, reconstructed the genetically-validated pedigree, conducted the quantitative genetic analysis and was the lead author on the manuscript. LIW conducted fieldwork, optimised the microsatellite array and provided genotype data from previously analysed samples. HH was instrumental in running the COLONY2 software on ICEBERG, the high performance computing resource in Sheffield, SJW provided significant guidance in conducting the quantitative genetic analyses, RTES supervised fieldwork under the management of WF, ACB and BJG. CC and BJG provided significant guidance in writing the manuscript and all co-authors provided useful comments.

List of abbreviations

Bp	Base pair
CCL	Curved carapace length
CITES	Convention on International Trade in Endangered Species of Wild Flora and Fauna
CF-IRMS	Continuous flow isotope ratio mass spectrometer
CMS	Convention on the Conservation of Migratory Species (Bonn convention)
CR	Expanded (~800bp) mtDNA control region sequence
ECF	Expected clutch frequency
FDR	False discovery rate
GLM	General linear model
GLMM	Generalised linear mixed model
H_E	Gene diversity
H_O	Observed heterozygosity
HR	Expanded (~800bp) mtDNA control region sequence concatenated to four mtSTRs
HSD	Tukey's honest significant difference
HWE	Hardy-Weinberg Equilibrium
IUCN	International Union for the Conservation of Nature
K	Number of alleles
LD	Linkage disequilibrium
mtDNA	Mitochondrial DNA
MTRG	Marine Turtle Research Group
mtSTRs	Short tandem repeats on the mitochondrial DNA
MU	Management unit
nDNA	Nuclear DNA
N_e	Effective population size
PCR	Polymerase chain reaction
PCoA	Principal Coordinate Analysis

PIC	Polymorphic information content
PIT	Passive integrated transponder (tag)
PTT	Platform terminal transmitter
REML	Restricted or Residual maximum likelihood
RMU	Regional management unit
SIA	Stable isotope analysis
SNP	Single nucleotide polymorphism
STAT	Satellite tracking and analysis tool

General introduction

Marine turtles are long-lived slow to mature and highly migratory marine megavertebrates. Much of their life-history remains cryptic as it is unfeasible to track an individual through to maturity and therefore much of their ecology is still unknown. There are seven extant species of marine turtles that are all classed as species of conservation concern including the green turtle (*Chelonia* mydas) which is listed as endangered by the International Union for the Conservation of Nature's (IUCN) *Red List* of Threatened Species (Seminoff 2004) and categorised under Appendix 1 in the Convention on the Conservation of Migratory Species (CMS, Bonn Convention) and the Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES). Many marine turtle rookeries are conservation-dependent (McClenachan et al. 2006) but a few have subsequently experienced a remarkable recovery as anthropogenic threats such as directed harvest, bycatch and habitat loss are removed (Broderick et al. 2006).

The green turtle nesting aggregations within the Mediterranean constitute the most endangered regional management unit (RMU) for this species (Wallace et al. 2010) as only 350 females are estimated to nest annually (Kasparek et al. 2001, Broderick et al. 2002, Stokes et al. 2015). The Mediterranean nesting aggregations were formally the only RMU to qualify as a subpopulation under the IUCN's guidelines based on unequivocal evidence for their reproductive isolation (Encalada et al. 1996). However, in 2006 they were officially delisted as a subpopulation (Mast et al. 2006, Mrosovsky 2006, Naro-Maciel & Formia 2006) when panmixia could not be rejected based on a global analysis of population connectivity based on four microsatellite loci (Roberts et al. 2004). Despite their removal as a subpopulation, several strong lines of evidence still support the reproductive isolation of the Mediterranean population from those in the Atlantic (Carreras et al. 2014, Seminoff et al. 2015) and all regional green turtle rookeries are considered to be conservation-dependent (Casale & Margaritoulis 2010).

In the Mediterranean, green turtle nesting is restricted to the Levantine basin with the largest rookeries situated in Turkey, Syria and Cyprus (Stokes et al. 2015). Northern

Cyprus is thought to host approximately 30% of regional green turtle nesting with the second largest rookery situated at Alagadi Beach (Casale & Margaritoulis 2010, Stokes et al. 2015). An intensive monitoring and nest protection programme initiated in 1992 has collated a long-term individual-based dataset and documented a recent recovery of this population that has been attributed to the reduction of terrestrial predation (Stokes et al. 2014). However, it can be challenging to identify the proximate causes for the change in population size without investigating at an individual-level as it can be difficult to distinguish between the effects of changes in breeding success, survival and immigration (Clutton-Brock & Sheldon 2010).

This thesis presents four chapters in which I investigate aspects of the green turtle's life-history strategy through indirect methods of assessment to determine if the population recovery is a result of local conservation efforts. I employ a multifaceted approach using molecular genetics, stable isotope analysis and satellite telemetry to provide a significant insight in to the spatial connectivity of the green turtles that nest at Alagadi and investigate their relationship structure and reproductive strategies. I have not included an overarching description of methodologies as these are described in detail within the main body of text or within the supplementary information for each individual chapter. I present the chapters as an intuitive sequence to comprehensively investigate four main research areas.

In **Chapter 1** "**Enhanced molecular tools reveal fine scale structuring in green turtles"** I evaluate stock structure among four green turtle rookeries in northern Cyprus to determine whether local demographics or immigration is the primary driver for recruitment at Alagadi. As previous regional stock assessments were confounded by low genetic variability within the region (Kaska 2000, Bagda et al. 2012) I employ 13 microsatellite loci and a high resolution (HR) haplotyping system that includes four hypervariable mtDNA short tandem repeats (mtSTRs, Tikochinski et al. (2012)) concatenated onto an expanded mtDNA control region (CR) haplotype (Abreu-Grobois et al. 2006). The statistical power of the HR haplotypes were compared to the CR haplotypes previously employed to provide evidence that the CR markers were not sufficiently variable to adequately determine regional stock structure. Furthermore, I

compare the statistical power of the 13 microsatellites employed in this study to the four microsatellites employed by Roberts et al. (2004) during the global analysis of stock structure to readdress the contentious issue of the Mediterranean RMU being reproductively isolated from the Atlantic RMUs. This work significantly improves our current knowledge on regional stock structure and the geographic scale of natal site fidelity for green turtles. I provide strong evidence that new regional and global assessments are required as the natal site fidelity of green turtles is likely to be greater than previously anticipated (Dethmers et al. 2006, Jensen et al. 2013).

In **Chapter 2** "**Satellite tracking and stable isotope analysis highlight differential recruitment among foraging areas in green turtles"** I evaluate the annual contributions from four broad-scale foraging areas to the Alagadi nesting aggregation. This chapter effectively demonstrates the utility of stable isotope analysis (SIA) to scale up the knowledge from, and guide satellite telemetry campaigns to ascertain a fundamental understanding of migratory connectivity between the breeding and non-breeding sites. I employ the temporal consistency of stable isotope ratios of females from skin samples collected over multiple nesting seasons to quantify foraging site fidelity and employ the long-term nesting data to evaluate foraging area dynamics over the last two decades. This work has significantly altered our perception of the relative importance of these four foraging areas to the Alagadi rookery and provides a baseline in which to monitor site specific levels of recruitment and survival probabilities.

In **Chapter 3** "**Carry-over effects influence the reproductive potential of green turtles"** I investigate the effects of differential foraging on five morphological and reproductive traits. I additionally use the genetic data generated for each individual in **Chapter 1** to evaluate the population sub-structuring of the Alagadi nesting aggregation among the four foraging areas using the SIA predictions and satellite telemetry data from **Chapter 2.** This chapter significantly improves our current knowledge on foraging area selection and the reproductive consequences resulting from foraging area effects.

In **Chapter 4** "**A wild marine turtle pedigree reveals unequal effective contribution to the next generation"** I investigate the relationship structure of the Alagadi nesting aggregation to determine if the effective contribution to the next generation is equal by

reconstructing a genetically-validated pedigree employing a full-likelihood sibship reconstruction approach. This work provides a novel insight into the kinship among individuals of the Alagadi nesting aggregation including the parent – offspring assignations that were used to estimate a minimum age to maturity. To evaluate the proportion of the phenotypic variability that can be explained by the genetic resemblance among close relatives, I employed a quantitative genetic analyses (Lynch & Walsh 1998) of five morphological and life-history traits. I analysed each trait in univariate animal models (Kruuk 2004, Wilson et al. 2010) with the phenotype conditioned on significant covariates and predictor variables from **Chapter 3** and thereby incorporating foraging area effects where appropriate. This is the first time that a full-pedigree likelihood methodology has been applied to reconstruct kinship in marine turtles and significantly improves our knowledge of local demographics and the effective contribution to the next generation.

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Chapter 1

Enhanced molecular tools reveal fine scale structuring among Mediterranean green turtle rookeries

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Abstract

Understanding the connectivity among populations is a key research priority for species of conservation concern. Genetic tools are widely used for this purpose, but the results can be limited by the resolution of the genetic markers in relation to the species and geographic scale. Here, we investigate natal philopatry in green turtles (*Chelonia mydas*) from four rookeries within close geographic proximity (~200km) on the Mediterranean island of Cyprus. We genotyped hypervariable mtSTRs, a mtDNA control region sequence (CR) and 13 microsatellite loci to genetically characterise 479 green turtles using markers with different modes of inheritance. We demonstrated matrilineal stock structure for the first time among Mediterranean green turtle rookeries. This result contradicts previous regional assessments and supports a growing body of evidence that green turtles exhibit a more precise level of natal site fidelity than has commonly been recognised. The microsatellites detected weak male philopatry with significant stock structure among three of the six pairwise comparisons. The absence of Atlantic CR haplotypes and mtSTRs amongst these robust sample sizes provide greater evidence for the reproductive isolation of Mediterranean green turtles and supports their status as a subpopulation. A power analysis effectively demonstrated that the genetic markers previously employed to evaluate regional stock identity and nuclear gene flow between the Mediterranean and Atlantic populations were confounded by an insufficient resolution considering the recent colonisation of this region. These findings improve the regional understanding of stock connectivity and illustrate the importance of using suitable genetic markers to define appropriate units for management.

Introduction

Identifying demographically independent populations is central to the management and conservation of natural populations (Palsböll *et al.* 2007) and a key research priority for species of conservation concern (Frankham 2010; Wallace *et al.* 2011). Moritz (1994) defined a Management Unit as a geographically differentiated population segment where contemporary gene flow still may exist with other management units despite being functionally independent. Molecular genetics have been used extensively to define demographically independent populations (reviewed in Waples & Gaggiotti 2006) for management and monitoring (Schwartz *et al.* 2007). However, populations are dynamic and alter in size and connectivity over contemporary and evolutionary time-scales.

The potential of a genetic marker to evaluate contemporary gene flow relies on its variability as this allows the detection of genetic differences in divergent lineages. The variability of a genetic marker across populations depends on marker specific rates of mutation, the number of immigrants exchanged among populations and the number of generations over which populations have diverged (Slatkin 1987). However, historical patterns of extinction and recolonization can mask contemporary levels of gene flow as they result in homogenising gene frequencies (Slatkin 1987). Thus, the mutation rate of the genetic marker can reflect different temporal depths of genetic subdivision. It is therefore of vital importance to select the appropriate genetic markers to define suitable management units (Wan *et al.* 2004; Karl *et al.* 2012). Genetic diversity in newly colonised or peripheral populations is expected to be lower than in older or more centrally located populations (Eckert *et al.* 2008) and only highly variable markers will allow proper discrimination among intraspecific populations. Microsatellites (Selkoe & Toonen 2006) and short tandem repeats on the mitochondrial DNA (mtSTRs) (Lunt *et al.* 1998; Tikochinski *et al.* 2012) are both highly variable as they acquire new mutations rapidly through slipped strand mispairing. However, the use of these hyper-variable markers is not free from drawbacks as, due to their mutation mechanisms (Di Rienzo *et al.* 1994), they can be subject to significant levels of homoplasy (Lunt *et al.* 1998). An additional caveat of using highly variable markers is that increased levels of within-

population heterozygosity depress among-population differentiation as alleles become saturated (Hedrick 1999). For these reasons, Selkoe & Toonen (2006) advise that markers with more than 25 alleles should be treated with caution. Thus, a greater number of microsatellites with fewer alleles per loci will be more likely to detect significant genetic structure among newly diverged populations. In summary, it is not having a marker with the most variability that is important, but to have a marker with the right amount of variability for the expected scale of differentiation.

Marine turtles are species of conservation concern that form distinct population segments as both sexes are known to exhibit natal site philopatry (Meylan *et al.* 1990; FitzSimmons *et al.* 1997). Philopatry is considered an evolutionary adaptation that ensures suitable reproductive conditions and mate choice (Refsnider & Janzen 2010) as well as maintaining local adaptations (Stiebens *et al.* 2013). Yet precise natal philopatry could be a recipe for extinction if some individuals did not stray to colonise new areas (Schroth *et al.* 1996). The nesting site fidelity of females defines reproductive populations, and thus units for management, as the loss of the females at a particular site will effectively result in a failure to reproduce (Bowen *et al.* 2005). Males on the other hand do not necessarily restrict their mating efforts to natal rookeries which is equally important for maintaining the adaptive potential of a population (Karl *et al.* 1992; Wright *et al.* 2012b). Therefore, genetic markers with different modes of inheritance are necessary to accurately evaluate the level of gene flow which connects marine turtle rookeries. To date, maternal stock structure has typically been evaluated through the use of mtDNA control region (CR) sequences due to their relatively rapid pace of evolution and non-recombining mode of inheritance (Avise 1995). Frequency-based analyses of CR haplotypes have been very informative for defining regional management units (RMU, Wallace *et al.* 2010) with significant genetic differentiation typically described among rookeries separated by >500 km (Dethmers *et al.* 2006; Bowen & Karl 2007). However, mtDNA in marine turtles evolves far more slowly than it does in other vertebrates (Avise *et al.* 1992) which makes it a comparatively insensitive marker at finer geographic scales or among more recently divergent populations (Formia *et al.* 2006). More recently, several studies have used more variable genetic markers to

reveal maternal genetic structure in populations which are genetically homogenous for CR haplotypes (Lee *et al.* 2007; Shamblin *et al.* 2015c). In contrast, the movement of males is generally inferred from microsatellites which have a higher mutation rate and a four-fold greater effective population size (Carreras *et al.* 2011 and refs therein). In general, less genetic structure is commonly detected using microsatellites which is commonly attributed to male-mediated gene flow (Karl *et al.* 1992; Bowen & Karl 2007). Gene flow is not the only explanation for lower genetic structure being detected within microsatellite markers (reviewed in Karl *et al.* 2012) but the extent of male-mediated gene flow might be less than previously estimated as stronger genetic structure is being detected by studies using a greater number of microsatellites providing increasing support for male philopatry (e.g. Dutton *et al.* 2013; Naro-Maciel *et al.* 2014). Thus, trying to define discrete management units based on the exact geographical specificity of natal homing in marine turtles has led to mixed conclusions dependent on the type and resolution of genetic markers (reviewed in Lee 2008; Jenson *et al.* 2013).

An example of this unclear resolution was raised when evaluating the fine scale regional genetic connectivity among populations of green turtles (*Chelonia mydas)* within the Mediterranean. This region has shown an unusual pattern, as a greater genetic structure has been detected in the males rather than the females (Bagda *et al.* 2012). The failure to detect maternal genetic structure could lead to the false assumption that all regional rookeries should be considered as a single management unit as it is the movement of the breeding females that delineates distinct turtle stocks (Bowen *et al.* 2005). However, the fact that biparentally inherited genetic structure does exist, and that the natal site fidelity of the females is expected to be greater than the males, there is clear evidence that the movement of females among these regional rookeries is likely to be more restricted than previously estimated from molecular studies (Kaska 2000; Bagda *et al.* 2012). Furthermore, the Mediterranean RMU (Wallace *et al.* 2010) is of great conservation interest as it formally constituted the only green turtle RMU to qualify as a subpopulation under the IUCN's *Red List* guidelines (Mrosovsky 2006). This specific regional listing was based on unequivocal evidence of the Mediterranean green turtle being genetically distinct from its conspecifics in the Atlantic based on short (400bp) CR

haplotypes (Encalada *et al.* 1996) as well as some morphological characteristics (Seminoff *et al.* 2015). However, this critically endangered subpopulation listing was subsequently removed in 2006 (Mast *et al.* 2006; Mrosovsky 2006; Naro-Maciel & Formia 2006) when panmixia could not be rejected among Mediterranean and Atlantic populations based on a study using four microsatellite loci (Roberts *et al.* 2004).

Therefore, we use higher resolution mtDNA markers and a greater number of microsatellites to address the fine scale genetic connectivity among four green turtle rookeries in northern Cyprus. Critically, we evaluate the statistical power of our markers against those previously used to assess regional connectivity as well as those used in the global assessment that led to the delisting of the Mediterranean green turtle as a subpopulation. These data will provide robust estimates of genetic structure in which to define appropriate units for management and conservation. Furthermore, we provide more accurate data which can be applied in mixed stock analyses of foraging areas (Bolker *et al.* 2007) and fisheries bycatch data (Laurent *et al.* 1998).

Methods

Sample collection and DNA extraction

We collected 479 tissue samples from green turtle adults or offspring from nests of unknown maternal origin during the 2001 – 2015 breeding seasons with permission obtained through the Society for the Protection of Turtles (SPOT) from the Department of Environmental Protection in northern Cyprus. Samples were collected from four rookeries in northern Cyprus: Akdeniz, Alagadi, North Karpaz and South Karpaz (Figure 1 & Table 1), all located within a 200 km stretch of coast. Tissue samples from adults comprised of a small skin biopsy (<0.5 cm^2) taken from the trailing edge of the fore flipper of nesting females shortly after laying. Females were flipper tagged and had passive integrated transponders (PIT tags) inserted into the shoulder to avoid pseudoreplication (Broderick *et al.* 2002). For clutches of unknown maternal origin, live or dead offspring were sampled post-hatching by taking a <1 mm wide biopsy section of the marginal scute proximal to the supra-caudal scute. Methodologies for working with

wild turtles were granted prior ethical approval (2014/492) through the University of Exeter, UK. All biopsies were stored in 96% ethanol until genomic DNA was extracted using the Qiagen DNeasy blood and tissue kit (Qiagen®) following the manufacturer's instructions or using an ammonium acetate precipitation method (Nicholls *et al.* 2000).

Mitochondrial DNA

A ~800 bp fragment of the mitochondrial DNA (mtDNA) control region was amplified by polymerase chain reaction (PCR) using the primer pair LCM15382 (5'-GCTTAACCCTAAAGCATTGG-3') and H950 (5'-GTCTCGGATTTAGGGGTTT-3') (Abreu-Grobois *et al.* 2006) in a 10µl reaction consisting of 4µl QIAGEN Multiplex PCR Master Mix (Qiagen®; including HotStar DNA *Taq* polymerase), 3µl ddH_2O, 1µl of forward and reverse primers (5uM) and 1µl of ~10ng template DNA. PCR parameters included an initial hot start denaturing step at 95˚C for 15 mins and then 35 cycles at 94˚C for 1 min, 52˚C for 1 min, 72˚C for 1 min 30 seconds and then a final extension step at 72˚C for 10 mins. Furthermore, a shorter 200bp fragment of the mtDNA control region which contains four hyper-variable dinucleotide (AT) short tandem repeats (mtSTRs) (Tikochinski *et al.* 2012; Shamblin *et al.* 2015c) was amplified using primer pair CM-D-1 F (5'-AGCCCATTTACTTCTCGCCAAACCCC-3') and CM-D-5 R (5'-GCTCCTTTTATCTGATGGGACTGTT-3') (Tikochinski *et al.* 2012). We used the same reaction as for the ~800bp mtDNA fragment but with the following PCR protocol: 95˚C for 15 mins and then 35 cycles at 94˚C for 30s, 56˚C for 30s, 72˚C for 1 min and then a final extension step at 72˚C for 7 mins.

PCR products were visualised by electrophoresis to ensure successful amplification. A total of 6µl of the PCR amplicon was purified using 2µl of ExoSAP-IT® (Affymetrix Inc.) and incubated as per manufacturer's instructions. Purified mtDNA amplicon was sequenced in forward and reverse directions using a Bigdye v3.1 Cycle Sequencing Kit (Applied Biosystems™) and loaded on an ABI 3730 DNA Analyser (Applied Biosystems™). All PCR reactions were run with positive and negative controls.

All forward and reverse sequence data were aligned in Geneious v6.17 (Biomatters Ltd). Mitochondrial DNA CR consensus sequences were compared against all nucleotide sequences in the National Centre for Biotechnology Information's database

(http://www.ncbi.nlm.nih.gov/) using BLAST and given the mtDNA CR haplotype designation prescribed by the Archie Carr Centre for Sea Turtle Research website (http://accstr.ufl.edu/cmmtdna.html). In cases of heteroplasmy of the mtSTRs, we took the major haplotype as the consensus sequence based on peak height and aligned them manually in BioEdit v7.2.5 (Hall 1999) to conform to the four short tandem repeats as described by Tikochinski *et al*. (2012). The mtSTRs were then concatenated to the end of the mtDNA CR haplotype sequence to construct a high resolution (HR) haplotyping system (Shamblin *et al.* 2015c) using the traditional nomenclature for green turtle CR haplotypes in the Atlantic (CM-A##) followed by the four digit repeat of the mtSTRs (e.g. CM-A13.1-6_8_8_4).

Nuclear DNA (nDNA)

Additionally, samples were genotyped at 13 polymorphic microsatellite loci using two multiplex reactions previously optimised by Wright *et al.* (2012a; b; Supplementary Information S1). PCR was carried out in 2µl reactions with 1µl of dried ~10ng template DNA, 1 µl QIAGEN Multiplex PCR Master Mix (Qiagen®, including HotStar DNA *Taq* polymerase) and 1µl of fluorescently labelled primer mix (6FAM, VIC and PET) at a final concentration of 1.8µM. Amplification was carried out in an MJ Research model PTC DNA Engine Tetrad thermal cycler according to the following protocol: 95˚C for 15 mins followed by 35 cycles of 94˚C for 30s, 58˚C for 1 min 30s and 72˚C for 1 min and finally one cycle of 60˚C for 30 mins. Allele sizes were assigned using an internal size standard (Genescan-500-LIZ; Applied Biosystems), an ABI 3730 DNA Analyser and ABI GeneMapper v3.7 software (Applied Biosystems). Samples which failed to amplify at all loci were re-amplified and re-scored. Error rate in allele size scoring was assessed by repeat marker amplification of 10% of the total sample size and comparing the number of incorrect allele calls divided by the total number of alleles (Selkoe & Toonen 2006).

Data analysis

We employed the programme Colony v2.0.5.0 (Jones & Wang 2010) to ensure all possible effects of pseudoreplication were removed by evaluating the offspring genotypes from nests of unknown maternal origin to identify full and half-sibship clusters. We cross referenced the HR haplotypes and the year in which the sample was

collected within sibship clusters and removed all possible variants which may relate to individuals being a first degree relative (Supplementary Information S2). Temporal tests were also conducted for rookeries where samples were collected over multiple seasons and all sample years that were not significantly different were pooled for further analysis (Supplementary Information S2)

Mitochondrial DNA

Haplotype diversity (*h*) based on Nei (1987), nucleotide diversity (π) and genetic structure were calculated using the programme Arlequin v3.5.2.3 (Excoffier & Lischer 2010). Genetic structure was assessed through exact tests of population differentiation conducted with default settings (Raymond & Rousset 1995a) and pairwise F_{ST} based on haplotype frequencies with significance calculated via 1000 permutation tests. All multiple tests were corrected using the modified False Discovery Rate (FDR) (Narum 2006). Pairwise F_{ST} values were used to perform a Principal Coordinate Analysis (PCoA) using the software GeneAlEx v6.5 (Peakall & Smouse 2012). All these analyses were conducted for both the CR and HR haplotype datasets. An unrooted parsimony network of relationships between mtDNA CR haplotypes was constructed using the Median Joining method implemented in Network v5.0 (Bandelt *et al.* 1999) with transversions weighted 3x that for transitions and indels given double weights as per the user guidelines. We included haplotypes previously detected in the Mediterranean (Bagda *et al.* 2012) but not found in this study as well as the two most abundant Atlantic haplotypes from 'lineage 1' and their derivatives (CM-A1.1- 1.4 and CM-A3.1 – 3.7, Shamblin *et al.* 2015a) as all known Mediterranean haplotypes originate within this lineage (Encalada *et al.* 1996).

Nuclear DNA

Analysis to detect deviation from Hardy Weinberg Equilibrium (HWE) and tests for linkage disequilibrium (LD) were conducted in Genepop on the web v4.2 (Raymond & Rousset 1995b; Rousset 2008) with significant P-values from multiple tests corrected using the FDR (Narum 2006). Evidence of null alleles was checked using Microchecker v2.2.3 software (Van Oosterhout *et al.* 2004). General diversity indexes were calculated for the four individual rookeries and for all rookeries combined, including the number

of alleles (k), observed heterozygosity (H_o) and genetic diversity (H_e) using GenAlEx v6.5 (Peakall & Smouse 2006, 2012). Allelic richness (AR) was calculated using the 'hierfstat' package (Goudet 2005) as implemented in R (https://www.r-project.org/). A non-parametric Kruskall-Wallis test was used to determine differences for these genetic diversity parameters among rookeries using R.

Global F_{ST} and pairwise tests for population differentiation were conducted in GeneAlEx v6.5 (Peakall & Smouse 2006, 2012) using frequency-based statistics including Wright's F_{ST} (1951) with Nei & Chesser's (1983) correction factor applied and Jost's estimate of differentiation (D_{est}) (Jost 2008; Heller & Siegismund 2009) with statistical significance ascertained via 999 permutation tests. Genetic differentiation based on D_{est} values were used to perform a PCoA and an isolation by distance Mantel test using the geographic distance among rookeries in kilometres following the shortest possible swimming distance along the coastline. Rookeries were checked for evidence of recent genetic bottlenecks using the one-tailed test for heterozygous excess (Cornuet & Luikart 1996) under the two-phase model for microsatellite mutation (Di Rienzo *et al.* 1994; Roberts *et al.* 2004) as implemented in Bottleneck v1.2.02 (Cornuet & Luikart 1996; Piry *et al.* 1999).

To assess whether clusters of genetically similar individuals could be identified from the whole nuclear dataset, we employed two Bayesian clustering algorithms in the software programmes STRUCTURE v2.3 (Pritchard *et al.* 2000) and Geneland (Guillot *et al.* 2005) (Supplementary Information S3).

The resolution of genetic markers to detect different levels of genetic differentiation were tested using the programme POWSIM v4.1 (Ryman & Palm 2006). To conduct a direct comparison of statistical power between our organelle mtDNA and nuclear DNA markers we followed the method of Larsson *et al.* (2009). Empirical sample sizes and allele frequencies were employed for all tests with the exception of the allele frequencies for the microsatellites used by (Roberts *et al.* 2004) which were approximated from the literature (Supplementary Information S4).

Results

All samples (n = 479) were successfully sequenced for HR haplotypes (thus including the ~800 bp CR haplotype and the mtSTRs) and genotyped across all 13 loci. The filtering process to remove putative close relatives reduced the sample sizes for South Karpaz by 13 individuals with a further 48 individuals removed from Akdeniz (Table 1). No significant temporal trends in haplotype frequencies were identified for the Alagadi or the Akdeniz rookeries and thus haplotype frequencies were pooled across years.

Mitochondrial DNA

A total of four CR haplotypes (Supplementary Information S5) were found among the four rookeries although two were unique to the South Karpaz. CM-A13.1 accounted for 94.3% of total samples and no additional variants were found using the longer mtDNA fragments compared to shorter fragments. These four CR haplotypes were previously known among Cypriot rookeries (Encalada *et al.* 1996; Kaska 2000; Bagda *et al.* 2012) although CMA-60.1 was only previously recorded as a case of heteroplasmy (Encalada *et al.* 1996) or from stranding data (Levin *et al.* 2008). Control region (CR) haplotype diversity was low for Alagadi, Akdeniz and North Karpaz (h = 0.024 - 0.073; S5) but much greater within the South Karpaz rookery (h = 0.437; S5). Nucleotide diversity was low among all sites ($\pi = 3E^{-5} – 5.7E^{-4}$; S5) as haplotypes only differ from CM-A13.1 through a single point mutation which included two transitions and one transversion (Supplementary Information S6). With the inclusion of the mtSTRs, the HR haplotype system revealed 33 haplotypes with the regionally common CM-A13.1 haplotype being subdivided into 28 unique haplotypes (Supplementary Information S5). Unsurprisingly, the genetic diversity among rookeries was much greater using HR haplotypes (h = 0.752 – 0.871; Table 2) with a marginal increase in nucleotide diversity ($\pi = 2.2E^{-3} – 3.3E^{-3}$, Table 2).

Significant stock structure was detected between the South Karpaz and the three other rookeries based on the CR marker but no stock structure was detected for any other pairwise combinations (Table 3). However, the HR haplotype marker revealed significant stock structure for four of the six pairwise comparisons which included significant

differentiation between Alagadi and all other rookeries as well as between Akdeniz and the South Karpaz (Table 3). Additional stock structure was also detected between the North Karpaz and Akdeniz based on the exact test of population differentiation (Non-differentiation exact P-value = 0.01415). The PCoA based on the pairwise F_{ST} values explained 100% of the genetic variation among populations in the first two axes (Figure 2A).

The unrooted parsimony network (Figure 3) supported the designation of CM-A13.1 as the ancestral haplotype giving rise to all known Mediterranean CR haplotypes. The haplotype network strongly suggests that the Mediterranean forms a discrete population segment as all known Mediterranean CR haplotypes are endemic and not shared with the Atlantic, with the exception of CM-A13.1 and CM-A27.1, although the latter was not found among these Cypriot rookeries.

Nuclear DNA

All loci were found to be highly polymorphic ranging between 4 and 14 alleles per locus among rookeries and up to 12 alleles per locus within rookeries (Supplementary Information S7). Genotypic differences were observed at 3 allele calls of 1340 (0.22% error) with the highest error rate recorded for locus B123 (1.9%). Loci B123 was subsequently sequenced on selected samples to evaluate the one base pair differences observed among some allele sizes which revealed a switching between one or two Adenine base pair insertions. All rookeries exhibited similar allelic richness, observed heterozygosity and gene diversity (Table 2) with no significant differences found among rookeries (Kruskall-Wallis, $P > 0.05$ for all tests). No signs of inbreeding were detected with observed heterozygosity equal to or greater than the expected levels of heterozygosity across loci in all cases. We found no evidence of null alleles or significant departures from HWE for individual rookeries across loci or when rookeries were pooled after correcting for multiple tests (All rookeries, $\chi^2_{104} = 134.27$, $P = 0.0245$). Some evidence of linkage disequilibrium (LD) was found for four pairs of loci within Alagadi where close relatives could be expected due to the near complete sampling of this rookery. These loci pairs did not remain significant among rookeries with the exception for loci pair Cm3 – B123 as the P-value within Alagadi was highly significant ($P < 0.000$).

However, no evidence for linkage was found for this loci pair within the other rookeries ($P > 0.05$) or in previous studies on the Alagadi rookery (Wright *et al.* 2012b; a) and so for these reasons we assumed all loci to be independent.

Global F_{ST} averaged across loci was 0.007 (range = 0.003 – 0.018; Supplementary Information S8) and weak but significant genetic differentiation was found for three of the six pairwise comparisons after correcting for multiple tests (Table 3). Significant stock structure was detected between Alagadi and both the Akdeniz and South Karpaz rookeries as well as between Akdeniz and the North Karpaz with marginally non-significant structure between the North and South Karpaz ($P = 0.052$; Table 3). The first two axis of the PCoA explained 100% of the genetic variation among rookeries and demonstrated a north-south divide with the two rookeries on the north coast being genetically more similar as were Akdeniz and the South Karpaz (Figure 2B). No evidence was detected for isolation by distance (Mantel test, $P = 0.323$) with a very weak correlation between D_{est} values and geographic distances ($R^2 = 0.0115$).

No genetic structure was inferred from the spatial and non-spatial Bayesian analyses with all individuals forming a single genetically similar cluster based on the approximation of the posterior probability for the STRUCTURE analysis ($L(K) = -14366.99$; Supplementary Information S9) and the highest log-likelihood of the posterior probability in Geneland (uncorrelated model = -12158.7; correlated model = -11967.2). However, ΔK from the STRUCTURE analysis (Evanno *et al.* 2005) suggested two ($\Delta K = 6.232$, S12) or four ($\Delta K = 6.026$, Supplementary Information S10) genetically similar clusters in turn which may relate to genetic sub-structuring as they failed to show any geographic coherence with low individual admixture coefficients within any single cluster. No evidence was found for recent genetic bottlenecks for any rookery under the two-phase model (one-tailed test for heterozygote excess $P > 0.05$ in all cases).

Analysis of statistical power for genetic markers to detect population structure

The POWSIM analysis revealed that the CR haplotype marker had insufficient statistical power to detect significant stock structure considering the levels of genetic differentiation found in this study. As predicted, the statistical power was greatly increased using the HR system and an acceptable Type 1 error rate was maintained

across the range of the F_{ST} values (Figure 4a). The microsatellite array used in this study exhibited the greatest statistical power among markers but there was a slight elevation in the Type 1 error rate. The microsatellite markers used by Roberts *et al*. (2004) revealed a greater statistical power than expected, yet these four microsatellites were less powerful than our microsatellite markers, and more critically, they would be unsuitable at detecting the levels of F_{ST} found within this study (Figure 4b). Negligible differences were detected in statistical power between Fisher's exact test and Pearson Chi-squared for the CR haplotypes and microsatellites whereas a mildly greater difference was observed for HR haplotypes.

Discussion

This study effectively demonstrates the need to employ genetic markers with the appropriate level of variability for both the study species and the geographic context of the studied populations. For the first time, through the use of higher resolution mtDNA markers we reveal matrilineal stock structure amongst Mediterranean green turtle rookeries. The four rookeries which were geographically separated by 60 - 200 km suggests that natal site fidelity for this population is much greater than previously suggested (Bagda *et al.* 2012). This provides additional evidence (Shamblin *et al.* 2012, 2015c) that female stock structure may be under-estimated amongst other rookeries worldwide which are genetically homogenous based on CR haplotypes. Evidence for male philopatry was detected, but F_{ST} values were an order of magnitude lower than those found with the HR haplotypes which suggest some male-mediated gene flow exists. The absence of shared CR haplotypes and mtSTRs provide strong evidence for the Mediterranean being designated as a discrete population from the Atlantic. This was further corroborated by a power analysis of the microsatellite markers previously employed by Roberts *et al.* (2004) which indicated that they would be unlikely to detect significant stock structure among these recently diverged populations.

