Molecular ecology of hawksbill turtles *Eretmochelys imbricata* in the Seychelles

Karl Peter Phillips

Thesis submitted for the degree of Doctor of Philosophy

University of East Anglia
UK

School of Biological Sciences

September 2013

Thesis abstract

Molecular genetics are an invaluable tool in whole-organism biology, allowing the indirect investigation of life history traits and evolutionary processes that are otherwise unobservable. In this thesis, I apply molecular genetic techniques to the study of the hawksbill sea turtle *Eretmochelys imbricata*, using a population in the Republic of Seychelles. My aim in this study was two-fold. Firstly, to better characterise certain key aspects of the hawksbill's life history and demography, with a view to better informing hawksbill conservation. Secondly, to test several hypotheses relating to broader evolutionary questions, e.g. regarding the forces shaping mating systems and the link between individual genetic variability and fitness. I found that the majority of females were fertilised by a single male each, and that they used stored sperm to fertilise all of their multiple clutches across a nesting season. There was no evidence of females biasing paternity towards males with particular genetic properties (variability, dissimilarity), suggesting that females are not using sperm storage to promote sexual selection. Males were rarely seen to fertilise more than one female in the study, suggesting low reproductive skew and a large male population that is mobile and/or dispersed. Females at nesting beaches spanning 450 km comprised a single genetic stock, but males were more dispersive than females. I found that the effective size of the population was relatively large, and did not show signs of inbreeding or loss of genetic variation following the substantial reduction in hawksbill numbers caused by historic overhunting. Finally, I found that both male genetic variability and parental genetic dissimilarity can predict nest success, but in a way that might select for a stabilised level of genetic variability. I discuss the implications of these results for both evolutionary biology and the ongoing conservation management of a species internationally listed as critically endangered.

Acknowledgements

Expressing one’s gratitude for a decent supervisor is a difficult task, but extremely decent David Richardson has been over these last four years, and extremely difficult it is to think how to thank him for all that. For his mentoring, for his patience (lots of this!), and for his ability to strip away [my] waffle (lots of this!) and expose the scientific bones underneath, I will be ever grateful. My thanks also to my second supervisor Tove Jorgensen for all her help in getting me this far. I apologise again for once using casual bad language in front of their children!

I thank all the members of the Richardson research group over my time at UEA. They are some of the best people I will ever meet, both as friends and as scientists: Lewis Spurgin, Emma Barrett, Dave Wright, Martijn Hammers, Sjouke Anne-Kingma, Cata Gonzalez-Quevedo, David Padilla, Ellie Fairfield, Danni Gilroy, Kat Bebbington, Ada Natoli, Michelle Simeoni and Helena Batalha. I also thank Tracey Chapman’s research group for helping me better ‘get’ mating systems in the early stages of my project – especially Amanda Bretman and Claudia Fricke.

I thank all of the other great characters and great friends (many both!) with whom I have spent considerable time at UEA. At the head of the list are Jacob Holland and Becky Laidlaw. We started together, finished together, and have shared each others’ ups and downs of Ph.D. life. They are two top-notch friends. In the office I also thank Janet Mason, Lucy Friend, Mohammad Albeshr, Henry Ferguson-Gow, Christiana Faria, and the immortal Conrad Gillett, for distractions, directions, diversions, and general companionship. In the wider corridors, and this is not an exhaustive list, I thank Alice, Alicia, Aly, Amy, Anna, Cat M, Damian, Danni P, Dave C, Freydis, Iain, Kitson (James), Lotty, Martin, Mike, Phil L, Phil S, Sarah M, Sarah Y, Siân, Tom, Will, and the faculty members, all forever burned into my consciousness in nothing but a positive way. A special extra ‘thank you’ to Kitson for the crash-course in word processing that helped me ‘beautify’ my thesis!

Acknowledgements

My thanks to an exceptional friend in Tim Huggins. One day somebody will write a book about him. Thank you also to Neil and Steve for being decent housemates, on the occasions I left UEA and went home!

Thanks to those variously mentioned above who have helped me through my more difficult times. I won’t name you again here – I just want to reaffirm my enduring appreciation for your support.

Away from UEA, I thank the staff and management of Cousine Island, Seychelles, for starting the project, for helping navigate Seychelles bureaucracy, for collecting so many of the samples, and for supporting my stay on the island. In particular, I thank the managers Jock and Janine Henwood, the owner Fred Keeley, and, most especially, the conservation wardens Kevin and San-Marie Jolliffe. Quite what they made of my scrawny, gangly, excruciatingly pale form when it first jumped on to Cousine’s beach, with a silly hat and large camera, I don’t know, but they showed it great hospitality nonetheless. It was a privilege to work with them, and to thereby be able to work with my study species ‘in the flesh’. I thank the current management team of Cousine, and the new conservation warden Julie Gane, for their continued support.

Also in Seychelles I thank Jeanne Mortimer, for advising on various research proposals, for helping with Seychelles bureaucracy, for sharing unpublished data, and for orchestrating the collection of samples from beyond Cousine. Across those other islands, I thank, P-A Adam, T Jupiter, A Nahaboo, D Jupiter, A Duhek, R Jeanne and Island Conservation Society (Desroches, Alphonse, St François Islands); D’Arros Research Centre for enabling Jeanne to collect samples on D’Arros and St Joseph Islands; G Canning and T Raposo (Frégate Island); L Vanherck (North Island); and J van de Crommenacker, N Bunbury and Seychelles Island Foundation (Aldabra).

I thank Sarah Vargas, Mike Jensen and Nancy FitzSimmons for sharing unpublished data with me.

Acknowledgements

I was blessed with spending several months at the NERC Biomolecular Analysis Facility at the University of Sheffield, where I performed the greater part of my lab work. Chiefest of thanks to Andy Krupa (for technical support and for once letting me camp in his garden), as well as to Deborah Dawson, Gavin Horsburgh, Alain Frantz, Lucy Wright (for the use of her primers!), and Terry Burke. I also thank Steph Hodges and Jodie Crane. By putting me up in their spare room they gave me a home-while-away-from-home, and I can't put a value on what that meant.

I thank the folk at the UEA Research Computing Service for supporting my use of the High Performance Computing Cluster, and answering all my inane 'this doesn't work' questions. Similar thanks go to Jinliang Wang of ZSL for answering my many, many questions about the programme COLONY.

I thank my thesis examiners Annette Broderick and Martin Taylor for three hours of challenging but enjoyable discussion on my project, and for their suggestions that improved the presentation of the finished product. I also thank Fred Janzen, Jon Slate, and two anonymous reviewers for helpful comments on chapter 2 during its peer review for publication.

I thank my funders: the UEA Dean of Science for my scholarship, the Natural Environment Research Council for the molecular work, and Cousine Island for considerable in-kind support. I also thank everybody whose financial support allowed me to attend and speak at several conferences: the John & Pamela Salter Charitable Trust, the Richard Warne Memorial Fund, the International Sea Turtle Symposium, and the International Society for Behavioral Ecology.

Finally, last but not least, I thank Old Mother Phillips, Father Bear, and my brother Kurt, for everything they've ever done for me that has allowed me to get to this point. I love them all.

Chapter contributions and publications

All parts of this thesis have been written by Karl Phillips, in consultation with David S. Richardson and Tove Jorgensen. Chapter 3 was also written in consultation with Jeanne Mortimer (Island Conservation Society and University of Florida). The idea of a hawksbill turtle molecular ecology project was initiated by David S. Richardson after a conversation with Jock Henwood of Cousine Island. Below are estimates of percentage contributions to the initial concepts, specific planning, data collection ('conducting'), and data analysis, as well as the publication status, for each chapter.

Chapter 2: published as Phillips KP, Jorgensen TH, Jolliffe KG, Jolliffe S-M, Henwood J, Richardson DS (2013) Reconstructing paternal genotypes to infer patterns of sperm storage and sexual selection in the hawksbill turtle. *Molecular Ecology* **22**: 2301-2312. In the thesis, the 'Methods' section from the publication has been replaced with a reference to the thesis 'General methods'.

- Concept: D Richardson 50%, **K Phillips 50%**
- Planning: D Richardson 30%, J Henwood 20%, T Jorgensen 10%, Cousine Island staff 10%, **K Phillips 30%**
- Conducting: Cousine Island staff 30% (field), **K Phillips 70% (20% field, 50% laboratory)**
- Analysis: **K Phillips 100%**

Chapter 3: in revision following rejection by *Biological Conservation*. To be re-submitted under authorship Phillips KP, Mortimer JA, Jolliffe KG, Jorgensen TH, Richardson DS.

- Concept: D Richardson 50%, **K Phillips 50%**
- Planning: D Richardson 30%, T Jorgensen 10%, J Mortimer 10%, **K Phillips 50%**
- Conducting: Cousine Island staff 30% (field), J Mortimer & colleagues 15% (field), **K Phillips 55% (5% field, 50% laboratory)**
- Analysis: **K Phillips 100%**

Chapter 4: not yet published. To be submitted under authorship Phillips KP, Jorgensen TH, Jolliffe KG, Richardson DS.

- Concept: D Richardson 50%, **K Phillips 50%**
- Planning: D Richardson 40%, T Jorgensen 10%, **K Phillips 50%**
- Conducting: Cousine Island staff 30% (field), **K Phillips 70% (20% field, 50% laboratory)**
- Analysis: **Karl Phillips 100%**

Chapter 5: in review at *Marine Turtle Newsletter* as Phillips KP, Jorgensen TH, Jolliffe KG, Richardson DS (*in review*) Potential inter-season sperm storage by a female hawksbill turtle.

- Concept: D Richardson 30%, **K Phillips 70%**
- Planning: D Richardson 25%, **K Phillips 75%**
- Conducting: Cousine Island staff 30% (field), **K Phillips 70% (20% field, 50% laboratory)**
- Analysis: **K Phillips 100%**

Thesis appendix 2: a report on the implications of this thesis for hawksbill turtle conservation in Seychelles, written for a non-scientific audience. To be sent to Seychelles Government and relevant NGOs under authorship Phillips KP, Jorgensen TH, Jolliffe KG, Richardson DS.

- The above chapters are predominantly set in an evolutionary biology context. This report summarises the results in a conservation context, so as the findings can be accessible to relevant stakeholders and managers, and thereby help inform conservation. It thus functions as knowledge exchange and public outreach.

CONTENTS

List of tables and figures

Chapter 1: General introduction

1.1 Background

1.1.1 Molecular ecology

Molecular ecology describes a broad range of sub-disciplines concerned with the application of molecular genetics to the study of ecology (Andrew *et al.* 2013). The field has expanded vastly since such studies first became possible, as a widening range of new technologies and analysis methods have extended the range of ecological questions that can be asked and the quality of answers that can be obtained (Beebee & Rowe 2004). Questions may range from those explicitly interested in the molecular data itself, such as quantitative trait mapping to link genotypes to phenotypes in the study of natural selection (Storfer 1996; McKay & Latta 2002), to those where genetic profiling is a tool for identifying kinship and testing adaptive behavioural hypotheses, such as those related to mating tactics and kin selection (e.g. Burke *et al.* 1989; Queller & Goodnight 1989). Molecular ecology thus provides biologists with a valuable toolkit for understanding a range of ecological and evolutionary processes.

An important sub-discipline of molecular ecology is conservation genetics, whereby the data and knowledge gleaned from molecular ecology is put to practical use in informing the conservation management of species (e.g. Frankham *et al.* 2002). Again, this encompasses a broad range of questions and processes. In captivity, managers might want to know which individuals should/should not be allowed to mate in order to minimise losses of fitness that might come about from inbreeding, as inbreeding depression has been acutely problematic in some captive species, e.g. primate populations (Ralls & Ballou 1982; Ralls *et al.* 1988; see also Frankham *et al.* 2002). Molecular techniques can also help managers avoid putting two individuals of the same sex together in species where gender is cryptic (Griffiths *et al.* 1998), and avoid inappropriately choosing subpopulations that are too divergent if crosses between separate subpopulations are desired. The latter has been implicated in the failure of captive breeding for the now-extinct dusky seaside sparrow *Ammodramus maritimus nigrescens* (Avise & Nelson 1989). In the wild, the practical applications range from the use of population genetics to define conservation management units (Moritz 1995; Crandall *et al.* 2000; Palsboll *et al.* 2007), to informing the use of translocation in establishing new populations of species – specifically, how many individuals should be taken to capture the genetic diversity of the source population and to maximise the persistence of that variation (Seddon *et al.* 2007; Weeks *et al.* 2011). There is also a role for studies that have less immediate practical application but which generate new information about a species' life history, demography or ecology. Such studies may help managers better understand important demographic processes that may be beyond their control, such as population cycles (Piertney *et al.* 1999; Piertney *et al.* 2008), but are also valuable from a precautionary perspective, as knowledge about a species' mating system or dispersal behaviour may become important to management at a later date.

The molecular ecologist's toolkit is growing all the time, with the continued advancement of laboratory techniques, statistical analysis, and the gathering of supporting observational data on behaviour and movements (e.g. tracking technologies, audiovisual recording; Andrew *et al.* 2013). Among molecular ecology's earliest applications was the discovery of regional differences in the frequencies of human ABO blood groups (e.g. see Allan 1963). Later, protein polymorphisms (Hubby & Lewontin 1966; Lewontin & Hubby 1966), especially allozymes (variants of enzymes coded for by different alleles at a given locus), became important markers for a range of molecular studies, including quantifying population divergence and studying the link between fitness and genetic variability (May 1992). DNA-focused studies were advanced by genetic 'fingerprinting' of highly variable, non-coding DNA (Jeffreys *et al.* 1985) and the development of the polymerase chain reaction (PCR) for amplifying target sections of DNA (Mullis & Faloona 1987). These were followed by several new DNA marker types, including amplified fragment length polymorphisms (AFLPs; Vos *et al.* 1995) and microsatellites, the latter also being known as short/simple sequence repeats or short/simple tandem repeats (SSRs or STRs; Tautz & Renz 1984; Queller *et al.* 1993). DNA sequencing (Sanger & Coulson 1975) also began to be applied to molecular ecology, from phylogenetics to population genetics (Beebee & Rowe 2004). We are currently entering the era of 'next generation sequencing', where very large amounts of sequence data can be generated relatively cost-effectively, allowing for finer-scale, sequence-based study of a range of molecular ecology questions, and potentially using entire genomes (e.g. Hudson 2008; Metzker 2010; Glenn 2011).