Marine turtle CR haplotypes accumulate new mutations at a very slow rate (0.2 $x10^{-8}$ My^{-1} Encalada *et al.* (1996)) which results in a low variability within recently colonised

areas. The Mediterranean region was likely to have been first colonised by the more temperate loggerhead turtle *Caretta caretta* (Clusa *et al.* 2013) and thus the subsequent colonisation by green turtles would have occurred within the last 10,000 yrs (Bowen *et al.* 1992). Given this limited generational time, the CR marker would be unlikely to have acquired a sufficient number of new mutations to detect recently divergent populations and this marker is more likely to reflect historical events as opposed to contemporary levels of gene flow (Formia *et al.* 2006). The power analysis clearly demonstrated that the CR marker is an insensitive marker with which to reject panmixia amongst these populations as it failed to detect significant stock structure even for relatively high levels of $F_{ST.}$ The number of haplotypes increased four-fold with the addition of the mtSTRs as they have a more rapid rate of mutation (Lunt *et al.* 1998) and are therefore a more appropriate marker to assess contemporary gene flow in this case. Thus, the significant stock structure detected between five of the six pairwise comparisons is more likely to represent distinct reproductive populations which should be considered as separate management units. This is the first evidence that matrilineal stock structure exists among Mediterranean green turtle rookeries and it is remarkable that it was detected amongst rookeries within such a close geographic proximity. The geographic extent of the genetic structure detected here is amongst the highest level of natal site fidelity recorded for any marine turtle species (Jensen *et al.* 2013). This has important implications as contemporary nester abundance is determined by local levels of reproduction and therefore these rookeries should be managed as discrete stocks. Although the HR haplotype frequencies indicated that a low level of immigration may occur between the North and South Karpaz, we would advocate for their management as independent units as the South Karpaz is demographically smaller, yet it holds unique mtDNA diversity. Furthermore, The HR haplotypes can also yield more accurate assignments of individuals to natal rookeries from mixed stock foraging areas. This is especially important within the Mediterranean as fisheries bycatch levels are considered unsustainably high (Casale 2011; Casale & Heppell 2016), and this would enable a more precise assessment of their impact on specific rookeries.

Some demographic exchange has been recorded among these rookeries through tag-recapture including the movement of females between the North Karpaz and Alagadi, albeit relatively rare (*pers.com.* Salihe Küsetoğlulları 2015). However, direct methods to assess connectivity can over-estimate gene flow and do not account for evolutionary time-scales (Slatkin 1987). The demographic exchange of females in marine turtles may not necessarily constitute effective gene flow as the offspring survival rate is notoriously low (Frazer 1986). Therefore, this study provides strong evidence that genetic structure exists among Mediterranean rookeries even though some movement of females has been detected.

The results from our mtSTR analysis build upon a growing body of evidence that the Mediterranean green turtles are reproductively isolated from their conspecifics in the Atlantic through the absence of shared mtSTRs (Shamblin *et al.* 2015b; c). So far, all 44 variants of the mtSTRs discovered within the Mediterranean appear near-endemic (Tikochinski *et al.* 2012; this study) which is further corroborated by the distinct absence of any Atlantic CR haplotypes, despite our robust sample sizes. The Mediterranean and Atlantic green turtle populations could be considered as peripatric populations as the former was colonised by the latter (Naro-Maciel *et al.* 2014). Although there is no physical barrier to dispersal (although see Revelles *et al.* (2007)) only two CR haplotypes are known to co-occur in both regions (Encalada *et al.* 1996; Kaska 2000; Shamblin *et al.* 2015b) suggesting that gene flow has been limited over an ecological time-scale. The CM-A13.1 CR haplotype is considered the colonising haplotype of the Mediterranean (Bowen *et al.* 1992) but this is found only at low frequencies amongst Florida rookeries (Shamblin *et al.* 2015a). The only other known Atlantic haplotype to occur within the Mediterranean is CM-A27.1, which was detected within a single individual among Turkish rookeries (Bagda *et al.* 2012). This CM-A27.1 haplotype was first represented as the hypothetical haplotype 'Hyp 1' in Lineage 1 by connecting the high frequency Atlantic (CM-A1) and Mediterranean (CM-A13) CR haplotypes through a single point mutation (Encalada *et al.* 1996). It is considered to have a similar Atlantic origin to CM-A13.1 where it is also only detected at low frequencies (Espinosa *et al.* 1999; Ruiz-urquiola *et al.* 2010; Anderson *et al.* 2013). The co-occurrence of the CM-A27.1

haplotype among these two genetically distinct regions most likely represents an historical yet more recent colonisation event.

Assumptions of sex-biased gene flow were confirmed as microsatellite markers revealed a lower, albeit significant, genetic structure among three of six pairwise comparisons. The F_{ST} values among rookery pairs were generally an order of magnitude lower than those found for mtDNA. However, this can be expected when comparing genetic markers with different modes of inheritance in addition to differences in marker evolution (reviewed in Karl *et al.* 2012). Male marine turtles are considered to exhibit a more relaxed level of philopatry as they do not need to find a suitable nesting site, only to find reproductive mates. Sex-biased levels of gene flow have been found among marine turtle rookeries globally (reviewed in Bowen & Karl 2007; Jenson *et al.* 2013) including those for green turtles (Karl *et al.* 1992). However, male turtles are still considered philopatric (FitzSimmons *et al.* 1997) and significant genetic structuring has previously been detected amongst green turtle rookeries in the Mediterranean (Bagda *et al.* 2012), as in the present study.

Our power analysis revealed that the array of 13 microsatellites were suitable to detect significant stock structure given the levels of F_{ST} found among rookeries. The stock structure indicated that males moved more frequently between Alagadi and the North Karpaz than to the more peripheral rookeries. This result supports expectations as these two rookeries are demographically the largest within this study (Stokes *et al.* 2015) and male turtles are considered to move among rookeries dependent upon the abundance or density of reproductive females (Fitzsimmons 1998; Zbinden *et al.* 2007; Wright *et al.* 2012b). More surprising was the lack of stock structure detected between Akdeniz and the South Karpaz as these two rookeries are the furthest apart geographically and are demographically the smallest. Although we cannot rule out the movement of males between these two rookeries, it might be unlikely for males to traverse between the two most distant rookeries via the south when greater opportunities to mate exist in closer proximity to the north. Therefore we suggest that allele size homoplasy or the retention of ancestral diversity might also explain the lack of genetic differentiation between Akdeniz and the South Karpaz (Roberts *et al.* 2004). However, the failure of the Bayesian

models to detect geographically–based genetic structure emphasise that only the most powerful suite of microsatellites can detect the level of genetic differentiation that delineates these rookeries.

In general, weak but significant genetic structure is increasingly being revealed within studies which employ greater numbers of microsatellites (e.g. Carreras *et al.* 2011; Dutton *et al.* 2013; Naro-Maciel *et al.* 2014). With such a low level of genetic differentiation detected among these rookeries, we evaluated the four highly polymorphic microsatellites employed by Roberts *et al.* (2004). The power analysis revealed that these four microsatellites were statistically more powerful than initially expected, but they would not be suitable to assess genetic structure within this region. The very high levels of heterozygosity (19 – 42 alleles) would further confound attempts to detect genetic structure as alleles become saturated through increased rates of homoplasy. The global assessment conducted by Roberts *et al.* (2004) was innovative and extremely informative on the molecular evolution of these markers. The markers used would have been suitable to detect differences between ocean basins as Atlantic and Pacific turtle populations have been evolving separately for approximately three million years (Bowen *et al.* 1992). However, and most critically, the Mediterranean was only colonised by turtles from the Atlantic during the last 10,000 years (Bowen *et al.* 1992; Clusa *et al.* 2013) and these findings suggest that the four microsatellites would have been unsuitable to detect significant levels of genetic differentiation between these two regions.

Conservation implications

With the advancements in molecular genetics over the last decade, it is time for a new global assessment of genetic connectivity among green turtle rookeries with a greater number of microsatellites or single nucleotide polymorphisms (SNPs). It is of vital importance for the Mediterranean green turtle that a new assessment is conducted with biparentally inherited nuclear DNA as it is a criteria of the IUCN's designation for a subpopulation (Naro-Maciel & Formia 2006). To afford the Mediterranean green turtle

the appropriate IUCN status, this criteria must be met as all other evidence including matrilineal stock structure (Encalada *et al.* 1996; Kaska 2000; Naro-Maciel & Formia 2006), satellite tracking (Stokes *et al.* 2015) and size morphology (Seminoff *et al.* 2015) suggest that Mediterranean green turtles are reproductively isolated from the Atlantic. Carreras *et al.* (2014 and refs therein) could not find any evidence of Atlantic and Mediterranean green turtles sharing foraging areas, or evidence of gene flow between regions in the more locally abundant loggerhead (Carreras *et al.* 2011).

We advocate new regional as well as international genetic assessments using higher resolution genetic markers as the contemporary level of matrilineal gene flow in the Mediterranean was previously overestimated. We advise that the green turtle rookeries in North Cyprus, and most likely the wider Mediterranean, should be considered as separate units for conservation and management.

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Tables and Figures.

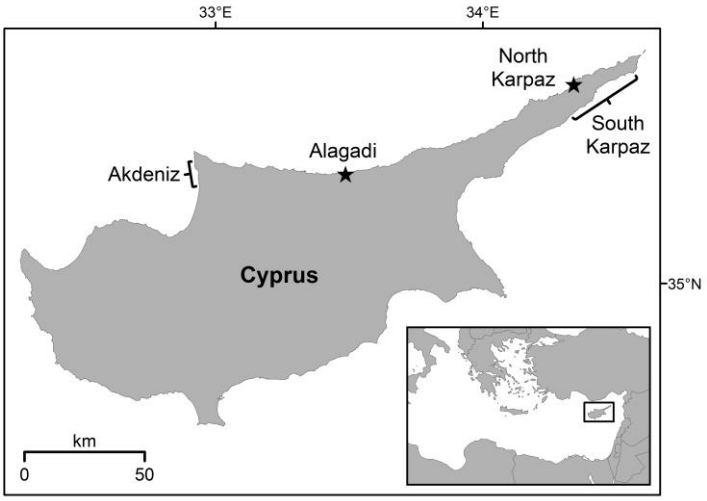

Figure 1 The location of the Mediterranean island of Cyprus and the four green turtle rookeries sampled at Akdeniz, Alagadi, North Karpaz and South Karpaz.

Table 1 Location and sampling information for the four green turtle rookeries in northern Cyprus

Location	Year	Cohort	Number of samples
Akdeniz	2013-2015	Offspring	132 (84)
Alagadi	2001-2015	Adults	234
N. Karpaz	2014	Adults	54
S. Karpaz	2013	Offspring	59 (46)

Detailed information of the samples obtained in this study, including the year that the sampling was conducted, the cohort from which the samples were obtained and the number of samples (filtered sample sizes in parenthesis).

Table 2 Genetic variability of the four green turtle rookeries

	Nests per season	mtDNA			nDNA			
		n	*h*	*π*	*k*	*AR*	*He*	*Ho*
Akdeniz	49 (4 - 125)	84	0.752 (0.024)	$2.2E^{-03}$ ($1.4E^{-03}$)	6.23	5.89	0.671	0.671
Alagadi	66 (8 - 236)	234	0.807 (0.066)	$2.9E^{-03}$ ($1.5E^{-03}$)	6.69	5.77	0.662	0.671
N Karpaz	104 (38 - 179)	54	0.871 (0.073)	$3.3E^{-03}$ ($2.0E^{-03}$)	6.08	5.82	0.655	0.655
S Karpaz	64 (35 – 107)	46	0.868 (0.438)	$3.1E^{-03}$ ($1.9E^{-03}$)	5.31	5.20	0.645	0.652

General demographic and genetic diversity indices of sampling locations for the different sets of markers used in the present study. Abbreviations codes, *n* = sample size, *h* = haplotype diversity, π = nucleotide diversity, *k* = mean number of alleles per locus, *AR* = allelic richness, *He* = gene diversity, *Ho* = observed heterozygosity. HR haplotype values reported for mtDNA with CR haplotype values in parenthesis. Nests per season are indicated as a mean with range in parenthesis from Stokes *et al.* (2015).

Table 3 Pairwise genetic distances among the four green turtle rookeries

	Alagadi	Akdeniz	N Karpaz	S Karpaz
Alagadi		**0.036** (0.001)	**0.039** (0.00)	**0.051 (0.286)**
Akdeniz	**0.003 (0.005)**		0.009 (0.00)	**0.046 (0.262)**
N Karpaz	0.003 (0.001)	**0.006 (0.010)**		0.010 **(0.162)**
S Karpaz	**0.005 (0.008)**	0.005 (0.002)	0.007 (0.008)	

Above diagonal pairwise F_{ST} values obtained from HR haplotypes (from CR haplotypes in parenthesis), below diagonal pairwise F_{ST} values based on nDNA microsatellites (D_{est} in parenthesis), Significant values are shown in bold after correcting for multiple comparisons (for a P-value < 0.05, FDR = 0.0204).

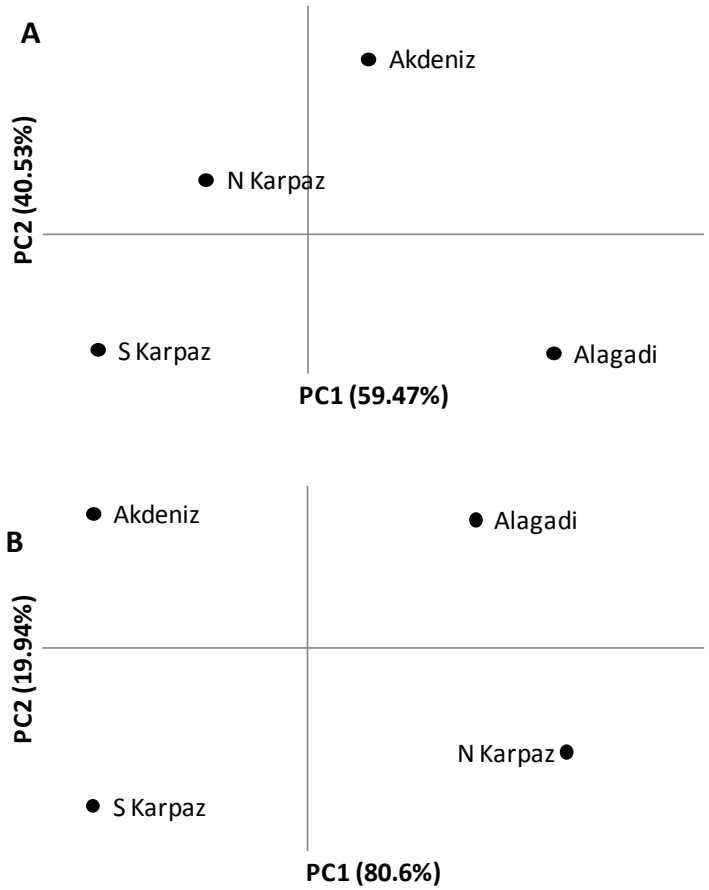

Figure 2 PCoA using a frequency based analysis of A) F-statistics for HR haplotypes and B) D_{est} values for nDNA microsatellites. The percentage of variability explained by each principal coordinate is shown in brackets.

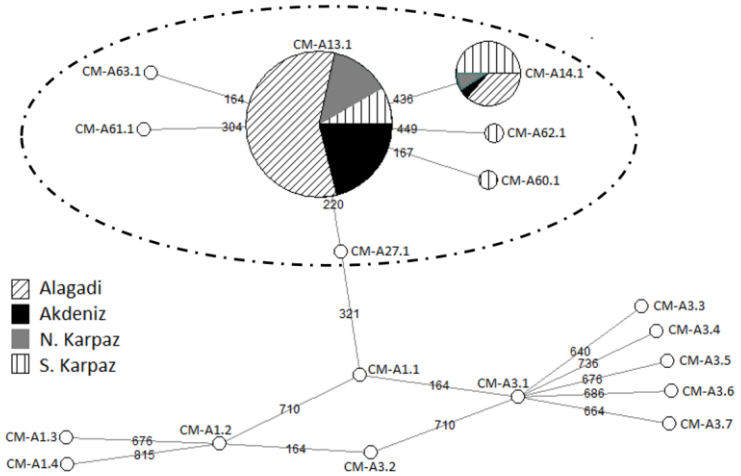

Figure 3 Unrooted parsimony network of CR haplotypes. Pie charts represent haplotype frequencies within each rookery and the size of the pie is representative of haplotype frequencies in our locations for illustrative purposes. Haplotypes represented in white were absent from the present study, haplotypes within the dashed line are known to be present within the Mediterranean while CM-A1.1 - 1.4 and CM-A3.1 – 3.7 represent the most common CR haplotypes from lineage 1 within the Atlantic.

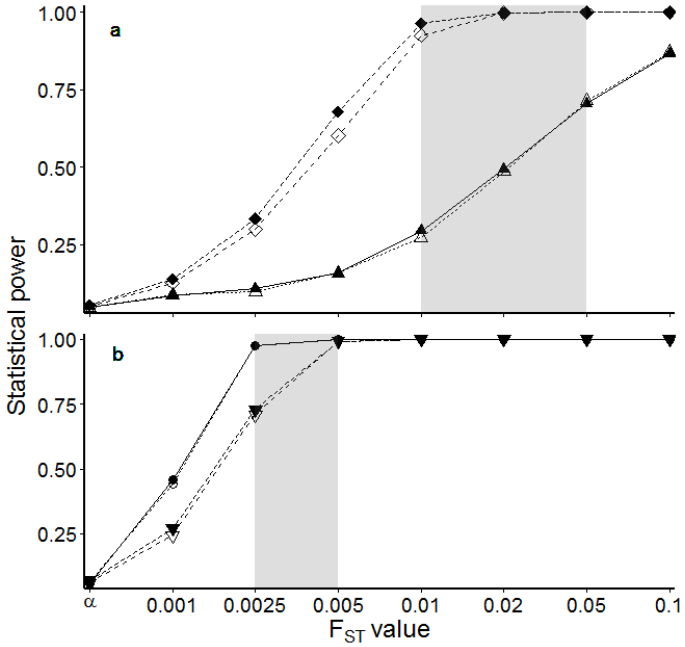

Figure 4 Statistical power of genetic markers to detect divergence at different levels of F_{ST} for a = mtDNA, (diamonds = HR haplotypes, triangles = CR haplotypes) and b = nDNA (circles = 13 microsatellites from this study, upside-down triangles = 4 microsatellites from Roberts *et al*. (2004)). Dark shapes = Chi-square, transparent shapes = exact test of population differentiation. Shaded areas indicate the range of F_{ST} values found within this study for a = mtDNA and b = microsatellites. Power is expressed as the proportion of significant values ($P < 0.05$) using empirical sample sizes for 13 microsatellites, CR and HR haplotypes from this study (Alagadi, n = 234; Akdeniz, n = 84; N Karpaz, n = 54; S Karpaz, n = 46) and for the 4 microsatellites (Roberts *et al*. 2004): 9 populations with sample sizes of (46, 21, 44, 21, 25, 15, 19, 7, 49).

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Data accessibility

The ‘mainparams’ file for the STRUCTURE analysis and a list of microsatellite genotypes with CR and HR haplotypes of all individuals (filtered and unfiltered), including individual ID, sampling location and cohort information as described in Table 1, is available through Dryad Digital Repository at XXXXXX.

Chapter 1 Supplementary methods

S1 Details of the microsatellite markers and multiplex sets used for the genotyping

Locus / Multiplex	Primer Sequence (5' – 3') (Flurodye included in forward sequence)	Size range (BP)	Repeat motif	Locus reference
A6/1	F **VIC**AGTGCAATAACCATCCTTACAC	121 - 138	(AG)n	1
	R GGGCTGAATAGAGCTACAGAC			
Cm3/1	F **PET**AATACTACCATGAGATGGGATGTG	166 - 200	(CA)n	2
	R ATTCTTTTCTCCATAAACAAGGCC			
Or7/1	F **FAM**GGGTTAGATATAGGAGGTGCTTGATGT	226 - 236	(TG)n(TG)n	3
	R TCAGGATTAGCCAACAAGAGCAAAA			
Cc28/1	F **VIC**AGCCCATATGTTTCCCTTCA	189 – 201	(CA)n(TA)n	4
	R TTGGCCCATCTTATTTCAGTG			
Cc7E11/1	F **PET**GTTTGAAGAGCTGACCCCATATAG	262 - 290	(AGAT)n	5
	R AAACACAGAAATGAGGGATAG			
CcP7D04/1	F **FAM**ATGAGCAAAGTAACCCTAACA	308 – 360	(AGAT)n	6
	R GTTTGGAGCCAAATTAGAGATCAAC			
D2/1	F **VIC**AGTCCCCACTACTCATACCC	276 - 334	(TAGA)n	1
	R GTTTCTTTTGTGTTACTTCGGTGTTTC			
Klk314/2	F **FAM**GGTGCCAAGGAGGACGCTG	109 - 119	(CA)n	7
	R CATGCTCGCCCCTGGAAAG			
Cm58/2	F **PET**GCCTGCAGTACACTCGGTATTTAT	136 - 156	(CA)n	2
	R TCAATGAAAGTGACAGGATGTACC			
B103/2	F **VIC**CAGTCCTTGTTGTGGTTAGAGT	150 - 162	(CAA)n	1
	R GTTTCTTTTTCCCTTTCATCTTCTGTC			
Cc2/2	F **PET**CCCCCATAACACCACATCTC	211 - 249	(TA)n(GA)n	4
	R AGGTCACAAATGGAGCAAGC			
C102/2	F **FAM**TAAAAAGGCAGCCAAGTAAG	235 - 255	(TACA)n(CA)n	1
	R GTTGCAGAACCAACAGAATAG			
B123/2	F **VIC**ATCCCAGACCAAACAC	216 - 225	(CAA)n	1
	R GGCACAAGCCTATCCAATA			

Locus reference: 1= Dutton & Frey 2009; 2 = FitzSimmons *et al.* 1995; 3 = Aggarwal *et al.* 2004; 4 = Monzón-Argüello *et al.* 2008; 5 = Shamblin *et al.* 2007; 6 = Shamblin *et al.* 2009; 7 = Kichler *et al.* 1999

S2 Removal of putative relatives to avoid pseudoreplication for the analysis of genetic structure

To evaluate the possible effect of pseudoreplication in the downstream analysis for rookeries where offspring from nests of unknown maternal origin were sampled, we ran the microsatellite genotypes through Colony v2.0.5.0 (Jones & Wang 2010) to identify full-sib clusters. Colony was run with the mating system set to polygamous for both sexes, without inbreeding, on a short run at low precision employing the FL-PLS algorithm (full-likelihood combined with pairwise-likelihood score) with a sibship complexity prior and without maternal or paternal candidates. All individuals identified within a full sibship cluster were cross referenced using their HR haplotypes and, where

appropriate, the season in which the sample was collected. If samples within a full sibship cluster had matching HR haplotypes and were sampled in the same year or non-consecutive years (as green turtles do not nest every year Miller 1997; Stokes *et al.* 2014) then we removed all but one individual to maintain all HR haplotypes although some rare microsatellite alleles may have been removed. If samples did not have matching HR haplotypes or samples were collected in consecutive years then all samples were included. Temporal tests were conducted using traditional F-statistics (Wright 1951) based on haplotype frequencies in Arlequin v3.5.2.3 (Excoffier & Lischer 2010) for all rookeries containing samples collected across multiple seasons. Years that were not statistically significant among years were pooled for further analysis.

S3 Bayesian analysis to detect genetic structure

STRUCTURE assigns the most likely number of populations or 'clusters' (*K*) which are in HWE and linkage disequilibrium based on multilocus genotypes without using prior information based on sampling location (Pritchard *et al.* 2000). We carried out 20 replicate runs for each value of *K* between 1 and 10 using an admixture model with alpha inferred from the data, lambda set to one and correlated allele frequencies but without assuming the same value of F_{ST} for different sub-populations (Falush *et al.* 2003; Evanno *et al.* 2005). Default values were assumed for all other parameters with a burnin period of 100,000 followed by 200,000 Markov Chain Monte Carlo (MCMC) iterations. The programme Structure Harvester (Earl & vonHoldt 2012) was used to visualise the STRUCTURE output and assign the most likely number of *K* value clusters using the natural logarithm of the posterior probability *L(K)* (Figure S9) and the ad-hoc statistic Δ*K* which calculates the number of clusters using the 2nd order rate of change in the log-probability of data between successive *K* values (Evanno *et al.* 2005, Figure S10). Furthermore, we used the programme CLUMPP (Jakobsson & Rosenberg 2007) to ascertain the admixture coefficients of individuals across the 20 replicate runs for population assignment by consensus analysis using the greedy algorithm with the G' matrix, random input orders and 200,000 repeats. Final output was visualised using Distruct (Rosenberg 2004).

Two spatial models were implemented in Geneland (Guillot *et al.* 2005) using Universal Transverse Mercator (UTM) coordinates for the four rookeries. Both models were run without admixture or linkage disequilibrium (Guillot *et al.* 2005) and with either uncorrelated or correlated allele frequencies (Falush *et al.* 2003) which are thought to over-and under-estimate the number of clusters, respectively, and is the suggested method of analysis (Guillot *et al.* 2005). The number of inferred clusters was taken as the run that had the highest log-likelihood posterior probability with concurrence across 10 replicate runs for each model.

S4 Power analysis for the genetic markers

We tested the resolution of our markers to detect different levels of genetic differentiation using the programme POWSIM v4.1 (Ryman & Palm 2006). POWSIM

employs a simulation approach to estimate statistical power (the probability of rejecting H_0 when it's false) for optional combinations of sample size, number of loci, alleles and allele frequencies considering any level of divergence (F_{ST}) (Ryman & Palm 2006). We used empirical allele frequencies and sample sizes whilst fixing Ne to 500 and altering the generation time. The Type 1 error rate was estimated (the probability of rejecting a false negative) by omitting the drift steps and fixing the divergence time (t) to zero (Ryman & Palm 2006). In addition to comparing the statistical power of the CR and HR markers we also compared the power of our microsatellite array to those employed by Roberts *et al.* (2004) using approximate allele frequencies and empirical sample sizes. Statistical power was calculated as the proportion of significant outcomes ($P<0.05$) per number of simulated runs in all cases. To conduct a direct comparison of statistical power between our organelle mtDNA and nuclear DNA markers we followed the method of Larsson *et al.* (2009).

Chapter 1 Supplementary results

S5 CR and HR haplotype frequencies and molecular diversity indices

Haplotypes	Akdeniz	Alagadi	North Karpaz	South Karpaz
CM-A13.1	83	226	52	33
CM-A14.1	1	8	2	11
CM-A60.1				1
CM-A62.1				1
n	84	234	54	46
h	0.0238 +/- 0.0231	0.0663 +/- 0.0221	0.0727 +/- 0.0476	0.4367 +/- 0.0700
π	0.000030 +/- 0.000113	0.000083 +/- 0.000190	0.000091 +/- 0.000202	0.000572 +/- 0.000569
CM-A13.1-5_7_6_4		1	3	
CM-A13.1-5_8_5_5		1		
CM-A13.1-5_8_6_4		3		
CM-A13.1-5_9_5_4			1	1
CM-A13.1-6_10_6_4			2	
CM-A13.1-6_11_5_4		1		
CM-A13.1-6_7_5_4		1	1	
CM-A13.1-6_7_6_4	2	2	1	
CM-A13.1-6_8_5_4	3	8	4	2
CM-A13.1-6_8_6_4	17	76	7	7
CM-A13.1-6_8_7_4	1	4		2
CM-A13.1-6_8_8_4	35	45	17	12
CM-A13.1-6_8_9_4		1	1	1
CM-A13.1-6_9_6_4	16	51	5	2
CM-A13.1-6_9_7_4		1		
CM-A13.1-6_9_8_4		2		
CM-A13.1-7_10_6_4		13		
CM-A13.1-7_11_5_4			1	
CM-A13.1-7_11_6_4		1		
CM-A13.1-7_7_7_4		1	1	2
CM-A13.1-7_8_5_4		2		
CM-A13.1-7_8_6_4	5	4	2	
CM-A13.1-7_8_7_4		3		1
CM-A13.1-7_8_8_4		1	1	
CM-A13.1-7_9_6_4	2	3	1	
CM-A13.1-8_6_6_4				1
CM-A13.1-8_7_7_4	1	1	4	2
CM-A13.1-8_9_6_4	1			
CM-A14.1-7_8_7_4	1	8	2	10
CM-A14.1-7_8_8_4				1

CM-A60.1-6_8_8_4				1
CM-A62.1-6_8_8_4				1
	n	84	234	54	46
	h	0.7516 +/- 0.0327	0.8066 +/- 0.0152	0.8707 +/- 0.0335	0.8676 +/- 0.0301
	π	0.0022 +/- 0.0014	0.0029 +/- 0.0015	0.0033 +/- 0.0020	0.0031 +/- 0.0019

Expanded CR haplotypes in shaded section. HR haplotypes in non-shaded section. Sample sizes and genetic diversity (mean ± SD) for each subpopulation is expressed as: *n* = sample size, *h* = haplotype diversity and π = nucleotide diversity.

S6 Polymorphic sites on the expanded control region (CR) haplotypes for green turtles within the Mediterranean and two common Atlantic haplotypes (CM-A1 and CM-A3) and their derivatives

Allard	81	84	137	221	238	353	366								
Abreu-Grobois	160	163	216	300	317	432	445	636	660	672	682	706	732	811	
CR haplotype*	164	167	220	304	321	436	449	640	664	676	686	710	736	815	Genbank
CM-A1.1	C	A	G	A	T	C	A	A	G	T	A	-	A	-	JF308465
CM-A1.2												A			JF308466
CM-A1.3										C		A			KT581616
CM-A1.4												A		C	KT581617
CM-A3.1	T														JN632497
CM-A3.2	T											A			HM365068
CM-A3.3	T							G							Shamblin #
CM-A3.4	T												G		Shamblin #
CM-A3.5	T									C					Shamblin #
CM-A3.6	T										G				Shamblin #
CM-A3.7	T								A						Shamblin #
CM-A13.1			A		C										JX306007
CM-A14.1			A		C	A									KR011754
CM-A27.1					C										AF366256
CM-A60.1		G	A		C										KR011755
CM-A61.1			A	G	C										JQ034602
CM-A62.1			A		C		G								JQ034603
CM-A63.1	T		A		C										JQ034604

*Base positions aligned with Allard *et al.* (1994) for bases 164 – 449
Shamblin# Sequences not published or submitted to Genbank

S7 Characterisation of microsatellite loci by population

Pop	Locus	Na	AR	Ho	He	uHe	F
Alagadi	A6.	6	5.83	0.782	0.741	0.743	-0.055
	Cc28.	4	4.00	0.709	0.720	0.721	0.014
	Cm3.	10	7.58	0.551	0.529	0.530	-0.042
	Cc7E11.	4	4.00	0.551	0.517	0.518	-0.066
	CcP7D04.	9	8.03	0.821	0.785	0.787	-0.045
	D2.	11	9.12	0.765	0.726	0.727	-0.054
	Or7.	5	4.97	0.688	0.660	0.661	-0.043
	Klk314.	4	3.35	0.453	0.439	0.440	-0.032
	B103.	5	4.90	0.641	0.658	0.659	0.026
	B123.	5	3.50	0.628	0.618	0.620	-0.016
	C102.	5	4.73	0.585	0.634	0.635	0.076
	Cc2.	12	8.38	0.756	0.768	0.769	0.015
	Cm58.	7	6.58	0.786	0.809	0.811	0.028
	Mean	6.692	5.77	0.671	0.662	0.663	-0.015
	SE	0.788	0.55	0.031	0.031	0.031	0.012
Akdeniz	A6.	6	8.79	0.714	0.735	0.739	0.028
	Cc28.	4	4.83	0.702	0.715	0.719	0.018
	Cm3.	10	8.56	0.571	0.527	0.530	-0.085
	Cc7E11.	4	8.81	0.619	0.585	0.589	-0.057
	CcP7D04.	6	4.98	0.667	0.745	0.750	0.105
	D2.	11	2.98	0.833	0.771	0.776	-0.081
	Or7.	5	5.00	0.607	0.635	0.639	0.044
	Klk314.	3	3.98	0.417	0.431	0.434	0.033
	B103.	5	4.98	0.714	0.687	0.691	-0.039
	B123.	5	7.81	0.690	0.650	0.654	-0.062
	C102.	5	6.85	0.631	0.668	0.672	0.055
	Cc2.	8	5.00	0.796	0.760	0.768	-0.047
	Cm58.	7	4.00	0.796	0.795	0.802	-0.002
	Mean	6.231	5.89	0.671	0.671	0.675	-0.002
	SE	0.744	0.56	0.030	0.030	0.030	0.016
N Karpaz	A6.	5	5.91	0.741	0.748	0.755	0.009
	Cc28.	4	4.00	0.704	0.711	0.718	0.010
	Cm3.	9	8.70	0.537	0.564	0.569	0.048
	Cc7E11.	5	4.00	0.463	0.446	0.450	-0.039
	CcP7D04.	9	6.00	0.704	0.762	0.769	0.077
	D2.	9	10.22	0.852	0.755	0.762	-0.128
	Or7.	5	4.99	0.648	0.622	0.627	-0.043
	Klk314.	3	2.55	0.389	0.435	0.439	0.106
	B103.	5	4.91	0.593	0.642	0.648	0.077
	B123.	5	3.87	0.667	0.649	0.655	-0.027
	C102.	5	4.98	0.667	0.621	0.627	-0.074
	Cc2.	11	9.48	0.738	0.749	0.753	0.014

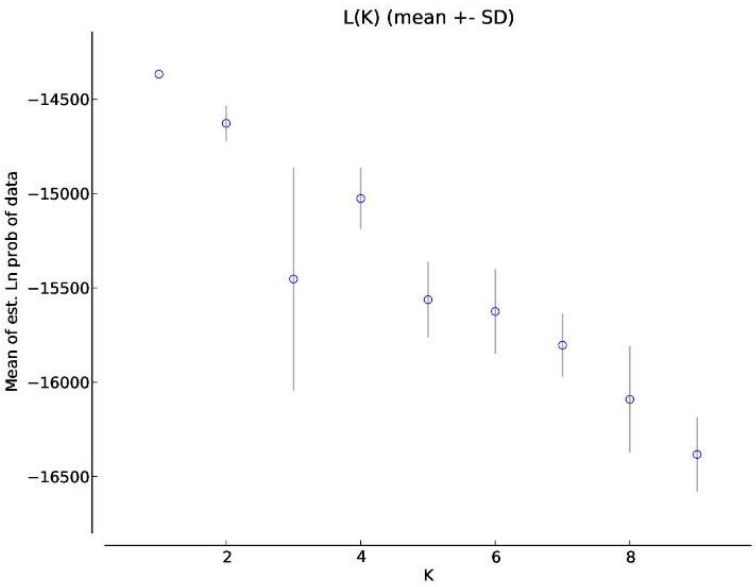
	Cm58.	6	6.00	0.821	0.821	0.826	-0.001
	Mean	6.077	5.82	0.658	0.655	0.661	-0.002
	SE	0.571	0.64	0.037	0.033	0.033	0.019
S Karpaz	A6.	5	5	0.696	0.739	0.747	0.059
	Cc28.	4	4	0.630	0.704	0.711	0.104
	A6.	5	5	0.696	0.739	0.747	0.059
	Cc28.	4	4	0.630	0.704	0.711	0.104
	Cm3.	6	6	0.413	0.468	0.473	0.117
	Cc7E11.	4	4	0.587	0.610	0.617	0.038
	CcP7D04.	7	7	0.783	0.754	0.763	-0.038
	D2.	8	8	0.652	0.731	0.739	0.108
	Or7.	5	5	0.652	0.543	0.549	-0.201
	Klk314.	2	2	0.326	0.375	0.379	0.130
	B103.	5	5	0.717	0.676	0.683	-0.061
	B123.	4	4	0.609	0.668	0.675	0.088
	C102.	5	5	0.652	0.584	0.591	-0.116
	Cc2.	8	8	0.804	0.711	0.719	-0.131
	Cm58.	6	6	0.870	0.816	0.825	-0.066
	Mean	5.308	5.20	0.645	0.645	0.652	0.003
	SE	0.472	0.42	0.041	0.035	0.035	0.031
Total	Mean	6.077	5.868	0.661	0.658	0.663	-0.004
	SE	0.326	0.265	0.017	0.016	0.016	0.010

Abbreviation code, Na = number of alleles, AR = allelic richness, Ho = observed heterozygosity, He = expected heterozygosity, uHe = unbiased expected heterozygosity, F = inbreeding coefficient.