Microsatellites are composed of a particular set of 2-5 DNA 'letters' (e.g. CAGA) repeated in tandem, and mutate by slippage of the DNA replication mechanism inserting or deleting one or more repeat units. This results in size polymorphism that can be easily visualised by PCR-amplifying a microsatellite locus, and then size-separating the product by gel electrophoresis. As molecular markers, a combination of characteristics give microsatellites numerous advantages over techniques such as allozymes and AFLPs, and may see them remain the marker of choice for some analyses (e.g. parentage) well into the 'next generation sequencing' era. They are codominant, meaning that one can identify both alleles carried by an individual at a locus. Their relatively fast mutation rate means that they are often highly variable, and are thus powerful discriminators when seeking to identify an individual from a DNA sample. For the most part, they are effectively neutral with respect to natural selection (but see Metzgar *et al.* 2000; Tóth *et al.* 2000; Li *et al.* 2002, 2004). They are relatively easy and cost-effective to characterise in large numbers, and the primers designed often show some degree of cross-species utility (primers can also be designed for cross-species utility, e.g. Dawson *et al.* 2010, 2012, 2013). Finally, multiple loci can be amplified in a single PCR, a so-called 'multiplex', improving the information yield per unit cost. On their limitations, their high mutation rate means that homoplasy (alleles/traits that look identical but have different phylogenetic histories) can become a problem, masking population differentiation as divergence in the system one is studying becomes stronger/deeper (e.g. Balloux *et al.* 2000). The mutation model itself is also not well known – specifically, the relative frequencies of mutations that add/delete only a single repeat unit and those that result in bigger steps. As indicated above, microsatellites may not be as universally selectively neutral as once thought, although this is likely to be heavily influenced by the location of the locus (e.g. expressed v. unexpressed, intron v. exon). Finally, it must always be remembered that they are only markers, and that although they can provide insight into a wide range of processes, they do not provide information on functional variation or the direct relationship between genotype and phenotype. Despite these cautionary notes, microsatellites remain the marker of choice for numerous ecological and evolutionary studies.

1.1.2 Mating systems

Mating systems are one of the most basic and important components of a species' life history, and their study is fundamental to understanding how evolution proceeds (Krebs & Davies 1993; Alcock 2005). However, mating behaviour is often cryptic, making the outcome (parentage) difficult to subject to evolutionary hypothesis testing. Molecular markers are thus essential to the study of mating systems, with one of their key utilities being the identification of parentage patterns. This has advanced understanding of a range of behaviours associated with mating systems, such as extra-pair copulation (Burke *et al.* 1989; Double & Cockburn 2003), intra-species brood parasitism ('egg dumping'; e.g. Lyon & Eadie 2008), cooperative breeding (Richardson *et al.* 2001), alternative mating tactics (Lank *et al.* 2002), and even parthenogenesis (Booth *et al.* 2011).

An important component of many species' mating systems is the ability of females to store sperm from a given mating and to utilise it at a later date. This ability may be central to the species' basic life cycle. For example, it may allow a female to mate in autumn, hibernate, and commence reproduction as soon as spring arrives, without the delay associated with seeking a mate (e.g. Gist *et al.* 1990 (Testudines); Hosken 1997 (Chiroptera)). In social Hymenoptera, it allows a 'queen' to mate on a nuptial flight and then to remain in effective isolation, dedicating the remainder of her life to egg production (Cole 1983; Boomsma *et al.* 2005). Sperm storage also promotes sexual selection, allowing a female to: A) 'trade up', only re-mating if a subsequent male is of superior genetic quality (Halliday 1983; Pitcher *et al.* 2003); B) promote sperm competition, whereby the ejaculates of multiple males compete directly to fertilise the female's eggs (Parker 1970; Birkhead & Hunter 1990); or C) practice cryptic choice, whereby she chooses which male to allocate paternity to after receiving and storing multiple inseminations (Eberhard 1996; Snow & Andrade 2005; Løvlie *et al.* 2013). Sperm storage is thus critical to understanding the evolutionary biology of mating systems. However, its prevalence and importance in wild populations of many taxa remains poorly characterised (Bretman & Tregenza 2005).

A mating system question closely associated with the concepts raised in the previous paragraph is why the females of some species mate with multiple males. This question can be traced back to Bateman's principle, where male reproductive success continues to increase with each additional female he mates with, whereas female reproductive success is limited by the number of eggs she can lay (Bateman 1948). In some systems, multiple mating may be a fertility insurance against the first male producing sperm of poor quality (Sheldon 1994). In other cases, she may obtain nuptial gifts from males that allow her to increase her egg production (Arnqvist & Nilsson 2000), although it is important to note that nuptial gifts can serve other functions (e.g. Wedell 1994). In other systems, a female may use multiple mating to enrol several males in helping to provision her offspring post-birth/hatching (Burke *et al.* 1989). However, in systems where males provide no parental care, the benefits to a female from engaging in mate choice cannot come about through direct means (e.g. nest-building, territory quality, or nuptial gifts), and must instead be indirect, genetic benefits. Genetic benefits models are founded upon the premise that males with particular genotypes or genotypic properties produce offspring of superior quality. These benefits may be through 'good genes', which can be additive (genes that are literally good; Birkhead & Møller 1992; Møller & Alatalo 1999) or non-additive (e.g. male genetic variability; Brown 1997; Fromhage *et al.* 2009), or through 'compatible genes', where the 'best' male genotype for a female to pair with is dependent upon her own genotype (e.g. selecting a partner of suitable genetic dissimilarity so as to maximise/optimise offspring genetic variability; Jennions 1997; Zeh & Zeh 1997). However, multiple paternity also occurs in systems where females do not appear to obtain fitness benefits. An alternative hypothesis to explain such cases is ‘convenience polyandry’, where the costs to a female of resisting male harassment exceed those of allowing additional males to mate (Weigensberg & Fairbairn 1994). Instead of a sexually-selected benefit, multiple mating thus becomes a sexual conflict (*sensu* Holland & Rice 1998), with each sex having a different optimum mating frequency.

1.1.3 Genetic variation: effects on fitness, and its maintenance in populations

Genetic variation is the raw material on which evolution by natural selection acts: between-individual variation in genotype correlates with between-individual variation in phenotype, with selection favouring certain phenotypes over others (e.g. Futuyma 1998; Freeman & Herron 2004). Understanding the relationships between genetic variation and fitness is thus an important goal in evolutionary biology (e.g. Chapman *et al.* 2009). Furthermore, the relationship between variation and fitness has ramifications at the population level, and thereby has implications for conservation management (Frankham *et al.* 2002). Natural selection is not, however, the only process acting on genetic variation. Mutation is constantly generating new variation, and ‘genetic drift’ causes stochastic changes in allele frequencies from one generation to the next. Given sufficient time and the absence of selection, mutation, or immigration, genetic drift will eventually lead to a given locus becoming fixed for a single allele in the population. This process will occur more rapidly in small populations, where it may overwhelm all-but-the-strongest selection (Lacy 1987; Grueber *et al.* 2013). A potential consequence of reduced population-level genetic variation may the lowering of that population’s ‘evolutionary potential’, i.e. its ability to adapt to future changes in the biotic (e.g. disease) or abiotic environment (e.g. climate change; Franklin 1980; Franklin & Frankham 1998).

Small populations are also vulnerable to inbreeding, which is when matings occur between genetic relatives. Although inbreeding does not lead to changes in population allele frequencies, it reduces levels of heterozygosity in the population. Because of this, inbred individuals tend to have lower fitness than the population average (poorer survival or lower reproductive output) due to increased expression of deleterious recessive alleles and loss of overdominance-associated benefits (heterosis) at a large number of fitness-affecting loci (Charlesworth & Charlesworth 1987, 1999). Such 'inbreeding depression' is a serious concern in conservation management, as the poor-quality individuals produced may restrict the ability of the population to recover, and may drive further declines. Such declines lead to the so-called 'extinction vortex', where declines drive further inbreeding, which drive further declines, until the remnant population is wiped out by a stochastic event (Gilpin & Soulé 1986). There are, however, several case studies of populations in apparently terminal decline being subject to 'genetic rescue', where individuals from a different population were allowed to mate with the focal population, introducing new genetic diversity and leading to a turnaround in the populations' fortunes – see, for example, the Florida panther *Puma concolor coryi* (Johnson *et al.* 2010) and the Smygehuk population of adders *Vipera berus* (Madsen *et al.* 1999, 2004). However, it is important to select an appropriate source population in such cases, as if the source is too divergent, the resulting disruption to coadapted gene complexes or local adaptation may have the reverse effect, so called 'outbreeding depression'. As mentioned earlier, this has been implicated in the failed genetic rescue of the dusky seaside sparrow *Ammodramus maritimus nigrescens* (Avise & Nelson 1989). More generally, inbreeding, genetic drift, and evolutionary potential are important considerations when seeking to use translocation to establish new populations of a species, and there is considerable debate and research into the issue of how many individuals to take from the source population in order both to capture population-level genetic variation and to maximise the persistence of that variation in the new location (Franklin 1980; Franklin & Frankham 1998; Weeks *et al.* 2011).

Genetic drift occurs more rapidly in small populations, but the rate of loss of genetic variation in real populations is almost always quicker than one would expect from census counts. This occurs because of violations of the ‘idealised population’ assumptions that underpin the estimation of rates of diversity loss based upon census data. These assumptions include low reproductive variance among individuals (variation is lost more quickly if a small number of individuals contribute disproportionately to the next generation), equal sex ratios (skew increases the rate of loss), random mating (mating preferences coinciding with genetic properties can change rates of loss), non-overlapping generations (can increase or decrease rates of loss), and a stable population size between years (if population size fluctuates, ‘low’ years carry greater weighting in the long-term effect on loss rates than do ‘high’ years). These processes underpin the concept of ‘effective population size’ or N_e (Wright 1931). The effective population size is that of an ‘idealised population’ that loses genetic diversity at a rate equivalent to that of the study population, and is almost always lower, often substantially so, than the census size (Hartl 1988). This can have important consequences for the balance between mutation, drift and selection. In applied terms, it means populations of conservation concern may be more at risk from deleterious processes such as drift and inbreeding than initially thought (Wright 1931; see also Hartl 1988; Frankham *et al.* 2002). To estimate effective population size from molecular data, one would ideally sample a population at a series of time steps and examine the loss of variation across that period (e.g. Waples & Do 2010). However, several methods have been derived that allow for estimation from a single sampling of a population, providing sufficient individuals are sampled and a large enough number of markers are examined (e.g. Robertson 1965; Tallmon *et al.* 2008; Waples & Do 2008; Wang 2009). Procedures also exist to allow researchers to test for recent or sharp changes in effective population size, such as population bottlenecks (Piry *et al.* 1999; Garza & Williamson 2001; Cornuet *et al.* 2008), thus revealing past demographic processes that might have substantial repercussions for present-day patterns of genetic diversity.

Inbreeding depression is a demonstration of the link between genetic variation and fitness on an individual level. The same is true of the ‘compatible genes’ and non-additive ‘good genes’ models of indirect benefits from mate choice discussed in the previous section. Substantial gaps remain in our knowledge of the mechanistic underpinning of this link, and studying the link is challenging in wild populations. In non-model systems, it is rarely possible to know how inbred an individual is, as to do so requires several generations of pedigree data. Furthermore, in populations with low variance in how inbred individuals are, the link between genetic variation and fitness may be more associated with the effects of a small number of loci that show relatively strong effects on fitness. Both inbreeding and single-locus effects can, in theory, be studied using heterozygosity-fitness correlations (HFCs; reviewed in Hansson & Westerberg 2002; Coltman & Slate 2003; Balloux *et al.* 2004; Chapman *et al.* 2009; Szulkin *et al.* 2010). Under this methodology, the ‘general effects’ model is linked to inbreeding depression, which reduces both genome-wide heterozygosity, as measured by a panel of molecular markers such as microsatellites, and fitness. The effect of any one locus is predicted to be small, possibly undetectable, but the cumulative outcome produces a detectable overall effect. In contrast, ‘direct effects’ and ‘local effects’ do not require inbreeding depression (e.g. Hansson *et al.* 2004), although inbreeding may affect the strength and probability of occurrence of such effects (Szulkin *et al.* 2010). Instead, these represent strong fitness effects associated with specific loci. In a ‘direct’ effect, a researcher is able to test for effects, additive and non-additive, of specific genotypes at a functional locus. In a ‘local’ effect, a neutral marker (e.g. a microsatellite) shows an HFC due to being in close linkage disequilibrium with one or several fitness-affecting loci. What ‘local effects’ represent remains a contentious topic, as they are usually found by testing a large number of neutral loci – though a small number in relation to the genome – for potential effects, rather than by screening a set of candidate loci. This creates several statistical difficulties, and may lead to local effects being over-reported (see Szulkin *et al.* 2010). Moreover, the linkage disequilibrium in a local effect does not have to be physical linkage, which leaves the result open to misinterpretation (Szulkin *et al.* 2010). However, ‘general effects’ are also contentious, as it is not clear as to how well marker sets represent inbreeding coefficients, how many loci are required for this representation, or why general effects are sometimes detected in cases where an explicit test for this representation is non-significant (see chapter 4). HFCs and the link between individual genetic variability and fitness remain an open question.

1.1.4 Population genetics

Over time, mutation and drift will lead to genetic differentiation between isolated populations at selectively neutral loci (Wright 1931), with the magnitude of differentiation related to the effective size of the subpopulations, historical demographic processes (e.g. founder events, bottlenecks), the strength of the isolation (migration rates), and the mutation rates of the markers used. The classic mathematical formalisations of population differentiation are Wright's F-statistics, which compare the observed frequency of heterozygosity at a marker to that expected under Hardy-Weinberg equilibrium (Wright 1965). A corollary of this is that population differentiation can help explain deviations from Hardy-Weinberg equilibrium, as the inadvertent pooling of divergent subpopulations will result in a significant deficit of heterozygosity, known as the Wahlund effect (e.g. see Hartl 1988). Wright's F-statistics remain an important tool in the study of population genetics, and their general principle has been extended to encompass additional marker types, mutation models, and demographic scenarios (e.g. see Excoffier *et al.* 1992; Meirmans & Hedrick 2011; Wang 2012). However, a range of other statistical techniques have also been developed, such as Bayesian clustering to detect cryptic population structure (Pritchard *et al.* 2000), and methods based on genotype probabilities and genotype similarities that can detect weaker structure signals than traditional approaches (Mossman & Waser 1999; Prugnolle & de Meeus 2002).

Careful application of population genetics allows ecologists to study the key demographic parameters of population isolation and connectedness without the use of tracking technology or capture-mark-recapture. From this, it then becomes possible to address a range of subsidiary evolutionary and ecological questions. For example, by comparing male- and female-specific markers, or sex-specific to biparental markers, one can infer whether one sex disperses more than the other (e.g. Scribner *et al.* 2001; Goudet *et al.* 2002). At hybrid zones, population genetics can help quantify the size of the contact zone and the magnitude of gene flow (e.g. Szymura & Barton 1986; Hewitt 1990; Haas *et al.* 2009). By comparing data from historical samples to the present day, one can infer whether species distributions fragmented by anthropogenic processes have disrupted gene flow and results in smaller, genetically-isolated populations (e.g. Martinez-Cruz *et al.* 2007). Perhaps its most practical application is to conservation management, where genetic data can help identify population boundaries and thereby assist in the designation of management units (Moritz 1995; Crandall *et al.* 2000; Frankham *et al.* 2002).

1.1.5 Marine turtles

The seven extant species of marine turtles form a single superfamily Chelonioidea (Fig. 1.1). At higher taxonomic levels, this sits within the suborder Cryptodira (all turtles, terrapins and tortoises that retract their head straight back), and in the order Testudines (all turtles, terrapins and tortoises). Two families comprise the present-day Chelonioidea: the Dermochelyidae, which has only a single extant species, the leatherback turtle *Dermochelys coriacea*, and the Cheloniidae, which holds the six hard-shelled species (Fig. 1.1). Although the seven species differ in many aspects of their ecologies, they are extremely similar in their basic life histories, being long-lived, slow-to-mature, migratory, and individually wide-ranging (Pritchard 1997; Plotkin 2002; but see van Dam *et al.* 2008). Adult females only come ashore to nest, and males do not habitually come ashore at all. Nests consist of a large (species means: 50-130; Miller 1997) number of eggs, buried in an egg chamber excavated on a sandy beach, above the high tide line. Adult turtles mate at sea, and no parental care beyond yolk provisioning by females is provided by either sex. Hatchlings dig out of the nest and crawl to the sea. Initial mortality is high, but declines as the individuals grow (Heppell *et al.* 2002). Marine turtles are slow to reach sexual maturity, ranging from 7-12 years for Kemp's ridley (*Lepidochelys kempii*; Turtle Expert Working Group

2000) to 45 years for loggerheads (*Caretta caretta*; Scott *et al.* 2012). Finally, adults of all species are highly migratory, with many making post-reproductive journeys of, or exceeding, several hundred kilometres (Plotkin 2002; but see van Dam *et al.* 2008). Unfortunately, when away from their nesting beaches, marine turtles are inherently difficult to study by direct observation. Returns of flipper tags have provided important insights into habitat use, nest site fidelity and the extent of migrations (e.g. Musick & Limpus 1997; Plotkin 2002). Satellite tracking, along with fitting depth gauges to tracker transmitters, have been something of a revolution to the studies of diving behaviour and post-reproductive migration, but such devices remain expensive and of limited lifespan (reviewed in Godley *et al.* 2008). In some species, there is also a tendency for tracking studies to be female-biased, owing to females being much easier to capture than males (Godley *et al.* 2008; Wright *et al.* 2012b). Furthermore, neither satellite transmitters nor flipper tags can be applied to hatchlings. Thus, from birth to reproduction, many fundamental aspects of marine turtle life history remained, at best, poorly characterised. Fortunately, the careful application of molecular techniques, applied to sufficient sample sizes, provide a powerful investigatory tool for examining these cryptic life history processes.

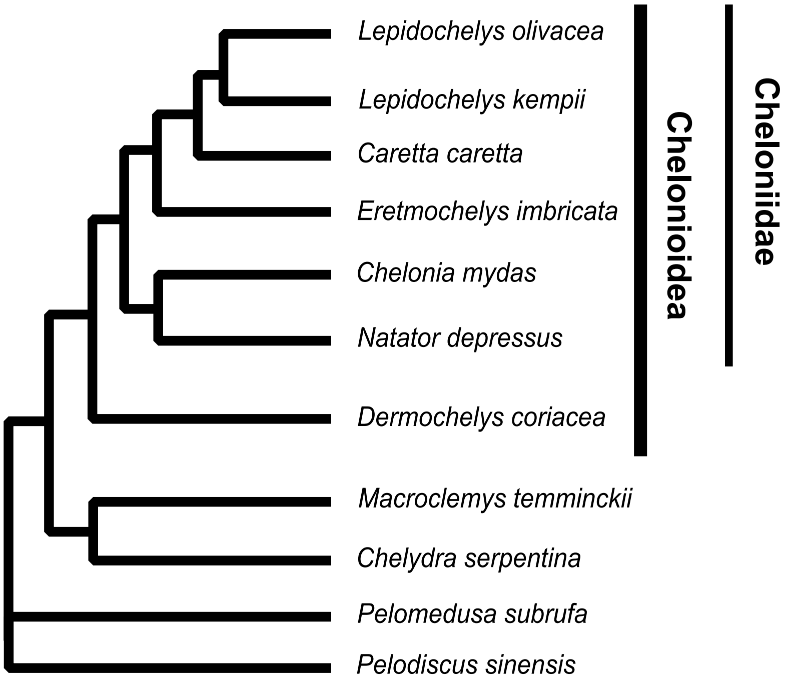

Figure 1.1. Phylogeny of extant marine turtles (Chelonioidea), based on nuclear and mitochondrial DNA (Naro-Maciel *et al.* 2008). Four non-marine species are included as outgroups. Branch lengths are not parametric.

Humans have a long history of exploiting marine turtles, both for food and to derive products from their shells and skeletons (Frazier 2002). Female marine turtles are extremely vulnerable when nesting, and clutches of eggs are often easily located. Furthermore, the long generation times of marine turtles make them particularly vulnerable to complacent overhunting: in theory, one could kill every female nesting at a particular location in a given year, but it could take in excess of two decades for the demographic consequences to be seen (Meylan & Donnelly 1999; Heppell *et al.* 2002). Additional human-derived threats faced by these species include accidental capture in fishing equipment, nest predation by domestic and feral animals (e.g. dogs, pigs), and development and disturbance at nesting sites, which affects both nesting females and emerging hatchlings (Lutcavage *et al.* 1997; Campbell 2002). Collectively, these have contributed to the extirpation of marine turtles from large areas of their former ranges, and to substantial reductions in population sizes in the locations where they still occur. Today, three species are listed by the IUCN as Critically Endangered (the leatherback *Dermochelys coriacea*, the hawksbill *Eretmochelys imbricata*, and Kemp's ridley *Lepidochelys kempii*), two as Endangered (the green turtle *Chelonia* *mydas*, and the loggerhead *Caretta caretta*), and one as Vulnerable (the olive ridley *L. olivacea*). The seventh species, the flatback *Natator depressus*, is listed as Data Deficient (IUCN 2013). These are creatures of international value, but to better appreciate this value, and to conserve them for future generations to value, we need to address the many outstanding questions that remain about their biology and ecology (Hamann *et al.* 2010).

The application of molecular genetics to the study of turtle life histories has, so far, predominantly proceeded along two routes of questioning. The first has concerned population structuring, and began with the testing of the natal philopatry hypothesis: do adult female turtles return to their regions of origin in order to nest. This was one of the longest-standing questions in marine turtle biology (Carr 1967; see also Bowen & Karl 1997), but, because hatchlings cannot be tagged, it was effectively unanswerable without genetic techniques. Meylan *et al.* (1990) were the first to test this, using green turtle rookeries (nesting grounds) in the west Atlantic. Their study, examining maternally-inherited mitochondrial DNA (mtDNA) samples collected from four rookeries, showed significant population structure between rookery pairs that was strongly indicative of female natal philopatry (Meylan *et al.* 1990). In loggerhead turtles in the North Atlantic, testing for mtDNA structure has highlighted long-distance migrations by young turtles. The rookeries of the North Atlantic show substantial population structuring (Bowen *et al.* 1993a; Laurent *et al.* 1993; Encalada *et al.* 1998), but structure is absent among pelagic post-hatching juveniles and extremely weak between aggregations of feeding sub-adults (Bowen *et al.* 2005). Furthermore, juveniles with western Atlantic haplotypes have been identified feeding in the eastern Atlantic and western Mediterranean (Bolten *et al.* 1998; Carreras *et al.* 2006). Collectively these results have serious implications for the conservation management of this species, as they mean that management decisions taken one area, e.g. the Mediterranean, could have repercussions that eventually filter through to affect nesting populations in another area, e.g. 7000 km away in North America. On a smaller scale, albeit one that still crosses numerous international borders, mtDNA structure has been used to highlight the connections between rookeries and juvenile feeding grounds in the

Caribbean population of hawksbills (Bowen *et al.* 2007a; Blumenthal *et al.* 2009). On occasion, this has led to intense debate over whom the turtles 'belong' to, and highlights the challenges faced by managing species populations across borders (Bowen *et al.* 2007a, 2007b; Godfrey *et al.* 2007; Mortimer *et al.* 2007a, 2007b).

In marine turtles, population genetic structure quantified from nuclear markers, such as microsatellites, tends to be weaker than that at mitochondrial DNA, which several authors have argued for as evidence of male-biased dispersal (for reviews, see Bowen & Karl 2007; Lee 2008). In cases where the difference is relatively small (e.g. Stiebens *et al.* 2013), this interpretation needs treating with caution, as mtDNA population structure is always expected to be stronger due to its having an effective population size a quarter that of nuclear DNA – mtDNA is haploid rather than diploid, and maternally-inherited rather than biparentally inherited. Caution is also needed because mtDNA and microsatellites have very different mutation models, making measures of differentiation difficult to compare between the marker classes. In other cases, though, where the difference in estimates of structure is very large, the results are more compelling (e.g. Bowen *et al.* 2005). Generalisations about the processes affecting population structure at nuclear markers in marine turtles are much more difficult to make than for mtDNA, as such studies are fewer, and molecular data from adult males are rarely available for comparison. FitzSimmons *et al.* (1997a, 1997b) found both mtDNA and nuclear structure between four major Australian nesting populations of green turtles, with the exception of between the two populations on the east coast. These showed structure at mtDNA but not microsatellites, potentially indicating dispersal by males. However, males captured in the vicinity of the nesting areas showed mtDNA more consistent with philopatry, and the researchers thus concluded that mating on migration routes, which overlap for this population pair, was responsible for the male-biased gene flow rather than male dispersal *per se*. On a finer scale, Lee *et al.* (2007) used male microsatellite genotypes reconstructed from paternity studies to suggest that female green turtles nesting on Ascension Island, Atlantic Ocean, engaged in natal philopatry whereas males did not.

The second major route of molecular research into marine turtle life histories has concerned the study of mating systems. Observations of wild turtle matings are inherently difficult to conduct (Booth & Peters 1972; Wood & Wood 1980), and even when this can be done, it is not possible to know how observed behaviours translate into paternity patterns. Among the questions that genetic studies of mating systems have been able to address are the number of males a female typically mates with, whether any males fertilise multiple females nesting on the same beach, and whether females store sperm (reviewed in Lee 2008; see also Theissinger *et al.* 2009; Joseph & Shaw 2011; Stewart & Dutton 2011; Wright *et al.* 2012a). The data from such studies provide important demographic information, including, if the sampling is comprehensive enough, on the number of males contributing to a breeding location. They also provide a foundation for testing hypotheses on the evolution of mating systems that will be of interest to the wider evolutionary biology community. For example, why does multiple paternity occur in marine turtle nests, why do some females show it but others do not, and why does its rate vary between populations and species? Multiple paternity in marine turtles has yet to be shown to benefit a female in terms of offspring production, although it is important to emphasise that this has rarely been explicitly tested, and that tests can only examine nest success parameters (Lee & Hays 2004; Lasala *et al.* 2013; Wright *et al.* 2013). However, several authors have argued that variation in multiple paternity rates between marine turtle populations may be correlated with population size (Ireland *et al.* 2003; Lee & Hays 2004; Jensen *et al.* 2006). The rationale behind this is that females experience more male harassment in larger populations, and that this drives higher rates of multiple paternity through convenience polyandry (see section 1.1.2). There are several difficulties with this model for marine turtles, such as whether population size is a good proxy for population density at the time of mating – the process could be confounded by large populations remaining dispersed or small populations aggregating more densely. Another difficulty is that it is unsafe to draw general conclusions from preliminary meta-analyses that treat each marine turtle paternity study as an independent data point (Ireland *et al.* 2003; Jensen *et al.* 2006), as although intra-species variation in rates of multiple paternity is large and may exceed inter-species variation (see Lasala *et al.* 2013), such tests do not control for the lack of phylogenetic independence (see Freckleton 2000) between data points. However, the convenience polyandry model demonstrates how studies on marine turtles have the potential to contribute to a variety of evolutionary discussions, including sexual selection and sexual conflict. In the only targeted test of the convenience polyandry hypothesis in marine turtles, Jensen *et al.* (2006) showed that multiple paternity rates were substantially higher among female olive ridley turtles nesting at an *arribada* (mass-nesting) site than at a non-*arribada* site (92% v. 30%). *Arribada* nesting is preceded by relatively dense aggregations of turtles offshore from the *arribada* site, which may create a strong selective pressure for convenience polyandry (Jensen *et al.* 2006). However, it is difficult to generalise from this study, as it is a comparison between two populations and thus has n = 1. Interestingly, Zbinden *et al.* (2007) report that bigger female loggerhead turtles in the Mediterranean are more likely to have been fertilised by multiple males. A similar effect was recently reported in loggerheads of the west Atlantic (Lasala *et al.* 2013). Zbinden *et al.* (2007) argue that bigger females might be preferentially targeted by males because bigger females lay more eggs and thus offer greater potential fitness returns for males mating with them. Again, this demonstrates the potential for marine turtles to inform much wider-ranging evolutionary discussions than simply those about turtles.