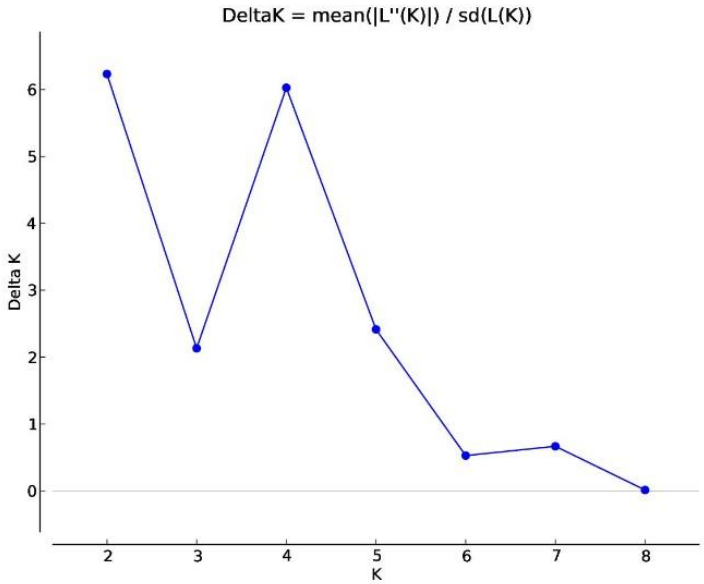
S8 F-statistics among rookeries for each locus

All Pops.	Locus	Mean He	Mean Ho	F_{IS}	F_{IT}	F_{ST}
	A6	0.741	0.733	0.010	0.014	0.004
	Cc28	0.712	0.686	0.036	0.041	0.005
	Cm3	0.522	0.518	0.007	0.011	0.004
	Cc7E11	0.540	0.555	-0.029	-0.010	0.018
	CcP7D04	0.762	0.743	0.024	0.032	0.008
	D2	0.746	0.776	-0.040	-0.031	0.008
	Or7	0.615	0.649	-0.055	-0.046	0.009
	Klk314	0.420	0.396	0.057	0.059	0.003
	B103	0.666	0.666	-0.001	0.005	0.006
	B123	0.646	0.649	-0.003	0.006	0.009
	C102	0.627	0.634	-0.011	-0.005	0.007
	Cc2	0.747	0.774	-0.036	-0.027	0.008
	Cm58	0.810	0.818	-0.010	-0.003	0.007
			Mean	-0.004	0.004	0.007
			SE	0.009	0.008	0.001

Abbreviation code, Mean He = Mean expected heterozygosity, Mean Ho = Mean observed heterozygosity, F_{IS} = inbreeding coefficient of an individual relative to the subpopulation, F_{IT} = inbreeding coefficient of the individual relative to the population, F_{ST} = inbreeding coefficient of alleles within the subpopulation relative to the population



S9 The most likely number of genetically similar clusters assigned using the mean approximation of the posterior probability from the STRUCTURE analysis



S10 The most likely number of genetically similar clusters assigned using Evanno's ΔK statistic from the STRUCTURE analysis

Chapter 1 Supplementary references

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Chapter 2

Satellite tracking and stable isotope analysis highlight differential recruitment among foraging areas in green turtles

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Abstract:

Identifying links between breeding and non-breeding sites in migratory animals is an important step in understanding their ecology. Recognising the relative importance of foraging areas and ascertaining site specific levels of recruitment can provide fundamental and applied insights. Here, satellite telemetry and the stable isotope ratios ($\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$) of 230 green turtles (*Chelonia mydas*) from a regionally important rookery in northern Cyprus were employed to evaluate the relative importance of four foraging areas. A preliminary analysis of stable isotope ratios suggested that a major foraging area had been missed through satellite telemetry as a large proportion of turtles had isotope ratios that did not correspond to sites previously identified. Stable isotope ratios were then employed to select five turtles to be fitted with platform terminal transmitters in 2015. All five turtles were subsequently tracked to the same location, Lake Bardawil in Egypt. Serially collected tissue samples from 45 females, ranging over two to four breeding seasons, suggested that foraging site fidelity was very common with 82% of females exhibiting extremely high temporal consistency in isotope ratios. Quantifying fidelity allowed an evaluation of foraging area specific contributions to each breeding cohort over the past two decades and demonstrated that recruitment was unequal among sites, and dynamic over-time, with Egypt now currently the major contributor to the nesting aggregation. This work demonstrates the utility of stable isotope analysis to elucidate the spatial ecology of cryptic taxa and illustrates how more robust baselines can be assembled against which to measure the success of future marine conservation initiatives.

Introduction:

Many species undertake migrations including ontogenetic shifts between successive life-stages (Bolten et al. 1998, Reich et al. 2007) or regular seasonal (Hobson & Schell 1998) and reproductive migrations (Rubenstein & Hobson 2004). Philopatric species (those animals that return to their natal region to breed) often form genetically distinct populations (Greenwood 1980, Meylan et al. 1990), but not all individuals from the breeding population necessarily migrate to the same non-breeding site (Webster et al. 2002, Bolker et al. 2007). Identifying these links between breeding and non-breeding sites is a priority for species conservation, but tracking migrating animals can be difficult. Large terrestrial species can often be observed or tracked using extrinsic markers (Rubenstein & Hobson 2004), although this is difficult with smaller more vagile species as detectability is low. Tracking animals in the marine environment is especially challenging as animals can move across great distances. Satellite telemetry has the ability to provide real time insight into animal movements including the large seasonal migrations of marine megavertebrates (Hart & Hyrenbach 2010, Block et al. 2011, Jeffers & Godley 2016), identifying stock connectivity (Bonfil et al. 2005, Heide-Jørgensen & Laidre 2006, Zerbini et al. 2006) and guiding the implementation of marine protected areas (Scott et al. 2012, Schofield et al. 2013, Revuelta et al. 2015) and time-area closures (Shillinger et al. 2008). Nevertheless, satellite telemetry is expensive and can entail direct costs to the study animals and therefore sample size is often limited (Wilson & McMahon 2006, Godley et al. 2008). However, the satellite data from a few individuals can be scaled-up to infer habitat use at a population level through the use of forensic chemical techniques such as stable isotope analysis (Hobson 2007, Jaeger et al. 2010, Zbinden et al. 2011, Robinson et al. 2016).

Stable isotope analysis (SIA) utilises the stable isotope ratios in the tissue of an animal to evaluate its resource use and migratory origin (Newsome et al. 2007, Hobson et al. 2010). The isotopic composition of a consumer's tissue reflect those of its diet after undergoing a predictable trophic enrichment (Graham et al. 2010) providing a natural intrinsic tag that can link an animal to a location (Hobson 2007). The time period over which the diet is assimilated depends on the tissue specific turn-over rates, and

metabolically active tissues can be selected dependent upon the time frame to be studied (Reich et al. 2008, Hobson et al. 2010). Most studies to date have employed the stable isotope ratios of carbon (^{13}C:^{12}C or $\delta^{13}C$) and nitrogen (^{15}N:^{14}N or $\delta^{15}N$) as dietary tracers as these elements are informative about foraging site location and the trophic level of the consumer (Peterson & Fry 1987, Hobson 1999). Carbon stable isotopes of a consumer reflect those of the primary producer as very little fractionation occurs through successive trophic levels (~1‰) (DeNiro & Epstein 1978). More specifically to the marine environment, ^{13}C can exhibit several strong spatial gradients relating to mean temperature and salinity as this influences primary production. In general, $\delta^{13}C$ values increase from higher to lower latitudes, as well as from oceanic to neritic ecosystems, and from pelagic to benthic food sources (Hobson 2007, Koch 2007). The $\delta^{15}N$ of a primary producer can be strongly influenced by the mode of nitrogen cycling (Hobson et al. 2010), and substantial trophic discrimination (~3.4‰) (DeNiro & Epstein 1981) enables assumptions to be drawn concerning the consumer's trophic level (Hobson & Welch 1992, Godley et al. 1998). Nitrogen cycling in coastal ecosystems is strongly influenced by anthropogenic inputs of nitrogen. Sources such as agricultural fertilisers and animal or human waste can elevate nitrate levels, resulting in an increase in ^{15}N of particulate organic matter that is reflected within the food web (Vander Zanden et al. 2005, Kendall et al. 2007).

Isotopic tracking at finer regional scales can be confounded in situations where there is ambiguity in source isotopic compositions (i.e. multiple geographically distinct areas share a similar isotopic profile), as discrete isotopic differences may not exist (Hobson 2007). In these circumstances, an additional isotope or trace element can be incorporated to supplement the carbon and nitrogen isotopes and possibly establish discrete differences among sites. The stable isotope ratio of sulphur (^{34}S:^{32}S or $\delta^{34}S$), for example, is particularly useful in differentiating between inshore and offshore feeding populations (Barros et al. 2010) and ontogenetic dietary shifts associated with successional developmental habitats (Cardona et al. 2009). Sulphur isotopes make ideal indicators for identifying the source of primary production as very little trophic discrimination occurs (Koch 2007). Sulphur is considered to truly discriminate between

neritic and oceanic ecosystems as phytoplankton and most macroalgae assimilate marine sulphate and are characterised by $\delta^{34}S$ values of ~21‰ (Cardona et al. 2009). Conversely, benthic primary producers such as seagrasses have a lower and more variable $\delta^{34}S$ value as ^{34}S from sulphide rich sediments is oxidised back to a sulphate within rhizospheres before being taken up by rooted plants (Fry et al. 1982, Peterson & Fry 1987, Moncreiff & Sullivan 2001).

There are some marine isoscapes (spatially explicit predictions for baseline isotope values) available, but these are generally of too coarse a scale to infer the foraging site of a species at a regional level (Hobson et al. 2010, Somes et al. 2010, McMahon et al. 2013). Thus, isotopic approaches to infer foraging area are often validated through the isotopic composition of satellite tracked individuals (e.g. Jaeger et al. 2010, Zbinden et al. 2011, Seminoff et al. 2012) that can then be used to create species specific isoscapes such as those developed for the loggerhead turtle (*Caretta caretta*) (Ceriani et al. 2014, Vander Zanden et al. 2015). However, a primary caveat of integrating SIA with satellite telemetry is the effective time lag between these techniques; SIA records the isotopic regime prior to tissue sampling whilst satellite telemetry tracks the animal after transmitter attachment (Seminoff et al. 2012). Therefore it is important to assess the foraging site fidelity of the study species before assuming that the isotopic composition of the tracked animal was assimilated at the finally determined foraging area.

Here we set out to fully categorise the foraging areas utilised by a green turtle (*Chelonia mydas*) population that has been the subject of long-term individual-based research (Stokes et al. 2014). Extensive satellite tracking has identified several key foraging sites for this population (Godley et al. 2002, Stokes et al. 2015) and repeat tracking of a small sample suggested that they exhibit fidelity to these sites (Broderick et al. 2007). We specifically set out to address four main research aims: 1) to infer the proportion of the nesting population which forage at each identified site, 2) to quantify foraging site fidelity among adult females, 3) to assess recruitment from each foraging area and 4) to evaluate the effectiveness of stable isotope ratios in monitoring the relative importance of foraging areas over time.

Methods

Satellite telemetry

Between 1998 and 2011, 23 Platform Terminal Transmitters (PTTs; see supplementary information S1) were attached to 21 female green turtles (Godley et al. 2002, Broderick et al. 2007, Stokes et al. 2015) and two males (Figure 1, Wright et al. 2012). All PTTs were attached on nesting beaches in northern Cyprus using standard protocols set out in Godley et al. (2002), with satellite data processing and conclusive endpoint destinations as determined in Stokes *et al.* (2015). Satellite tracking identified four distinct regions as important foraging areas for Mediterranean green turtles that include several sites around Turkey and Cyprus (hereafter termed Turkey-Cyprus), the Gulf of Sirte and the Libya - Tunisia border (hereafter termed West Libya), the Gulf of Bomba (in eastern Libya) and Egypt (Figure 1, Stokes et al. (2015)). Subsequent to a preliminary analysis of the stable isotope ratios, we targeted five specific females during the 2015 breeding season for the attachment of Wildlife computer SPOT-293A tags (see supplementary information S1).

Tissue sample collection

A total of 323 tissue samples were collected from 230 green turtles on Alagadi beach in northern Cyprus (35˚19’56.17”N; 33˚28’57.59”E) between 2006 and 2015. Tissue samples were collected from post-nesting females during the breeding season (mid-May until end of July) with the exception of one male encountered at Alagadi beach still coupled to the emergent female (the other satellite tracked male was not tissue sampled, see supplementary information S1). Tissue samples comprising of a small epidermal biopsy (<0.5 cm^2) were taken from the trailing edge of the fore flipper and stored in 96% ethanol until sample preparation. All turtles were individually marked using both external flipper tags and Passive Integrated Transponder (PIT) tags (Stokes et al. 2014).

Stable isotope analysis

We analysed the stable isotopes of carbon, nitrogen and sulphur from green turtle epidermal tissue samples (Seminoff et al. 2006, Reich et al. 2008) following a standard protocol (Ceriani et al. 2014) with the exception that samples were dried at +60°C for 48

– 72 hours. Approximately 0.7mg ± 0.1mg of each sample was weighed into a tin capsule, sealed and analysed for carbon and nitrogen. Isotope analysis was performed at the Stable Isotope Facility of the Environment and Sustainability Institute (ESI; University of Exeter, Penryn Campus) via a continuous flow isotope ratio mass spectrometer (CF-IRMS) using a Sercon Integra2 stable isotope analyser. A greater sample mass was required for sulphur isotope analysis; with approximately 5mg ± 0.5mg of sample sealed into a tin capsule together with a small amount (<1mg) of vanadium pentoxide to aid combustion of the larger sample quantity. The analysis of sulphur isotopes was conducted at Elemtex in Launceston, UK using an ANCA SL attached to a Sercon 2020 CF-IRMS.

Stable isotope ratios are expressed using a conventional notation as δ values defined as parts per thousand or permil (‰) according to the following equation as per Bond & Hobson (2012):

$$\delta X = [(R_{sample}/R_{standard})-1]$$

Where *X* is ^{15}N, ^{13}C, or ^{34}S; R_{sample} is the corresponding ratio of the heavier to lighter isotopes (^{15}N:^{14}N; ^{13}C:^{12}C or ^{34}S:^{32}S) and $R_{standard}$ is relative to the international standards of atmospheric nitrogen, Pee Dee Belemnite and Vienna Cañon Diablo Trolite, respectively. The standard deviation of the laboratory reference material among runs for $\delta^{15}N$ were: 0.18 ‰ for IAEA N1 ($\delta^{15}N$ = +0.4 ‰) and 0.25 ‰ for IAEA N2 ($\delta^{15}N$ = +0.25 ‰), $\delta^{13}C$: 0.10 ‰ for IAEA CH6 ($\delta^{13}C$ = -10.45 ‰), 0.16 ‰ for IAEA Isvec ($\delta^{13}C$ = -46.6 ‰) and 0.19 ‰ for IAEA nbs-18 ($\delta^{13}C$ = -5.01 ‰) and for $\delta^{34}S$: 0.32 ‰ for IAEA S1 ($\delta^{34}S$ = -0.3 ‰), 0.29 ‰ for IAEA S2 ($\delta^{34}S$ = +22.7 ‰), 0.42 ‰ for USGS 42 ($\delta^{34}S$ = +7.8 ‰) and 0.26 ‰ for USGS 43 ($\delta^{34}S$ = +10.21 ‰).

Selecting samples

Tissue samples were available for some females over multiple breeding seasons and these were employed to quantify foraging site fidelity. However, to avoid pseudoreplication when inferring foraging area use at a population scale, we selected a single epidermal tissue sample for each turtle. A more defined criteria was employed for selecting tissue samples for satellite tracked turtles to ameliorate the time lag between

satellite telemetry and SIA. For satellite tracked turtles we selected tissue samples using the following criteria in order of preference: 1) sample mass available to analyse all three isotopes; 2) sample collected during the breeding season subsequent to satellite tracking, 3) sample collected during the PTT deployment or 4) sample collected temporally closest to when the turtle was satellite tracked (see supplementary information S1). No tissue samples were available for four satellite tracked turtles and so these were omitted from this study (see supplementary S1). When multiple tissue samples were available for turtles that were not satellite tracked, we selected the most recent sample available to minimise any temporal variation in baseline isotopic values that might occur over long time frames.

Control of possible methodological biases

To evaluate additional sources of variation, we analysed 20 paired samples to determine if lipid extraction was necessary. Lipids are commonly extracted from tissues before conducting SIA as they have a more negative $\delta^{13}C$ value than proteins and carbohydrates and therefore the lipid content within a tissue can introduce a potential source of bias (Post et al. 2007). Paired t-tests were conducted on lipid extracted and non-lipid extracted samples (see supplementary information S2) with no significant differences found for $\delta^{15}N$ values (paired t-test, $t_{19} = 1.70$, $p = 0.11$, S2a). Statistically significant differences were detected between paired samples for $\delta^{13}C$ (paired t-test, $t_{19} = - 4.0$, $p < 0.001$, S2b), but the mean difference in $\delta^{13}C$ due to lipid extraction (mean = - 0.18 ‰, range = - 0.27 – +0.09 ‰) was judged biologically irrelevant considering the mean difference in $\delta^{13}C$ among sites (+1.67 ‰). Thus, lipid extraction was deemed unnecessary.

Some disparity exists within the literature concerning the effect that a greater than 70% ethanol concentration can have on the isotopic values of stored tissue samples (Hobson et al. 1997, Tillberg et al. 2006, Barrow et al. 2013, Kaufman et al. 2014). Therefore, as tissue samples for this study were stored in a 96% ethanol concentration, we conducted paired t-tests on tissue samples collected simultaneously from 33 individuals and stored in 96% and 70% ethanol concentration for up to five months (see supplementary information S3). We found no significant differences between samples ($\delta^{15}N$, paired t-

test, t_{32} = 0.67, p = 0.51, S3a; $\delta^{13}C$ values, paired t-test, t_{32} = - 0.13, p = 0.90, S3b), meaning that no consistent enrichment or depletion was observed. Possible sources of variation in $\delta^{34}S$ values were not investigated due to limitations in tissue sample availability.

Analysis and Results

A broad range in stable isotope values was found ($\delta^{15}N$ = +1.99 ‰ – +12.98 ‰, $\delta^{13}C$ = - 16.32 ‰ – - 4.90 ‰ and $\delta^{34}S$ = +0.17 ‰ – +20.21 ‰, Figure 2) and pairwise comparisons found all pairs of isotopes to be significantly correlated (Pearson's product-moment correlation coefficient, $p < 0.001$ in all cases, $\delta^{13}C$ & $\delta^{15}N$, r = - 0.26; $\delta^{15}N$ & $\delta^{34}S$, r = 0.23; $\delta^{13}C$ & $\delta^{34}S$, r = - 0.75, see supplementary information S4). Turtles tracked to Bomba were $\delta^{13}C$-enriched and $\delta^{34}S$-depleted, whereas the turtles tracked to Egypt were generally $\delta^{15}N$-enriched compared to all other foraging areas. Turtles tracked to Turkey-Cyprus and West Libya were nearly isotopically indistinguishable in terms of $\delta^{13}C$ and $\delta^{15}N$, but individuals from Turkey-Cyprus exhibited higher $\delta^{34}S$ values compared to those from West Libya providing isotopic differentiation between these sites.

Inferring foraging area use

Nominal assignment approaches are commonly used to predict the foraging locations of a population using stable isotope signatures calibrated from the satellite telemetry of a subset of individuals (Wunder 2012). We broadly followed previously described methods (Pajuelo et al. 2012, Ceriani et al. 2012, Vander Zanden et al. 2015) to predict foraging area using a discriminant function analysis (see supplementary information S5). The initial composition of the data, validated by the 19 turtles satellite tracked before 2015, strongly suggested that the pre-defined foraging areas did not fully characterise the isotope ratios of the turtle population (Figure 2). Thus, we hypothesised that a foraging area had been missed, or under-represented, through previous satellite tracking effort.

To substantiate this hypothesis, we conducted a preliminary discriminant analysis using $\delta^{13}C$ and $\delta^{15}N$ isotope ratios to obtain prediction probabilities for turtles that might forage in the area not previously characterised. We selected three turtles with isotope

ratios corresponding to this uncalibrated isospace, in addition to the 19 satellite tracked turtles, to calibrate a discriminant analysis and predict the putative foraging area for 184 turtles (see supplementary information S6). We then produced a list of 48 turtles that were likely (at a greater than 80% probability) to forage in the isotopically uncharacterised foraging area (Figure 2).

This list of 48 turtles was subsequently employed during the 2015 breeding season to select five females for PTT deployment based on their prediction probabilities. Eight of those 48 turtles nested at Alagadi that season, of which six had a greater than 90% probability of foraging in the uncharacterised area and were specifically targeted for PTT deployment. On their next successful nesting attempt, five of these six turtles were fitted with PTTs and tracked for 58 – 146 days (mean ± SD = 80.6 ± 37.13, supplementary information S1). All five turtles were tracked to the same foraging area, Lake Bardawil in Egypt (Figure 1 & supplementary information S1 & S1a) where the PTTs then failed most likely due to the hypersaline conditions (Abd Ellah & Hussein 2009, pers. comm. Kevin Ng Wildlife Computers 2015). Early PTT failure was also thought to have resulted in the short tracking duration of the two turtles (one male and one female) previously tracked to the same location (Wright et al. 2012, supplementary information S1).

Subsequent to the 2015 breeding season, and with the full isotopic composition of the turtle population now validated through the satellite telemetry of 23 turtles, we conducted a second discriminant analysis with the addition of a third stable isotope ($\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$). Tissue samples collected in 2015 from previously unsampled females (n = 27) were incorporated within this analysis whilst some females (n = 42) from the preliminary analysis were excluded as sample mass was not adequate to analyse the greater quantity necessary for sulphur; which simultaneously excluded one turtle (G044) satellite tracked to West Libya. Discrete differences were found in the combined isotopic values (MANOVA, Pillai's trace test, $F_{3,19} = 6.54$, $p < 0.001$), yet multiple pairwise comparisons conducted with Tukey's Honest Significant Difference (HSD) still failed to identify discrete differences among all foraging areas (Table 1). For this reason, we combined the two foraging areas that were not discretely differentiated (Turkey-Cyprus and West Libya; TCWL) to establish discrete isotopic differences among

three groups (Bomba, Egypt and TCWL; Table 1). With normal distributions found for the three isotopes and the variance among foraging areas homogenous, we employed a linear discriminant function analysis. We conducted the analysis with non-uniform priors using the number of turtles tracked to each site from the satellite telemetry including those tracked during 2015, although these were near uniform (Royle & Rubenstein 2004, Vander Zanden et al. 2015), and a posterior probability of assignment set at 80%. The discriminant analysis was evaluated using the leave-one-out cross validation method with 95.7% of turtles from the training data correctly reclassified and no differences were found for foraging area assignments based on the type of priors employed. The putative foraging area was predicted for 132 of 165 turtles (80%) with 65 turtles (39%) predicted to forage in Egypt, 22 (13%) in Bomba, and 45 (27%) to the combined foraging area of Turkey-Cyprus and West Libya (TCWL).

We then subjected the isotope ratios from the 45 turtles assigned to TCWL to a secondary classification method (Wunder 2012) similar to that of Zbinden *et al.* (2011). We selected $\delta^{34}S$ as the discriminating criterion as it showed the greatest statistical differences among sites (Table 1). The pooled means and 95% confidence intervals (CI) for the turtles satellite tracked to each foraging area were used to create an overlap in which turtles could not be reliably assigned to either foraging area; these were then included with the turtles unassigned from the discriminant analysis (Figure 3). Those turtles with a $\delta^{34}S$ value greater or lower than the overlap created by the CI were assigned to Turkey-Cyprus or West Libya, respectively. This resulted in 11 turtles (7%, n=165) predicted to forage in Turkey-Cyprus, 19 (12%) in West Libya and a further 15 unassigned turtles (9%) included with the 33 turtles (20%) unassigned from the discriminant analysis (Figures 1, 4 & supplementary information S7).

Foraging site fidelity

Evidence for foraging site fidelity has already been demonstrated for this population of green turtles through the repeat satellite tracking of three individuals (Broderick et al. 2007). To further investigate foraging site fidelity among a broader sample, we used serially collected samples from 45 females, with 33 sampled over two seasons, 9 sampled over three seasons and 3 sampled over four seasons. Samples for 42 of the

females were collected from consecutive breeding seasons including all females sampled for more than two seasons, three females were sampled for two breeding seasons but these were not consecutive as they were not sampled for a single intermediate breeding season. To investigate fidelity we used a repeatability analysis to test the temporal consistency in isotope ratios with the identity of the turtle as the grouping factor and their predicted foraging area as a covariate (see supplementary information S8). The $\delta^{13}C$ and $\delta^{15}N$ values were found to be remarkably consistent over multiple seasons with highly significant repeatability estimates ($\delta^{15}N$: R = 0.65 ± SE = 0.09, 95% CI = 0.46 – 0.79, p = 0.001, Figure 5a; $\delta^{13}C$: R = 0.74 ± SE = 0.07, 95% CI = 0.58 – 0.84, p = 0.001, Figure 5b). The $\delta^{15}N$ values were more variable than $\delta^{13}C$ values with 65% of samples differing less than 1 ‰ and 76.7% less than 1.5 ‰ in subsequent sampling, (always two or more years apart) within an overall range in $\delta^{15}N$ within the population of 8.8 ‰. In contrast, 91.7% of the $\delta^{13}C$ values differed less than 1 ‰ and 96.7% less than 1.5 ‰ within an overall range of 6.08 ‰. As carbon isotopes are a more accurate predictor for the source of primary production (Hobson 2007, Hobson et al. 2010), we contrasted the difference in $\delta^{13}C$ among serially collected samples to the mean difference in $\delta^{13}C$ among sites (+1.67 ‰). This resulted in a conservative estimate of 82% of females (37 out of the 45 females) remaining site faithful as these females did not exhibit a greater than 1 ‰ difference in $\delta^{13}C$ among seasons. Thus, we assumed that foraging site fidelity is extremely common within this population. Only two females exhibited differences greater than 1.5 ‰ in $\delta^{13}C$ among seasons (4.5%) suggesting that plasticity in foraging site fidelity does exist, albeit relatively rarely (Figures 5a & 5b). An isotopic mismatch was also noted for one turtle satellite tracked to West Libya in 2003 as it had isotopic values more suggestive of the Gulf of Bomba when tissue sampled during the subsequent breeding season (Figure 2, turtle G055). However, this turtle then appeared to remain faithful to Bomba for the subsequent two interbreeding intervals.

Evaluating foraging area specific annual contributions to the breeding cohort

As foraging site fidelity is typical within this population we used the individually-based nesting data collected at Alagadi since 1992 (Stokes et al. 2014) to retrospectively evaluate the foraging area specific contributions to each breeding cohort based on the

turtle's unique identification. For each year a turtle nested, they were included as a contributor from their respective foraging area with some females recorded for up to ten reproductive seasons. Although we were limited to nesters that had been satellite tracked or their foraging area inferred through SIA, we were able to gain significant insight into foraging area dynamics for more than two decades. We found that the contributions to the annual breeding cohort from each foraging area were unequal among years (glm, $F_{3,84} = 8.91$, $p < 0.001$) with a general biannual variation characteristic among foraging areas (Figure 6 and supplementary information S9). The trends identified for each foraging area suggest that Bomba was historically the major contributor to the annual breeding cohort but current trends suggest that there has not been any substantial increase in nester abundance from this site (Figure 6a). Egypt, and in particular Lake Bardawil, may have only contributed a few individuals to each breeding cohort until 2008 but then recruitment significantly increased the nester abundance from this site resulting in Egypt, presently, being the single most important foraging area for the Alagadi rookery (Figure 6b). The trends for Turkey-Cyprus and West Libya suggest that these foraging areas only contribute a few individuals to each breeding cohort which is in stark contrast to the inferred importance of these sites from the satellite telemetry (Stokes et al. 2015, Figure 6c & 6d, respectively).

Discussion

Stable isotope analysis calibrated by satellite tracking has great potential to unveil the ecology of migratory species and forms part of a suite of tools to predict where individuals forage at a population scale. Based on our experience here, we advocate the use of SIA prior to, and during, satellite tracking campaigns and discuss in turn the major insights we have gained from the current study:

1. **Selecting the elements for stable isotope analysis**

Stable isotopes are now commonly used to track animal migration across broad spatial scales for both terrestrial and marine species (Rubenstein & Hobson 2004, Michener & Kaufman 2007). However, this study and others (e.g. Tucker et al. 2014) did not find discrete differences among all non-breeding sites at a regional level as the two most

geographically separated foraging areas (Turkey-Cyprus and West Libya) were the most similar for $\delta^{13}C$ and $\delta^{15}N$. The predictable increase of ^{13}C towards the lower latitudes (Hobson 2007, Koch 2007) was confounded as our foraging areas were located on the north and south continental faces. Without the addition of the sulphur isotope ratios, we could not have reliably predicted the foraging area for a large proportion of turtles within this population.

The strong negative correlation found between $\delta^{13}C$ and $\delta^{34}S$ was previously undescribed among seagrass habitats and resulted in sulphur being the more informative for this study population. Sulphur was specifically selected for this study as green turtles are thought to feed predominantly on seagrasses in the Mediterranean (Cardona et al. 2010) that derive their nutrients from the marine sediments as opposed to the open ocean environment. Benthic macroalgae and seagrasses therefore can vary considerably among sites as the marine sedimentary cycle (reviewed in Thode 1991) produces a wide range in ^{34}S values as the reduction of seawater sulphate to H_2S in shallow sediments is influenced by rock type and accretion rates. Thus, we considered that these factors should produce variable ^{34}S values at a local level despite the similarity in habitat type. However, strong intra-site differences have been found in the $\delta^{34}S$ values of seagrasses attributed to the interaction of particulate organic matter and oxygen levels exuded by seagrass roots (Oakes & Connolly 2004). Seagrass samples taken only hundreds of metres apart can have as great a difference in $\delta^{34}S$ as samples taken thousands of kilometres apart (Connolly et al. 2004). Nevertheless, such variation over small geographic scales is incorporated within large megavertebrates such as green turtles that forage over 10's of squared kilometres (Broderick et al. 2007, Christiansen et al. 2017) and the diet assimilated provided distinct isotopic differences among these foraging areas. In a similar study, Tucker et al. (2014) did not find $\delta^{34}S$ in loggerhead turtles (*Caretta caretta*) to be informative as the intra-foraging site variation in $\delta^{34}S$ values encompassed a much greater range (11-15 ‰ at several sites) effectively masking among-site differences. However, loggerhead turtles consume a broader diet over a greater range of depths than green turtles and critically, they do not necessarily forage in food webs based on benthic primary production.

Sulphur isotopes are considered alongside carbon to distinguish more differences among producers than any other element within marine food webs (Connolly et al. 2004). This provided additional evidence to explain the statistical outlier that exhibited severely depleted $\delta^{13}C$ values in association with highly enriched levels of $\delta^{34}S$ (see supplementary information S4). The combination of depleted ^{13}C and enriched ^{34}S strongly suggest that this turtle foraged predominantly within a food web where the primary producers obtained their nutrients from the open ocean as they were not representative of an ecosystem with seagrass as the primary producer (Cardona et al. 2010). Unusual dietary preferences have been evoked previously to explain statistical outliers (Seminoff et al. 2007) and several alternative hypotheses may explain such an isotopic profile. These include: 1) a diet primarily consisting of macroalgae growing on a rocky substrate (Cardona et al. 2010), 2) a greater proportion of invertebrates such as cnidarians and ctenophores within their diet (Burkholder et al. 2011, Lemons et al. 2011) or 3) open ocean foraging (Hatase et al. 2006).

2. The power of using stable isotope analysis to target satellite tracking

The application of SIA validated by satellite telemetry is almost routine now when evaluating foraging areas (Rubenstein & Hobson 2004, Hobson et al. 2010), but the SIA is commonly conducted subsequent to the satellite telemetry. This study has effectively demonstrated that SIA conducted prior to, or during, satellite telemetry campaigns can greatly augment the study by providing scientific guidance to identify specific groups of individuals for PTT attachment and the most likely number of transmitters necessary to identify geographically discrete foraging sites.

3. Assigning turtles to their foraging area

The combined results of the nominal assignment approaches predicted the foraging areas for 71% of the turtles sampled. This provided a sample size of 117 turtles from the 165 analysed in which to assess the relative importance of the foraging areas using SIA, and yielded substantially different results than those inferred from satellite telemetry (Figure 1). The satellite telemetry conducted before 2015 inferred that 65% of green turtles from northern Cyprus were foraging in Libya (35% in West Libya and 30% in the Gulf of Bomba), 17% in Turkey-Cyprus, 13% in Egypt and 5% were undetermined due to

PTT failure (Stokes et al. 2015). In contrast, SIA suggested that 25% foraged in Libya (13% in the Gulf of Bomba and 12% in West Libya), 7% in Turkey-Cyprus, 39% in Egypt but with 29% undetermined. The difference between these two techniques arise from several factors including the limited sample size associated with satellite tracking relative to SIA sampling, interannual variations in the relative contributions from each foraging area, and most importantly in this case, the recent demographic shift causing an increase in turtles recruiting from Lake Bardawil. The observed differences in results from these techniques underlines the need to conduct SIA, in addition to satellite tracking, over sufficient time frames to prevent erroneous conclusions as both the relative contributions from foraging areas and baseline isotopic values are dynamic (see supplementary information S10). These techniques should complement each other as SIA will never be as accurate as satellite telemetry but satellite telemetry will rarely incorporate such robust samples sizes. Thus, a sustained tissue sampling protocol should be supported by satellite telemetry as resources permit.

4. Ascertaining foraging site fidelity

Serially collected tissue samples encompassing multiple seasons can quantify foraging site fidelity through the temporal consistency of stable isotope values (Lowther et al. 2011, Tucker 2014, Wakefield et al. 2015). This work builds upon a growing body of evidence that green turtles (Broderick et al. 2007, Vander Zanden et al. 2013, Shimada et al. 2014, 2016) and other marine turtle species (Schofield et al. 2010, Vander Zanden et al. 2010, 2016, Thomson et al. 2012, Tucker et al. 2014, Pajuelo et al. 2016) show high levels of fidelity to non-breeding sites. The ability to isotopically track some individuals for up to four breeding seasons, a temporal frame of approximately two to eight years, presented clear evidence for a high degree of fidelity to the pre-defined foraging areas. We consider our estimate of 82% of females exhibiting fidelity to be conservative as only two females (4.5%) exhibited substantial differences in $\delta^{13}C$ (> 1.5 ‰) more indicative of a move over a broad spatial scale. However, plasticity does exist and Stokes et al. (2015) also noted evidence from satellite telemetry of secondary movements after turtles had taken up residency, but these were also relative exceptions (four individuals out of a total of 29 tracked conclusively to foraging grounds). These movements were

generally between neighbouring foraging sites, and in some cases only temporary, but this suggests that foraging site fidelity is not hard-wired and is most likely subject to external variables such as resource availability.