1.1.6 The hawksbill turtle

The hawksbill turtle *Eretmochelys imbricata* L. (Figs 1.2-1.7) occurs throughout the world's tropical oceans, where the adults feed predominantly on sponges and soft corals. In this environment, there is evidence that hawksbills fill a keystone role in reef ecosystems by affecting the competitive balance between sponges and corals (Bjorndal & Jackson 2002; Leon & Bjorndal 2002). Precise population size estimates for hawksbills, as for all marine turtles, are difficult to obtain, owing to long generation times, non-annual nesting, and males remaining at sea, but census data compiled for the species' IUCN Red List assessment in 2008 put the global number of hawksbill females nesting in 2005 at just over 10,000 (Mortimer & Donnelly 2008). Globally, hawksbills have traditionally been hunted for their shells, the scutes of which, more than any other species, are used to make ‘tortoiseshell’ or ‘bekko’ products (Mortimer 1984; Mortimer & Donnelly 2008; e.g. Fig. 1.8). Overhunting has driven substantial population declines across the species’ distribution, declines that have contributed to the hawksbill being classified as Endangered by the IUCN since 1968, and upgraded to Critically Endangered in 1996 (see Meylan & Donnelly 1999). Hawksbill products from all ocean basins have been listed on Appendix I of the UN Convention on International Trade in Endangered Species (CITES) in 1977, although Japan retained a reservation until 1992.

Figure 1.2. Nesting female hawksbill turtle, Cousine Island. Photo by the author.

Figure 1.3. Nesting female hawksbill turtle, Cousine Island. This individual has a damaged shell. Photo by the author.

Figure 1.4. Hawksbill female returning to sea after nesting, Cousine Island. Photo by the author.

Figure 1.5. Hawksbill female returning to sea after nesting, Cousine Island. Photo by the author

Figure 1.6. Hawksbill turtle hatchling, Cousine Island. Photo by the author.

Figure 1.7. Hawksbill turtle hatchlings heading to the sea, Cousine Island. Photo by the author.

Figure 1.8. Example of a hawksbill turtle shell product. This candle holder belonged to my great aunt. The right-hand image shows the warm translucence and textured colouring for which hawksbill shell is prized, and for which it has been overhunted in many parts of its distribution. Photo by Conrad Gillett.

Despite their global distribution and internationally high-priority conservation status, many aspects of hawksbill life history and ecology remain poorly known. Furthermore, the data that are available are heavily biased towards a few, better-studied areas, especially the Caribbean. Outside these locations, including in some of the most important hawksbill population areas, knowledge is poorer still. In this thesis, I conduct a molecular genetics study into the hawksbill population of the Republic of Seychelles in the western Indian Ocean, one of only five global hawksbill populations that held over 1000 females at the end of the 20th Century (Meylan & Donnelly 1999). Precise estimates of the historic size of the Seychelles hawksbill population are difficult to obtain, but the current population is estimated to be 30-95% lower than that of three hawksbill generations earlier (3 × 35 years; Mortimer & Donnelly 2008). Hawksbills have only been fully protected under Seychelles law since 1994, but several islands (Aride, Cousin, Curieuse, the St Anne Marine Park, and D’Arros/St Joseph) have enacted local protection since the late 1960s and 1970s, with two others (Cousine and Bird) doing the same in 1992 (Mortimer & Donnelly 2008; Figs 1.9, 1.10). My first objective in this study was to use molecular techniques to better characterise certain aspects of the hawksbill’s life history and demography. Specifically, I wanted to characterise the population’s mating system, test for population structure, and consider the implications for these and other molecular inferences for hawksbill effective population size and ongoing hawksbill conservation in Seychelles. My second objective was to use the data from these life history/demographic characterisation studies to test more general evolutionary principles. These included sexual selection processes operating in the hawksbill mating system, and the role of individual genetic diversity in predicting reproductive fitness.

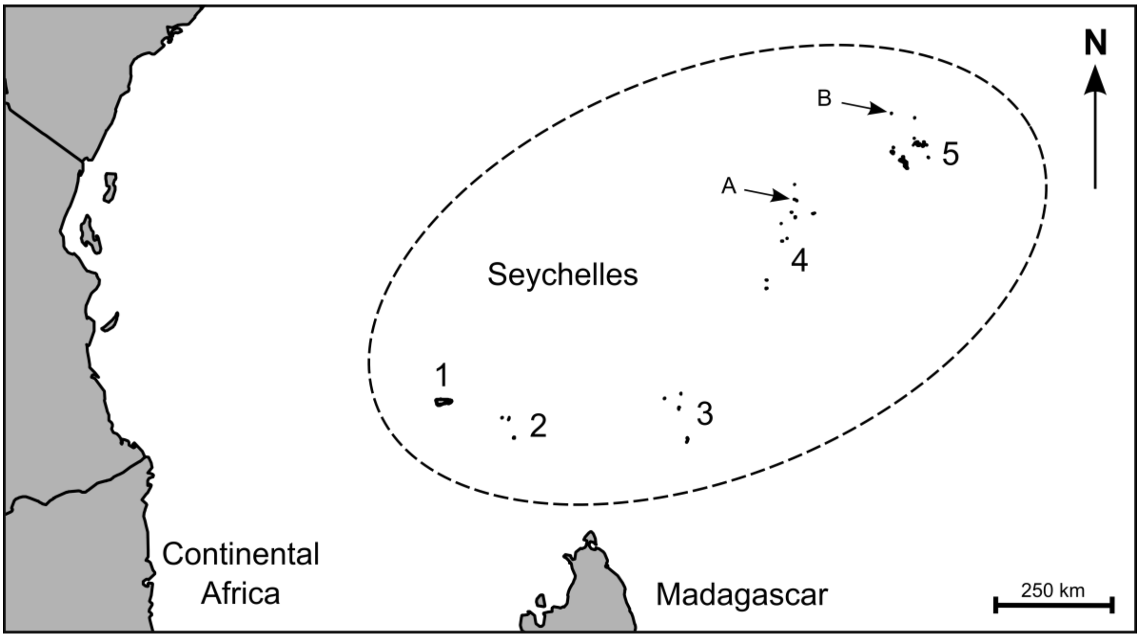

Figure 1.9. Map of Seychelles highlighting major island groups. 1 = Aldabra Atoll; 2 = Cosmoledo group; 3 = Farquhar group; 4 = Amirantes group; 5 = Granitic group. Also indicated are two islands that enacted hawksbill turtle protection prior to the national legislation of 1994: A = D'Arros Island and St Joseph Atoll; B = Bird Island. See also Fig. 1.10.

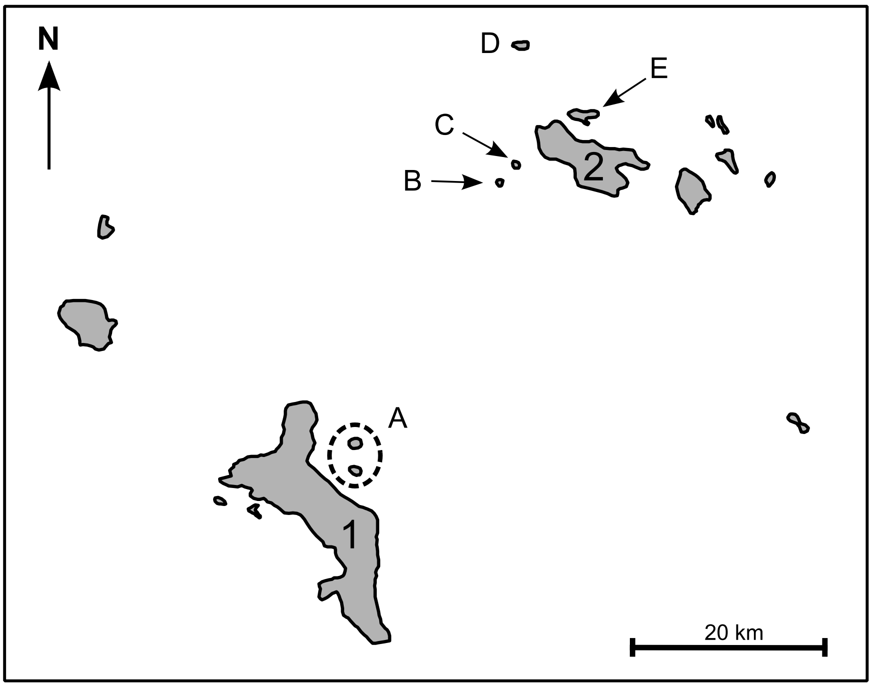

Figure 1.10. Map of Granitic Seychelles. The two most populous islands of Mahé (1) and Praslin (2) are highlighted, along with five sites that enacted hawksbill turtle protection prior to the national legislation of 1994: A = St Anne Marine Park; B = Cousine Island; C = Cousin Island; D = Aride Island; E = Curieuse Island.

1.2 Methods

The greater part of the methods used in this thesis are common to all data chapters, especially with regards to fieldwork, laboratory work, and the statistical procedures used in parentage analysis and paternal genotype reconstruction. Rather than describe these three times, or have the reader refer back to a specific data chapter, I describe them in greater detail here. Chapter-specific variations and additions remain in their relevant chapters.

1.2.1 Field methods

Chapters 2, 4 and 5 are based entirely on studying the hawksbill turtles nesting on Cousine Island, Republic of Seychelles (04° 21′ S, 55° 38′ E; Figs 1.9-1.11), whereas chapter 3 also draws on data from samples collected from other Seychelles islands (Fig. 3.1). Here, I outline the turtle study methods as used on Cousine, and give some background on this specific island. Details of the additional islands sampled for chapter 3, and variations on the field methods deployed on those islands, are given in that chapter's "Methods" section (3.3).

Cousine is a small (25 ha), granitic island, located 40 km northeast of the Seychelles' largest and most populous island of Mahé, and 5 km from the second largest and second most-populous island, Praslin (Figs 1.10, 1.11). Between Cousine and Praslin lies Cousin Island, an important hawksbill nesting site that has been protected as a nature reserve since 1968 (Diamond 1976; Mortimer & Bresson 1999; Allen *et al.* 2010). Since 1991, Cousine has been owned by Mr Fred Keeley, a South African businessman. The island is currently run as both a luxury guest resort and a nature reserve. It is proud of its ecotourism credentials, supporting important populations of seabirds, as well as terrestrial birds, reptiles, invertebrates, and plants that are endemic to Seychelles. At the time of my fieldwork, Cousine had 12-16 staff in residence, including two full-time conservation wardens (Kevin and San-Marie Jolliffe).

Figure 1.11. Cousine Island from Google Earth. The island is approximately 1.0 km long, and 0.4 km wide at its widest point. This image was taken during the northeast monsoon season, as the beach is narrow in the north and broad in the south, and features a lagoon. During the southwest monsoon, the lagoon is absent, and beach material seaward of it is redistributed to the north.

Monitoring of hawksbill nesting on Cousine increased through the 1990s, but the population was poorly known before this point (see Hitchins *et al.* 2004). However, a long-term study on neighbouring Cousin has documented an increase in excess of eight-fold in the number of hawksbill females nesting on that island since the 1970s (Allen *et al.* 2010). This increase has been reflected on Cousine since systematic monitoring began, and a present-day nesting season is likely to see 70-200 nests laid by 30-130 females, and 15-35 new females fitted with unique titanium tags. Tagging on Cousin and Cousine has previously established that an individual female may nest on both islands within and between seasons, that Cousin/Cousine-tagged turtles are recorded nesting on other nearby islands relatively rarely, and that females tagged elsewhere in Seychelles are only rarely seen on Cousin or Cousine (Allen *et al.* 2010). Turtle tags in Seychelles are issued by Seychelles Islands Foundation.

The nesting season for hawksbill turtles in Seychelles is September to February, although occasional nests are laid in other months (K Jolliffe & S-M Jolliffe, pers. comm.). During this period, Cousine's conservation wardens patrol the island's 900 m beach hourly from 6 am to 6 pm to locate and identify nesting females. In contrast to elsewhere in its global range, hawksbill turtles in the western Indian Ocean nest almost exclusively during the day (Diamond 1976). For our study, all females observed during these patrols were measured (curved carapace length, measured by tape measure to the nearest millimetre), tagged (see above), and sampled (6 mm sterile biopsy punch from trailing edge of forelimb), either when laying or as they returned to the sea (Fig. 1.12). The biopsy tool was heat- and ethanol-sterilised after each use. The hawksbill nesting season in Seychelles straddles the reversal of the prevailing monsoon winds, from southwest to northeast. During this time, a large amount of Cousine's beach material is transported from one end of the island to the other over a very short time, to the effect that the beach all but disappears from the northeast corner of the island (Fig. 1.11). To prevent loss of hawksbill nests to this process, the conservation wardens relocate the majority of clutches to 'erosion safe' zones of the beach, and line egg chambers with nets to limit predation from *Oocypode* crabs. Nest relocation is a controversial practice in the marine turtle literature, owing to its potential to increase embryo mortality and distort primary sex ratios (Pintus *et al.* 2009). However, the losses of hawksbill nests to seasonal beach erosion on Cousine are considered too great to justify no intervention, given the present reduced size of the regional hawksbill population. A long-term ambition is to scale back this intervention when the population has increased sufficiently. The numbers of eggs laid by a female were counted by digging up nests within 12 hrs of laying (later disturbance can increase embryo mortality; Limpus *et al.* 1979; Parmenter 1980). Because of the size of Cousine's beach, the wardens are able to locate almost every nest laid in a season and to observe the female at the nest for the majority of these.