5. Monitoring forage site contribution over time

A sustained tissue sampling regime provided a useful technique to monitor foraging area specific contributions and recruitment to the breeding cohort (Vander Zanden et al. 2014, Ceriani et al. 2015). Significant temporal change in the number of individuals originating from foraging areas can be informative of foraging area dynamics without the need to conduct site-based surveys. Some evidence suggests that foraging areas in Turkey-Cyprus may be less productive than those in north Africa as these turtles did not exhibit such a prominent bi-annual nesting pattern (Hays 2000, Hatase & Tsukamoto 2008) that can be indicative of some foraging sites as green turtles do not breed annually as they require an adequate body condition before they can reproduce. Future research should evaluate foraging area specific differences in correlates of reproductive success as phenotypic responses to varying levels of resource abundance have been found among other marine turtle populations (e.g. Zbinden et al. 2011, Hatase et al. 2013, Cardona et al. 2014, Vander Zanden et al. 2014, Ceriani et al. 2015).

Foraging area specific trends in the annual contribution to each nesting cohort clearly demonstrate that the increase in the number of females nesting at Alagadi is primarily being driven by recruitment of turtles that forage in Egypt (Lake Bardawil). It is unclear at present what is driving this increase but it is worthy of further investigation. Several alternative and not mutually exclusive drivers could result in a foraging area specific increase in recruitment at Lake Bardawil. These include an increase in the survival probabilities of juveniles and sub-adults as industrial fisheries are excluded (Casale 2011, Casale & Heppell 2016), greater productivity reducing the age to sexual maturity (Bjorndal et al. 2013), temporal oscillations in sea surface currents, such as those dictated by the Cyprus eddy (Zodiatis et al. 2005) varying the distribution of pelagic-stage juveniles and thus the number of individuals recruiting to each foraging area (Gaspar et al. 2012, Scott et al. 2014, 2017) or a change in the ecological conditions within the hypersaline lake as the channels have been routinely dredged to improve

conditions for the local fisheries (Mehanna 2006, Abd Ellah & Hussein 2009). The maintenance of these channels has substantially reduced the salinity levels over the past few decades which has allowed Cymodocea nodosa, the primary dietary item of the green turtle within the Mediterranean (Cardona 2010), to colonise and now dominate the shallow western basin (EL-Bana et al. 2002, Abd Ellah & Hussein 2009). Therefore, the conditions within Lake Bardawil might not have been tolerable or have provided adequate foraging for green turtles before the channels were maintained but this lake might now provide ideal foraging conditions.

The knowledge that a high proportion of recruits are originating from a single site is a critical development in our understanding of foraging area dynamics. At present, the conservation efforts undertaken on the beaches of northern Cyprus have been effective in increasing the number of hatchlings reaching the water (Stokes et al. 2014) with a possible rise in the number of juveniles reaching a reproductive age. Through the continued tissue sampling of nesting females, we can evaluate which drivers are most likely to result in this foraging area specific increase in recruitment. For example, if an increase in juvenile survivorship or a change in ecological conditions within Lake Bardawil are primarily responsible then we are unlikely to observe a similar increase among foraging areas. In contrast, a delayed increase in recruitment from one or more of the other foraging areas points to one of the other three suggested hypotheses. However, ensuring the continuance of the current trends in recruitment may largely depend on the adequate protection of the turtles foraging within Lake Bardawil which might be challenging as some human-turtle conflict has been reported (Nada et al. 2013) and turtles are still exploited by fisheries if over-wintering in deeper water off the Egyptian coast (Broderick et al. 2007, Boura et al. 2016). International co-operation is necessary to protect turtles foraging in Egypt in addition to those from the key recognised sites in Libya, namely the Gulf of Bomba and the Gulf of Sirte (Casale 2011, Stokes et al. 2015, Casale & Heppell 2016).

Conclusion

Through the analysis of stable isotopes calibrated by satellite telemetry we have answered several important questions for the conservation of marine turtles (see Hamann et al. 2010, Rees et al. 2016). These include identifying and assessing the relative importance of all major foraging sites utilised by green turtles nesting at Alagadi, quantifying foraging site fidelity and gaining a critical insight into foraging area dynamics.

This work builds upon a detailed, long-term monitoring programme following a marked population (e.g. Broderick et al. 2001, 2003, Stokes et al. 2014, 2015) that emphasises the true value that such individual-based data can provide. The long-term nesting data was utilised to evaluate the annual contributions to the rookery from each foraging area for over two decades. These data can now be employed in monitoring site specific in-water survival probabilities that could identify specific areas to direct conservation efforts (García-Cruz et al. 2016) or provide a more detailed assessment on their successful implementation (e.g. Bourjea et al. 2015). We stress the importance of having a balanced satellite telemetry campaign, supported by long-term SIA, as contributions from foraging areas to the breeding cohort are unequal among years, and importantly, these proportions can shift dynamically over time.

These data can provide essential baseline evidence to advise and monitor marine conservation efforts such as establishing marine protected areas, formulating site specific management plans and increasing international cooperation through the identification of important migratory links. A caveat to this type of foraging area assessment is that males are poorly represented. Evidence suggests that some foraging areas can be highly female biased, reflecting primary sex ratios (Jensen et al. 2016) and targeted efforts are needed to collect more tissue samples from males. Future research will evaluate the reasons for the substantial shift in the relative importance of foraging areas and the root cause(s) for the increase in recruitment from Lake Bardawil.

Tables and Figures

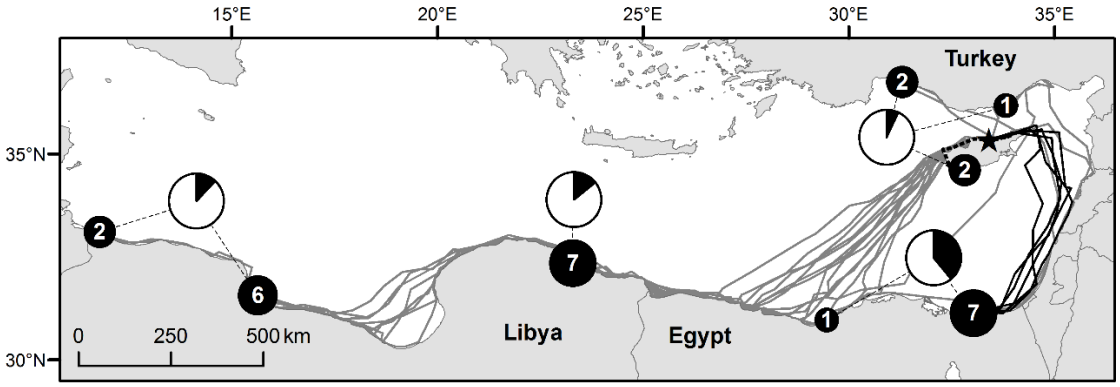

Figure 1 Post nesting green turtle satellite tracks from Cyprus to four broad scale foraging areas including 'Turkey-Cyprus' that combines several foraging sites clustered around Turkey and Cyprus, 'West Libya' that combines two sites (The Gulf of Sirte and a site on the Libya – Tunisia border), the Gulf of 'Bomba' in east Libya and 'Egypt' that combines two sites (Gulf of Arab and Lake Bardawil). Light grey tracks = individuals satellite tracked between 1998 and 2011 from Stokes et al. (2015) and Wright et al. (2012), black-broken track = previously unpublished male tracked to southern Cyprus (PTT = 52818), black = individuals satellite tracked in 2015 to Lake Bardawil, Egypt. Numbers indicate how many individuals were satellite tracked to each foraging area. Pie charts segmented to represent the proportion of individuals assigned to each foraging area based on their stable isotope composition from the 165 turtles of unknown origin within the 2015 analysis. Black section of each pie = proportion of turtles assigned to that foraging area.

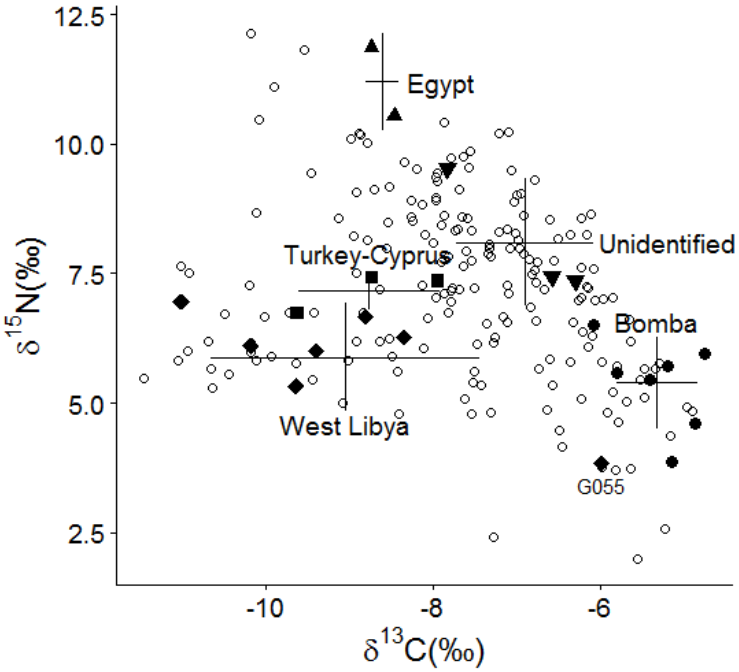

Figure 2 Bivariate plot of $\delta^{13}C$ and $\delta^{15}N$ values for green turtles included in the preliminary discriminant analysis. Large crosses represent the mean ± SD of isotopic values for satellite tracked turtles used to calibrate each foraging area. Circles = turtles satellite tracked to Bomba (n = 7), triangles = satellite tracked to Egypt (n = 2), squares = satellite tracked to Turkey-Cyprus (n = 3), diamonds satellite tracked to West Libya (n = 7), upside-down triangles = individuals selected to characterise the unidentified foraging area (n = 3), open circles = individuals of unknown foraging area (n = 186). Note: One data point removed for greater graph clarity ($\delta^{13}C$ < -14‰). G055 highlighted as an isotopic mismatch; satellite tracked to West Libya but exhibited an isotopic signature corresponding to Bomba.

Table 1 Tukey HSD results comparing stable isotope values in green turtles among foraging areas.

Foraging areas	Nitrogen	Carbon	Sulphur
Bomba - Egypt	**<0.001**	**0.01**	**<0.001**
Bomba - Turkey-Cyprus	0.56	**<0.001**	**<0.001**
Bomba - West Libya	0.66	**<0.001**	**<0.001**
Egypt - Turkey-Cyprus	0.1	0.32	**0.04**
Egypt - West Libya	**0.011**	0.36	0.31
Turkey-Cyprus - West Libya	0.98	0.98	0.5
Bomba - Egypt	**<0.001**	**<0.001**	**<0.001**
Bomba - TCWL	0.32	**<0.001**	**<0.001**
Egypt - TCWL	**<0.001**	0.12	**0.045**

Upper section = Tukey HSD results among the four foraging areas, lower section shaded grey = Tukey HSD results among three foraging areas, TCWL = Turkey-Cyprus and West Libya combined. Significant p-values adjusted for multiple tests in bold.

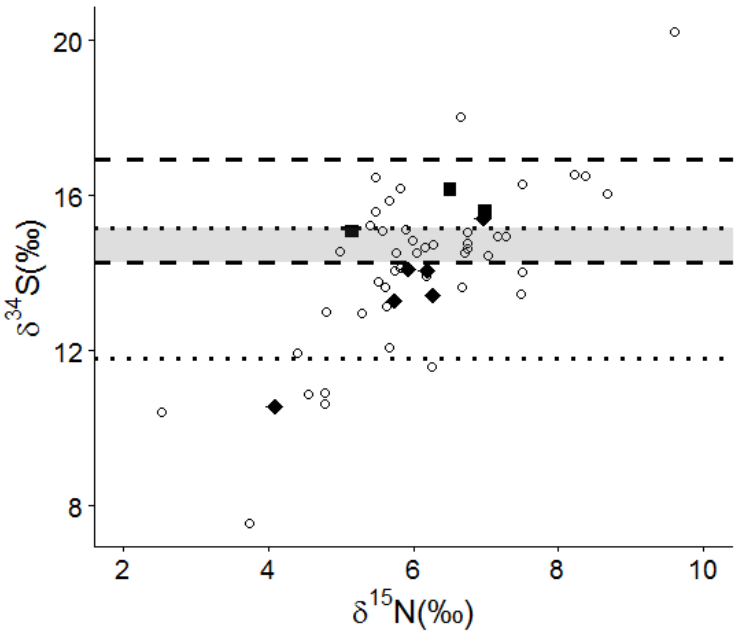

Figure 3 Classification of individuals to Turkey-Cyprus or West Libya from the combined foraging area TCWL based on the 95% CI of $\delta^{34}S$ values of satellite tracked turtles used to calibrate Turkey-Cyprus = squares (n = 3) and West Libya = diamonds (n = 6), dashed lines = upper and lower CI for Turkey-Cyprus, dotted lines = upper and lower CI for West Libya, open circles = turtles to be assigned. Individuals within the grey overlapping region were unassigned (n = 15), individuals above the grey region were assigned to Turkey-Cyprus (n = 11) and those below were assigned to West Libya (n = 19).

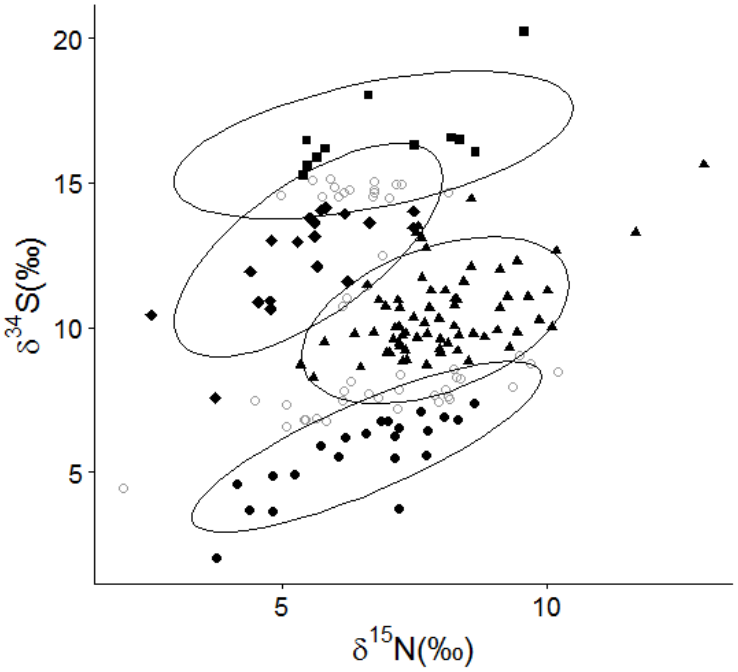

Figure 4 $\delta^{15}N$ and $\delta^{34}S$ values for green turtles predicted to forage in: closed circles = Bomba (n = 22), triangles = Egypt (n = 65), squares = Turkey-Cyprus (n = 11), diamonds = West Libya (n = 19), open circles = unassigned (n = 48). Ellipses set at 95% CI, (total n = 165).

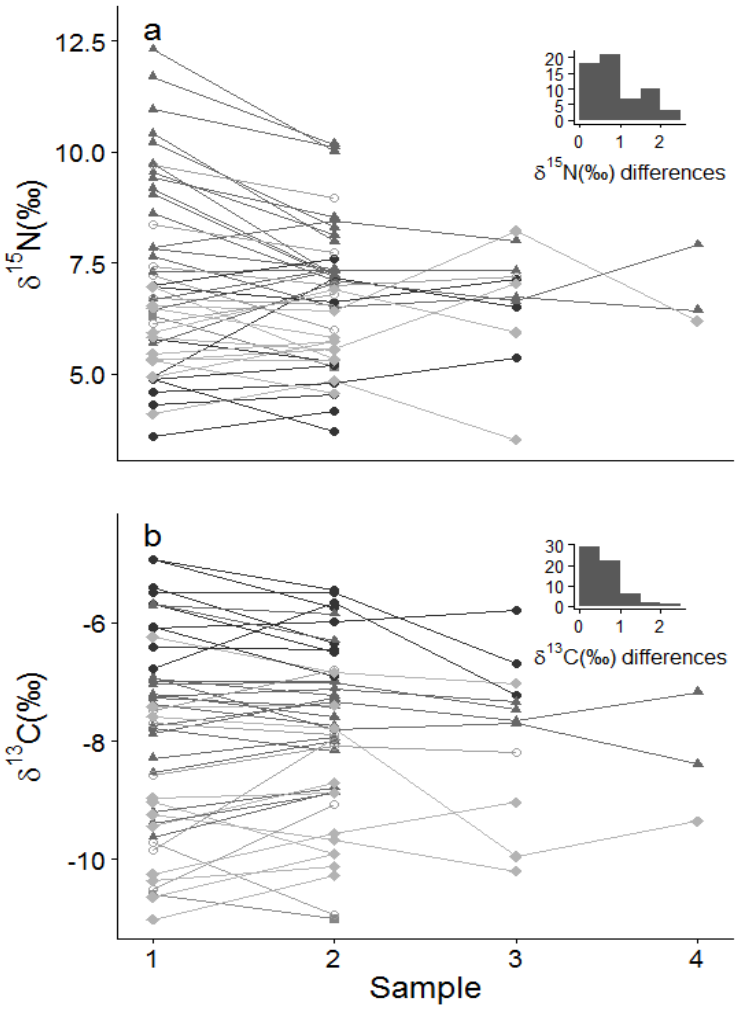

Figure 5 Temporal consistency of isotopic values for serially collected tissue samples of green turtles over successive breeding seasons (n = 45) for a = $\delta^{15}N$ and b = $\delta^{13}C$ values. Subplots represent within individual absolute differences among serially collected samples using the first sample as a reference. Legend: closed circles = Bomba, triangles = Egypt, squares = Turkey-Cyprus, diamonds = West Libya, open circles = unassigned.

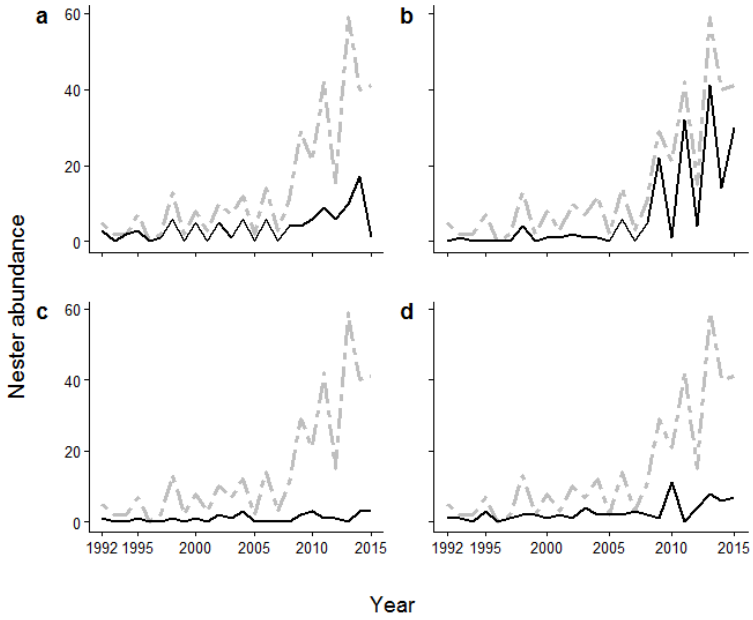

Figure 6 Total nester abundance at Alagadi (1992 – 2015) = grey broken line with foraging area specific contributions to the breeding cohort from a = Bomba, b = Egypt, c = Turkey-Cyprus and d = West Libya.

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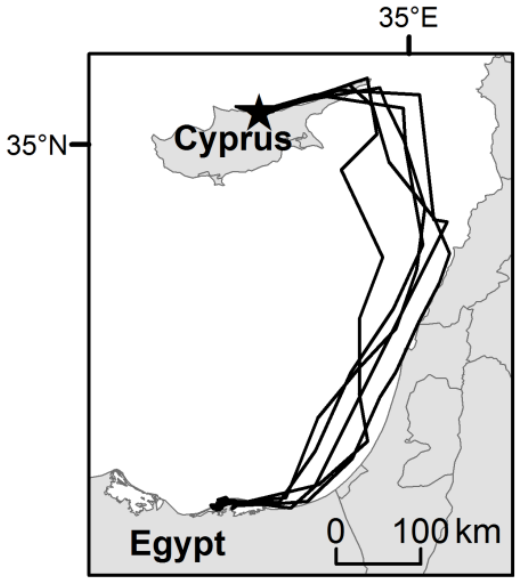
Chapter 2 Supplementary information

S1 data from 28 green turtles satellite tracked from northern Cyprus.

ID	PTT	Year	Days	Foraging area	Calibration	Sample 1	Sample 2	Sample 3	Sample 4	Reference
G077	4150	1998	294	Libya (Bomba)	✓	2006*	2011*	2014		1,2,3
G078	4149	1998	222	Libya (Bomba)	✓	2006*	2014			1,2,3
G082	4148	1998	289	Turkey	✗					1,2,3
G125	6598	1999	243	West Libya (Sirte)	✗					1,2,3
G059	4405	2002	403	Turkey	✓	2012				2,3
G055	36638	2003	364	West Libya (Sirte)	✓	2006	2010*	2015		2,3
G008	36639	2004	395	West Libya (Sirte)	✓	2012				2,3
G076	49815	2004	61	Turkey	✗					2,3
G002	49816	2004	357	Libya (Bomba)	✓	2010	2014*			2,3
G044	49813	2004	311	West Libya (Sirte)	(C&N only)	2007*	2010*	2013*		2,3
Randall	95099	2009	81	Egypt (Bardawil)	✗					4
G015	95097	2009	486	West Libya (Sirte)	✓	2009	2013			3
G157	95101	2009	715	Libya (Bomba)	✓	2009				3
G166	95098	2009	116	Egypt (Gulf of Arab)	✓	2006				3
G189	95102	2009	110	Egypt Bardawil)	✓	2009	2011			3
G058	52820	2010	751	Libya (Bomba)	✓	2006*	2010	2014		3
G009	86898	2010	475	Libya (Bomba)	✓	2010*	2014			3
G163	52846	2010	348	West Libya (Tus)	✓	2005*	2007	2010*	2013	3
G080	52827	2010	407	West Libya (Tus)	✓	2010	2013*			3
G087	52949	2010	478	Libya (Bomba)	✓	2006*	2010*	2014		3
G006	86900	2010	412	S. Cyprus	✓	2010*	2015			3
G172	52888	2010	122	West Libya	✓	2007*	2010	2013		3
Pepsi K	52818	2011	134	S. Cyprus	✓	2011				NA
G217	150429	2015	146	Egypt (Bardawil)	✓	2009*	2013	2015		NA
G252	150427	2015	68	Egypt (Bardawil)	✓	2011	2015			NA
G020	150430	2015	73	Egypt (Bardawil)	✓	2009	2011	2013	2015	NA
G201	150431	2015	58	Egypt (Bardawil)	✓	2008*	2011	2013*	2015	NA
G254	150428	2015	58	Egypt (Bardawil)	✓	2011	2015			NA

ID = turtle identification (bold = male), PTT = platform terminal transmitter, Days = number of tracked days, Foraging area = conclusive end point where turtle was deemed resident, Calibration = calibrated the discriminant analysis, Samples 1 - 4 = year tissue sample collected for SIA, underlined = sample that calibrated the discriminant analysis, * = only analysed for $\delta^{13}C$ & $\delta^{15}N$. Reference, 1 = Godley et al. (2002), 2 = Broderick et al. (2007), 3 = Stokes et al. (2015), 4 = Wright et al. (2012) and NA = unpublished data.

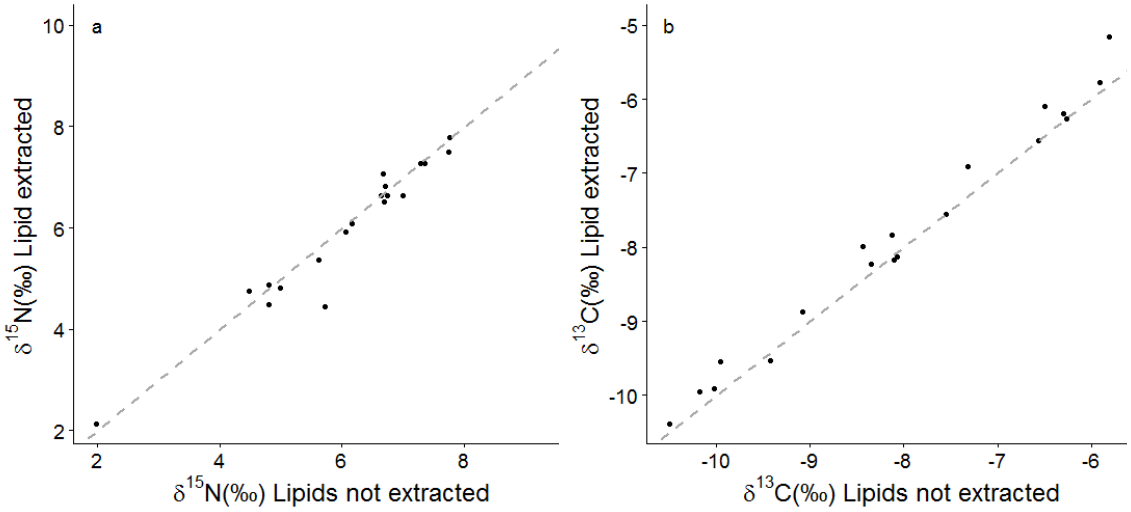


S1a Post nesting green turtle satellite tracks recorded in 2015 from Cyprus to Lake Bardawil, Egypt. These five turtles were specifically selected for PTT attachment based on their δ13C and δ15N values.

S2 Lipid extraction

Samples from 20 green turtles that nested among years (2009 – 2014) were used to determine whether lipid extraction was necessary by subdividing the sample so that half were not lipid-extracted whilst the other half were lipid-extracted using a 2:1 chloroform:methanol ratio in a Soxlet apparatus and heated for one hour.

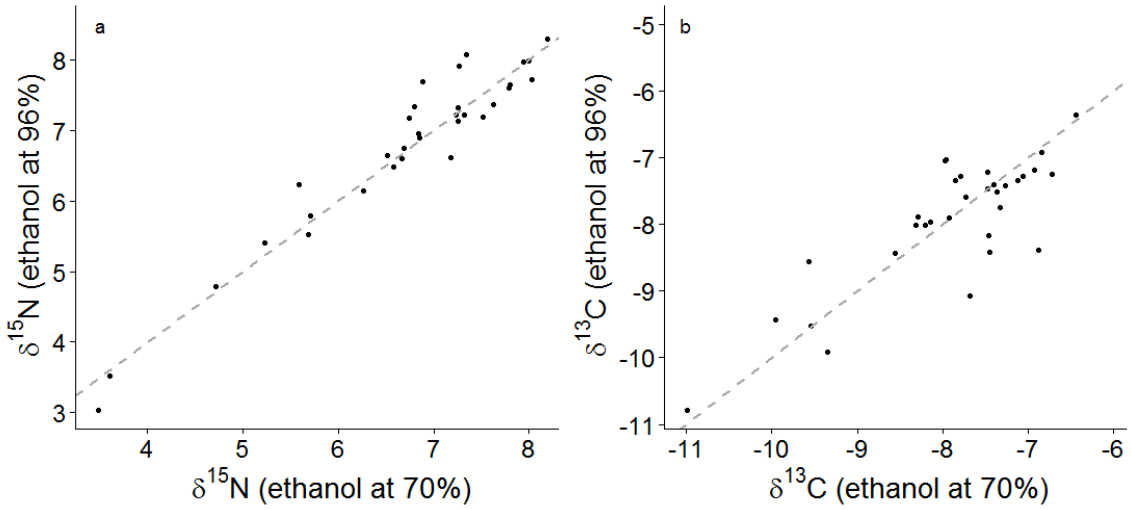
The selected samples had a pre-extraction C:N ratio of 2.68 (±SD = 0.06). No significant differences were found between untreated and lipid-extracted tissue samples for $\delta^{15}N$ (paired t-test, $t_{19} = 1.70$, $p = 0.11$, S2a). Statistically significant differences were found for $\delta^{13}C$ (paired t-test, $t_{19} = -4.0$, $p < 0.001$, S2b) with a mean difference of -0.18 (range =-0.27 – 0.09). However, the differences in $\delta^{13}C$ values between lipid extracted and untreated samples were not substantially different considering the mean difference in $\delta^{13}C$ among foraging areas (1.68‰) and lipid extraction was not considered necessary for the whole dataset.



S2 Differences in stable isotope values between untreated and lipid extracted paired samples for a = $\delta^{15}N$ and b = $\delta^{13}C$ (n = 20). Grey dashed line = no difference (y = x).

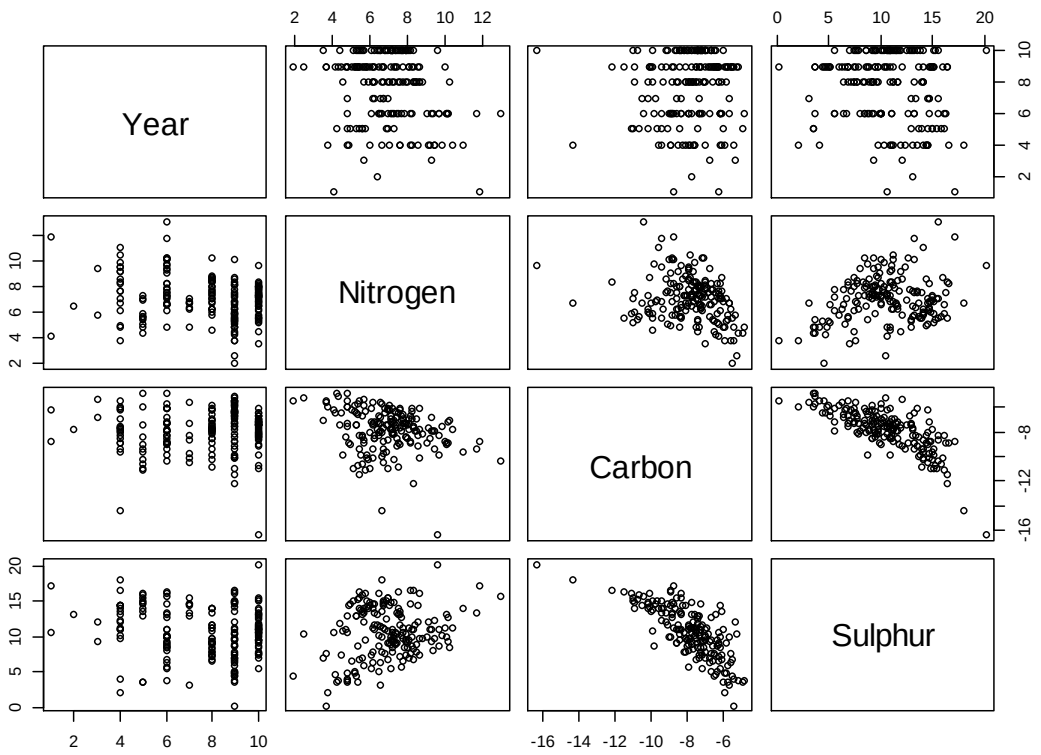
S3 Storage concentration of ethanol

Paired epidermal tissue samples were collected simultaneously from 33 nesting females post-oviposition and stored in a 96% and 70% ethanol concentration for up to five months. The concentration of ethanol had no significant effect on $\delta^{15}N$ (paired t-test, t_{32} = 0.673, p = 0.506, S3a) or $\delta^{13}C$ values (paired t-test, t_{32} = -0.129, p = 0.8981, S3b) as no consistent enrichment or depletion of $\delta^{15}N$ or $\delta^{13}C$ values was found among samples.



S3 Comparison of stable isotope values for paired green turtle epidermis samples (n = 33) stored in either 70% or 96% ethanol concentration for a = $\delta^{15}N$ values, b = $\delta^{13}C$ values. Grey dashed line = no difference (y = x).

S4 Isotopic composition of the study population



S4 Pairwise collinearity plot for the year the sample was collected and the $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values of the turtle epidermis for individuals included within the second discriminant analysis conducted after the 2015 satellite tracking (n = 188). All pairwise comparisons for isotopic values were found to be significantly correlated (Pearson's product moment correlation coefficient, $p < 0.001$ in all cases $\delta^{13}C$ & $\delta^{15}N$, r = -0.26; $\delta^{15}N$ & $\delta^{34}S$, r = 0.23; $\delta^{13}C$ & $\delta^{34}S$, r = -0.75).

S5 Predicting foraging area

This study adopted standard methods (Pajuelo et al. 2012, Ceriani et al. 2012, Vander Zanden et al. 2015) to predict foraging areas using a discriminant function analysis evaluated by the leave-one-out cross validation method. Discrete differences in the combined isotopic values were assessed with a multivariate analysis of variance (MANOVA) with multiple pairwise comparisons conducted with Tukey's Honest Significant Difference to identify significant differences among foraging areas. Non-uniform priors were used based on the number of turtles tracked to each foraging area as they can improve the accuracy of assignment (Royle & Rubenstein 2004, Vander Zanden et al. 2015). We set a posterior probability of assignment at 80% or greater to

maintain consistency among studies (Pajuelo et al. 2012, Seminoff et al. 2012, Vander Zanden et al. 2015) as this provides a 8 - 12 fold improvement in assignment over random odds considering three or four foraging areas, respectively (Wunder 2012, Vander Zanden et al. 2015).

S6 Preliminary discriminant analysis to identify the origin of turtles from the foraging area not characterised through previous satellite tracking (1998 – 2011)

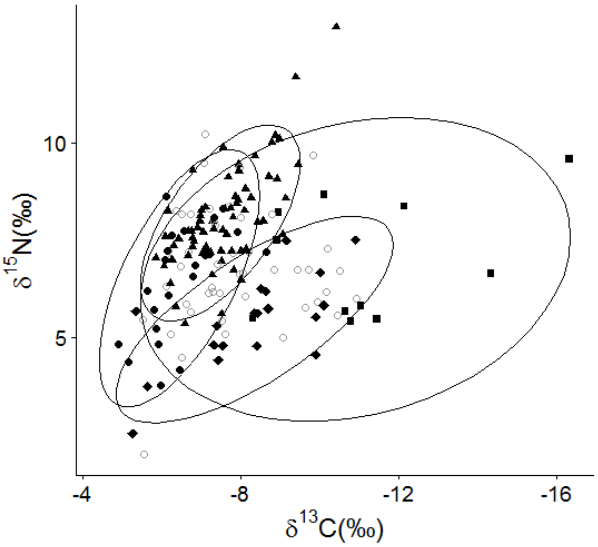
Stable isotope analyses conducted prior to satellite tracking can identify isotopic clusters to target foraging areas with specific isotopic profiles. When clusters are not evident, then groups of isotopic signatures can be selected to characterise the isotopic composition of the population. These groups can be used as pseudo-satellite tracked animals to calibrate a discriminant function analysis and obtain prior prediction probabilities for animals foraging in an area characterised by specific isotopic values.

For this study, we identified an area of isospace encompassing a large proportion of isotopic signatures which were not characterised by the pre-defined and calibrated foraging areas. To identify the origin of these isotopic values we selected three turtles which had temporal consistency in isotopic values over two breeding seasons and defined this region as the 'unidentified' foraging area. These turtles were used in addition to the 19 satellite tracked turtles to calibrate a discriminant analysis using $\delta^{15}N$ or $\delta^{13}C$ values. We predicted the putative foraging area for 186 turtles using this technique and produced a list of 48 turtles which were likely to forage at a greater than 80% probability in the 'unidentified' foraging area.

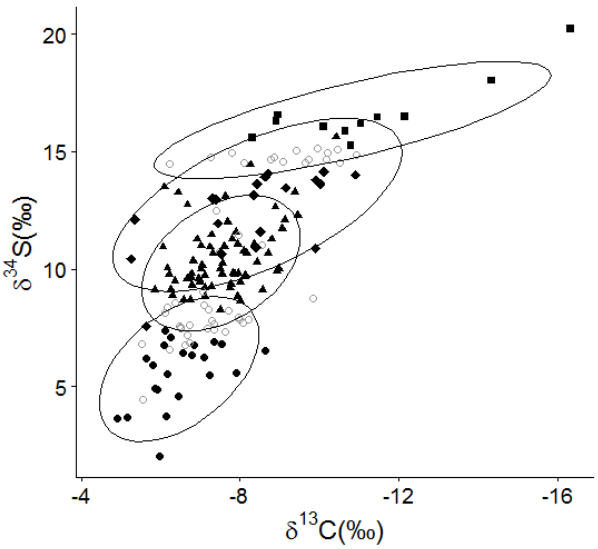
S7 Plots for final predictions of where turtles forage

The most discriminating isotopic criterion for turtles among foraging areas was visualised in a bivariate plot incorporating $\delta^{34}S$ and $\delta^{15}N$ (see Chapter 2 Figure 4). Here we present alternative plots incorporating the isotopic combination of $\delta^{13}C$ and $\delta^{15}N$

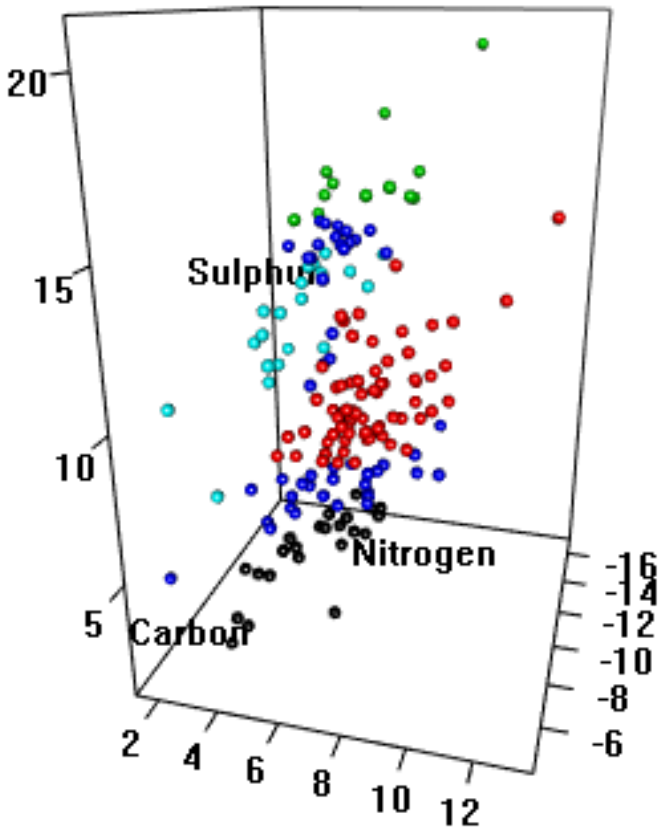
(S7a), $\delta^{13}C$ and $\delta^{34}S$ (S7b) and the full isotopic composition of the turtles ($\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$) predicted to forage in each area (S7c).



S7a $\delta^{13}C$ and $\delta^{15}N$ values for green turtles predicted to forage in: closed circles = Bomba (n = 22), triangles = Egypt (n = 65), diamonds = West Libya (n = 19), squares = Turkey-Cyprus (n = 11), open circles = unassigned (n = 48). Ellipses set at 95% CI, (total n = 165).



S7b $\delta^{13}C$ and $\delta^{34}S$ for green turtles predicted to forage in: closed circles = Bomba (n = 22), triangles = Egypt (n = 65), diamonds = West Libya (n = 19), squares = Turkey-Cyprus (n = 11), open circles = unassigned (n = 48). Ellipses set at 95% CI, (total n = 165).



S7c $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$for green turtles predicted to forage in: black circles = Bomba (n = 22), red circles = Egypt (n = 65), cyan circles = West Libya (n = 19), green circles = Turkey-Cyprus (n = 11) and blue circles = unassigned (n = 48), (total n = 165).

S8 Analysing foraging site fidelity

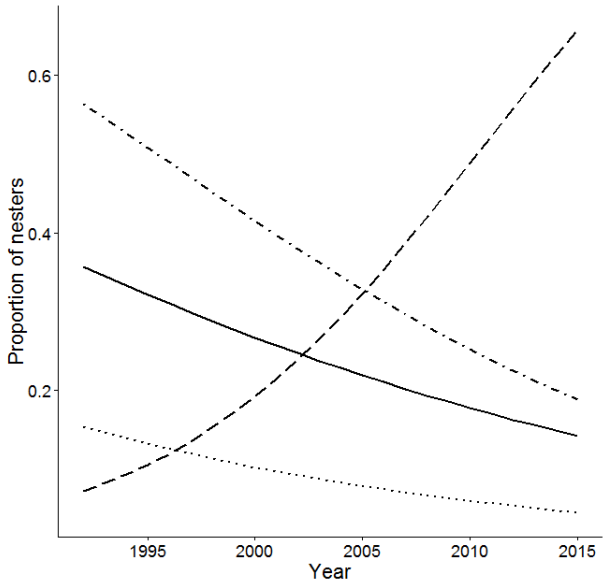
Foraging site fidelity was evaluated using a repeatability analysis employed in the R statistical package 'rptR' (Nakagawa & Schielzeth 2010) using a linear mixed-effects model based estimation for Gaussian data fitted with restricted maximum likelihood (REML). The identity of the turtle was the grouping factor and we controlled for the variance attributed to where a turtle forages by including this as a covariate, although six of the 45 turtles were of unknown foraging area. Confidence intervals (CI) were set at 95% and calculated through 1000 bootstrap statistics with asymptotic p-values calculated by 1000 permutations.

The differences in $\delta^{13}C$ and $\delta^{15}N$ values among serially collected samples was calculated using the first sample as a reference. The mean difference in $\delta^{15}N$ = 0.91 ‰ (upper & lower quantiles = 0.34 – 1.36 ‰, range = 0.02 – 2.50 ‰) and $\delta^{13}C$ = 0.61 ‰

(upper & lower quantiles = 0.27 – 0.81 ‰, range = 0.00 – 2.36 ‰, Figure 5 in main text)

S9 Evaluating foraging area specific contributions to the breeding cohort

We employed linear and non-linear mixed effects modelling to evaluate foraging area specific contributions to the breeding cohort. We evaluated autocorrelation through generalised least squares estimation models (GLS) within the R statistical package nlme (Pinheiro et al. 2016) as a general bi-annual pattern in foraging area contributions was observed. However, only the GLS model for turtles foraging in Egypt was significantly more accurate when accounting for autocorrelation based on AICc model selection (R statistical package MuMin for multi-model selection based on information criteria). Therefore, we did not account for autocorrelation within the full model incorporating all foraging areas. We employed a general linear model with a quasibinomial error structure to determine if the proportion of nesters from each foraging area to the breeding cohort significantly differed among years. The model was fitted with a proportional dependent variable based on the number of nesters from each site (number of nesters from x / total number of nesters – number of nesters from x) with year (also fitted as a quadratic variable) and foraging area as interacting fixed effects. A Tukey test of Honest Significant Differences (HSD) revealed that three out of six pairwise comparisons were significantly different (Figure S9) with Egypt exhibiting a strong positive trend in the proportion of nesters contributed to the rookery whereas the other three sites showed a negative trend.



S9 Prediction from the GLM for the proportion of the nesting cohort contributed from each foraging area from 1992 – 2015. Dot-dash line = Bomba, dashed line = Egypt, dotted line = Turkey-Cyprus and solid line = West Libya.

S10 detecting temporal trends in stable isotope ratios

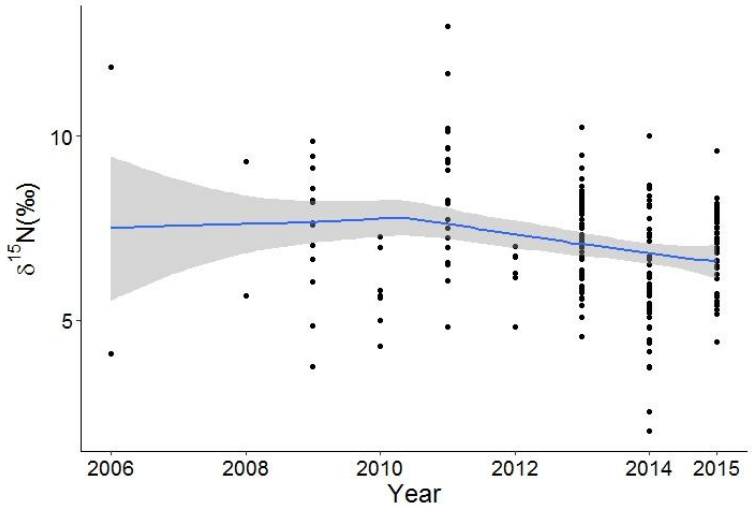
Temporal trends in the stable isotope ratios of nitrogen, carbon and sulphur were evaluated using general linear models to detect significant among year variation for all turtles employed in the post 2015 analysis to predict where turtles foraged.

This revealed a negative trend in $\delta^{15}N$ values over successive years (GLM, $F_{1,188} = 8.22$, $p = 0.003$) with a linear decrease in $\delta^{15}N$ of -0.17 ‰ per year, although the standard errors for samples collected prior to 2009 are substantially larger than those for samples collected more recently (S10a). Furthermore, the inter-annual variability in $\delta^{15}N$ was highly significant (GLM, $F_{1,181} = 6.681$, $p < 0.001$) with 7 out of 36 pairwise comparisons being significantly different, although these did not suggest any long-term trends (Table S10a).

In contrast, no significant trend was detected for $\delta^{13}C$ values over successive years (GLM, $F_{1,188} = 0.512$, $p = 0.471$, Figure S10b) although low but significant inter-annual variability was found (GLM, $F_{1,181} = 2.09$, $p = 0.039$) even though no pairwise comparisons were significantly different (Tukey HSD >0.05 in all cases, Table S10b).

A significant trend in $\delta^{34}S$ was detected (GLM, $F_{1,188} = 4.446$, $p = 0.036$) with a linear decrease in $\delta^{34}S$ values of -0.27 ‰ per year, although this trend was not significant after omitting the samples collected in 2006 (GLM, $F_{1,186} = 2.952$, $p = 0.087$, Figure S10c). Evidence of significant inter-annual variability was also found (GLM, $F_{1,181} = 3.992$, $p < 0.001$) with 4 of 36 pairwise comparisons being significantly different (Table S10c).

These results suggest that stable isotope analysis should be calibrated with additional satellite tracking to cover the timeframe of the study in order to monitor the baseline isotopic values and ensure the continued accuracy of foraging area predictions.

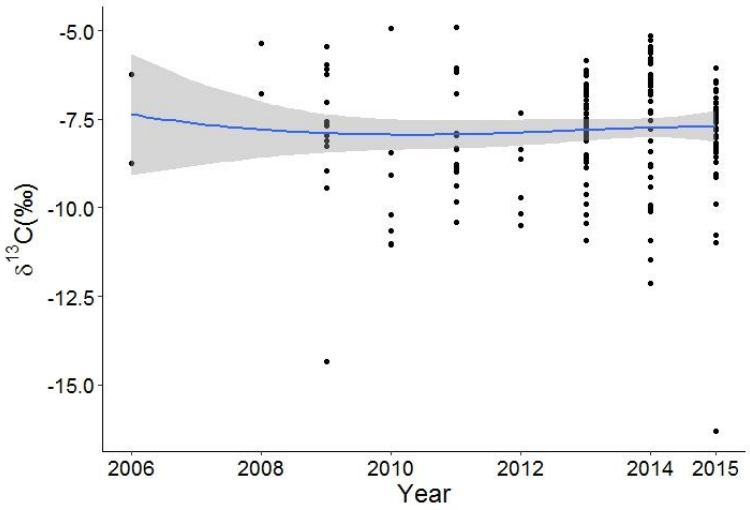


S10a temporal consistency in the $\delta^{15}N$ values of green turtles sampled among foraging habitats

Table S10a Tukey test of honest significant differences (HSD) for $\delta^{15}N$ values of green turtles sampled among years and foraging habitats

Year comparison	Estimate	Std.Error	z value	Pr(>|z|)	
2008 - 2006	-0.4878	1.484	-0.329	0.99999	
2009 - 2006	-0.3726	1.1218	-0.332	0.99999	
2010 - 2006	-2.1762	1.1898	-1.829	0.61526	
2011 - 2006	0.6216	1.1006	0.565	0.99966	
2012 - 2006	-1.6964	1.2117	-1.4	0.87514	
2013 - 2006	-0.6744	1.0686	-0.631	0.99923	
2014 - 2006	-1.9541	1.0735	-1.82	0.6211	
2015 - 2006	-0.9953	1.074	-0.927	0.9888	
2009 - 2008	0.1152	1.1218	0.103	1	
2010 - 2008	-1.6883	1.1898	-1.419	0.86665	
2011 - 2008	1.1094	1.1006	1.008	0.98079	
2012 - 2008	-1.2086	1.2117	-0.997	0.98201	
2013 - 2008	-0.1865	1.0686	-0.175	1	
2014 - 2008	-1.4662	1.0735	-1.366	0.88995	
2015 - 2008	-0.5075	1.074	-0.472	0.99991	
2010 - 2009	-1.8036	0.687	-2.625	0.14579	
2011 - 2009	0.9942	0.5171	1.922	0.54763	
2012 - 2009	-1.3238	0.7241	-1.828	0.61569	
2013 - 2009	-0.3018	0.4451	-0.678	0.99871	
2014 - 2009	-1.5815	0.4566	-3.463	0.01215	*
2015 - 2009	-0.6227	0.458	-1.36	0.89237	
2011 - 2010	2.7977	0.6517	4.293	<0.001	***
2012 - 2010	0.4797	0.8256	0.581	0.99958	
2013 - 2010	1.5018	0.5961	2.519	0.18662	
2014 - 2010	0.2221	0.6048	0.367	0.99999	
2015 - 2010	1.1809	0.6058	1.949	0.52877	
2012 - 2011	-2.318	0.6908	-3.356	0.01773	*
2013 - 2011	-1.2959	0.3885	-3.336	0.01878	*
2014 - 2011	-2.5756	0.4017	-6.413	<0.001	***
2015 - 2011	-1.6169	0.4032	-4.01	0.00152	**
2013 - 2012	1.0221	0.6386	1.6	0.76888	
2014 - 2012	-0.2576	0.6467	-0.398	0.99998	
2015 - 2013	0.7011	0.6477	1.083	0.9701	
2014 - 2013	-1.2797	0.3033	-4.219	<0.001	***
2015 - 2013	-0.3209	0.3053	-1.051	0.97503	
2015 - 2014	0.9588	0.3219	2.978	0.05747	

Significance codes: * < 0.05, ** < 0.01, *** < 0.001

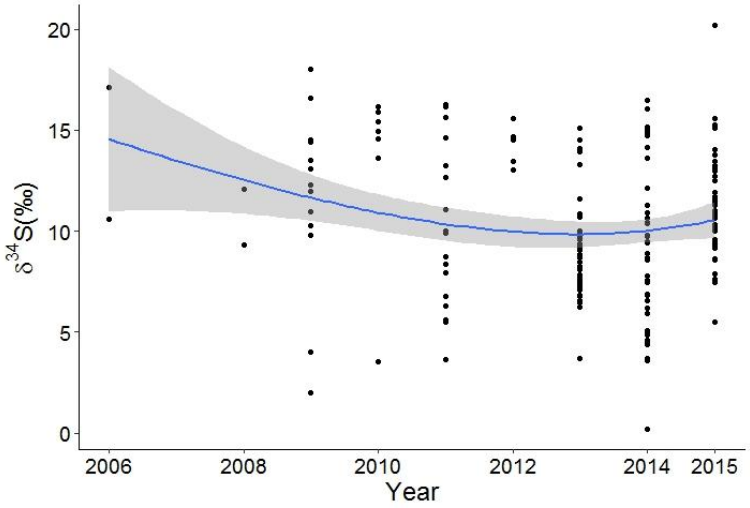


S10b temporal consistency in the $\delta^{13}C$ values of green turtles sampled among foraging habitats

Table S10b Tukey HSD for $\delta^{13}C$ values of green turtles sampled among years and foraging habitats

Year comparison	Estimate	Std.Error	z value	Pr(>|z|)
2008 - 2006	1.422024	1.610251	0.883	0.9919
2009 - 2006	-0.41833	1.217235	-0.344	1
2010 - 2006	-1.84475	1.291073	-1.429	0.8622
2011 - 2006	-0.49434	1.194194	-0.414	1
2012 - 2006	-1.62429	1.314764	-1.235	0.9354
2013 - 2006	-0.14841	1.159513	-0.128	1
2014 - 2006	0.079996	1.164798	0.069	1
2015 - 2006	-0.50328	1.165414	-0.432	1
2009 - 2008	-1.84035	1.217235	-1.512	0.82
2010 - 2008	-3.26678	1.291073	-2.53	0.1826
2011 - 2008	-1.91637	1.194194	-1.605	0.766
2012 - 2008	-3.04632	1.314764	-2.317	0.2864
2013 - 2008	-1.57044	1.159513	-1.354	0.8946
2014 - 2008	-1.34203	1.164798	-1.152	0.9567
2015 - 2008	-1.92531	1.165414	-1.652	0.7365
2010 - 2009	-1.42643	0.745401	-1.914	0.554
2011 - 2009	-0.07601	0.561118	-0.135	1
2012 - 2009	-1.20596	0.785722	-1.535	0.8073
2013 - 2009	0.269917	0.482933	0.559	0.9997
2014 - 2009	0.498324	0.495488	1.006	0.981
2015 - 2009	-0.08495	0.496934	-0.171	1
2011 - 2010	1.350413	0.70715	1.91	0.5572
2012 - 2010	0.220462	0.895861	0.246	1
2013 - 2010	1.696343	0.646863	2.622	0.147
2014 - 2010	1.92475	0.656289	2.933	0.0651
2015 - 2010	1.341472	0.657382	2.041	0.4631
2012 - 2011	-1.12995	0.749531	-1.508	0.8223
2013 - 2011	0.34593	0.4215	0.821	0.995
2014 - 2011	0.574337	0.435827	1.318	0.9086
2015 - 2011	-0.00894	0.437471	-0.02	1
2013 - 2012	1.475881	0.692942	2.13	0.4019
2014 - 2012	1.704288	0.701749	2.429	0.2283
2015 - 2013	1.121009	0.702771	1.595	0.7722
2014 - 2013	0.228407	0.329115	0.694	0.9985
2015 - 2013	-0.35487	0.331289	-1.071	0.9719
2015 - 2014	-0.58328	0.349337	-1.67	0.725

Significance codes: * < 0.05, ** < 0.01, *** < 0.001



S10c temporal consistency in the $\delta^{34}S$ values of green turtles sampled among foraging habitats

Table S10c Tukey HSD for $\delta^{34}S$ values of green turtles sampled among years and foraging habitats

Year comparison	Estimate	Std.Error	z value	Pr(>|z|)	
2008 - 2006	-3.14	3.331864	-0.942	0.9875	
2009 - 2006	-1.98929	2.518652	-0.79	0.9962	
2010 - 2006	-0.39143	2.671434	-0.147	1	
2011 - 2006	-3.4985	2.470976	-1.416	0.8682	
2012 - 2006	0.471667	2.720455	0.173	1	
2013 - 2006	-4.54065	2.399216	-1.893	0.5698	
2014 - 2006	-4.55047	2.410151	-1.888	0.5727	
2015 - 2006	-2.655	2.411426	-1.101	0.9669	
2009 - 2008	1.150714	2.518652	0.457	0.9999	
2010 - 2008	2.748571	2.671434	1.029	0.9781	
2011 - 2008	-0.3585	2.470976	-0.145	1	
2012 - 2008	3.611667	2.720455	1.328	0.9048	
2013 - 2008	-1.40065	2.399216	-0.584	0.9996	
2014 - 2008	-1.41047	2.410151	-0.585	0.9996	
2015 - 2008	0.485	2.411426	0.201	1	
2010 - 2009	1.597857	1.542353	1.036	0.9772	
2011 - 2009	-1.50921	1.161041	-1.3	0.9149	
2012 - 2009	2.460952	1.625783	1.514	0.8191	
2013 - 2009	-2.55136	0.999265	-2.553	0.1731	
2014 - 2009	-2.56118	1.025242	-2.498	0.1965	
2015 - 2009	-0.66571	1.028235	-0.647	0.9991	
2011 - 2010	-3.10707	1.463205	-2.123	0.4068	
2012 - 2010	0.863095	1.853678	0.466	0.9999	
2013 - 2010	-4.14922	1.338463	-3.1	0.0403	*
2014 - 2010	-4.15904	1.357966	-3.063	0.0445	*
2015 - 2010	-2.26357	1.360228	-1.664	0.7287	
2012 - 2011	3.970167	1.550898	2.56	0.1703	
2013 - 2011	-1.04215	0.87215	-1.195	0.9465	
2014 - 2011	-1.05197	0.901796	-1.167	0.9534	
2015 - 2011	0.8435	0.905198	0.932	0.9884	
2013 - 2012	-5.01232	1.433806	-3.496	0.0109	*
2014 - 2012	-5.02213	1.452029	-3.459	0.0125	*
2015 - 2013	-3.12667	1.454145	-2.15	0.3888	
2014 - 2013	-0.00982	0.680992	-0.014	1	
2015 - 2013	1.885648	0.68549	2.751	0.1068	
2015 - 2014	1.895465	0.722833	2.622	0.1471	

Significance codes: * < 0.05, ** < 0.01, *** < 0.001

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Chapter 3

Carry-over effects influence the reproductive potential of green turtles

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Abstract

Understanding foraging site selection and the resultant ecological consequences are of fundamental interest. Foraging sites are likely to vary in resources and environmental conditions, variation that may have profound implications for an individual's reproductive success. Here, we investigate foraging site selection and evaluate morphological and reproductive traits of green turtles (*Chelonia mydas*) from a nesting aggregation in Cyprus that disperse among four geographically distinct feeding sites. Possible population sub-structuring of the nesting aggregation by foraging area was evaluated by employing a mtDNA control region sequence, four mtSTRs and 13 microsatellite markers. No evidence for genetic clustering was found and therefore foraging area location is most likely learnt from previous life experience. Foraging area effects were evaluated across five morphological and reproductive traits with significant among site differences revealed for the body size of recruits, interbreeding intervals and the date of the first nest of the season. Foraging area did not significantly affect the size or number of clutches laid. Substantial regional differences in the interbreeding interval were attributed to resource related carry-over effects from foraging in areas that differ in their ecological and environmental conditions. This resulted in a considerable difference in life-time reproductive potential with individuals foraging in Turkey and Cyprus estimated to lay half as many eggs over a 20 year period as those from Egypt and approximately 40% fewer than those feeding in Libya. This work emphasises the utility of long-term monitoring at the individual-level and demonstrates the possibility of foraging site condition being inferred without conducting site-based surveys.

Introduction

Alternative life-histories often exist within breeding populations and across a species range (Bolnick et al. 2003). These can include key life-history trade-offs (Stearns 1992) with alternative strategies linked to habitat heterogeneity as individuals adapt to their environment (Suryan et al. 2009). Resource availability and environmental factors can have important implications for the reproductive potential of individuals through carry-over effects (Harrison et al. 2011) as healthier and more fecund individuals are associated with richer foraging sites (Studds & Marra 2005). Here we define a carry-over effect as per O'Connor et al. (2014) as "any situation in which an individual's previous history and experience explains their current performance in a given situation". Therefore, carry-over effects can stem from previous life decisions, such as foraging site selection, that influence reproductive success within and among seasons (Norris 2005, Inger et al. 2010) resulting in substantial variation in fecundity for species that exhibit high fidelity to those sites.

Green turtles (*Chelonia mydas*) are long-lived, slow to mature iteroparous marine reptiles (Miller 1997) that exhibit natal site philopatry (Meylan et al. 1990). Offspring leave the natal beach shortly after hatching and spend several years drifting in the open-ocean feeding on planktonic prey before recruiting to neritic foraging areas (Reich et al. 2007). Water circulation patterns are widely considered to be the primary driver for the broad scale dispersion of pelagic-stage juveniles and thus determining the habitats that they encounter (Carreras et al. 2006, Hays et al. 2010, Putman & Naro-maciel 2013, Scott et al. 2014), however population substructuring has rarely been evaluated to determine if close relatives aggregate within feeding sites (although see Watanabe et al. 2011). The shift from the pelagic to neritic environment may be facultative (McClellan & Read 2007, Williard et al. 2017) and juveniles may undergo several ontogenetic shifts among nearshore foraging areas before maturity (Bowen et al. 2005, Hamabata et al. 2015). Habitat shifts may be related to an increase in dietary specialism (Cardona et al. 2009, 2010) with mature individuals eventually becoming established in the most optimal foraging habitats encountered thus far (Gaspar et al. 2012, Scott et al. 2014). Therefore mature individuals from a breeding population can become aggregated within a number of geographically discrete foraging areas where they exhibit high fidelity (Chapter 2,

Broderick et al. 2007, Shimada et al. 2016). Mature green turtles are primary consumers feeding on seagrass and macroalgae although some level of omnivory is retained throughout their life (Godley et al. 1998, Cardona et al. 2010).

The study of carry-over effects in marine turtles has expanded rapidly as stable isotope analysis, calibrated by satellite tracking, has provided a technique to predict the foraging area location for large numbers of individuals within a randomly breeding population (Hobson et al. 2010, Chapter 2). Carry-over effects are likely to influence the breeding frequency and fecundity of marine turtles as they are capital breeders (Stearns 1992) and thus require a sufficient energy store prior to leaving the foraging area in order to reproduce (Hamann et al. 2003). Foraging area effects were found to influence the size morphology and reproductive success within breeding populations of loggerhead (*Caretta caretta)* (e.g. Zbinden et al. 2011, Hatase et al. 2013, Vander Zanden et al. 2014, Ceriani et al. 2015) and leatherback (*Dermochelys* coriacea) (Wallace et al. 2006) turtles with variability assumed to result from differences in resource availability. The effect of foraging area location on the morphology and reproductive traits of green turtles has not been widely studied but their reproductive potential may be more closely linked to ecological and environmental conditions compared to that of other turtles as they feed at a lower trophic position (Godley et al. 1998). However, no morphological differences were detected among mature green turtles exhibiting either a pelagic or neritic foraging strategy (Hatase et al. 2006) which is in stark contrast to the morphological differences among loggerheads exhibiting a similar foraging dichotomy (Hatase et al. 2002, Eder et al. 2012).

Here, we investigate foraging area selection and the effect of differential feeding on a nesting aggregation of green turtles located at Alagadi on the Mediterranean island of Cyprus. The Alagadi turtle rookery is currently thought to be in recovery as a large number of neophytes (new breeders) is driving a positive trend in nester abundance (Stokes et al. 2014). It is unclear what is driving the recovery but conservation activities that commenced in 1992 to reduce terrestrial predation is likely to be the root cause (Stokes et al. 2014). But a recent study (Chapter 2) highlighted that the recovery was linked to a high level of recruitment from a single foraging area in Egypt where few turtles were previously thought to forage (Stokes et al. 2015). Therefore we aim to

evaluate foraging area selection by investigating the population substructuring of the Alagadi nesting aggregation among the four foraging areas to determine if close relatives aggregate within sites and to disentangle genetic similarities from phenotypic responses to foraging area effects. Foraging area effects will then be determined for five morphological and reproductive traits.

Methods

Study site and data collection

Green turtles (*Chelonia mydas*) nesting at Alagadi beach, Cyprus (35˚19'56.17"N; 33˚28'57.59"E) were monitored from 1992-2015. Data collection and tissue sampling were conducted through night time patrols undertaken at a sufficient frequency throughout the breeding season (end of May to mid-August) to maintain a 99% encounter rate for all nesting green turtles (Stokes et al. 2014). Females were individually marked using external flipper tags and internal Passive Integrated Transponder (PIT) tags enabling an individually-based data set to be compiled over multiple seasons (Broderick et al. 2002). Post tagging, a skin sample was taken from the fore flipper of the turtle and morphometric data were collected. All tissue samples were stored in a 96% ethanol solution until laboratory preparation. Data on clutch size were collected during morning patrols when nests were excavated approximately two days after hatching (Broderick et al. 2003).

Determining foraging area

The foraging site location was determined for 139 female green turtles through stable isotope analysis and satellite telemetry. This includes 25 turtles satellite tracked between 1998 and 2015 (Godley et al. 2002, Broderick et al. 2007, Stokes et al. 2015, Chapter 2) and 114 turtles whose foraging area was inferred through their stable isotope ratios of $\delta^{13}C$ (carbon), $\delta^{15}N$ (nitrogen) and $\delta^{34}S$ (sulphur) (Chapter 2). From these combined sources, we deduced that 14 turtles foraged in Turkey-Cyprus, 25 in West Libya, 29 in Bomba and 71 in Egypt (Figure 1).

Genetic markers

To evaluate the genetic structure of the Alagadi turtles which use the different foraging areas, we employed three genetic markers that effectively defined genetic structure among four rookeries in northern Cyprus, including the Alagadi nesting population (Chapter 1). All individuals were genetically characterised using the high resolution (HR) haplotyping system described in this study; thus including an ~800bp sequence from the 5' end of the mtDNA control region (Abreu-Grobois et al. 2006) concatenated with a series of short tandem repeats at the 3' end of the mtDNA control region (mtSTRs) (Tikochinski et al. 2012), and 13 highly polymorphic microsatellite loci. All marker checks and evaluations of genetic structure using Wright's F_{ST} (1951) were conducted as in Chapter 1 or refer to Supplementary information S1 – S2. All P-values from multiple tests were corrected using the false discovery rate (FDR) (Narum 2006).

Analysis of reproductive parameters

Foraging area effects for interbreeding intervals, clutch size, expected clutch frequency and date of the first nest of the season (hereafter termed 'first nest phenology') were evaluated using Generalised Linear Mixed Models (GLMM) using the R statistical package lme4 (Bates et al. 2015). GLMMs allow for the analysis of data that are not normally distributed through the use of link functions and exponential family distributions (Bolker et al. 2009). 'Turtle identity' was included in all GLMMs as a categorical random effect to incorporate repeated measures from the same female within and among seasons with 'Year' included to account for environmental factors correlated within year. Recruitment size was analysed as a General Linear Model (GLM) as all data were independent. The significance of fixed effects for all models were determined through stepwise deletion using likelihood ratio tests with a threshold set at $p = 0.05$. Model residuals were checked for overdispersion, normality and homoscedasticity. Foraging area location was included as a fixed effect within all models and where this was found to account for significant variability we determined the groups responsible by the post-hoc Tukey test of honest significant differences (HSD) using the R statistical package multcomp (Hothorn et al. 2008). All statistical analysis was conducted using R statistical software (R Core Team 2013).

To reduce the chance of a misclassification, females first observed nesting prior to 2000 were classed as 'reproductive status unknown' for their first breeding season for two reasons: (i) external flipper tags were frequently lost and the accuracy of neophyte / remigrant identification was increased through the introduction of PIT tags in 1997 (Stokes et al. 2014), and (ii) to reduce the occurrence of females with long interbreeding intervals being interpreted as neophytes as they last nested prior to the onset of monitoring. Turtles that were first observed nesting from 2000 were classed as 'neophytes' for their first year (Stokes et al. 2014) with all turtles classified as 'remigrants' from their second breeding season. The rationale and model fit for each morphological and reproductive trait is described here and summarised in Table 1:

1. **Recruitment size:** A measure of body size was selected as it is positively correlated with clutch size (Bjorndal & Carr 1989) and therefore larger females have the ability to lay larger clutches of eggs (Broderick et al. 2003). However, we selected to analyse the size at recruitment as green turtles can continue to grow for up to ten years after maturity (Omeyer Unpublished) which would bias results among the foraging areas with the greatest proportion of recent recruits. Females that recruited after 1999 (to ensure their true neophyte status) were measured to the nearest 0.1 cm using the minimum curved carapace length (CCL, notch to notch) during each nesting event and a mean CCL calculated for their first reproductive season. The year that the female recruited was included as a fixed effect to account for any temporal variation in recruitment size.

2. **Interbreeding interval:** Interbreeding intervals refer to the time in between reproductive migrations. These were taken as empirical values due to the high observer effort and frequency of encounter for nesting green turtles on Alagadi (Stokes et al. 2014). Variation in the length of interbreeding intervals is likely to reflect habitat quality, environmental conditions (Broderick et al. 2001, 2003, Solow et al. 2002), physiology (Cardona et al. 2010) and energetics (Hatase & Tsukamoto 2008). Fixed effects included the CCL of the female taken during each nesting event and averaged within year for each reproductive season observed (CCL-female), the expected clutch frequency (ECF, described below) and the total

number of eggs produced for each nesting season prior to the measured interbreeding interval (Prior reproduction).

3. **Clutch size:** Clutch size was determined by counting hatched shells and unhatched eggs during nest excavation as this is highly correlated with the number of eggs at the time of laying (Broderick et al. 2003). The date that the nest was made (DOY) was included as an additional random factor as clutch size is positively correlated with clutch frequency and therefore clutches laid later in the season tend to be larger (Broderick et al. 2003). Clutch size is also positively correlated with the size of the turtle (Broderick et al. 2003) and therefore CCL-female was included as a fixed effect with clutch frequency for that year and the female's reproductive status (neophyte, remigrant or unknown).

4. **Expected clutch frequency (ECF):** The observed number of clutches laid each season was adjusted to account for long intervals between nesting events indicative of females laying clutches on nearby beaches. Therefore, inter-nesting intervals of 20 days or greater resulted in the addition of an extra clutch to the observed clutch frequency for that season (Broderick et al. 2002, Stokes et al. 2014). Fixed effects included CCL-female, first nest phenology as late nesters are likely to lay fewer clutches and whether a female was a recruit or exhibited a two year or greater than two year interbreeding interval prior to the measured reproductive season.

5. **First nest phenology:** Variation in first nest phenology may result from differences in the migratory distance travelled and the thermal regime and other environmental factors experienced at the foraging area (Mazaris et al. 2009). Fixed effects included CCL-female and the known reproductive status as neophyte or remigrant with females of unknown status omitted for that year.

To quantify the foraging area effect on the life-time reproductive potential of a turtle, we estimated the cumulative reproductive output as the total number of eggs produced for a remigrant breeder from each foraging area taking into account the among site differences for each reproductive parameter. Estimates were based on a reproductive life span of 20 years using the following equation:

$$reproductive\ output = \frac{reproductive\ lifespan}{interbreeding\ interval}\ x\ clutch\ size\ x\ clutch\ frequency$$

Values for the interbreeding interval, clutch size and clutch frequency were mean foraging site specific estimates from the GLMM models. We standardised the reproductive output for comparison by dividing the total number of eggs produced by a turtle from each foraging site by the number of eggs produced by a 'typical turtle' where the effect of foraging area was removed from the GLMM models.