Figure 1.12. Karl Phillips and Kevin Jolliffe collecting a tissue sample from a hawksbill female as she returns to the sea. Picture by San-Marie Jolliffe.

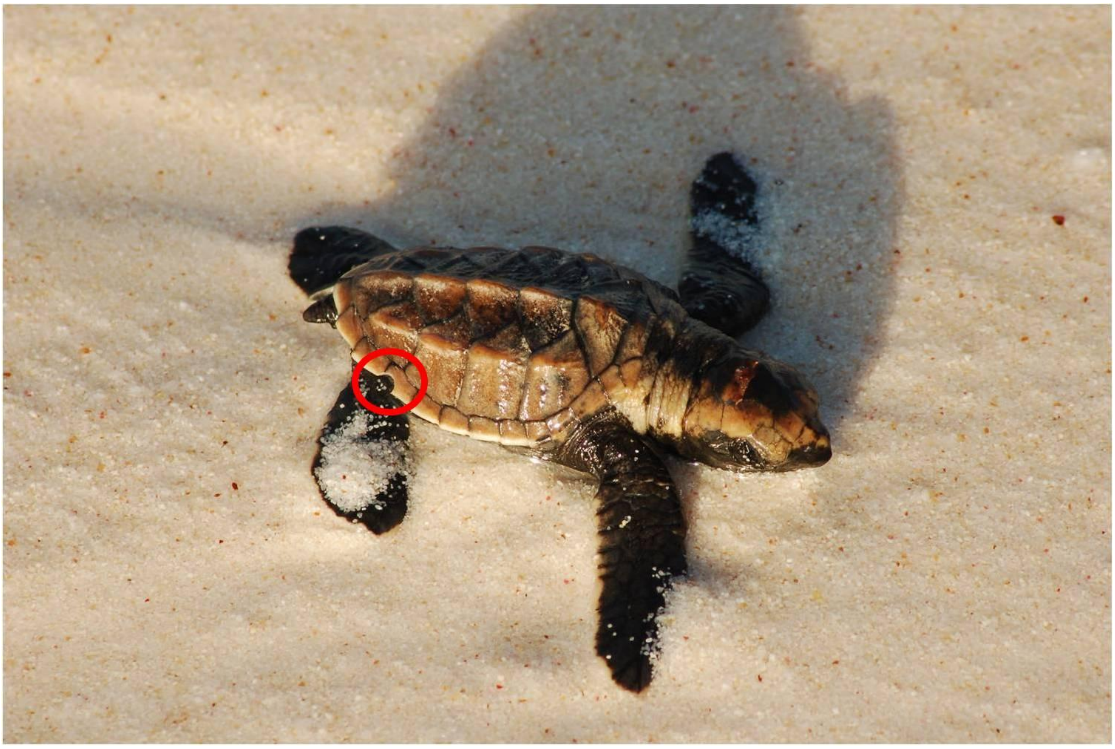

Figure 1.13. Sampled hawksbill hatchling, with tissue sample mark circled.

After 50 days of incubation, nests are checked daily for signs of activity. This is especially important for relocated nests, as the net lining could prevent escape if the nest hatched early. On Cousine, incidence of nests hatching before the 50-day mark was < 1/500 (K Jolliffe & S-M Jolliffe, pers. comm.). Upon release of a nest, live hatchlings were counted into a bucket, and, from these, a tissue sample was taken from up to 20 randomly-chosen individuals per nest. All live hatchlings were sampled from a nest if fewer than 20 hatched. Hatchling tissue samples were taken from the marginal scute above the rear limb using a sterile 2 mm biopsy tool (Fig. 1.13). The tool was cleaned with 99.8% ethanol between sampling each hatchling, and heat-sterilised between nests. Clutches were released by emptying the bucket of hatchlings 4-6 m from the sea, after chasing away any nearby crabs, and allowing hatchlings to crawl the remainder of the way to the water. After release, the numbers of unhatched eggs and dead hatchlings remaining in the nest chamber were counted. Unhatched eggs were examined for any obvious sign of embryo development, and were classes as either ‘developed’ (embryo in evidence) or ‘undeveloped’ (no evident embryo). We have deliberately refrained from referring to undeveloped eggs as ‘infertile’, as we could not distinguish between genuine infertility and very early embryo mortality (Miller 1997; see also Birkhead *et al.* 2008).

All tissue samples were preserved in 1 ml of 99.8% ethanol. Although our tissue sampling techniques were well established in the literature prior to our using them, and were considered to be ultimately harmless, we were nonetheless pleased to see a study of captive loggerhead turtles that indicated such tissue biopsies cause no long-term harm (Bjorndal *et al.* 2010).

1.2.2 Laboratory methods

DNA was extracted using an ammonium acetate method (Nicholls *et al.* 2000). For a full protocol, see Thesis Appendix 1. Briefly, a section of each tissue sample was digested with Proteinase K (Thermo Scientific) and SDS in a Tris-EDTA-NaCl buffer at 55°C for 3 hrs. To avoid cross-contamination, sections were cut on a grid-marked glass plate with a razor blade that was heat-sterilised between samples. Non-DNA cellular material was removed from digested tissue by adding ammonium acetate, centrifuging the mixture, and pouring the supernatant liquid to a new tube. Ice-cold 99.8% ethanol was then added to precipitate the DNA, and the sample centrifuged. The supernatant was poured away, and the DNA pellet was washed with 70% ethanol and re-centrifuged. The supernatant was poured away again, and the tube and pellet were allowed to dry. Dry DNA was re-suspended in a weak Tris-EDTA solution (10 mM Tris, 0.1 mM EDTA) – 50 µl for hatchlings and 100 µl for adults. DNA quality was assessed by running samples on agarose gels. DNA concentrations were then measured by spectrophotometer (‘NanoDrop’, Thermo Scientific), and samples were diluted to a working concentration of 7-10 ng/µl accordingly.

1.2.3 Microsatellite genotyping

Using published primers, I designed three polymerase chain reaction (PCR) microsatellite multiplexes for use on hawksbill turtles. I initially tested all published microsatellite primers previously designed for the hawksbill turtle (FitzSimmons *et al.* 1995; Lin *et al.* 2008; Miro-Herrans *et al.* 2008). Because marine turtle microsatellite primers show high cross-species utility, I tested a further 51 primer sets designed from other marine turtle species. Many of these primers were selected because they were in use by colleagues for other projects (M Simeoni, LI Wright), and 12 of them had not previously been tested on hawksbills (Table 1.1). I also tested seven primer sets that I re-designed from published hawksbill microsatellite flanking sequences (Table 1.2).

After each major batch of genotyping, I re-checked all loci for null allele frequencies, Hardy-Weinberg equilibrium, and linkage equilibrium. Close physical linkage between markers seems unlikely, given that hawksbills have 56 chromosomes (Bickham 1981). After the first batch of genotyping, locus *Cc*P7C08 was found to have too high a null allele frequency to make its inclusion in further analyses safe, and was dropped. The final 32 loci had an average expected heterozygosity of 0.69 ± 0.13, and a combined false inclusion probability of 5.41×10^{-31} (Table 1.3).

Table 1.1. Details of 12 published green turtle microsatellite primers (*Chelonia mydas*; Dutton & Frey 2009) tested here on hawksbill turtles for the first time.

Locus	**Alleles**	**Size range**	**N**	**Comment**
A6	1	114	4	
B103	≥2	149-151	4	Genotype trace difficult to score
B108	1	173	4	
B116	1	220	4	
B123	0	*Failed*	4	
C102	1	226	4	
D1*	12	203-239	All samples	
D105	≥2	105-155	4	>2 alleles per individual
D108	5	240-270	4	
D110*	7	113-133	All samples	
D115	0	*Failed*	4	
D2	≥2	105-155	4	>2 alleles per individual

* Indicates primers used in downstream work

All primers were tested at a concentration of 0.2 µMol, in duplexes (two loci per reaction) wherever possible. For all PCR amplifications, I used Qiagen Multiplex PCR kit in 2 µl reactions (Kenta *et al.* 2008). These consisted of adding 1 µl of suspended DNA (7-10 ng/µl) to a PCR plate well, briefly centrifuging this to ensure the droplet was at the bottom of the well, and then allowing the DNA to air-dry. To this, I then added 1 µl of 2 × Qiagen Multiplex PCR Master Mix and 1 µl of primer mix, and overlaid the reagents with 10 µl of mineral oil. Following this step, the PCR plate was again briefly centrifuged to ensure all reagents were at the bottom of their well, with no air bubbles. The thermal conditions for the PCR were 36 cycles of 94°C for 30 s, a 90 s annealing step with the temperature varied to suit different loci, and 72°C for 60 s, plus an initial 15 min at 95°C and a final 15 min at 60°C. PCR products were separated and sized using an ABI 3730 automated sequencer with ROX 500 size standard (both Applied Biosystems). I then scored the resulting genotype electropherograms in GeneMapper 3.7 (Applied Biosystems).

Table 1.2. Sequences for microsatellite primers redesigned from published sequences.

Primer	**Original name**	**Reference**	**GenBank accession no.**	**New forward sequence (5’-3’)**	**New reverse sequence (5’-3’)**	**Old size range**	**New size range**
*Eim*11(b)	*Eim*11	Miro-Herrans *et al.* (2008)	EU216603	TTCCTCAGGTCTGGAGAAGG	GGCCTGACCCTGTCACAC	216-244	115-144
*Eim*38(b)	*Eim*38	Miro-Herrans *et al.* (2008)	EU216609	TCTTCTGGTGGAGAATGCAAG	TAGCAGCTGAGGTTCGTTCC	340-344	387-413
*Eim*41(b)	*Eim*41	Miro-Herrans *et al.* (2008)	EU216611	AGACCAAAGCACTGAAGAAGC	AAGGCATGTAATTAGTCTGTGGTG	335-355	111-137
HKB31(b)	HKB31	Lin *et al.* (2008)	FN824823	TGAATGTATTAGTTTCTCATATTTGTTC	TCACCTCATAGCGTGAAAGAC	361-409	93-106

Table 1.3. Summary of microsatellite loci properties and PCR multiplexes. Allele counts are from COLONY; expected heterozygosity (HE), observed heterozygosity (HO), and number of individuals (N) are from CERVUS; significance testing was conducted in GENEPOP. All locus properties are based on the chapter 3 data set, with the exception CcP7C08 (chapter 2 data only). Multiplex 1 annealing temperature = 56°C; multiplex 2 = 57°C; multiplex 3 = 58°C.

Locus	**Multiplex**	**Conc. (µMol)**	**Dye**	**Ref.**	**Alleles**	**Size range**	**H_E**	**H_O**	**N**
*Cc*117	1	0.460	6FAM	4	3	213-227	0.477	0.478	375
*Cc*P7C08	1	0.214	HEX	9	6	264-280	0.712	0.571***	42
*Cc*P7E11	1	0.346	NED	8	14	256-313	0.882	0.872	373
*Eim*38(b)†	1	0.378	6FAM	6	10	387-413	0.499	0.502	373
*Eim*41(b)†	1	0.082	NED	6	11	111-137	0.768	0.782	375
HKB26	1	0.119	6FAM	5	6	283-293	0.730	0.709	374
HKB29	1	0.081	NED	5	8	187-206	0.547	0.553	373
HKB30	1	0.620	HEX	5	5	396-407	0.605	0.589	372
Or4	1	0.076	HEX	1	7	107-124	0.781	0.750	374
Or7	1	0.050	6FAM	1	25	85-165	0.734	0.743	372
Or18	1	0.384	HEX	2	14	166-202	0.772	0.793	372
*Cc*1	2	0.110	6FAM	7	13	159-185	0.792	0.801	375
*Cc*P1G03	2	0.203	6FAM	8	6	244-265	0.632	0.639	375
*Cc*P2H12	2	0.676	NED	8	19	306-374	0.918	0.901	368
*Cc*P7D04	2	0.128	6FAM	9	13	315-375	0.839	0.865	373
D1††	2	0.435	NED	3	12	203-243	0.881	0.882	371
*Ei*8	2	0.254	HEX	4	28	179-286	0.788	0.783	373
*Eim*11(b)†	2	0.078	NED	6	8	115-153	0.687	0.683	371
*Eim*17	2	0.349	HEX	6	19	245-295	0.892	0.869	370
HKB17	2	0.273	6FAM	5	4	404-410	0.470	0.430	372
HKB31(b)†	2	0.271	6FAM	5	6	93-118	0.563	0.577	373
Or14	2	0.060	HEX	2	4	130-136	0.581	0.577	375
*Cc*2	3	0.734	HEX	7	12	392-412	0.619	0.684	370
*Cc*13	3	0.143	NED	7	5	221-228	0.658	0.680	374
*Cc*28	3	0.101	HEX	7	10	199-225	0.741	0.767	374
*Cc*P8E07	3	0.115	6FAM	8	17	238-303	0.848	0.851	368
*Cm*58	3	0.205	NED	4	6	120-140	0.648	0.673	375
D110††	3	0.056	HEX	3	7	112-133	0.555	0.551	375
*Eim*19	3	0.325	HEX	6	6	290-300	0.536	0.497	373
*Eim*31	3	0.250	NED	6	10	302-326	0.685	0.688	371
HKB24	3	0.089	6FAM	5	10	91-115	0.564	0.576	374
HKB25	3	0.172	6FAM	5	6	353-364	0.620	0.606	374
Or2	3	0.189	6FAM	1	16	148-182	0.851	0.832	375

References: 1 = Aggarwal *et al.* (2004); 2 = Aggarwal *et al.* (2008); 3 = Dutton & Frey (2009); 4 = FitzSimmons *et al.* (1995); 5 = Lin *et al.* (2008); 6 = Miro-Herrans *et al.* (2008); 7 = Monzón-Argüello *et al.* (2008); 8 = Shamblin *et al.* (2007); 9 = Shamblin *et al.* (2009).