Results

Genetic structure

We successfully sequenced 130 individuals for the HR haplotype and genotyped 131 individuals at all 13 microsatellite loci. Haplotype diversity was similar among foraging areas with the greatest haplotype diversity found among the individuals foraging in West Libya and the lowest among those foraging in Bomba (0.73 – 0.885, Table 2 and Supplementary information Table S1). No evidence for deviations from Hardy-Weinberg Equilibrium, linkage disequilibrium or null alleles were found within the microsatellite data. Turkey-Cyprus was found to have the greatest mean number of alleles among loci and all foraging areas except Egypt had a greater than expected level of heterozygosity (Table 2). No significant genetic structure was found for the Alagadi turtles distributed among the foraging areas with either the maternally (HR haplotypes) or biparentally (microsatellites) inherited genetic markers (Table 3).

Reproductive parameters

Foraging area location significantly influenced the size of recruits, interbreeding intervals and the first nest phenology that are summarised here in turn. Table 1 contains a summary of all significant and non-significant terms for each trait with correlations among parameters of reproductive success summarised in Supplementary information S3.

Size at recruitment: CCL for the 116 recruits ranged from 73.5 – 103 cm with a mean ± SD of 85.7 ± 5.33 cm. CCL was significantly influenced by the interaction between foraging area and year (GLM, $F_{3,108} = 6.98$, $p<0.001$, Figure 2) meaning that the average

size of recruits from Bomba and West Libya are getting smaller over successive seasons and there is no change, or a marginal increase in the size of recruits from Turkey-Cyprus and Egypt, respectively. We predicted a general downward shift in the mean CCL of recruits among foraging areas from 89.0 cm (± SE = 1.73) in 2000 to 84.7 cm (± 0.74) in 2015 with a substantial reduction in the size range of recruits.

Interbreeding intervals: Interbreeding intervals ranged from one to ten years with a mean ± SD of 3.42 ± 1.48. Foraging area location accounted for a significant proportion of the observed variability (GLMM, $\chi^2_{(3)} = 19.04$, $p < 0.001$) with turtles from Turkey-Cyprus exhibiting significantly longer interbreeding intervals (5.5 ± 2.68 years) than turtles foraging elsewhere (Bomba = 3.38 ± 1.22, Egypt = 2.92 ± 1.42, West Libya = 3.41 ± 0.73, Tukey HSD, $p < 0.01$ in all cases). All other fixed effects that we tested were not significant but we selected to visualise the significant differences among foraging areas regressed against 'Prior reproduction' as this was only marginally not significant at alpha = 0. 05 ($\chi^2_{(1)} = 3.34$, $p = 0.068$, Figure 3).

First nest phenology: The date that females made their first nest of the season ranged from May 29th until July 30th with a significant foraging area effect (GLMM, $\chi^2_{(3)} = 14.86$, $p < 0.001$, Figure 4) as turtles from West Libya made their first nest approximately ten and eight days later than turtles from Bomba or Egypt, respectively (Tukey HSD, $p < 0001$ in both cases). Although turtles from West Libya also made their first nest on average six days later than turtles from Turkey-Cyprus, this result was not significant (Tukey HSD = 0.25). The reproductive status of the mother was highly significant (GLMM, $\chi^2_{(1)} = 29.22$, $p < 0.001$) with neophytes making their first nest approximately eight days after remigrants irrespective of where they previously foraged. The year was also highly significant (GLMM, $\chi^2_{(1)} = 10.68$, $p < 0.001$) as mean first nest phenology was approximately ten days earlier in 2015 than in 1992.

Cumulative reproductive output: The total number of eggs produced by an average remigrant green turtle over the course of 20 years from each site equated to 1358, 2765, 2183 and 2205, respectively for Turkey-Cyprus, Egypt, Bomba and West Libya. These values were standardised for comparison with the number of eggs produced by a 'typical turtle' (1986) whereby the effect of foraging area location had been removed from the GLMM. This produced a comparative cumulative reproductive output for the number of

eggs of 0.68, 1.39, 1.10 and 1.11, respectively, for Turkey-Cyprus, Egypt, Bomba and West Libya.

The results for the models in which foraging area was not significant (clutch size and expected clutch frequency) can be found in Supplementary information (S4 – S5).

Discussion

Genetic studies of foraging areas are usually limited to identifying the migratory links between the mixed stock feeding aggregations and the source rookeries (Bolker et al. 2007, Jensen et al. 2013). Here, we investigated population sub-structuring based on the distribution of the Alagadi nesting population among the four foraging sites. Our results concurred with those of Watanabe et al. (2011) who similarly did not detect significant genetic structure among nesting loggerhead (*Caretta caretta*) females in Japan that displayed alternative feeding and habitat use. The absence of population sub-structuring suggests that foraging area effects can significantly influence the size of recruits, interbreeding intervals and the first nest phenology. This provides evidence that carry-over effects can influence the phenotype and reproductive success of a green turtle with important implications for their life-time reproductive potential.

Predicting the foraging area location of the nesting females using stable isotope analysis calibrated by satellite telemetry is comparable to conducting a rookery centric mixed stock analysis (MSA). The rookery centric MSA approach could be extended to all green turtle rookeries within the Mediterranean as evidence suggests that they share these same foraging sites (Stokes et al. 2015). This can be facilitated through the collection of a single fresh egg from each nest as this can provide a suitable voucher of the mother's stable isotope signature (Kaufman et al. 2014) and DNA (Shamblin et al. 2011). This may prove to be a suitable method to evaluate the spatial ecology of the green turtle within the Mediterranean as the genetic sampling of turtles at some foraging areas may not be appropriate considering the current political climate. This could also allow for the genetic composition of the foraging areas to be inferred and compared to future many–to–many MSA's (Bolker et al. 2007). The absence of genetic clustering indicates that foraging area selection is unlikely to be heritable in marine turtles which is unsurprising

considering the absence of parental care. This could have been partially assumed as no deviation from Hardy-Weinberg Equilibrium was detected for this aggregation as could be expected with multiple demes within the population (Chapter 1). Still, these results support the Learning Migration Goal (LMG) theory whereby turtles take up residence at foraging areas encountered during developmental migrations (Gaspar et al. 2012, Scott et al. 2014). Therefore, local sea surface currents including the location and diameter of the Cyprus eddy may vary the dispersal of pelagic-stage juveniles on a monthly, annual or decadal scale (Zodiatis et al. 2005, Scott et al. 2017).

There are many factors linked to resource availability and physiology that can influence the developmental rate of juveniles (Bjorndal et al. 2000, 2003, Suryan et al. 2009, Avens & Snover 2013) with considerable variation in adult body size within and among populations (Bjorndal et al. 2013). Here, we observed a negative correlation between the number of recruits at Alagadi (Stokes et al. 2014) and the downsizing of females at maturity for two of the feeding aggregations. We acknowledge that sample sizes for turtles recruiting prior to 2008 were necessarily limited by the tissue sampling regime, and imprecise natal philopatry means that some turtles may have previously reproduced elsewhere, but a similar trend was detected in other populations of green (García-Cruz et al. 2016, Piacenza et al. 2016) and snake-necked (*Chelodina rugose*) (Fordham et al. 2007) turtles where mature individuals were subjected to high mortality. In these cases, the downsizing of recruits were attributed to compensatory density-dependent mechanisms. This is a common response in over-exploited marine stocks (Rose et al. 2001) whereby the removal of larger, older individuals reduces competition and increases the resources available resulting in faster growth and earlier maturation of the younger age classes (Jonsson et al. 1984, Trippel 1995, Landers Jr et al. 2001). But a better access to resources in green turtles results in more rapid somatic growth (Bjorndal et al. 2000, 2003) and earlier maturation, but not necessarily at a smaller size (Bjorndal et al. 2013). Small conspecifics are more commonly associated with resource limitation in green turtles (Seminoff et al. 2008) but we found no evidence to suggest that turtles foraging in West Libya or Bomba were resource limited based on their reproductive traits. Evolutionary control provides an alternative explanation as the removal of larger, older conspecifics can shift selection for an early maturing, relative to

a later or larger maturing genotype (Olsen et al. 2004, DiBattista et al. 2009). Mediterranean green turtles were subject to intense harvest during the last century (Sella 1982) with contemporary levels of fisheries by-catch (Casale 2011) resulting in a short reproductive life-span (9.4 yrs, Casale & Heppell 2016). The selection of an early maturating genotype should increase recruitment as this can equalise, or extend the reproductive life-time of an individual. As a greater number of offspring are produced from the early maturing compared to late maturing individuals, the subsequent increase in recruitment will be accompanied by a rapid evolution to the earlier or smaller maturing genotype (Olsen et al. 2004).

The marked dichotomy in the cumulative reproductive output was primarily linked to the long interbreeding intervals for turtles foraging in Turkey-Cyprus compared to those foraging in north Africa. We assume that this difference is attributed to the failure of turtles in the northern foraging areas to acquire a suitable body condition as rapidly as those foraging elsewhere based on ecological and environmental differences among sites (Hays 2000, Hatase & Tsukamoto 2008). These unusually long interbreeding intervals could alternatively be linked to a lower natal site fidelity as the Turkey-Cyprus foraging areas are in closer geographic proximity to other nesting aggregations (Stokes et al. 2015). However, fine-scale genetic structuring detected in northern Cyprus suggests that natal site fidelity is high within this population (Chapter 1).

It is challenging to draw distinct ecological contrasts among these sites as systematic habitat assessments have not been conducted and nor have any conclusive dietary analyses of the turtles that forage there. However, *Cymodocea nodosa* is the most important dietary item for green turtles within this region (Cardona et al. 2010, Bentivegna et al. 2011) and therefore the quantity and quality of these seagrass plots (Bjorndal & Jackson 2003, Christianen et al. 2014) are likely to determine the amount of nutrition available at each site. *Cymodocea nodosa* occurs in a greater abundance along the sandy shallows of north Africa compared to the foraging areas in Turkey-Cyprus (Lipkin et al. 2003, den Hartog 2006) with plots now dominating the shallow western basin of Lake Bardawil in Egypt (El-bana et al. 2002, Nada et al. 2013). This is interesting as Lake Bardawil is the foraging area responsible for the increase in recruitment to

Alagadi (Chapter 2) and these turtles also exhibit the greatest life-time reproductive potential.

Environmental factors can also play an important role as the breeding frequency of green turtles is tightly linked to primary productivity (Broderick et al. 2001) and seagrass growth is influenced by light, temperature and nutrient availability (Lee et al. 2007). Furthermore, green turtles in the Mediterranean are at the northern-most part of their geographic range where they are not so physiologically adapted to the temperate conditions (Naro-Maciel et al. 2014, Seminoff et al. 2015) and therefore their nutrient uptake may be adversely affected (Cardona et al. 2010) at sites near their thermal tolerance.

Turtles in the northern foraging areas commenced nesting later than turtles from Egypt and Bomba even though they had less migratory distance to travel (Turkey-Cyprus ~280 km, Egypt ~630 km and Bomba ~1030 km, Figure 1). This could be mediated by dietary or environmental (e.g. day length) cues as it is not likely to be thermally driven as mean annual sea surface temperatures in Turkey-Cyprus are not cooler than in Bomba or West Libya (Stokes et al. 2015 Supplementary information).

Conclusion

This work suggests that foraging area selection in marine turtles is more likely to be learned through previous life experiences. The observed variability in size morphology and the interbreeding intervals was attributed to foraging area effects with important consequences for the life time reproductive potential of a turtle. The geographical distribution of green turtles within the Mediterranean (Stokes et al. 2015) suggests that they occupy a narrow ecological niche determined by temperature in addition to suitable foraging habitat. Therefore Mediterranean populations of green turtles may benefit under current climate change scenarios through increased fecundity and a range expansion despite concerns about female-biased primary sex ratios (Wright et al. 2012).

This study emphasises the importance of long-term individual-based studies to analyse intrapopulation variability in reproductive success (Clutton-Brock & Sheldon 2010). Individuals from a breeding population that are distributed among a heterogeneous landscape may not be reproductively equivalent. Therefore, foraging area effects should be investigated as they can inform conservation strategy and provide important information on the condition of feeding sites.

Figures and Tables

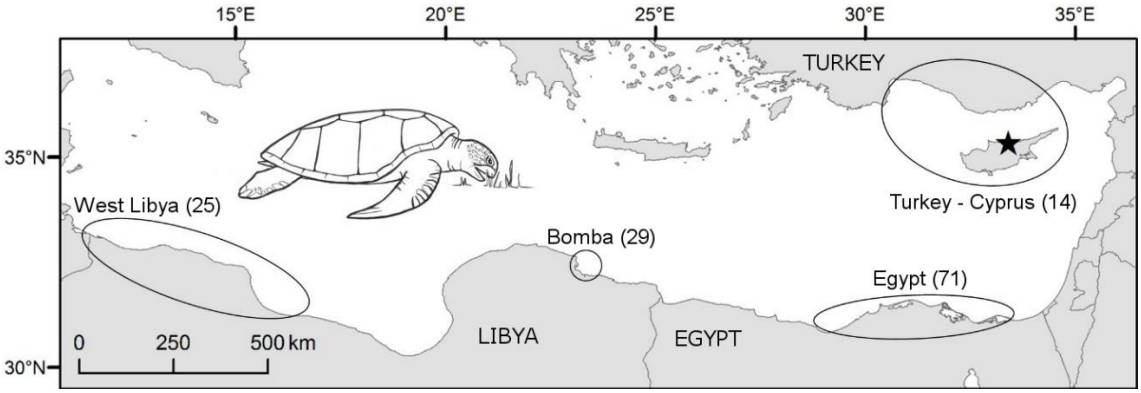

Figure 2 The four broad scale foraging areas (circled) utilised by the 139 green turtles from the Alagadi nesting population in northern Cyprus denoted by the star. Sample sizes for each site are in parenthesis and derived from Chapter 2. Turtle Illustration by Emma Wood.

Table 1 Characterisation of the GLM and GLMM models selected to evaluate foraging area effects on the morphological and reproductive traits.

Trait	N	Family (link)	Random effects (levels)	Fixed effects (levels)	P-value
Recruitment size	116	Gaussian (Identity)		Foraging (4) * Year	0.056
				Year	0.087
				Foraging (4) * Year (^2)	< **0.001**
Interbreeding interval	169	Poisson (Log)	Identity (63) Year (21)	CCL-female	0.988
				ECF for previous season (6)	0.944
				Prior reproduction	0.104
				Foraging (4)	< **0.001**
Clutch size	896	Gaussian (Identity)	Identity (137) Year (22) DOY	ECF (6)	0.981
				Foraging (4) * CCL-female	0.467
				Reproductive status (3)	0.466
				CCL-female	0.081
				Foraging (4)	0.105
				CCL-female (^2)	< **0.001**
Expected clutch frequency (ECF)	310	Poisson (Log)	Identity (139) Year (23)	Foraging (4) * First nest phenology	0.694
				Foraging (4) * Interbreeding interval (3)	0.760
				Foraging (4)	0.467
				First nest phenology	0.131
				Interbreeding interval (3)	**0.012**
				First nest phenology (^2)	< **0.001**
First nest phenology	287	Gaussian (Identity)	Identity (136) Year (23)	CCL-female	0.384
				Foraging (4) * Reproductive status (2)	0.187
				Foraging (4)	**0.002**
				Year	**0.001**
				Reproductive status (2)	< **0.001**

Trait = selected morphometric or reproductive parameter, N = number of observations, Family = probability distribution, Link = relationship between linear predictor and mean of distribution function, Random effects = grouped (not-independent) data, Fixed effects = independent variable, P-value = significance values. Fixed effects ordered as removed from the model by least significant term first, underlined = standardised covariate, (^2) = quadratic term, * = interaction fitted between fixed effects. Identity = number of individuals, Year = number of years of data, DOY = day of year for nesting event.

Table 2 Genetic variability of the Alagadi nesting green turtles grouped by the foraging area used (Figure 1).

	mtDNA			nDNA			
	n	*h*	*π*	*n*	*k*	*He*	*Ho*
Bomba	28	0.730 (0.07)	$3.3E^{-03}$ ($2.0E^{-03}$)	27	5.23	0.644	0.678
Egypt	68	0.774 (0.031)	$3.8E^{-03}$ ($2.2E^{-03}$)	69	6.0	0.654	0.651
Turkey-Cyprus	11	0.818 (0.119)	$3.7E^{-03}$ ($2.4E^{-03}$)	11	6.08	0.635	0.671
West Libya	23	0.885 (0.031)	$5.5E^{-03}$ ($3.1E^{-03}$)	24	5.31	0.662	0.699

Abbreviations codes, *n* = sample size, *h* = haplotype diversity, π = nucleotide diversity, *k* = mean number of alleles per locus, *He* = gene diversity, *Ho* = observed heterozygosity.

Table 3 Pair-wise genetic comparison of the green turtles from the Alagadi nesting population grouped by the foraging areas used (Figure 1).

	Bomba	Egypt	Turkey-Cyprus	West Libya
Bomba	-	-0.008	-0.030	0.041
Egypt	0.006	-	-0.005	0.016
Turkey-Cyprus	0.015	0.016	-	0.005
West Libya	0.009	0.007	0.012	-

Above diagonal pairwise F_{ST} values from mtDNA HR haplotypes, below diagonal pairwise F_{ST} values from microsatellites. All p-values for F_{ST} values were greater than the FDR threshold for significance (P < 0.0204)

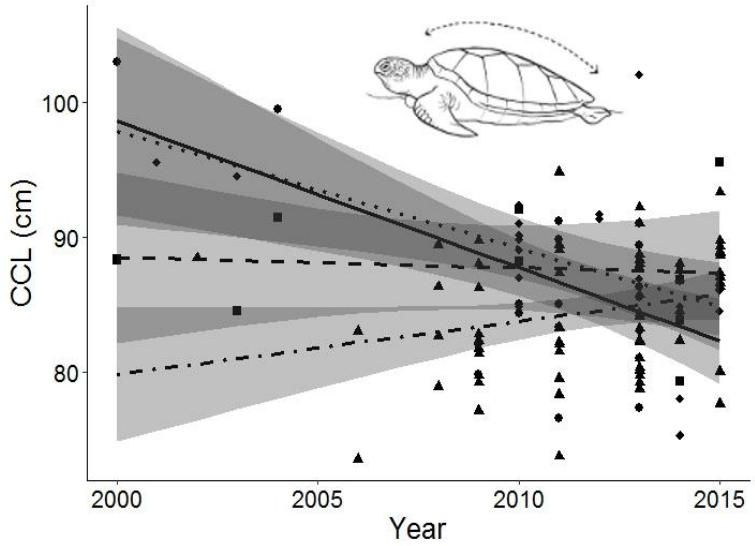

Figure 3 Body size (CCL) for green turtles at the time of recruiting to the breeding population at Alagadi from 2000 – 2015 that use each of the four foraging areas considered in this study: Bomba (n = 22) = circles / solid line, Egypt (n = 66) = triangles / dot-dash line, Turkey-Cyprus (n = 9) = squares / dashed line and West Libya (n = 19) = diamonds / dotted line, shaded areas = 95% confidence intervals (total n = 116). Turtle Illustration by Emma Wood.

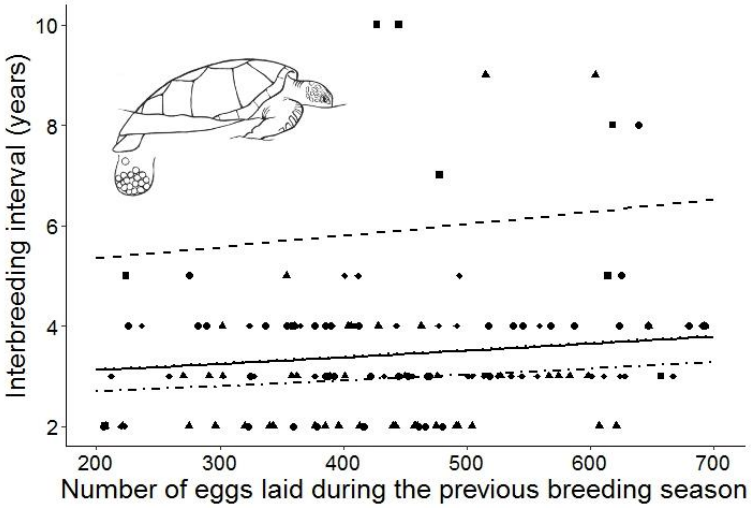

Figure 3 Interbreeding intervals exhibited by green turtles at Alagadi that use each of the four foraging areas regressed against the total number of eggs laid during the previous breeding season: Bomba (n = 52) = circles / solid line, Egypt (n = 56) = triangles / dot-dash line, Turkey-Cyprus (n = 13) = squares / dashed line and West Libya (n = 48) = diamonds / dotted line, (total n = 169). Turtle Illustration by Emma Wood.

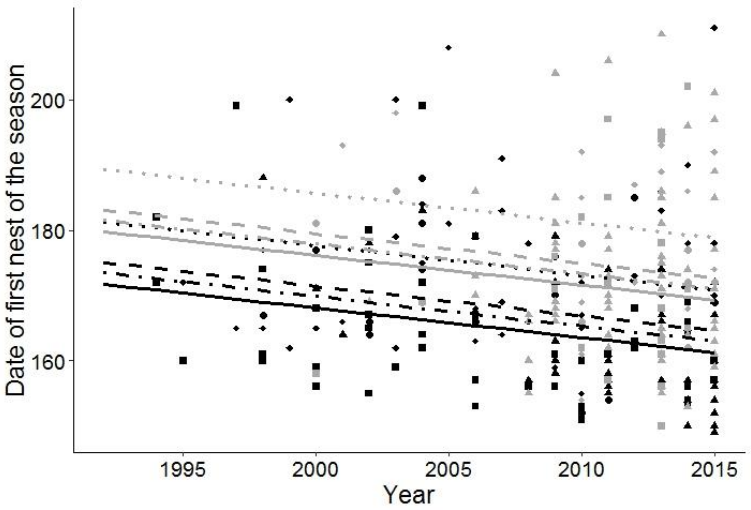

Figure 4 First nest phenology for green turtles at Alagadi from 1992 – 2015 that use each of the four foraging areas: Bomba (n = 75) = circles / solid line, Egypt (n = 122) = triangles / dot-dash line, Turkey-Cyprus (n = 23) = squares / dashed line and West Libya (n = 67) = diamonds / dotted line with neophytes = grey and remigrants = black, (Total n = 287).

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Chapter 3 Supplementary information

S1 Mitochondrial DNA markers

A ~800 bp fragment of the mitochondrial DNA (mtDNA) control region was amplified by polymerase chain reaction (PCR) using the primer pair LCM15382 (5'-GCTTAACCCTAAAGCATTGG-3') and H950 (5'-GTCTCGGATTTAGGGGTTT-3') (Abreu-Grobois *et al.* 2006) in a 10µl reaction consisting of 4µl QIAGEN Multiplex PCR Master Mix (Qiagen®; including HotStar DNA *Taq* polymerase), 3µl ddH_2O, 1µl of forward and reverse primers (5uM) and 1µl of ~10ng template DNA. PCR parameters included an initial hot start denaturing step at 95˚C for 15 mins and then 35 cycles at 94˚C for 1 min, 52˚C for 1 min, 72˚C for 90s and then a final extension step at 72˚C for 10 mins. Furthermore, a shorter 200bp fragment of the mtDNA control region that contains four hyper-variable dinucleotide (AT) short tandem repeats (mtSTRs) (Tikochinski *et al.* 2012; Shamblin *et al.* 2015) was amplified using primer pair CM-D-1 F (5'-AGCCCATTTACTTCTCGCCAAACCCC-3') and CM-D-5 R (5'-GCTCCTTTTATCTGATGGGACTGTT-3') (Tikochinski *et al.* 2012). We used the same reaction as for the ~800bp mtDNA fragment but with the following PCR protocol: 95˚C for 15 mins and then 35 cycles at 94˚C for 30s, 56˚C for 30s, 72˚C for 1 min and then a final extension step at 72˚C for 7 mins.

PCR products were visualised by electrophoresis to ensure successful amplification. A total of 6µl of the PCR amplicon was purified using 2µl of ExoSAP-IT® (Affymetrix Inc.) and incubated as per manufacturer's instructions. Purified mtDNA amplicon was sequenced in forward and reverse in an ABI 3730 DNA Analyser (Applied Biosystems™). All PCR reactions were run with positive and negative controls.

All forward and reverse sequence data was aligned in Geneious v6.17 (Biomatters Ltd). Mitochondrial DNA CR consensus sequences were compared against all nucleotide sequences in the National Centre for Biotechnology Information's database (http://www.ncbi.nlm.nih.gov/) using BLAST and the mtDNA CR haplotype designation prescribed by the Archie Carr Centre for Sea Turtle Research website (http://accstr.ufl.edu/cmmtdna.html). In cases of heteroplasmy of the mtSTRs, we took the major haplotype as the consensus sequence and aligned them manually in BioEdit

v7.2.5 (Hall 1999) to conform to the four short tandem repeats described by Tikochinski *et al*. (2012). The mtSTRs were then concatenated to the end of the mtDNA CR haplotype sequence to construct a high resolution (HR) haplotyping system (Shamblin *et al.* 2015) using the traditional nomenclature for green turtle CR haplotypes in the Atlantic (CM-A##) followed by the four digit repeat of the mtSTRs (e.g. CM-A13.1-6_8_8_4, Table 2).

Haplotype diversity (*h*) based on Nei (1987), nucleotide diversity (π) and genetic structure were calculated using the programme Arlequin v3.5.2.3 (Excoffier & Lischer 2010) (Table 2). Genetic structure was assessed through pairwise tests of F_{ST} based on haplotype frequencies with significance calculated via 1000 permutation tests.

Table S1 mtDNA high resolution (HR) haplotype diversity of the Alagadi green turtle nesting aggregation grouped by the four foraging areas used.

HR haplotypes	Bomba	Egypt	Turkey-Cyprus	West Libya
CM-A13.1-5_7_6_4			1	
CM-A13.1-5_8_6_4				1
CM-A13.1-6_11_5_4	1			
CM-A13.1-6_7_5_4		1		
CM-A13.1-6_7_6_4			1	
CM-A13.1-6_8_5_4	1	2	1	3
CM-A13.1-6_8_6_4	13	25	5	5
CM-A13.1-6_8_7_4		1		
CM-A13.1-6_8_8_4	3	14	1	4
CM-A13.1-6_9_6_4	7	16	1	3
CM-A13.1-6_9_7_4		2		
CM-A13.1-6_9_8_4		1		
CM-A13.1-7_10_6_4		3		4
CM-A13.1-7_8_6_4	1	1		
CM-A13.1-7_8_7_4	1		1	1
CM-A13.1-7_8_8_4				
CM-A13.1-7_9_6_4	1	1		
CM-A14.1-7_8_7_4		1		2
n	28	68	11	23
h	0.730 +/- 0.068	0.774 +/- 0.031	0.818 +/- 0.119	0.885 +/- 0.031
π	0.003 +/- 0.002	0.004 +/- 0.002	0.004 +/- 0.002	0.006 +/- 0.003

Sample size and genetic diversity (mean ± SD) for each subpopulation is expressed as: *n* = sample size, *h* = haplotype diversity and π = nucleotide diversity.

S2 Nuclear DNA markers

Samples were genotyped at 13 polymorphic microsatellite loci in two multiplex reactions previously optimised by Wright *et al.* (2012a; b, Table S2). PCR was carried in 2µl reactions with 1µl of dried ~10ng template DNA, 1 µl PCR Mastermix (Qiagen®) and 1µl of fluorescently labelled primer mix (6FAM, VIC and PET) at a final concentration of 1.8µM. Amplification was carried out in an MJ Research model PTC DNA Engine Tetrad thermal cycler according to the following protocol: 95˚C for 15 mins followed by 35 cycles of 94˚C for 30s, 58˚C for 90s and 72˚C for 1 min and finally one cycle of 60˚C for 30 mins. Allele sizes were assigned using an internal size standard (Genescan-500-LIZ; Applied Biosystems), an ABI 3730 DNA Analyser and ABI GeneMapper 3.7 software (Applied Biosystems). Samples that failed to amplify at all loci were re-amplified and re-scored. Error rate in allele size scoring was assessed by repeat marker amplification of 10% of the total sample size and comparing the number of incorrect allele calls divided by the total number of alleles (Selkoe & Toonen 2006).

The microsatellite dataset was evaluated as a whole, and in groups relating to the individual foraging areas for deviations from Hardy Weinberg Equilibrium and tested for linkage disequilibrium (LD) using Genepop on the web v4.2 (Raymond & Rousset 1995; Rousset 2008). Evidence of null alleles were checked using Microchecker v2.2.3 software (Van Oosterhout *et al.* 2004). Genetic structure was assessed using Wright's F_{ST} (1951) in a frequency-based analysis in GeneAlEx 6.5 (Peakall & Smouse 2006, 2012).

Table S2 Details of the microsatellite markers and multiplex sets used for the genotyping.

Locus / Multiplex	Primer Sequence (5' – 3') (Flurodye included in forward sequence)	Size range (BP)	Repeat motif	Locus reference
A6/1	F **VIC**AGTGCAATAACCATCCTTACAC	121 - 138	(AG)n	1
	R GGGCTGAATAGAGCTACAGAC			
Cm3/1	F **PET**AATACTACCATGAGATGGGATGTG	166 - 200	(CA)n	2
	R ATTCTTTTCTCCATAAACAAGGCC			
Or7/1	F **FAM**GGGTTAGATATAGGAGGTGCTTGATGT	226 - 236	(TG)n(TG)n	3
	R TCAGGATTAGCCAACAAGAGCAAAA			
Cc28/1	F **VIC**AGCCCATATGTTTCCCTTCA	189 – 201	(CA)n(TA)n	4
	R TTGGCCCATCTTATTTCAGTG			
Cc7E11/1	F **PET**GTTTGAAGAGCTGACCCCATATAG	262 - 290	(AGAT)n	5
	R AAACACAGAAATGAGGGATAG			
CcP7D04/1	F **FAM**ATGAGCAAAGTAACCCTAACA	308 – 360	(AGAT)n	6
	R GTTTGGAGCCAAATTAGAGATCAAC			
D2/1	F **VIC**AGTCCCCACTACTCATACCC	276 - 334	(TAGA)n	1
	R GTTTCTTTTGTGTTACTTCGGTGTTTC			
Klk314/2	F **FAM**GGTGCCAAGGAGGACGCTG	109 - 119	(CA)n	7
	R CATGCTCGCCCCTGGAAAG			
Cm58/2	F **PET**GCCTGCAGTACACTCGGTATTTAT	136 - 156	(CA)n	2
	R TCAATGAAAGTGACAGGATGTACC			
B103/2	F **VIC**CAGTCCTTGTTGTGGTTAGAGT	150 - 162	(CAA)n	1
	R GTTTCTTTTTCCCTTTCATCTTCTGTC			
Cc2/2	F **PET**CCCCCATAACACCACATCTC	211 - 249	(TA)n(GA)n	4
	R AGGTCACAAATGGAGCAAGC			
C102/2	F **FAM**TAAAAAGGCAGCCAAGTAAG	235 - 255	(TACA)n(CA)n	1
	R GTTGCAGAACCAACAGAATAG			
B123/2	F **VIC**ATCCCAGACCAAACAC	216 - 225	(CAA)n	1
	R GGCACAAGCCTATCCAATA			

Locus reference: 1= Dutton & Frey 2009; 2 = FitzSimmons *et al.* 1995; 3 = Aggarwal *et al.* 2004; 4 = Monzón-Argüello *et al.* 2008; 5 = Shamblin *et al.* 2007; 6 = Shamblin *et al.* 2009; 7 = Kichler *et al.* 1999.

S3 Correlation of morphologial and life history traits and the year that they were sampled

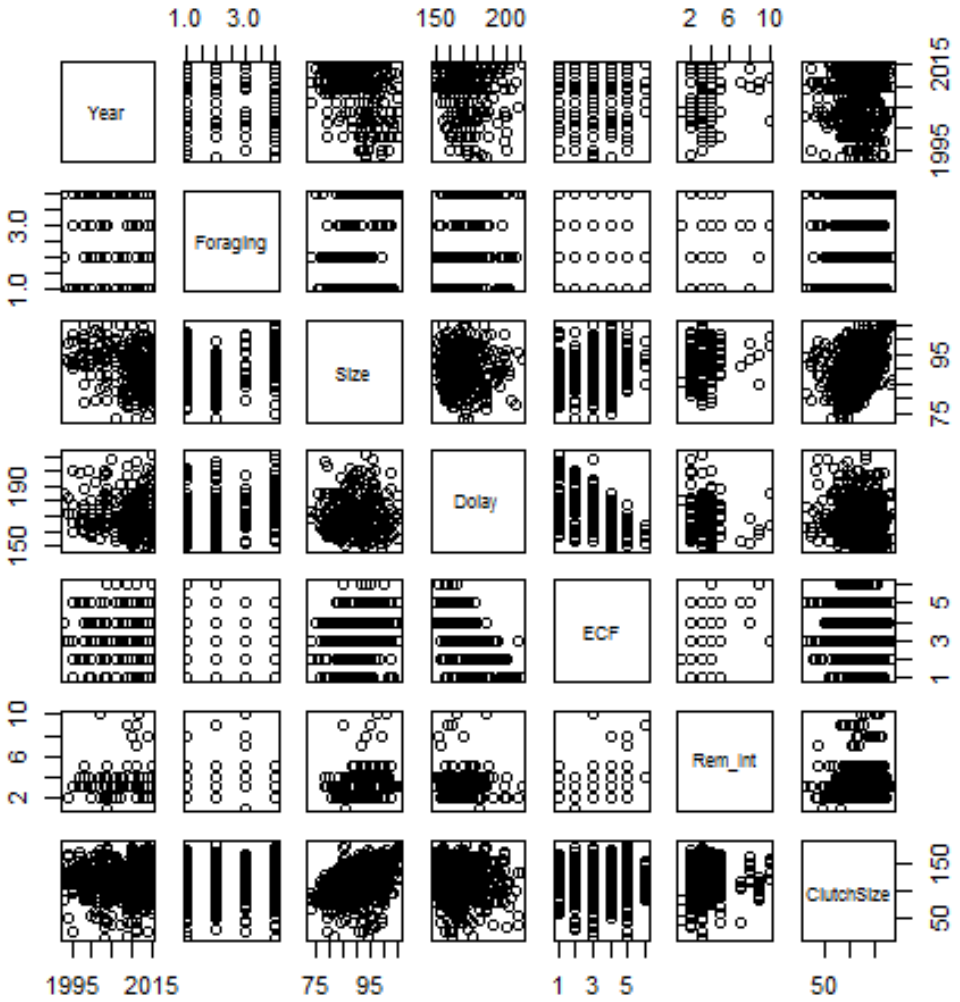

Figure S3 Pairwise collinearity plot for Year = year when sample was taken, Foraging = one of the four foraging areas ordered as Bomba, Egypt, Turkey-Cyprus and West Libya, Size = female body size (CCL), Dolay = day that the first nest of the season was made, ECF = expected clutch frequency, Rem_Int = interbreeding intervals and ClutchSize = number of eggs per clutch.

Table S3 Pearson's product moment correlation coefficients of the five morphological and reproductive traits and the year that they were sampled.

	Female body size	First nest phenology	ECF	Clutch size	Interbreeding interval
Female body size					
First nest phenology	-0.004				
ECF	**0.17***	**-0.55**			
Clutch size	**0.48**	-0.05	**0.12**		
Interbreeding interval	**0.42**	**-0.27**	**0.37**	**0.24**	
Year	**-0.35**	**-0.22**	-0.07	0.03	-0.04

Significant values in bold < 0.001, * < 0.01

S4 Clutch sizes ranged from 14 – 187 eggs and were significantly influenced by the covariate of female body size (CCL-female, GLMM, $\chi^2_{(1)} = 79.23$, $p < 0.001$, Figure S4). All other fixed effects were found to be non-significant.