† Indicates primers redesigned from published sequences for better use in multiplex (Table 1.2)

†† Indicates primers not previously tested on hawksbill turtles (Table 1.1)

*** $P < 0.001$

I designed the multiplexes in Multiplex Manager 1.1 (Holleley & Geerts 2009), using locus size ranges and measures of discriminatory power derived from genotyping one offspring per nest from the nests of 30 separate females. Selected loci had null (i.e. non-amplifying) allele frequencies < 0.1 (CERVUS 3.0; Marshall *et al.* 1998), and did not deviate significantly from Hardy-Weinberg or linkage equilibria after correcting for multiple tests (GENEPOP 4.1; Raymond & Rousset 1995; Rousset 2008). Design parameters for Multiplex Manager were that complementarity between any pair of loci in a multiplex could be no greater than six, and that at least 30 base pairs of space be left between loci tagged with the same fluorescent dye. I then tested proposed multiplexes on 30 individuals whose genotypes at each locus had been established in earlier primer trials, experimenting with a range of locus combinations, annealing temperatures, and adjustments to the concentrations of individual primers. This eventually resulted in three multiplexes, each amplifying 11 loci (Table 1.3). Annealing temperatures for these multiplexes were 56°C, 57°C and 58°C.

1.2.4 Parentage analysis and paternal genotype reconstruction

I performed parentage analysis in COLONY 2.0 (Wang & Santure 2009), which uses a maximum likelihood method to cluster offspring into full- and half-sibling groups, and to then assign parentage based on these groups. Critically, if the programme cannot find a match in the list of candidate parents provided, it will assign parentage to an unknown individual, and suggest possible genotypes for that individual. Thus, if one parent is known and a sufficient number of its offspring is sampled, COLONY can be used to reconstruct the full multilocus genotype of the unsampled parent. When both parents are unknown, multilocus genotypes cannot be reconstructed. However, the programme is still able to determine the parental composition of such families (i.e. how many males and females contributed to the sample). In my case, that meant identifying whether the offspring of an unsampled female were of single or multiple paternity. For each parentage analysis, I ran the data through COLONY three times, each time with a different random number seed. All runs used ‘medium’ duration and ‘medium’ likelihood precision, under the ‘full likelihood’ approach. Both sexes were allowed to be polygamous, and the programme was allowed to account for inbreeding (I later found this option to make no difference to the analysis). I provided no prior on sibship size, and set the prior probability of an offspring’s mother’s genotype being among the candidates as 0.5. Finally, I allowed the programme to adjust allele frequencies as the analysis proceeded, to take account of the inferred relatedness structure of the sample. The discriminatory power of the microsatellite array means that any two individuals sampled will almost certainly be genetically distinct, and that the probability of detecting multiple paternity in a clutch is dependent only on the number of hatchlings sampled and the magnitude of reproductive skew.

A valuable feature of COLONY is its ability to account for genotyping error, which it divides into mistyping and dropout errors. The former can arise through sizing errors of PCR products, manual errors in naming alleles, and actual mutation, whereas the latter represents both systematic dropout (e.g. null alleles) and accidental dropout (e.g. large alleles that occasionally fail to amplify, or heterozygotes falsely called as homozygotes because of a ‘stuttered’ genotype electropherogram). We estimated per-locus measures of error by repeating PCR and genotyping on 96 randomly-selected samples. We also compared all offspring genotypes to those of their mothers to identify errors. Per-locus dropout ranged from 0.003-0.023, and per-locus mistyping error rates ranged from 0.003-0.009. As part of the output, COLONY highlights genotypes it suspects of containing errors.

COLONY reconstructs parental genotypes on a locus-by-locus basis and provides a confidence value for each reconstruction. It is therefore up to the user to decide which loci to include when assembling multilocus genotypes. I only incorporated single-locus genotypes with confidence ≥ 0.90, and only used assembled multilocus genotypes in downstream analyses if they contained ≥ 29/32 loci and were reconstructed from ≥ 10 offspring. Setting a minimum number of offspring minimises the possible bias arising from heterozygous paternal genotypes requiring fewer offspring for confident reconstruction than homozygous genotypes.

1.2.5 Ethical statement

Tissue samples were collected from hawksbill turtles under a permit issued by Seychelles Bureau of Standards, and the project was approved by the University of East Anglia's School of Biological Sciences Ethics Committee. Samples were exported from the Republic of Seychelles to the UK in accordance with the Convention on the International Trade in Endangered Species (CITES).

Chapter 2: Reconstructing paternal genotypes to infer patterns of sperm storage and sexual selection in the hawksbill turtle

2.1 Abstract

Post-copulatory sperm storage can serve a range of functions, including ensuring fertility, allowing delayed fertilisation, and facilitating sexual selection. Sperm storage is likely to be particularly important in wide-ranging animals with low population densities, but its prevalence and importance in such taxa, and its role in promoting sexual selection, are poorly known. Here we use a powerful microsatellite array and paternal genotype reconstruction to assess the prevalence of sperm storage and test sexual selection hypotheses of genetic biases to paternity in one such species, the critically endangered hawksbill turtle, *Eretmochelys imbricata*. In the majority of females (90.7%, N = 43), all offspring were sired by a single male. In the few cases of multiple paternity (9.3%), two males fertilised each female. Importantly, the identity and proportional fertilisation success of males were consistent across all sequential nests laid by individual females over the breeding season (up to five nests over 75 days). No males were identified as having fertilised more than one female, suggesting that a large number of males are available to females. No evidence for biases to paternity based on heterozygosity or relatedness was found. These results indicate that female hawksbill turtles are predominantly monogamous within a season, store sperm for the duration of the nesting season, and do not re-mate between nests. Furthermore, females do not appear to be using sperm storage to facilitate sexual selection. Consequently the primary value of storing sperm in marine turtles may be to uncouple mating and fertilisation in time and avoid costly re-mating.

2.2 Introduction

The ability of females to store sperm and utilise it at a later date is a critical component of many mating systems across taxa, driving the evolution of male and female sexual behaviours and physiologies (Birkhead & Møller 1998; Kleven *et al.* 2009). Sperm storage allows a female to separate copulation and fertilisation in time, which is important when the biology of a species necessitates substantial separation between the two (e.g. Gist *et al.* 1990; Stanback *et al.* 2002; Boomsma *et al.* 2005). Coupled with multiple mating, it also allows a female to ensure fertility (Sheldon 1994), to promote 'trading-up' (i.e. mating with a new, better male; Halliday 1983) and postcopulatory sexual selection (Parker 1970; Eberhard 1996), and to optimise offspring genetic variability (e.g. Jennions 1997; Zeh & Zeh 1997). However, although the literature on sperm storage and its evolutionary implications is substantial, especially for model organisms (Parker 1970; Pizzari *et al.* 2003; Boomsma 2009; Collet *et al.* 2012), the prevalence of sperm storage as a reproductive tactic, and its consequences for downstream aspects of mating systems, is still unknown for most taxa, especially in the wild (see Bretman & Tregenza 2005).

Genetic benefits of facilitating sexual selection through sperm storage may be particularly important in systems where males provide no resources or parental care, and where any benefits to females of particular mating strategies must therefore be indirect (e.g. Kempenaers *et al.* 1992; Kokko *et al.* 2003). Debate over the importance of indirect genetic benefits has been considerable (see Arnqvist & Kirkpatrick 2005; Griffith 2007), but their presence has been indicated in numerous taxa (e.g. Tregenza & Wedell 1998; Richardson *et al.* 2005; Cohas *et al.* 2006; Rogers *et al.* 2008). Several hypotheses have been suggested for these benefits, all of which centre on the premise that males with particular genotypic properties produce offspring of better quality (reviewed in Jennions & Petrie 2000; Kokko *et al.* 2002). The 'good genes' hypothesis predicts that paternity will be biased to males of high genotypic quality, either because of additive 'good genes' *per se* (e.g. Birkhead & Møller 1992; Møller & Alatalo 1999) or high heterozygosity (e.g. Brown

1997; Fromhage *et al.* 2009). Alternatively, paternity may be biased towards genetically dissimilar males to avoid inbreeding or to optimise offspring genetic diversity ('genetic compatibility' – see Jennions 1997; Zeh & Zeh 1997). Direct testing for genetic benefits is difficult without extensive genetic and fitness data, but paternity biases can be explored with a large panel of neutral loci.

The mating systems of wide-ranging species with low population densities are poorly known, but sperm storage is likely to be an important component. For example, little is known about sexual selection in marine turtles, which are long-lived, wide-ranging species of international conservation priority (Wallace *et al.* 2011). The ability of some testudines (turtles, tortoises and terrapins) to store sperm is well documented from dissection studies (Owens 1980; Gist & Jones 1989), from genetic studies of terrestrial and freshwater species (e.g. Palmer *et al.* 1998; Pearse *et al.* 2001; Cutuli *et al.* 2013), and from captive females that have produced non-parthenogenetic offspring after prolonged isolation from males (Ewing 1943; Whitaker 2006; Murphy *et al.* 2007). However, the prevalence of sperm storage within and among wild populations is not well known for most marine species, and the ramifications of sperm storage have received limited attention. Furthermore, although multiple paternity has been recorded in all seven species of marine turtle (Lee 2008; Theissinger *et al.* 2009; Joseph & Shaw 2011), the drivers of the substantial variation in multiple paternity frequency observed across populations and species remain unclear (Lee & Hays 2004; Jensen *et al.* 2006; Zbinden *et al.* 2007). No studies have tested for genetic biases to paternity in these taxa.

The application of molecular techniques has transformed our ability to study the mating systems of wide ranging and cryptic animals where observational data on mating is extremely difficult to collect. However, the mating system of the hawksbill turtle (*Eretmochelys imbricata*), a Critically Endangered species (IUCN Red List) distributed across the world’s tropical oceans (Mortimer & Donnelly 2008), is still poorly understood. A recent study based on just ten females identified the presence of multiple paternity in this species (Joseph & Shaw 2011), but most parameters of the mating system have not yet been determined. Here, we quantify the prevalence of sperm storage and explore its potential role in sexual selection in marine turtles after determining patterns of paternity in a large sample of hawksbill turtle nests. Specifically, we a) determine the frequency of polyandry and polygyny; b) infer the frequency, duration and viability of sperm storage; and c) use reconstructed male genotypes to test for genetic biases to paternity based on the good genes and genetic compatibility hypotheses. These hypotheses predict that fertilisation will be biased towards males of higher heterozygosity, or to males that are more genetically dissimilar to females, respectively. To our knowledge, our work will provide the first test for genetic biases to parentage in marine turtles. Also, by assessing the mating system of one of the most poorly studied turtle species, we substantially improve general understanding of marine turtle mating systems.

2.3 Methods

Sampling was conducted on Cousine Island during the 2007/08 hawksbill nesting season. Field methods, laboratory methods, and parentage analysis were conducted as described in section 1.2.

To explore within-year temporal patterns of paternity in females with more than one multiple-paternity nest, we plotted the proportional paternity of the primary male (the male with the greatest share of paternity) against nest number for each female. Because proportional paternity values are based on a sample of offspring rather than an entire nest, we calculated confidence intervals for each estimate using the Wilson method (Wilson 1927; Newcombe 1998). Simulations were used to assess the likelihood that any observed fluctuations in paternity proportions over a female's nests occurred by chance (details in Appendix 2.1).

To test for changes in successful egg development across a female's consecutive clutches that potentially result from depletion of sperm quality and/or quantity, we divided the number of undeveloped eggs in a clutch by the total clutch size for each nest and built a linear mixed model of proportional egg viability (Box-Cox transformed: $\lambda = 0.194$; Box & Cox 1964) in R (R Development Core Team 2008). The mean-centring approach of van de Pol & Wright (2009) was used to calculate two time parameters for use as fixed effects: a) date of the midpoint (mean) of a female's observed laying sequence, in days from the laying of the first nest of the 2007/08 season, as a between-individual term; b) date of a given clutch relative to the female's mean laying date as a within-individual term. Female identity was included as a random effect. The within-individual term tests for changes in a female's egg development success through time, and the between-individual term tests and controls for any general effect of time in the season on egg development. To provide a comparative assessment of model fit, the analysis was re-run with only the intercept and random effect terms. We have deliberately avoided referring to undeveloped eggs as 'infertile' because it is not possible to separate genuine infertility from early embryo mortality in the field (Miller 1997).

To test whether paternity is biased towards males with higher genetic variability, we calculated 'homozygosity by loci' (HL – Aparicio *et al.* 2006) for all genotyped females and all reconstructed males. HL is the proportion of loci at which an individual is homozygous, weighting the contributions of loci by expected homozygosity, and is one of several metrics that better correlate with genome-wide heterozygosity than simple mean heterozygosity of typed loci (see also Amos *et al.* 2001; Wang 2011a; but see Chapman *et al.* 2009). Following tests of assumptions, male and female HL were compared using an independent samples t-test (two-tailed). If paternity is biased towards males with higher heterozygosity, mean male HL should be significantly lower (because HL measures homozygosity) than mean female HL. This test assumes that males and females come from the same population, that the females are a random sample from this population, and that male and female HL would not differ significantly under random mating.

Selection against mating with genetically similar individuals should lead to observed male-female pairings having lower similarity scores than randomly generated male-female pairings. To quantify genetic similarity between males and females, we calculated pairwise relatedness (*r*) for all possible male-female dyads in the data set following (Queller & Goodnight 1989). Calculations were conducted in COANCESTRY 1.0 (Wang 2011b) using allele frequencies estimated by COLONY. Dyads were partitioned into 'observed' and 'potential' pairings, and the difference between the groups tested using COANCESTRY's bootstrap estimator of *P*-values (10,000 bootstraps, two-tailed test). Simulations were conducted in MATLAB v. 7.11 (The MathWorks Inc. 2010) and Excel to explore the effect sizes detectable by the above tests, given our sample size (details in Appendix 2.2).