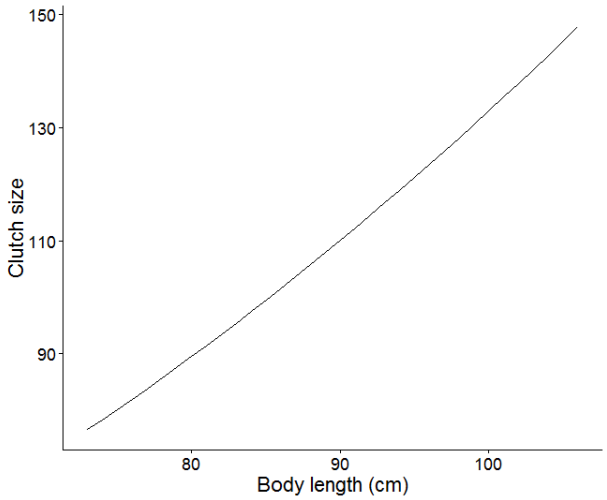

Figure S4 Clutch size regressed against female body size (CCL-female). Number of observations = 896 (Bomba = 226, Egypt = 384, Turkey-Cyprus = 70 and West Libya = 216).

S5 The expected clutch frequency for green turtles within this population varied between one and six clutches in a season. The expected clutch frequency was significantly influenced by the date that the turtle first nested (GLMM, $\chi^2_{(1)} = 39.01$, $p < 0.001$) and if the mother was a recruit or had a two year or greater than two year interbreeding interval (GLMM, $\chi^2_{(2)} = 8.37$, $p = 0.012$). Turtles that started nesting earlier in the season made a greater number of nests which then decreased for turtles that started to nest later in the season (Figure S5). Neophytes laid significantly fewer clutches

than turtles that exhibited a greater than two year remigration interval (Tukey HSD, p = 0.015) with two year remigrant turtles nesting early in the season laying on average one extra clutch compared to neophytes (Figure S5).

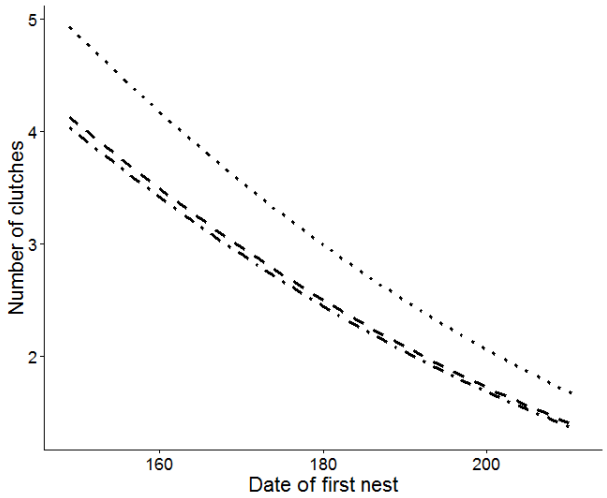

Figure S5 The expected clutch frequency regressed against the date that the turtle first nested for neophytes = dot-dash line, two year remigrants = dashed line and more than two year remigrant = dotted line. Number of observations = 310 (Bomba = 82, Egypt = 127, Turkey-Cyprus = 28 and West Libya = 73).

Chapter 3 Supplementary references

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Chapter 4

A wild marine turtle pedigree reveals unequal effective contribution to the next generation

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Abstract

Pedigrees are often used to investigate key issues in evolutionary biology and the effectiveness of the breeders by uncovering kinship among individuals. Obtaining accurate pedigree information can be challenging for wild populations although relationships can be reconstructed though the use of molecular genetics. Here, we reconstruct a genetically-validated green turtle (*Chelonia mydas*) pedigree consisting of 245 individuals from a rookery in Cyprus that has been the subject of intensive monitoring for 24 years. Multilocus genotypes were employed to jointly assign parentage and partition the offspring into full-and half-sibship clusters using a full-pedigree likelihood methodology. This revealed a strong relationship structure, with 93 full-sibship and 646 half-sibship clusters, 408 maternal and 238 paternal. The effective contribution to the next generation was highly variable among breeders, with a maximum cluster size of full- and half-siblings of 8 and 14, respectively. Furthermore, up to 76% of females that have bred for over 20 years were yet unassigned parentage. This work emphasises the importance of individual-based monitoring and reveals that the recent recruitment that has led to the recovery of this nesting population has arisen from local demographics. However, the unequal contribution of the breeders to the next generation means that the effective population size is likely to be less than the census size with important conservation implications for the management of small populations.

Introduction

Genetically reconstructing relationships can be a powerful tool in wild populations for understanding the effective contribution of the breeders and investigating key parameters in evolutionary biology including extra-pair copulation (Griffith et al. 2002, Cohas & Allainé 2009), multiple paternity (reviewed in Avise et al. 2002, Uller & Olsson 2008) and inbreeding avoidance (Nielsen et al. 2012, Sanderson et al. 2015). Numerous methods exist for inferring genealogical relationships among individuals based solely on genetic marker information (reviewed in Blouin 2003, Jones & Ardren 2003) with estimates of relationships commonly conducted on a pairwise basis. However, pairwise based relatedness estimators can be quite imprecise as they ignore other individuals within the sample (Van de Casteele et al. 2001, Wang 2004, Pemberton 2008). Group-likelihood approaches can substantially improve the accuracy of inferred relationships as they consider all individuals and simultaneously partition them in to full- (siblings that share both parents) and half-sibship (siblings that share either a mother or a father) clusters whilst jointly allocating parentage (Wang 2004, Pemberton 2008, Jones & Wang 2010). Such methods are often limited to two generational pedigrees (Koch et al. 2008), but multigenerational pedigrees can be reconstructed by sampling at a sufficient time-scale to ensure that individual birth cohorts are known, and then additional data can be augmented on a cohort by cohort basis (DiBattista et al. 2009).

Genetically validated pedigrees also allow the adaptive potential of a wild population to be evaluated through a quantitative genetic analysis. Quantitative genetics allows the estimation of the heritability of phenotypic traits that vary continuously and thus investigating how they may respond to selection (Falconer & Mackay 1996, Lynch & Walsh 1998). The phenotypic variance can be used to estimate the narrow-sense heritability of a trait which is the proportion of the variability that is explained by the degree of resemblance between relatives (Thomas & Hill 2000, Wilson et al. 2010). This can be critical for informing conservation strategies as anthropogenic activities such as over-harvesting and habitat fragmentation can be key drivers for rapid evolution as species face a higher extinction risk if they are unable to adapt (Stockwell et al. 2003).

However, quantitative genetics requires reliable information on the relationship structure which can be challenging to obtain in wild populations.

Some primary requirements to ensure the accuracy of a genetically-validated pedigree include the adequate sampling of the candidate parents and offspring, sufficient marker information to uncover true relationships and evaluating and accounting for genotyping error rates within the analyses (Jones & Ardren 2003, Pemberton 2008). Ecological and behavioural data can be useful to augment genetic data as specifying interactions can improve the accuracy of the relationship structure (Pemberton 2008). But obtaining behavioural information or collecting genetic samples from a sufficient number of individuals can be challenging in cryptic or highly mobile marine species. The philopatric behaviour of some marine vertebrates, however, does present an opportunity as offspring return to their natal areas to breed. Therefore their relationship structure can be reconstructed if genetic sampling is undertaken over a sufficient time-frame to incorporate parents and offspring for an adequate proportion of the population (Dutton et al. 2005, Herbinger et al. 2006, DiBattista et al. 2009).

Green turtles (*Chelonia mydas*) are long-lived, slow to mature and highly fecund marine vertebrates that exhibit natal site fidelity (Meylan et al. 1990). Several aspects of their reproductive biology makes this an interesting species in which to perform a pedigree reconstruction as: (i) both sexes are polygamous with multiple paternity common within clutches (Pearse & Avise 2001, Bowen & Karl 2007, Wright, Fuller, et al. 2012), (ii) paternity cannot be inferred as copulation is rarely observed and females can store sperm from multiple mating events (Pearse & Avise 2001), and (iii) information regarding the specific birth cohort is difficult to obtain as the survival rate of hatchlings to adulthood is very low, compared to the number of hatchlings produced (Frazer 1986, Casale & Heppell 2016), which prohibits researchers obtaining a genetic voucher or marking every offspring at hatching. However, the green turtle nesting aggregation at Alagadi beach in northern Cyprus presents a rare opportunity as this population has been intensively studied since 1992 (Stokes et al. 2014). Saturation tagging conducted during night patrols has maintained a greater than 99% encounter rate of nesting females producing one of the longest and most complete individual-based datasets for

a marine turtle population (Stokes et al. 2014). During the last decade, this nesting aggregation has experienced a significant population recovery that is attributed to an increase in recruitment to the breeding population (Stokes et al. 2014). Furthermore, molecular genetics has established the reproductive isolation of this nesting aggregation (Chapter 1, Tikochinski et al. Unpublished) and thus a strong relationship structure could be expected. For these reasons the Alagadi nesting aggregation provides an ideal situation in which to employ the multilocus genotypes of the nesting females to reconstruct a genetically-validated wild pedigree.

Here, we employ a full-pedigree likelihood methodology using sibship reconstruction to infer the relationship structure and (i) determine the effective contribution of the breeding individuals to the next generation, (ii) estimate the minimum age at recruitment, and (iii) employ a quantitative genetic analysis to estimate the narrow-sense heritability of morphological and life-history traits.

Methods

Sample collection

Beach monitoring at Alagadi Beach in northern Cyprus (35˚19’56.17”N; 33˚28’57.59”E) has been ongoing since 1992 (Broderick et al. 2002, Stokes et al. 2014). Females are given a unique identity using external flipper tags and passive integrated transponders (PIT tags; since 1997) upon first encounter with morphometric and reproductive data collected during every nesting event (Broderick et al. 2002, Stokes et al. 2014). Tissue samples were collected from 243 nesting green turtles and two males during the breeding seasons from 2001 to 2015 thereby sampling remigrating females that have bred since monitoring was initiated (Figure 1). Tissue samples comprising of a small skin biopsy (<0.5 cm^2) were taken from the trailing edge of the fore flipper shortly after oviposition. All biopsies were stored in 96% ethanol until genomic DNA was extracted using the Qiagen DNeasy blood and tissue kit (Qiagen®) following the manufacturer’s instructions or using an ammonium acetate precipitation method (Nicholls et al. 2000).

Microsatellite characterisation

All individuals were successfully genotyped at 13 polymorphic microsatellite loci using previously described methods (Chapter 1, Wright, Fuller, et al. 2012). Error rates were investigated by the repeat marker amplification of 10% of the total sample size (Selkoe & Toonen 2006) with genotypic differences observed at three allele calls of 1340 resulting in a class II error rate of 0.22% per loci (Wang 2004). Class I error rates (Wang 2004) from null alleles (F(Null)) and general diversity indexes for the microsatellite array, including the number of alleles per locus (*k*), observed (H_O) and expected heterozygosity (H_E), polymorphic information content (PIC) and exclusion probabilities were calculated in Cervus v3.0.7 (Marshall et al. 1998) and summarised in Table 1. Average exclusion probabilities were 0.986 for a single parent and greater than 0.999 for a second parent, a parent pair and sib-identity. Analysis in Genepop on the web v4.2 (Raymond & Rousset 1995, Rousset 2008) using default parameters found no evidence for deviations from Hardy Weinberg Equilibrium for any single locus or across loci with significant P-values from multiple tests corrected using the false discovery rate (Narum 2006). Three pairs of linked loci were identified (Cc7E11 – Klk314, A6 – Or7 and Cc28 – CcP7DO4) that were not linked among three neighbouring rookeries (Chapter 1) and therefore all loci were assumed to be independent. No evidence was found for null alleles using Microchecker v2.2.3 software (Van Oosterhout et al. 2004).

Pedigree reconstruction

We employed the programme COLONY *v2.0.5.0* (Wang 2004, Wang & Santure 2009, Jones & Wang 2010) to reconstruct the wild green turtle pedigree as it jointly infers parentage and full- and half-sibships using a maximum likelihood algorithm considering the entire pedigree configuration whilst allowing for genotyping errors (Jones & Wang 2010). This can be particularly beneficial when reconstructing pedigrees for wild populations where complete sampling of candidate parents is often logistically difficult as 'dummy' parents can be assigned and their genotypes reconstructed (Wang 2004, Jones & Wang 2010). All independent COLONY2 runs were performed on ICEBERG, a central high performance computing resource at Sheffield University.

COLONY2 data input

The multilocus genotypes of sampled females were included as candidate offspring and candidate mothers as we could not guarantee the true neophyte status of all females despite the high observer effort (Stokes et al. 2014). Thus, any female within the pedigree 'could' be the mother to any other turtle. Only two males were included as candidate fathers as males are rarely encountered. Maternity and paternity was excluded based upon parent-offspring sharing expanded mtDNA control-region haplotypes obtained from Chapter 1. The probability that the true mother and father were included in the sample was set at 0.5 (Figure 1) and 0.1, respectively. No data were available regarding 'Known Maternal sibs' or 'Known Paternal sibs' and so these data files were omitted. Marker error rates corresponding to Wang (2004) were calculated as stated under microsatellite characterisation.

Model parameters and replicate runs

Four independent COLONY2 runs were performed with the mating system defined as polygamous for both sexes (Wright, Fuller, et al. 2012) and the species specified as diploid and dioecious. No evidence for inbreeding was detected in COANCESTRY (Wang 2011) during a preliminary analysis and therefore inbreeding was omitted from the COLONY2 analysis. All analyses were conducted using the full-likelihood algorithm, set on long length at high precision. We applied the sibship complexity prior as this reduces the possibility of erroneously misclassifying unrelated or loosely related specimens as half-siblings which is advised in the context of weak family structure, polygamy and limited genotypic resolution power (Meraner et al. 2013). The four pedigrees were visualised in Pedigree Viewer (Kinghorn 1994) and characterised using the 'pedigreeStats' function in the R statistical package PEDANTICS (Morrissey & Wilson 2010). To avoid unintentional bias, 'Pedigree A' was predetermined as the relationship structure for estimating the age to maturity and conducting the quantitative genetics analysis.

Animal models

To investigate trait heritability we employed a type of mixed effects model known as the 'animal model' that partitions the phenotypic variance (V_P) into genetic (V_G) and

environmental components (V_E) with the residual variance (V_R) accounting for any unmeasured environmental effects (Falconer & Mackay 1996, Lynch & Walsh 1998, Wilson et al. 2010):

$$V_P = V_G + V_E + V_R$$

The genetic component (V_G) can be decomposed further but additive dominant and epistatic sources are difficult to estimate in non-experimental settings (Wilson et al. 2010). Therefore the additive genetic variance (V_A) was measured by estimating the phenotypic similarity of relatives (Falconer & Mackay 1996, Kruuk 2004, Wilson et al. 2010) with the relationship data obtained from the 'COLONY2 .BestConfig' output file from 'Pedigree A' and transformed as described in Wilson et al. (2010):

$$V_P = V_A + V_E + V_R$$

The environmental variance (V_E) was decomposed by including random effects to account for the non-independence of data and to estimate the phenotypic variance corresponding to that component (Wilson et al. 2010). These included 'year' to account for any temporal variance for V_P in the population, 'individual identity' to account for among individual variance by grouping repeated observations of the same individual within and among years and the mother 'dam' to quantify any remaining between-individual variance over and above that due to additive genetic effects that might be related to maternal-half sibs (Falconer & Mackay 1996, Lynch & Walsh 1998, Wilson et al. 2010)

The narrow-sense heritability (h^2) is the 'proportion of phenotypic variance of a trait that is explained by the added genetic variance' (Falconer & Mackay 1996, Lynch & Walsh 1998):

$$h^2 = V_A / V_P$$

The proportion of phenotypic variance of a trait for each environmental component was estimated in a similar manner:

$$r^2 = V_E / V_P$$

where V_E was either 'year', 'individual identity' or 'dam'. When analysing the narrow-sense heritability of clutch size, the clutch 'order' was also included as an additional environmental component as clutch size tends to increase over successive nests in green turtles (Broderick et al. 2003).

Variance components were estimated using ASReml-R by residual maximum likelihood (REML) (Butler et al. 2007). Significant additive genetic and environmental components were tested by comparing models with and without the effect of interest using a likelihood ratio test as per Wilson et al. (2010). The significance of fixed effects were revaluated using the 'Wald method' subsequent to the model simplification of the variance components (Butler et al. 2007).

Traits of interest

Variance components were estimated for two morphological (female body size and hatchling mass) and three life-history (interbreeding intervals, clutch size and clutch frequency) traits in univariate animal models (see Supplementary information for trait descriptions). Phenotypic variance was conditioned on the significant covariates and predictor variables from the Generalised Linear Mixed Models (GLMMs, Bolker et al. 2009) previously employed to evaluate foraging area effects from Chapter 3 by including these as fixed effects within the animal models (Wilson et al. 2010). Foraging area location was a significant predictor variable for the size of recruits and the length of interbreeding intervals (Chapter 3) and therefore included as a fixed effect within animal models evaluating female body size and interbreeding intervals. Foraging area location for each individual was determined from Chapter 2, but as the foraging area could not be predicted for all individuals within the pedigree, the sample sizes were necessarily reduced. However, because a temporal interaction was found between the foraging area location and the size of recruits (Chapter 3), we analysed female body size twice, with and without foraging area location as a fixed effect. All models are summarised in (Table 2) and described in Supplementary information.

Results

The four COLONY2 runs inferred a strong relationship structure with 93 ± 13.0 (mean ± SD, range = 81 – 111) full sibship clusters amongst the 243 candidate offspring with 407.8 ± 17.1 (range = 392 – 432) and 237.5 ± 8.1 (range = 228 – 246) maternal and paternal half-sibship clusters (Table 3). Mean maternal and paternal sibship size was, 4.2 ± 0.2 (range = 1 – 14) and 3.2 ± 0.1 (range = 1 – 10), respectively (Table 3). The mean number of assigned maternities and paternities were 60 ± 2.4 (range = 57 – 62) and 78.5 ± 2.1 (range = 76 – 81), respectively of which 9.3 ± 1.9 and 1 ± 1.2 were true parents from the sample as opposed to reconstructed genotypes. The depth of the pedigree varied from three to five generations with the first generation made up of reconstructed genotypes (founders). The pedigree in which the age to maturation and estimates of trait heritability 'Pedigree A' is graphically displayed in Figure 2 whilst pedigrees 'B - D' are displayed in Supplementary information Figures S1 – S3.

Of the females first observed nesting within the 1990's, 7 ± 0.7 (range = 6 – 8) out of 25 were assigned maternity of 35.25 ± 3.2 (range = 32 – 38) candidate offspring (Figure 3) with the remaining 17 – 19 females failing to effectively contribute offspring thus far. No links were found between fecundity and the effective contribution to the next generation as no significant differences were found between those assigned maternity and those which were not for the mean number of eggs produced annually by each female (Mann-Whitney U test, W_6 = 61.5, difference = 14.12, p = 0.80) or the mean hatching success of the nests (Mann-Whitney U test, W_6 = 56.5, difference = -2.15, p = 1).

The effective population size (N_e) estimated from sibship assignments assuming random mating was 126.5 ± 5.5 (range = 122 – 141, Table 3). However, N_e in this case refers to the founder generation that produced the candidate offspring as overlapping generations will increase the frequency of full sibships and thus it is termed as N_b (Wang 2009).

Age to maturation

The age to maturity was to be estimated through the temporal difference in the neophyte status of parent – offspring relationships. However, we were unable to estimate an accurate age to maturity as we could not ensure the true neophyte status of the mother and therefore they might have been nesting previous to the onset of monitoring (Figure 3).

Genetic variances

In general, we were unable to obtain heritability estimates as large standard errors around the variance components were suggestive that we did not have sufficient relationship and / or phenotype data (Bérénos et al. 2014). A significant narrow-sense heritability estimate was obtained for female body size (mean ± SE, $h^2 = 0.26 \pm 0.01$) when analysed using all relationship information but we did not find evidence for added genetic variance when conditioning the phenotypic variance on foraging area location (Table 2). All traits that were estimated from repeated measures had significant common environmental effects with the exception of the mother's phenotype (dam) which was not significant for any model.

Discussion

Here, we present the first genetically-validated wild marine turtle pedigree inferred by sibship reconstruction using a maximum likelihood algorithm considering the entire relationship configuration. This provided a significant insight into the relationship structure among the females of the Alagadi nesting aggregation and an in-depth assessment of local demographics. We revealed that the effective contribution to the next generation was highly variable with up to 8 and 14 offspring assigned to full and half-sibship clusters, respectively whilst only 24% of females nesting in the 1990's were yet assigned parentage. Unfortunately, at present we are unable to accurately estimate the minimum age to maturity as a greater number of parent – offspring pairs are necessary where we can have enhanced surety of the true neophyte status of the mother.

Although the relationship data were complemented with phenotype data encompassing near complete coverage of every green turtle nesting event since 1992, this still proved to be insufficient to provide estimates for the narrow-sense heritability of life-history traits. A moderate heritability estimate was obtained for female body size but not after conditioning the phenotypes on foraging area effects. Researchers for other long-lived and cryptic species are likely to encounter similar limitations when recreating a genetically-validated wild pedigree and so here we discuss the challenges highlighted by this study.

There are several aspects of the green turtle's (*Chelonia* mydas) life-history that make it a challenging species in which to reconstruct a genetically-validated pedigree. These include (i) the necessity of sampling the reproductive cohort that contains overlapping generations as the candidate offspring, (ii) the inability to identify individual birth cohorts, and (iii) the logistical challenge of sampling candidate parents due to the longevity of life stages. However, in this case, the inherent problem of sampling the candidate parents (Pemberton 2008, Aykanat et al. 2014) will diminish over-time as nearly all nesting females have been genetically sampled since 2007 and thus a high proportion of candidate mothers for future offspring. The presence of overlapping generations caused several types of erroneous assignments among highly related individuals. These generally consisted of parent – offspring relationships being misinterpreted whereby the offspring was assigned parentage of the true parent, and the 'sib paradox' whereby full siblings are misinterpreted as parent – offspring as they share more alleles than the true parent and offspring (Thompson & Meagher 1987). In most cases, a sufficient increase in marker information should prevent full siblings from being misinterpreted as parent - offspring as they differ in the way that they share alleles by descent (Blouin 2003). The use of 14 and 28 microsatellites in a hypothetical pedigree increased the number of true parental links from 67% to 95.2% (Aykanat et al. 2014). This could also reduce the number of Type I errors, where truly unrelated individuals are classed as related which can be common in pedigrees with a strong relationship structure consisting of many small half-sib families (Thomas & Hill 2000). Using a greater number of microsatellites is preferable to more variable microsatellites as genotyping errors, null alleles and mutations contribute to false exclusions (Jones & Ardren 2003,

Selkoe & Toonen 2006). An increase in marker information would not be able to prevent offspring from being falsely interpreted as the parent and therefore a strict temporal exclusion criteria would be necessary in this case. This is not an easy concept for marine turtles as natal site fidelity can be imprecise (Schroth et al. 1996) and therefore a female might not be a true neophyte when first encountered. Consequently, a strict exclusion criteria could increase the risk of Type II errors where truly related individuals are classed as unrelated (Thomas & Hill 2000). Nonetheless, a strict exclusion policy could reduce the occurrence of misinterpreted relationships and thereby reducing the variability in the generational depth.

The occurrence of some missassigned parentage in a genetically-validated wild pedigree is unlikely to cause any downward bias for the heritability estimates within the quantitative genetic analysis as the erroneously assigned parent is generally closely related to the true parent (Bérénos et al. 2014). However, these types of analyses typically require very large sample sizes encompassing multiple generations (Kruuk & Hill 2008, Wilson et al. 2010) and whilst our dataset might be large considering the percentage of the population covered and its longevity, it is small considering the multigenerational pedigrees consisting of thousands of individuals that are commonly employed (Kruuk 2004). Therefore more accurate estimates of trait heritability can be obtained with a sufficient increase in relationship and phenotype data, but this may involve several decades of additional data collection and genetic sampling. Nevertheless, some evidence was found for the heritability of body size suggesting an adaptive potential to local conditions. Body size plasticity might be evident from the variability of green turtles among geographically discrete nesting aggregations (Bjorndal et al. 2013) but this moderate heritability estimate was not found after conditioning female body size on foraging area location. This may be a result of the necessary reduction in sample size for individuals with relationship and phenotype data as foraging area location could not be predicted for all individuals within the pedigree. But the estimation of quantitative genetic traits in wild populations can be confounded as environmental variation frequently obscures the underlying evolutionary patterns (Kruuk 2004). Obtaining heritability estimates can be even more difficult for the life-history traits as they often encompass more residual variance than morphological traits

as directional selection for fitness can erode added genetic variance (Mousseau & Roff 1987, Coltman et al. 2005). Thus, the weak migratory connectivity between marine turtle nesting aggregations and geographically discrete foraging areas (Webster et al. 2002) may further challenge the estimation of trait heritability without a prohibitively large number of candidate individuals within the pedigree in comparison to that of an insular mammalian population where all individuals share environmental conditions (e.g. Poissant et al. 2008).

Next generation sequencing could be a promising tool as this allows for the genotyping of thousands of markers with single nucleotide polymorphisms (SNPs) (Mardis 2008). SNPs benefit from fewer genotyping errors as they acquire new mutations through single base pair substitutions. In addition, SNPs also allow for genome-wide association studies (GWAs) that evaluate the direct correlation between the genotype and phenotype without the need to build a pedigree (Korte & Farlow 2013) which diminishes problems associated with disentangling discrete relationships.

Despite the limitations discussed here, the genetically-validated pedigree provided some significant insights into green turtle population dynamics. Importantly, it revealed that the individual effective contribution to the next generation was unequal. Unequal family groups with high number of females failing to contribute to the next generation implies that the effective population size (N_e) of the Alagadi rookery could be less than expected considering its census size under the assumption of equal contribution (Frankham 1995). The presence of overlapping generations (Phillips et al. 2014) and the fluctuating population size (Sella 1982, Stokes et al. 2014) could cause an upwards or downward bias in the estimates of N_e (Frankham 1995). However, the estimate of N_e was greater than the revised estimates to minimise the effects of inbreeding (≥ 100) although considerably lower than what is considered the minimum for the long-term persistence of genetic variation (≥ 1000, Frankham et al. 2014). Still, if these results of unequal contribution are common among marine turtles, thus implying that effective population sizes are lower than the census sizes, we would need to reconsider what would be the optimal census size of a genetically healthy population of these organisms.

Although we were unable to conduct a quantitative analysis on trait heritability, we suggest that fecundity may not be an adequate measure of reproductive success as we failed to detect an association between egg production or hatching success and the effective contribution of the female to the next generation. This indicates that juvenile survivorship may have a greater evolutionary importance and that parents that effectively contribute more progeny may produce better quality offspring rather than a larger quantity. However, individual fitness is difficult to measure and survivability might be linked to foraging area location for this population and the associated levels of predation and anthropogenic sources of mortality (Casale 2011). Quantitative genetic analysis should be re-examined when more individuals can be included within the pedigree.

Conclusion

This work emphasises the type of in-depth research that becomes attainable when long-term individual-based monitoring is conducted over a sufficiently long time-frame (Clutton-Brock & Sheldon 2010). This is the first genetically-validated wild marine turtle pedigree to be reconstructed using a maximum likelihood algorithm considering the entire relationship configuration. Some limitations were identified, but these can be ameliorated through additional sampling, greater marker information and a more defined parentage exclusion criteria. Nonetheless, the genetically-validated pedigree still provided an in-depth assessment of the relationship structure and highlighted that the effective contribution to the next generation is unequal. This raises some concerns for management as the effective population size is likely to be smaller than previously anticipated. However, the strong relationship structure provides evidence that the recent population increase is likely to result from local demographics. Future research needs to address the reproductive movement of males as it is presently unclear whether a large dispersive population of males exist within the Mediterranean or if each rookery has a large number of philopatric males (Chapter 1, Wright, Stokes, et al. 2012, Wright, Fuller, et al. 2012). The number of males has important implications for the effective

contribution to the next generation as it may be currently underestimated as males were not included as candidate offspring.

Figures and Tables

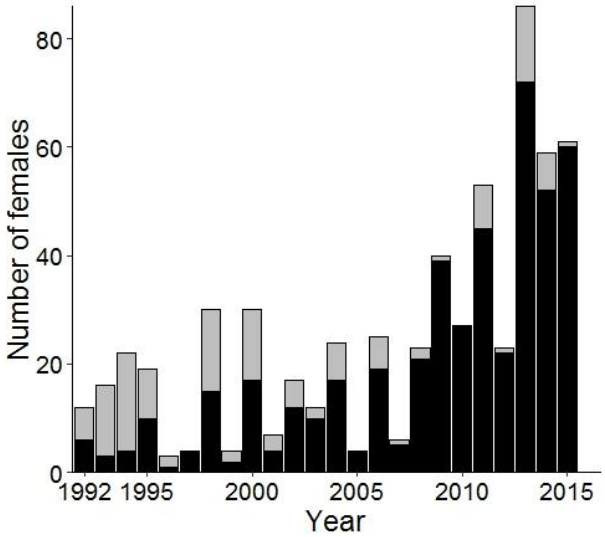

Figure 1 Number of female green turtles nesting at Alagadi that were genotyped for the pedigree. Black = genotyped, grey = not genotyped, whole bar length = total number of females nesting that year.

Table 1 Characterisation of microsatellite loci for 243 female green turtles from the Alagadi rookery

Locus	k	H_O	H_E	PIC	F(Null)	Reference
A6	6	0.770	0.741	0.698	-0.019	Dutton & Frey (2009)
B103	5	0.642	0.662	0.607	0.010	Dutton & Frey (2009)
B123	5	0.630	0.619	0.56	-0.013	Dutton & Frey (2009)
C102	5	0.588	0.633	0.569	0.044	Dutton & Frey (2009)
D2	11	0.765	0.727	0.696	-0.023	Dutton & Frey (2009)
Cm3	10	0.556	0.535	0.512	-0.020	FitzSimmons et al. (1995)
Cm58	7	0.790	0.812	0.784	0.014	FitzSimmons et al. (1995)
Klk314	4	0.453	0.445	0.367	-0.012	Kichler et al. (1999)
Or7	5	0.700	0.663	0.617	-0.023	Aggarwal et al. (2004)
Cc2	12	0.753	0.769	0.735	0.011	Monzón-Argüello et al. (2008)
Cc28	4	0.704	0.723	0.668	-0.012	Monzón-Argüello et al. (2008)
Cc7E11	4	0.547	0.518	0.471	-0.038	Shamblin et al. (2007)
CcP7D04	9	0.815	0.783	0.749	-0.021	Shamblin et al. (2009)
Mean	6.69	0.67	0.66	0.62		

K = number of alleles, H_O = observed heterozygosity, H_E = gene diversity, PIC = polymorphic information content, F(Null) = frequency of null alleles

Table 2 Variance component estimates from single trait animal models

Trait	Family (link)	Fixed effects (levels)	N (females)	Mean	SD	V_A	h^2 (mean ± SE)	Common environmental variable	V_E	R^2 (mean ± SE)	V_R
CCL (cm)	Gaussian (Identity)	Reproductive status (2)	469 (242)	89.29	6.21	4.87	0.26 ± 0.01	Individual Id	12.71	**0.68 ±0.15**	1
								Year	0.23	**0.01 ±0.01**	
CCL-2 (cm)	Gaussian (Identity)	Foraging area (4), Reproductive status (2)	268 (125)	90.02	6.69	NA	NA	Individual Id	17.57	**0.94 ±0.01**	1
								Year	0.18	**0.01 ±0.01**	
Hatchling Mass (g)	Gaussian (Identity)	Date of Nest* CCL*	934 (213)	19.22	1.99	NA	NA	Individual Id	0.63	**0.35 ±0.04**	1
								Year	0.17	**0.09 ±0.04**	
Interbreeding Interval (yrs)	Poisson (Log)	Foraging area (4)	150 (56)	3.41	1.51	NA	NA	Individual Id	NA	NA	NC
								Year	NA	NA	NC
Clutch size	Gaussian (Identity)	CCL*	1317 (242)	111.38	28.15	NA	NA	Individual Id	0.29	**0.20 ±0.03**	1
								Year	0.06	**0.04 ±0.02**	
								Order	0.07	**0.05 ±0.03**	
Clutch Frequency	Gaussian (Identity)	First nest phenology* Interbreeding intervals (11)	469 (243)	2.93	1.32	NA	NA	Individual Id	0.57	**0.41 ±0.07**	1
								Year	NA	NA	

Family (link) = error distribution and link function for model fit, V_A = additive genetic variance component, h^2 = narrow-sense heritability estimate, V_E = permanent environmental variance component, R^2 = repeatability estimate, V_R = residual variance component, * standardised covariates (mean centred and scaled by standard deviations), NA = Not applicable (variance component was not significant), NC = Model fit failed to converge, grey = morphometric traits, white = life-history traits. Significant values for heritability and repeatability at $p < 0.001$ in bold, at $p < 0.05$ underlined. Note, the phenotype of the mother was also included as a common environmental variable but was non-significant in all models and therefore omitted here.

Table 3 Summary statistics from PEDANTICS for the four genetically-validated pedigrees reconstructed from the multilocus genotypes of 243 green turtles in COLONY2

	Pedigree A	Pedigree B	Pedigree C	Pedigree D
Total maternities	243	243	243	243
Total paternitites	243	243	243	243
Full sibships	93	81	87	111
Total maternal sibships	498	473	519	513
Total maternal half-sibships	405	392	432	402
Total paternal sibships	327	309	329	357
Total paternal half-sibships	234	228	242	246
Total maternal grandmothers	37	36	42	46
Total maternal grandfathers	37	36	42	46
Total paternal grandmothers	0	0	0	0
Total paternal grandfathers	0	0	0	0
Pedigree depth (generations)	4	3	5	4
Founders	125	131	123	122
Mean maternal sibship size	4.26	4.05	4.42	4.05
Mean paternal sibship size	3.20	3.07	3.16	3.28
Non-zero inbreeding coefficients	0	0	0	0
Mean pairwise relatedness	0.00760	0.00700	0.00788	0.00832
RC: Unrelated (0)	75720	68210	64903	64320
RC: 0.025	24	0	84	111
RC: 0.05	102	80	176	271
RC: First cousins (0.125)	249	180	286	353
RC: Half siblings (0.25)	747	714	773	774
RC: Full siblings / P - O (0.5)	579	567	573	597
pairwise relatedness ≥ 0.125	0.02332	0.02095	0.02443	0.02597
pairwise relatedness ≥ 0.25	0.01964	0.01837	0.02015	0.02064
pairwise relatedness ≥ 0.5	0.00857	0.00813	0.00858	0.00899
N_b	133 (104 - 169)	141 (112 - 178)	129 (103 - 163)	122 (94 - 157)

RC = relationship categories, N_b = effective population size of founder generation (mean ± 95% confidence interval in parenthesis), shaded area = Pedigree A which was employed for estimating the age at maturation and conducting the quantitative genetic analysis.

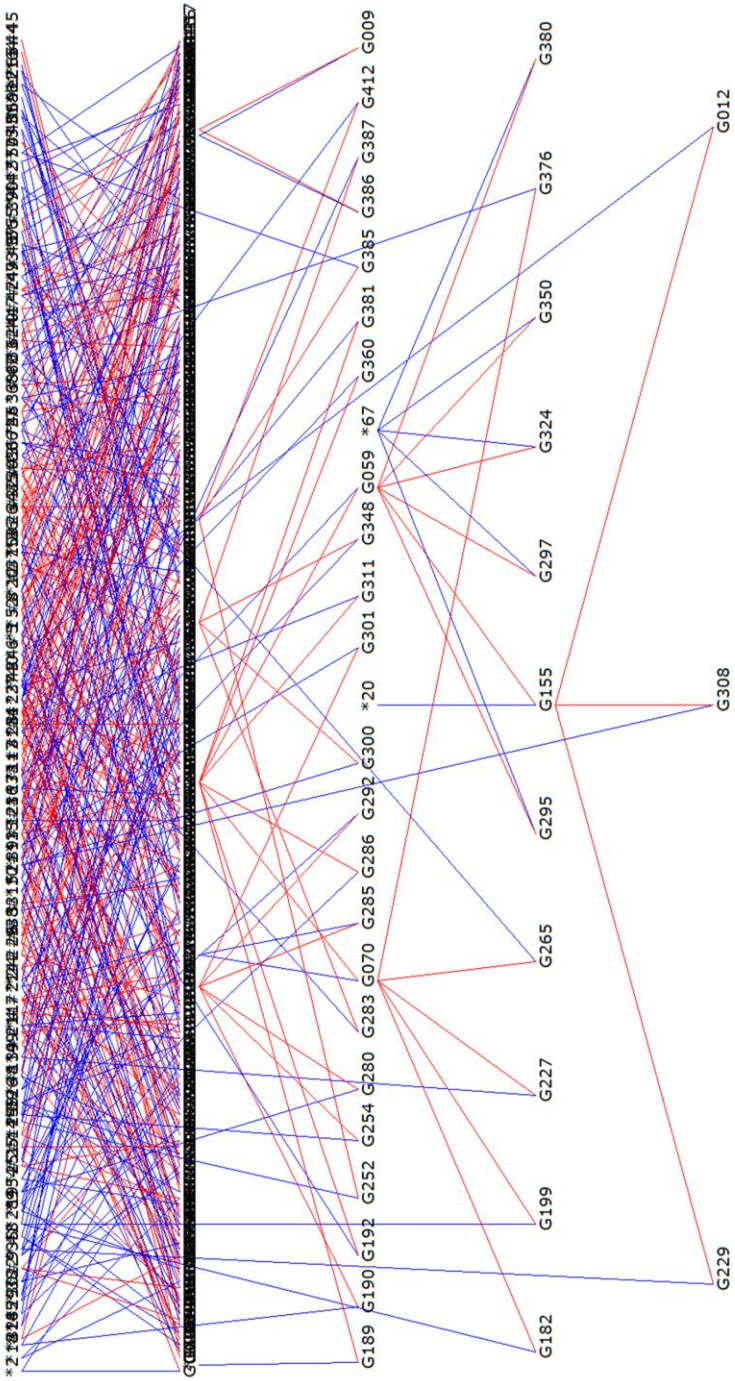

Figure 2 Genetically-validated pedigree 'A' reconstructed from multilocus genotypes and visualised in Pedigree Viewer. Red lines = maternal links, blue lines = paternal links, Alphanumeric code (i.e G308) denotes turtle identification in order of encounter (i.e. G001 was the first turtle observed in 1992), star-numeric (i.e *20) = reconstructed paternal genotype, hashtag-numeric (i.e. #45) = reconstructed maternal genotype. Top level (level 0) = founder generation of reconstructed genotypes, subsequent levels (1 – 4) = inferred relationship structure of sampled candidate offspring and reconstructed parental genotypes in proposed generations.

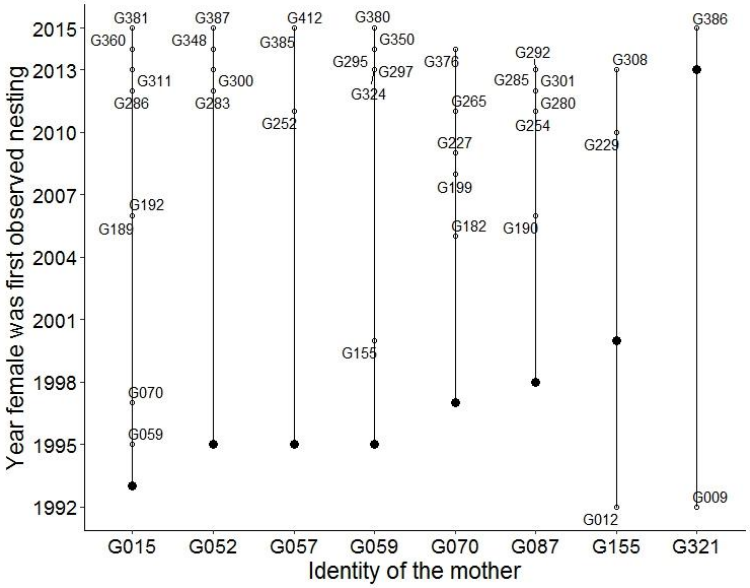

Figure 3 Parent-offspring assignations from the reconstructed 'Pedigree A' with the year that each mother = black circle and offspring = open circle were first observed nesting at Alagadi. Offspring identity denoted on graph, mother's identity denoted as x axis labels.

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Chapter 4 Supplementary Information

Description of each trait of interest and the corresponding 'animal model'

1. **Female Size**: The curved carapace length was measured notch to notch (CCL min, Bolten (1999)) to the nearest 0.1 cm subsequent to each nesting event and averaged over the season for each breeding season a female was observed. The quantitative genetic analysis for female body size was conducted twice with the phenotype conditioned on the females 'Reproductive status' categorised as a neophyte or remigrant within both analyses as females are smaller when they are neophytes than when they return as remigrants. In the second analysis (CCL-2, Table 2 main text) we also included foraging area location as evidence was found for females to mature at different sizes depending on birth year and foraging area location (Chapter 3).

2. **Hatchling mass:** Hatchlings were prevented from leaving the nesting site after emergence by placing a wire cage around the nest from 20:30 – 05:30; cages were left open during the day. Nests were checked for hatching events every 20 - 30 minutes during the night. When a nest hatched, approximately ten hatchlings were selected at random (range = 1 – 124) and weighed to the nearest 0.1 g and a mean calculated for each nest. Hatchling mass can be influenced by the size of the mother in addition to the clutch size and the incubation temperature of the egg (Bjorndal & Carr 1989; Booth et al. 2004). Therefore we conditioned the phenotype on the size of the mother (CCL) and the date that the nest was made (Date of Nest) as this will influence the incubation temperature of the egg.

3. **Interbreeding interval:** Empirical interbreeding intervals were used as observer effort is sufficient to allocate 99% of green turtles nests to a specific female (Stokes et al. 2014). The interbreeding interval reflects the time taken to attain a suitable body condition for reproduction and other foraging area specific environmental factors (Chapter 3, Broderick et al. 2002; Broderick et al. 2003; Hays 2000). Therefore we conditioned the phenotype on foraging area location from Chapter 2.

4. **Clutch size:** Clutch sizes were calculated from nest excavations as they are highly correlated with number of eggs counted at the time of laying (Broderick et al. 2003). The phenotype was conditioned on the size of the mother as clutch size is correlated with, and limited by, female body size (Chapter 3, Bjorndal & Carr 1989). As clutch size significantly increases throughout the season at the population level (Bjorndal & Carr 1989) and over successive clutches at the individual level (Broderick et al. 2003) we included clutch order (1 – 6) as an additional common environmental variable.

5. **Expected clutch frequency:** Observed clutch frequencies were adjusted within season as per Broderick et al. (2002) and Stokes et al. (2014) to incorporate imprecise natal

site fidelity (Chapter 3). The phenotype was conditioned on the length of the interbreeding interval previous to the nesting season and the date that the first nest of the season was made (First nest phenology) as fewer nests are laid by females which start nesting later in the season (Chapter 3).

Genetically-validated Pedigrees B - D

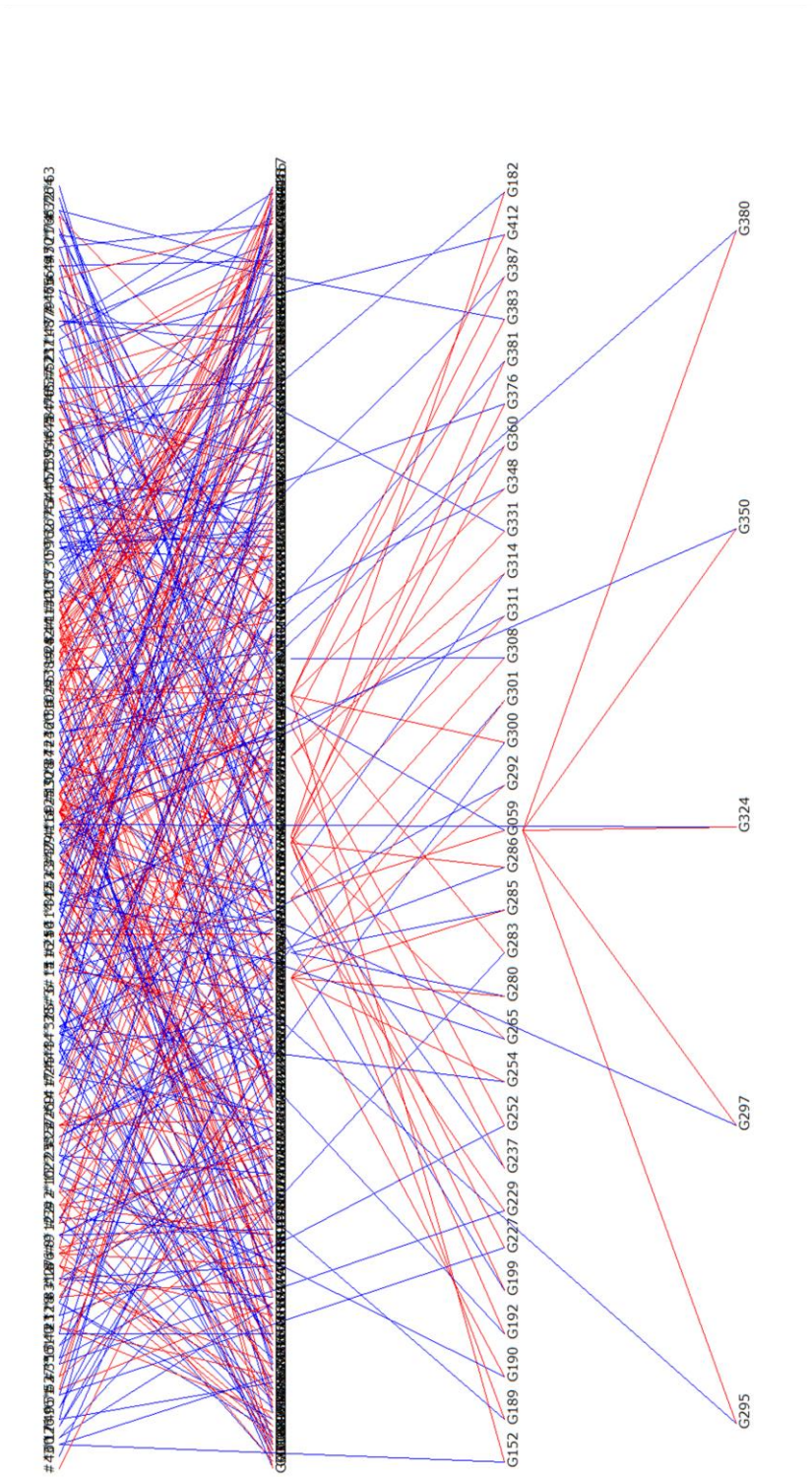

Figure S1 Genetically-validated 'Pedigree B' reconstructed from multilocus genotypes and visualised in Pedigree Viewer. Red lines = maternal links, blue lines = paternal links, Alphanumeric code (i.e G308) denotes turtle identification in order of encounter (i.e. G001 was the first turtle observed in 1992), star-numeric (i.e *20) = reconstructed paternal genotype, hashtag-numeric (i.e. #45) = reconstructed maternal genotype. Top level (level 0) = founder generation of reconstructed genotypes, subsequent levels (1 – 3) = inferred relationship structure of sampled candidate offspring and reconstructed parental genotypes in proposed generations.

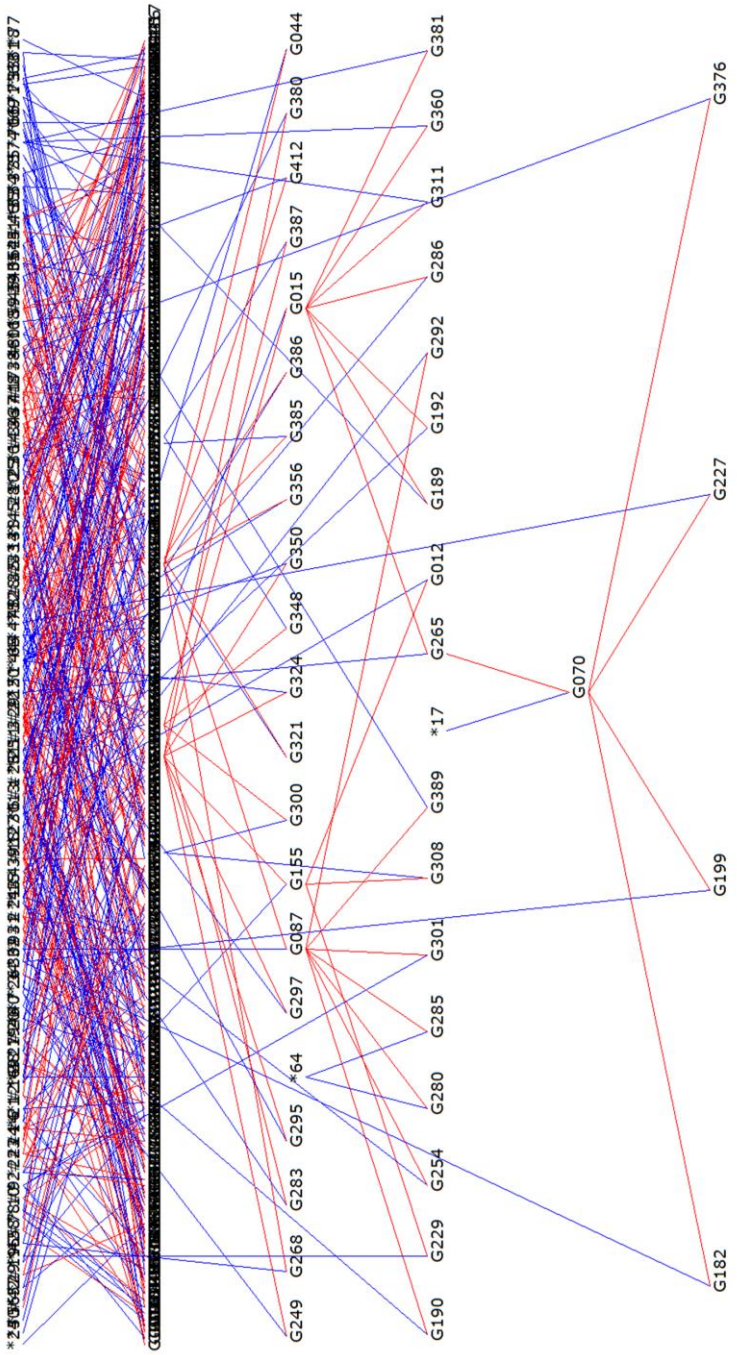

Figure S2 Genetically-validated 'Pedigree C' reconstructed from multilocus genotypes and visualised in Pedigree Viewer. Red lines = maternal links, blue lines = paternal links, Alphanumeric code (i.e G308) denotes turtle identification in order of encounter (i.e. G001 was the first turtle observed in 1992), star-numeric (i.e *20) = reconstructed paternal genotype, hashtag-numeric (i.e. #45) = reconstructed maternal genotype. Top level (level 0) = founder generation of reconstructed genotypes, subsequent levels (1 – 5) = inferred relationship structure of sampled candidate offspring and reconstructed parental genotypes in proposed generations.

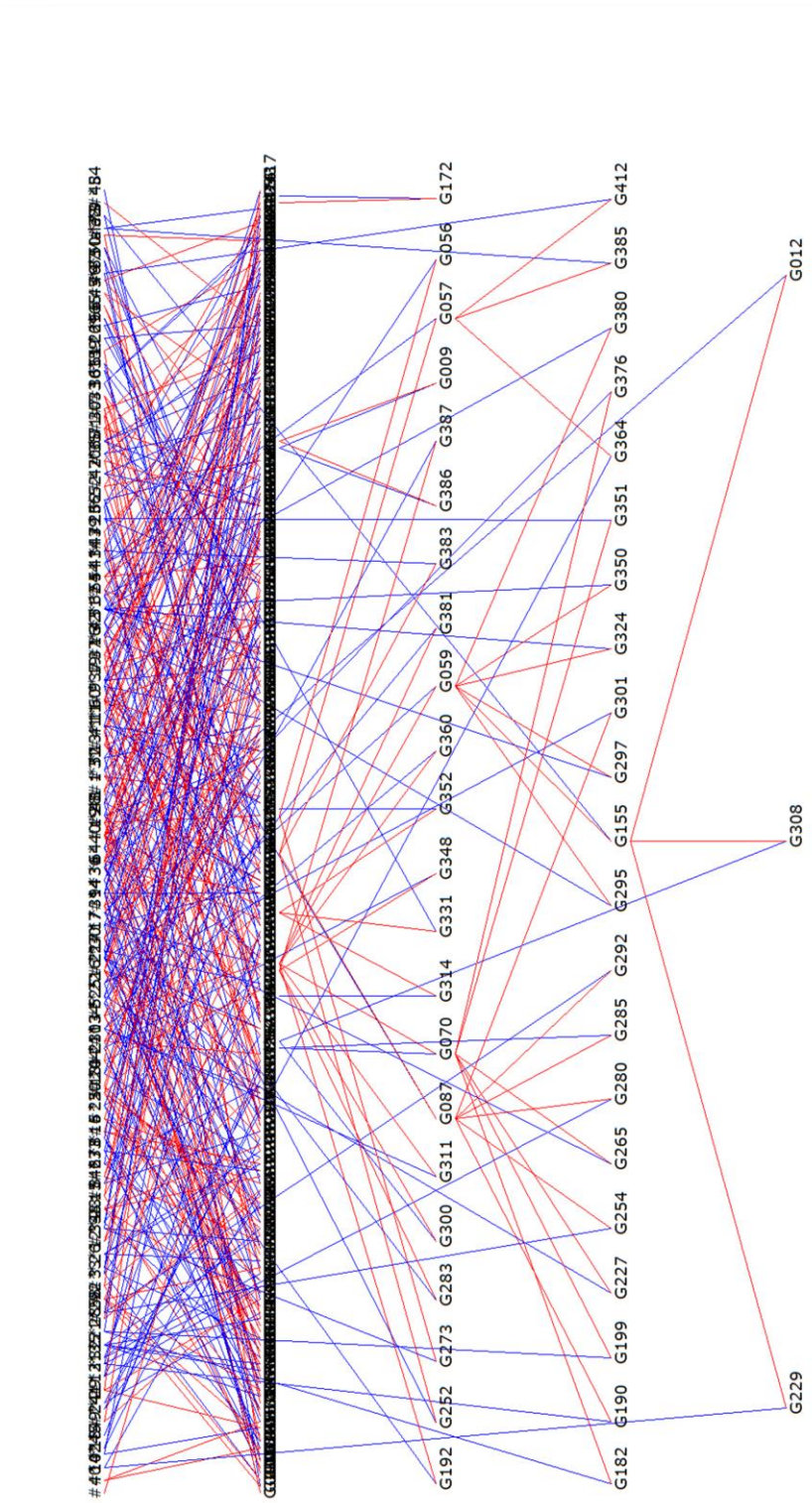

Figure S3 Genetically-validated 'Pedigree D' reconstructed from multilocus genotypes and visualised in Pedigree Viewer. Red lines = maternal links, blue lines = paternal links, Alphanumeric code (i.e G308) denotes turtle identification in order of encounter (i.e. G001 was the first turtle observed in 1992), star-numeric (i.e *20) = reconstructed paternal genotype, hashtag-numeric (i.e. #45) = reconstructed maternal genotype. Top level (level 0) = founder generation of reconstructed genotypes, subsequent levels (1 – 4) = inferred relationship structure of sampled candidate offspring and reconstructed parental genotypes in proposed generations.

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General discussion

In this thesis I effectively demonstrate how indirect methods of assessment can answer fundamental questions concerning the life-history strategy of the green turtle (*Chelonia mydas*). These include priority research questions about their reproductive biology, biogeography and population ecology that have significantly improved our understanding of their population and migratory connectivity and several aspects concerning their reproductive fitness (Hamann et al. 2010, Rees et al. 2016). Although this work focuses on the green turtle nesting aggregation at Alagadi beach in northern Cyprus, these techniques can be applied among marine turtle populations and other cryptic taxa that exhibit a similar life-history (philopatry, long-distance migrations and fidelity to foraging sites). In particular this work should be extended to incorporate all Mediterranean green turtle rookeries to help inform a cohesive regional conservation strategy. This could easily be facilitated through international cooperation and the collection of a single egg from each nest to provide a sample of DNA (Shamblin et al. 2011) and a voucher for the stable isotope signature of the mother (Zbinden et al. 2011, Kaufman et al. 2014, Carpentier et al. 2015), possibly augmented with additional satellite telemetry.

A primary aim for this thesis was to investigate the recent population recovery of the green turtle nesting aggregation at Alagadi (Stokes et al. 2014) by evaluating aspects of their life-history that might influence an individual's contribution to the next generation. To address this question I used a multifaceted approach to investigate rookery and migratory connectivity (**Chapters 1** & **2**), foraging site fidelity and dynamics (**Chapter 2**), phenotype variability from foraging area effects (**Chapter 3**) and trait heritability (**Chapter 4**). Here I highlight what I have learnt and discuss the broader management implications of this research.

Summary of key findings

In **Chapter 1** I employed a high resolution (HR) haplotyping system consisting of a long sequence (~800bp) mtDNA control region sequence concatenated to a series of mtSTRs (Tikochinski et al. 2012) to identify matrilineal stock structure, for the first time, among green turtle rookeries in the Mediterranean. As a result of this research, the four geographically discrete nesting aggregations can now be considered and managed as functionally

independent breeding populations. Furthermore, the stock structure that I detected was among four rookeries within a close proximity that challenges previous conceptions on the geographic distance which delineates green turtle breeding stocks (Dethmers et al. 2006, Jensen et al. 2013). The detection of genetic differentiation among these four rookeries is not only important for the Mediterranean populations, but this also indicates that global marine turtle populations may require a new assessment using higher resolution genetic markers as more studies are detecting significant stock structure among rookeries previously considered to be genetically homogenous (Shamblin et al. 2012, 2015). Evidence for male philopatry was also detected, albeit at a lower level of differentiation than that found with the mtDNA markers. The detection of fine scale genetic structure using maternally and biparentally inherited DNA markers is especially important within this region as it provides greater evidence to readdress the contentious issue of the IUCN subpopulation status of the green turtle in the Mediterranean (Mrosovsky 2006a, b, Naro-Maciel & Formia 2006). I provide evidence for the reproductive isolation of the Mediterranean green turtle populations from those in the Atlantic through the absence of shared mtDNA sequences and a lack of appropriate statistical power in the microsatellite loci employed by Roberts *et al.* (2004) which led to the original delisting. Thus, the green turtle in the Mediterranean is likely to constitute an evolutionary significant unit (Moritz 1994) and should receive an appropriate conservation status to ensure long-term viability.

In **Chapter 2** I used satellite telemetry and stable isotope analysis (SIA) to evaluate the relative importance of four foraging areas in the Mediterranean to the Alagadi nesting aggregation. The novel use of the stable isotope signatures of the turtles to determine which animals to track via satellite telemetry was noteworthy and crucial in the identification of Lake Bardawil as the single most important foraging area for this population. Consistency of stable isotope ratios in stable isotope ratios suggested that at least 82% of the females exhibited a high fidelity to foraging sites over multiple seasons. Quantifying foraging site fidelity is necessary especially important as this allowed the individual-based nesting data to be employed to monitor the annual contribution from the foraging site to each breeding cohort since 1992. Evaluating the foraging area contributions was critical as it highlighted that the recent increase in recruitment was not occurring equally among foraging areas but was primarily occurring from a single site which was previously underrepresented during a 15 year satellite

telemetry campaign. This work emphasised that foraging areas are dynamic and that they need to be continuously monitored as this can reveal site-specific changes in management or ecological and environmental conditions. Furthermore I highlight that Egypt is now a critical foraging site for the green turtle and this information should be utilised to initiate stakeholder discussions and to consider the most appropriate conservation measures.

In **Chapter 3** I investigated the population sub-structuring of the Alagadi nesting aggregation by foraging area using the haplotype and genotype information from **Chapter 1**. The absence of genetic structure among foraging areas provides support for the Learning Migration Goal theory whereby older juveniles and mature individuals take up residence within foraging areas previously encountered during their developmental migrations (Gaspar et al. 2012). Furthermore, as close relatives are not aggregated within foraging areas, the among site variability in turtle phenotype most likely arises from a foraging area effect. To investigate foraging area effects on the phenotype of a turtle, I selected five morphological and life-history traits. Significant foraging area effects were found for body size at maturity, interbreeding intervals and the date that the first nest of the season was made. No phenotypic variability was found for the size or number of clutches laid. Among site phenotypic variability was attributed to resource related carry-over effects from foraging in areas that differ in their ecological and environmental conditions. This work is the first study investigating the phenotypic plasticity of green turtles which forage among geographically discrete neritic habitats and augments a growing body of literature that suggests differential foraging has important implications for the morphology and reproductive fitness of marine turtles. Furthermore, this work demonstrates the possibility that the ecological conditions at each site can be inferred from the breeding frequency of the individuals that forage there.

In **Chapter 4** I investigated the phenotypic similarity among close relatives by uncovering the relationship structure at Alagadi through the reconstruction of a genetically-validated pedigree. The pedigree was both novel and interesting as this is the first time a marine turtle pedigree has been reconstructed using a full-likelihood sibship approach. This work highlighted some of the challenges with reconstructing relationships for a long-lived cryptic species but time and financial constraints prevented me from improving the pedigree accuracy as this required the identification of new microsatellite markers and the

optimisation of the multiplexes. However, the pedigree still provided a significant insight in to the relationship structure of the Alagadi breeding population and directly measured the effective contribution to the next generation. I tentatively provide an estimate of 16 years as the minimum age to maturity but additional sampling is required over a longer time-frame to increase the number of parent – offspring assignations where the true neophyte status of the mother is more certain, although this can never be known. One of the key aims for this thesis was to evaluate if some lineages are more successful than others and if individuals with a similar genotype exhibit a similar reproductive strategy. Therefore I conducted a quantitative genetic analysis to provide the first estimates for the narrow-sense heritability of several morphological and life-history traits using an animal model approach (Kruuk 2004, Wilson et al. 2010). However, I was unable to obtain any meaningful heritability estimates due to sample size constraints. Nonetheless, the pedigree demonstrated that the effective contribution of the breeders to the next generation is highly variable. This raised some concerns for rookery management, especially for small populations, as the effective population size is likely to be lower than the census size.

In summary, this thesis has significantly advanced our current knowledge on the ecology and connectivity of the green turtle in the Mediterranean. This information is useful for informing a regional conservation strategy in order to adequately protect this species, and what I believe should be considered as a critically endangered subpopulation

Broader implications of this research for setting management priorities:

Understanding a species distribution and the connectivity among geographically discrete populations is fundamental for conservation and management. Marine turtle species such as those of the green, loggerhead (*Caretta caretta),* hawksbill (*Eretmochelys imbricata*), olive ridley (*Lepidochelys olivacea*) and leatherback (*Dermocheyls coriacea*) turtle are circumglobally distributed with geographically discrete populations exhibiting varied population trajectories. During glacial and interglacial cycles the abundance, distribution and range of marine turtle populations will have fluctuated with many rookeries experiencing a series of colonisation and extirpation events. Genetic analysis has been vital for elucidating the historical processes responsible for shaping the geographic distributions of these species and revealing their population structure (e.g. Bowen *et al.* 1994, 1998; Encalada *et al.* 1996;

Dutton *et al.* 1999; Bowen & Karl 2007; Leroux *et al.* 2012; Naro-Maciel *et al.* 2014). However, marine turtle DNA evolves very slowly (Avise et al. 1992, FitzSimmons et al. 1995) and new mutations may not reach a sufficient frequency to differentiate among newly diverged populations. This is likely to have resulted in an underestimation of contemporary population structure as it is likely that many rookeries might not have existed for more than the last 10,000 years since sea level rise subsequent to the last glacial maxima (Bowen et al. 1992). This was effectively demonstrated with the Mediterranean case study in **Chapter 1** when the HR haplotyping system demonstrated significant population structure among four rookeries that would have been considered as genetically homogenous using the traditional short or long mtDNA control region haplotypes that have traditionally defined marine turtle population structure globally (Bowen & Karl 2007, Jensen et al. 2013). This raises serious concerns for management as many marine turtle rookeries are conservation-dependent (McClenachan et al. 2006) with small populations facing additional problems associated with the loss of genetic diversity and an increase in the risk of inbreeding depression (Gilpin & Soulé 1986). Therefore it is critical that the population structure of marine turtles is reassessed using genetic markers with a suitable variability considering the demographic history and the geographic context of the studied populations. Determining population structure might be especially relevant as the marine turtle pedigree from **Chapter 4** suggested that the effective population size (N_e) is likely to be lower than current census estimates as family sizes are unequal. However, the paucity of data on the breeding males presents an additional challenge as molecular studies on stock structure and parentage can provide some conflicting views. This might be the case for the green turtle in the Mediterranean as evidence for male philopatry (**Chapter 1**, Bagda *et al.* 2012) does not conform to the large number of sires and a dispersive male population suggested by a robust study on parentage (Wright, Stokes, et al. 2012, Wright, Fuller, et al. 2012).

In addition to defining independent breeding stocks as management units, it is critical that a coherent network of marine protected areas (MPAs) is established within the Mediterranean as fisheries bycatch is unsustainably high with 132,000 reported captures of green and loggerhead turtles per year (Casale 2011, Casale & Heppell 2016). These figures are likely to be a minimum estimate as they do not account for artisanal fisheries (Godley et al. 1998, Snape & Beton 2013) and the exact number of captures from industrial fisheries is unknown

in the areas where green turtles are the most abundant (Casale & Margaritoulis 2010). Inferring the proportion of a nesting aggregation which forage at each site can provide essential scientific guidance to prioritise areas which can achieve the greatest conservation benefits for marine turtles. The effectiveness of these MPAs, or the consequences of not establishing them, can be further monitored through the annual contributions from each site to the natal area if the females are encountered or individuals are inferred through their multilocus genotypes. The research conducted in **Chapter 2** could be extended to incorporate all regional rookeries and this could be easily facilitated as the foraging areas contain mixed stocks (Stokes et al. 2015) and they have already been isotopically characterised**.** However, it is vital to establish the number of males which forage at each site as without this knowledge we might fail to adequately protect them when determining priority areas for conservation.

Establishing the link between the recovery of the Alagadi nesting aggregation and the high level of recruitment from Lake Bardawil was a critical finding of this research. It is unclear at present whether Lake Bardawil represents a sanctuary for green turtles where juvenile survival is increased through the exclusion of industrial fisheries or if foraging conditions are more favourable. Although these hypotheses are not mutually-exclusive, conditions within Lake Bardawil may have prohibited foraging by green turtles prior to the 1990's as salinity levels often exceeded 100 ‰ (Abd Ellah & Hussein 2009) and *Ruppia cirrhosa* was the only seagrass to be recorded within the lake when surveyed in the 1970's (El-bana et al. 2002). Since this time, the channels that connect Lake Bardawil to the Mediterranean have been routinely dredged to improve the Lake's ecological conditions and develop the local fisheries (El-bana et al. 2002, Abd Ellah & Hussein 2009). This reduced salinity levels and allowed *Cymodocea nodosa* to colonise and now dominate the shallow western basin (El-bana et al. 2002). The large area covered by *C. nodosa* in combination with the warm summer temperatures (El-bana et al. 2002, Nada et al. 2013) provides ideal foraging conditions for green turtles. However, the increase in the number of marine turtles within Lake Bardawil has caused some human-turtle conflict with reports of turtles depredating fish from nets and becoming entangled causing damage (Nada et al. 2013). These issues need to be addressed to ameliorate any negative impacts through discussion involving local and international stakeholders as the marine turtle populations in Lake Bardawil are likely to expand further.

Protection of green turtle foraging habitats will provide additional conservation benefits as seagrasses are primary producers which form important and complex habitats that provide a number of ecosystem services (Larkum et al. 2006). The extent of seagrass ecosystems have decreased globally as anthropogenic impacts disproportionally effect coastal areas (Green & Short 2003) and the protection of seagrass beds can offer additional economic benefits as they provide a nursery ground for many commercially-caught species (Larkum et al. 2006). The restoration of healthy green turtle populations is likely to benefit seagrass habitats (Bjorndal & Jackson 2003) promoting a positive feedback system that can be monitored through the annual contributions of turtles to the nesting aggregation (**Chapter 2**) and the phenotype plasticity in interbreeding intervals (**Chapter 3**).

Future research

Determining the number and movement of the breeding males should be a research priority as this has important implications for $N_{e,}$ and the adaptive potential and viability of marine turtle populations. Collecting tissue samples and attaching platform terminal transmitters to males will entail their in-water capture which is beyond the scope of many marine turtle monitoring projects. However, this is a vital area of research that might also explain the apparent inconsistencies between the primary and operational sex ratios (Wright, Fuller, et al. 2012).

It is important to evaluate the effects of human activities on animal populations including the effectiveness of conservation approaches in order to prioritise and efficiently use the limited resources available. This can be challenging for long-lived species as there can be a considerable time lag between an event and an associated demographic response. A key aim for this was research was to determine if the recent population recovery of the Alagadi rookery was a result of the land-based conservation activities initiated in 1993. Thus, it was important to determine the minimum age to maturity as a baseline in which to evaluate this trend. However, due to the longevity of life stages, I was unable to obtain an accurate estimate for the age to maturity and therefore I cannot determine if the nest screening has been successful thus far. The stock structure detected in **Chapter 1** and the strong relationship revealed by the reconstructed pedigree in **Chapter 4** strongly suggest that recruitment is from this breeding population and not due to immigration from other rookeries. Nevertheless, if

green turtles at Alagadi matured at approximately 16 – 20 years of age then I would expect to observe an increase in recruitment among foraging areas if the nest protection activities were the primary driver for the population recovery. An absence of a an increase in recruitment among foraging area indicates that either the maturity estimate is much lower than the actual age to maturity or that high levels of fisheries bycatch might render land-based conservation measures ineffective. This will be elucidated within the near future as additional sampling will augment the marine turtle pedigree allowing a more accurate estimation for the age to maturity.

This thesis emphasises the utility that long-term individual-based monitoring can provide in identifying trends and investigating phenotype variability and aspects of evolutionary biology (Clutton-Brock & Sheldon 2010, Stokes et al. 2014). Measuring fitness through morphological and life-history traits requires dedicated field survey effort over considerable time-scales making them particularly valuable for a quantitative genetic analysis. However, the long generational time of some species such as the green turtle may preclude the animal model approach in favour of genome-wide association studies as the rapid advancement of next generation sequencing will negate the need for the genetic sampling of multiple generations. Therefore future research should focus on developing a suitable panel of single nucleotide polymorphisms (SNPs) to evaluate trait heritability.

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