2.4 Results

Genotypes were obtained from 1600 out of 1610 sampled hatchlings, representing the progeny of 85 nests. Sample size per nest ranged from 3-20, with 20 samples for 65 nests, 15-19 for 15 nests, and only five nests with < 15 samples (n = 3-7). Fifty-three females were genotyped.

2.4.1 Parentage

Field maternity data was available for 51/85 nests. One female genotype was omitted from analyses in COLONY because of mismatches with hatchling genotypes attributable to degraded maternal DNA in a poorly preserved sample. All three COLONY runs returned identical kinship structure for the data set. Of the 34 nests without field data on maternity, 23 were assigned by COLONY to sampled females, with the remaining 11 explained by seven unsampled females. In total, 43 females contributed offspring to the sample, with 1-4 nests per female (mean ± SD = 1.98 ± 1.19), although the length of intervals between clutches indicated some females had probably laid a fifth clutch on another island. Summing across nests, 36 females had ≥ 20 genotyped offspring (mean = 41.9 ± 23.1; max. = 80), four had 15-19, and only three had < 15 (n = 4-7). Sixteen additional sampled and genotyped females were not assigned any maternity by COLONY.

For the majority of females (39/43, 90.7%), including 18 with data from more than one nest (7 with 2 nests, 5 with 3, and 6 with 4), a single father explained all offspring across all nests (36/40, 90.0%, if the three females with < 20 genotyped offspring, and thus a lower chance of detecting multiple paternity, are excluded). For the remaining four females (9.3%), two fathers per female explained all offspring across all nests. This included three females with multiple nests (1 with 2 nests, 2 with 4), with both males having offspring across nests in all three cases. In one case the male with the lower share of paternity was not detected in the second of a female’s four nests, but given the very low representation of this male over the female’s other three nests (1-2 offspring per nest), this seems likely to be a sampling artefact. Overall, no males were detected as having fertilised the offspring of more than one female, meaning a total of 47 males fathered all the offspring sampled.

The average paternity share of the dominant male in a mixed-paternity clutch, weighted by female rather than nest, was 81.3% (± 13.1%). The proportion of offspring fathered by each male varied little across the nests of each multiply-fertilised female, and neither of the females with four nests appeared to show any consistent unidirectional change in paternity proportions across clutches (Fig. 2.1). The maximum difference between nests in the proportion of paternity obtained by a primary male was 0.19. This set of nests also showed the maximum change in paternity proportion between first and last nest (-0.15). Simulations indicated that all observed variation in proportional paternity among females’ nests fell within the ranges expected under random fluctuations – the lowest probability for an observed value of either variation measure (maximum among-nests change in paternity proportion, and change in proportion between first and last nest) was 0.20.

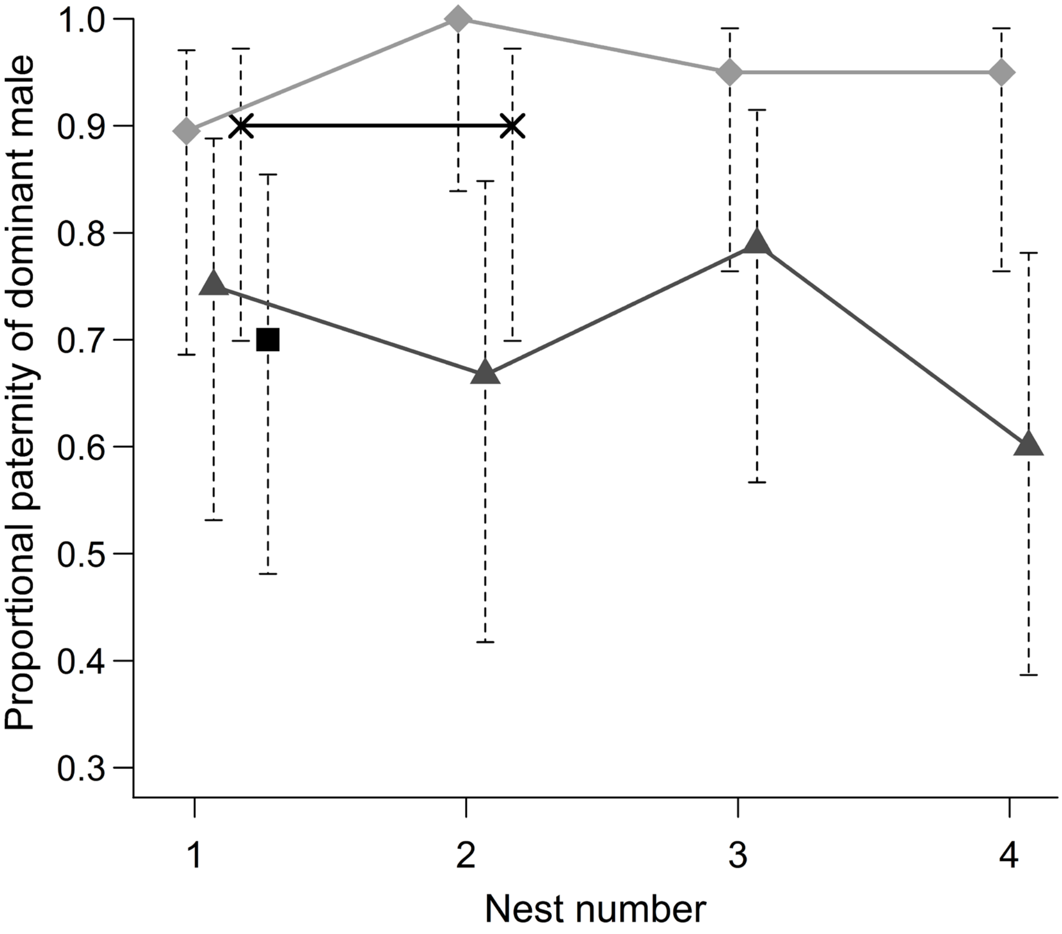

Figure 2.1. Proportions of paternity allocated to dominant hawksbill turtle males across nests of females showing multiple paternity. Each symbol denotes the nests of a particular female. Error bars are 95% CIs for estimates of proportions.

The linear mixed model of proportional egg development success showed no within-individual effect (effect size ± SE = 0.000 ± 0.005), suggesting no temporal trend across the nests of individual females. There was also no significant between-individual effect (0.008 ± 0.005), suggesting that proportional egg development success is not affected by the time of season around which a female's laying is focussed. The random effect term (individual female) explained 36.2% of random-effects variance (0.301/0.832). However, this model had poorer fit statistics than the null model (e.g. Akaike Information Criterion (Akaike 1974): model = 239.8, null = 221.2), in which the random effect (individual female) explained 44.5% of variance (0.393/0.884).

2.4.2 Genetic biases to paternity

Of the 47 inferred breeding males, 34 had multi-locus genotypes that met our confidence criteria (genotype $P \geq 0.9$, at ≥ 29 loci, reconstructed from ≥ 10 offspring). Genotypes could not be confidently reconstructed for the remaining 13 males either because they were mated to unsampled females with single-paternity nests (n = 6) or because they did not father sufficient offspring (n = 7).

There was no significant difference between the HL of sampled females and inferred males (mean ± SD = 0.29 ± 0.09 v. 0.28 ± 0.08, n = 52 v. 34 respectively; t-test: t = 0.51, d.f. = 84, $P = 0.61$; Fig. 2.2). Mean relatedness among observed male-female dyads was -0.02 ± 0.10 (n = 34), compared with -0.01 ± 0.11 (n = 1734) for 'potential' dyads (Fig. 2.3). This difference (-0.01) was not statistically significant (10,000 bootstraps: $P = 0.99$; critical difference for rejection of the null hypothesis = -0.04), and thus there was no evidence for genetic biases to paternity. Simulations to explore the effect sizes detectable by these analyses indicated that the Type II error rate fell below 0.2 for both HL and r with relatively strong paternity biases, equivalent to the third of the male population with the lowest heterozygosity, or to whom a female was most genetically similar, receiving no paternity (details in Appendix 2.2).

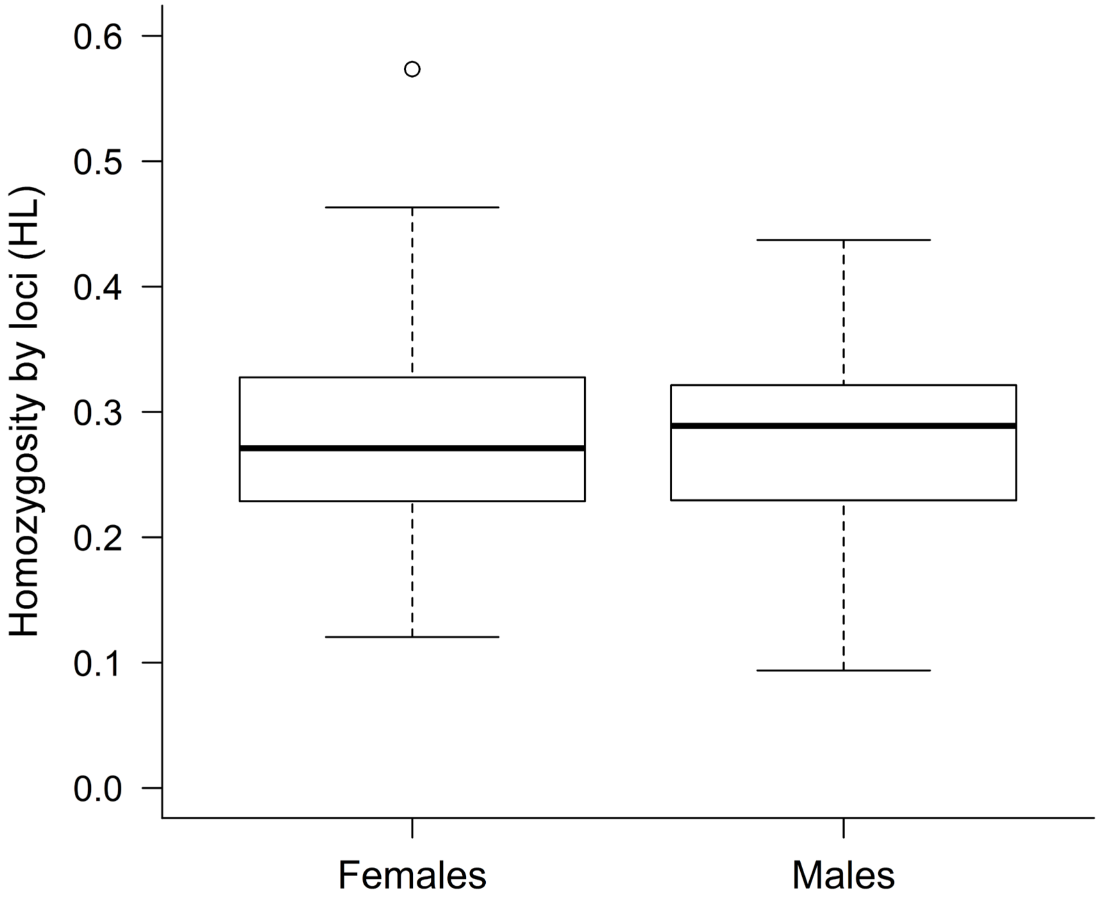

Figure 2.2. Homozygosity by locus (HL) of sampled hawksbill turtle females (n = 52) and inferred males (n = 34). Maximum whisker length = 1.5 × inter-quartile range.

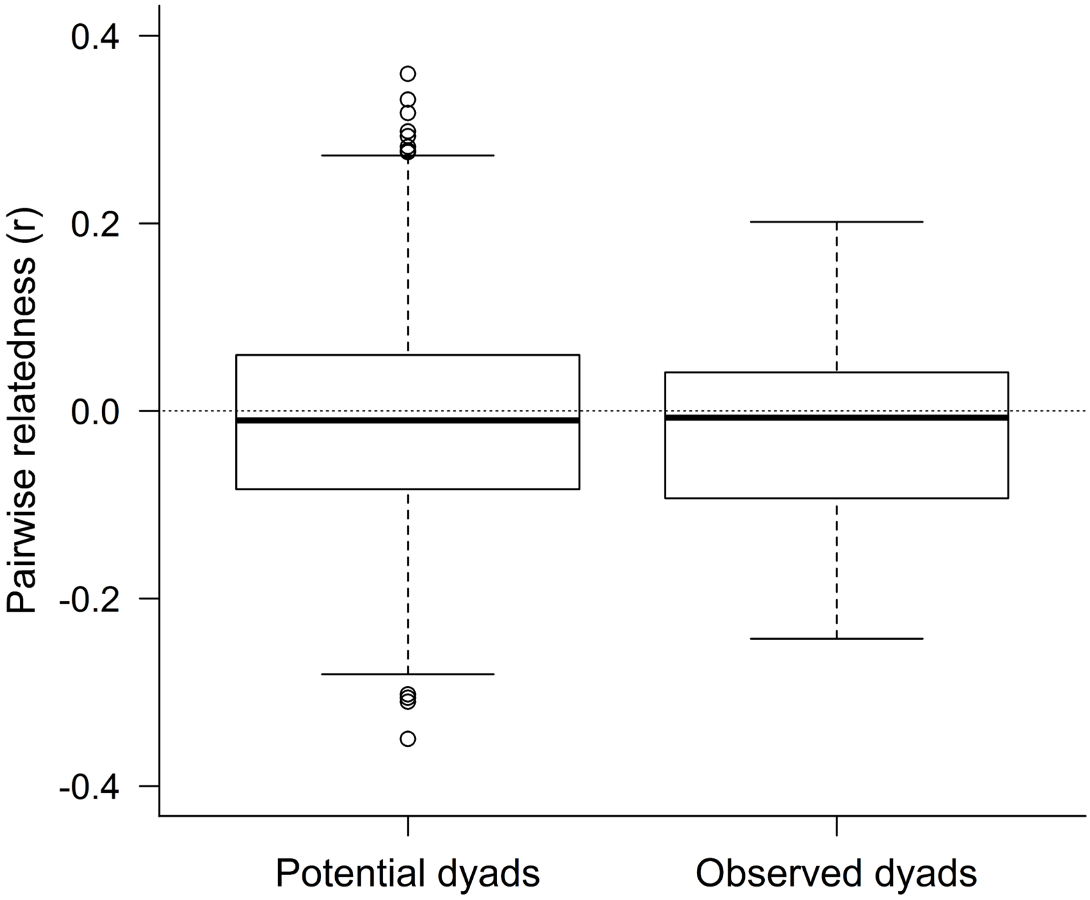

Figure 2.3. Pairwise relatedness scores for observed (n = 34) and potential (n = 1734) hawksbill turtle male-female dyads. Maximum whisker length = 1.5 × inter-quartile range.

2.5 Discussion

In this study of hawksbill turtles, the majority of females (*ca* 91%) showed single paternity in all clutches. Multiple paternity was uncommon, and in all cases could be explained by just two males per female. No male was found to have fertilised the eggs of more than one female, meaning a total of 47 males and 43 females contributed offspring to the sample. Importantly, each female was fertilised by the same unique male across all her clutches, or by the same two males in cases of multiple paternity (1-4 nests per female). For multiply-fertilised females, the proportion of paternity allocated to each male remained consistent across nests. There was no indication that proportional egg development success changed over the season.

The frequency of multiple paternity detected in this population (approximately 10%) is low compared with previous marine turtle studies (15-93%; reviewed in Lee 2008; see also Theissinger *et al.* 2009; Stewart & Dutton 2011; Wright *et al.* 2012a; Wright *et al.* 2012b; but see Crim *et al.* 2002), including an earlier, smaller study on hawksbills in Malaysia (Joseph & Shaw 2011). Why multiple paternity is infrequent in our population is not clear. Several studies (Ireland *et al.* 2003; Lee & Hays 2004; Jensen *et al.* 2006) have argued that one of the main drivers of variation in marine turtle multiple paternity is 'convenience polyandry', whereby a female should allow an additional male to mate if the costs of refusing, such as persistent harassment, exceed the costs of accepting (Weigensberg & Fairbairn 1994). The costs of refusing mating are predicted to increase as females encounter more males, which will happen at higher population densities (Weigensberg & Fairbairn 1994; Uller & Olsson 2008). The frequency of multiple paternity in a population would thus be an expression of sexual conflict (*sensu* Holland & Rice 1998) over mating frequency set in the context of local population density, rather than a strategy that benefits females *per se* (Ireland *et al.* 2003; Lee & Hays 2004; Jensen *et al.* 2006). Consequently, the low incidence of multiple paternity observed in our study may be indicative of a low encounter rate between males and females, possibly as a result of a widely dispersed population at the time of mating. Variation in opportunity for mating is considered a major driver for variation in reptile multiple paternity more generally (Uller & Olsson 2008; Faria *et al.* 2010; While *et al.* 2011). However, testing the density-dependent convenience polyandry model is beyond the scope of the present study.

That no males gained paternity with multiple females suggests that a large number of males exist in the population, although it is not clear how many males any given female may encounter and potentially mate with. If mating takes place close to the nesting areas, one would expect to see some degree of polygyny, as some females are likely to encounter the same male, but this is never the case in our sample. Moreover, if a large local population existed, the high population density would be difficult to reconcile with the low rate of multiple paternity we observed. It therefore seems likely that mating is either very dispersed relative to nesting beaches or that there is a high local turnover of males, whereby a large number of males pass through the area but only a small number are present at a time. The latter is more consistent with recent satellite tracking data from other marine turtle species, which have indicated that males can be highly mobile during the mating season (Hays *et al.* 2010; Wright *et al.* 2012b), although it is also known for male green turtles to hold courtship territories in some locations (Limpus 1993). Little is known about the movements of male hawksbill turtles (Godley *et al.* 2008), but data that are available suggest that males do not travel far from the nesting beaches between breeding attempts (6/8 travelled < 100 km; van Dam *et al.* 2008), raising the possibility that each male may concentrate his searching for mates on a specific area. These tentative inferences could be further explored by satellite tracking of males and/or identifying the males contributing paternity at other hawksbill nesting sites across the Seychelles.

In the present study, the absolute consistency of paternal identity across each female's nests, including for the multiply-fertilised females, indicates that females are utilising sperm stored from copulations prior to the nesting season and are not seeking re-fertilisation between clutches. Previous non-molecular studies, predominantly of green turtles (*Chelonia mydas*), have raised the suspicion that sperm storage is important in marine turtles (Frazier 1971; Booth & Peters 1972; Wood & Wood 1980), and the physiological capability of female turtles to store sperm in small tubules throughout the oviduct is well established from dissections (Owens 1980; Gist & Jones 1989; Gist & Congdon 1998). Furthermore, several molecular studies of marine turtle have remarked on patterns of paternity indicative of sperm storage (Table 2.S1), but these have often been limited by containing small numbers of multiple-nesting females, or lacking confident resolution of family structure. However, the collective indication of these previous studies when combined with the present, intensive study on hawksbill turtles, is that intra-season sperm storage is an important and widespread reproductive tactic among these long-lived, low population density, highly migratory taxa.

Paternity patterns can be affected by the mode of sperm storage. If sperm from multiple males is stratified in a female's sperm storage organs, patterns of male precedence should exist based on mating order (Birkhead & Hunter 1990). In the absence of sperm stratification, paternity outcome will be more of a 'sperm raffle', biased by ejaculate parameters such as sperm number and motility (Parker 1990; Parker & Pizzari 2010), or by genetic properties of the sperm, the so-called 'loaded raffle' model (Parker 1990; Griffith & Immler 2009). Temporal patterns of paternity precedence in multiply-fertilised female turtles have not been well resolved because the accurate assignment of offspring to particular fathers has often been limited by the resolution of the analysis (a limited marker panel and/or number of sampled offspring per male). Piecing together the suitable data available from previous studies (Crim *et al.* 2002; Theissinger *et al.* 2009; Stewart & Dutton 2011; Wright *et al.* 2012a) suggests that the proportion of paternity between nests is normally relatively constant in marine turtles. In the present study, our results show that this is consistently the case in hawksbill turtles. These patterns suggest that sperm from multiple males is mixed rather than stratified during sperm storage in marine turtles. Although such an inference does not rule out an effect of mating order, the mixing of ejaculates does increase the potential for sperm competition on the grounds of sperm/ejaculate quality (Parker 1970; Birkhead & Hunter 1990).

Indirect benefits of mate choice and polyandry have been a major focus of evolutionary biology over the last couple of decades (e.g. Kempenaers *et al.* 1992; Kokko *et al.* 2003; Slatyer *et al.* 2012), but these questions have received limited attention in marine turtles. Lee & Hays (2004) found no evidence for fitness benefits associated with multiple paternity *per se* in green turtles (although their interpretation would be confounded if females only re-mate when initially paired with a low-quality male, to recover fitness that would otherwise be lost, e.g. Brouwer *et al.* (2010); Michalczyk *et al.* (2011); Varian-Ramos & Webster (2012)). Similarly, multiple paternity was not found to affect hatching success in flatback turtles (*Natator depressus*), although this study had a small sample size of single-paternity females (n = 3; Theissinger *et al.* 2009). In the present study, we found no indication that paternity is biased according to male heterozygosity or male-female genetic similarity. These results suggest female hawksbill turtles do not bias mating towards specific males, nor are they using their ability to store sperm to promote post-copulatory sexual selection. It may be that, despite the apparently large male population, the frequency with which females encounter males, and therefore the opportunity for such sexual selection, is restricted. This low encounter rate may be normal for this species, or could be because of the substantial population declines suffered following decades of overhunting (Mortimer & Donnelly 2008). Alternatively, females may not encounter enough low-quality males for sexual selection processes to be favoured in the face of the costs of seeking additional matings. For example, if males migrate between populations, or if females mate non-locally (e.g. FitzSimmons *et al.* 1997a; FitzSimmons *et al.* 1997b; reviewed in Bowen & Karl 2007; Lee 2008), the overall population will be larger and have greater genetic variability. Consequently, a female is less likely to encounter a male of low genetic variability or high genetic similarity to herself, and thus any randomly encountered male may be a 'good enough' mate (e.g. as in Hansson *et al.* 2007; Jamieson *et al.* 2009). What is clear is that exploring sexual selection in marine turtles is extremely challenging: data are only available on the outcome of any selective process (i.e. fertilisations), making it impossible to determine the number or characteristics of males a female rejects, or that she mates with but which fail to gain fertilisations. The potential for genetic benefits could be assessed by estimating relatedness or genetic variability in random males sampled across the entire available male population, but this requires extensive sampling and is beyond the scope of this study. However, despite the inherent difficulties, it is important that these processes be studied in marine turtles, both to provide insights into sexual selection across a range of life histories, and to advance knowledge of the basic biology of these conservation-priority species.

Although sperm storage may not be used to promote sexual selection in hawksbill turtles, it may provide other benefits. For example, it may allow a female to separate mating and fertilisation in time (Birkhead & Møller 1993; Palmer *et al.* 1998), thus making her less dependent on finding a male while simultaneously migrating to nesting grounds. Sperm storage also allows a female to avoid the potential costs of re-mating, e.g. prolonged courtship, or risk of injury during mating (Booth & Peters 1972; Wood & Wood 1980; Miller 1997). Such a strategy may be risky if laying in excess of 800 eggs in a season (five clutches, mean eggs/clutch = 166 ± 32 (SD)) depletes sperm stocks, or if storage (up to 75 days between first and last nests) reduces sperm quality (Birkhead & Møller 1992). In our study we found no decline in the proportion of eggs that developed across the sequential nests of individual females, and sperm storage in hawksbill turtles thus appears to be sufficient to ensure fertilisation success over an entire season. This finding is consistent with evidence of long-term sperm viability in terrestrial and freshwater Testudines (e.g. Pearse *et al.* 2002; Johnston *et al.* 2006), but unremarkable compared with some taxa, especially eusocial insects, where a single mating can service a female with sufficient sperm to last across decades and produce offspring numbering in the millions (Cole 1983; Hölldobler & Wilson 1990; Boomsma *et al.* 2005).

Comprehensive studies, with large samples sizes, are needed across other marine turtles and other populations of hawksbill turtles to assess the accuracy and generality of the findings of this study. Furthermore, robust tests for genetic biases to paternity, such as heterozygosity and male-female relatedness, will require larger panels of markers than many previous studies, especially in regards to assessments of heterozygosity (Balloux *et al.* 2004; Slate *et al.* 2004; but see Csilléry *et al.* 2006). It would also be highly informative to assess paternity patterns with respect to candidate loci, such as the major histocompatibility complex (MHC), as paternity may be random with respect to similarity at neutral loci but biased with respect to functional loci (e.g. Landry *et al.* 2001; Richardson *et al.* 2005).

In summary, we have characterised a mating system where season-long sperm storage is a ubiquitous reproductive tactic, but where a low frequency of multiple paternity and no evident genetic biases to paternity suggest that females are not using sperm storage to promote sexual selection. The absence of evident sexual selection, despite indications of there being a large number of males within the population, may reflect a low encounter rate between males and females that does not favour the evolution of such selection. The primary utility of sperm storage to this long-lived, low population density, migratory species may, instead, be separating mating and fertilisation and avoiding the costs of re-mating.

2.6 Appendices

2.6.1 Appendix 2.1: Modelling fluctuations in paternal share across multiple-paternity nests

Across the consecutive nests of females with offspring of multiple paternity, proportional paternity share of contributing fathers was not perfectly constant, so simulations were used to assess how likely observed fluctuations in proportional paternity were to have occurred by chance. For each female, her offspring were shuffled randomly across her nests, thereby keeping average paternity proportions constant but varying them within and between nests. We performed 10,000 randomisations per female, each time calculating, from the perspective of the male with the greater share of paternity, a) the maximum change in paternity proportion observed between any pair of a female's nests, and b) the change in paternity proportion between a female's first and last nest (for females with two nests, both measures are the same). We then recorded the position of the observed values for both difference types on their respective simulated distributions.

2.6.2 Appendix 2.2: Simulations to explore detectable effect sizes for tests of genetic biases to paternity

Simulations were conducted in MATLAB v. 7.11 (The MathWorks Inc. 2010) and Excel. For the heterozygosity (HL) test, 1000 female and 1000 male genotypes were simulated from population allele frequencies, and their HL values calculated. A sample was drawn from a) females at random, and b) males at random, but from below a given percentile of the population distribution of HL to represent selection against the most homozygous males. Female and male samples were compared using a t-test. The process was repeated 10,000 times for each tested selection percentile (0.50-0.95 in increments of 0.05), and the proportion of tests returning significant differences was calculated (two tailed, $P < 0.05$).

For the pairwise relatedness test, the simulation was based on re-sampling from the observed data set. A subsample of 34 females (the number of reconstructed male genotypes that met our confidence criteria) was drawn from all available females. Each selected female was then paired with a male chosen at random from among those males to whom the female had a relatedness score below a given threshold. A range of thresholds were tested, taken from percentiles (0.50-0.95 in increments of 0.05) of the population distribution of *r*-values. The difference between the average male-female relatedness of these simulated 'observed' pairings and the average for 'potential' pairings in the actual sample was calculated, and tested against the critical value estimated by the main COANCESTRY (Wang 2011) analysis. We repeated this process 10,000 times for each given percentile and calculated the proportion of the 10,000 repeats that returned significant results.

Table 2.S1. Marine turtle studies in which paternity patterns have been assayed for multiple nests from individual females. N_M = number of females for which multiple nests were tested; N_T = total number of females in the study.

Species	**Reference**	**Markers**	**N_M**	**N_T**
Loggerhead (*Caretta caretta*)	Harry & Briscoe (1988)	3 allozymes	21	24
	Zbinden *et al.* (2007)	4 microsatellites	5	15
Green (*Chelonia mydas*)	FitzSimmons (1998)	5 microsatellites	9	13
	Wright *et al.* (2012)	12 microsatellites	16	20
Leatherback (*Dermochelys coriacea*)	Crim *et al.* (2002)	3 microsatellites	20	20
	Stewart & Dutton (2011)	7 microsatellites	12	12
Hawksbill (*Eretmochelys imbricata*)	Joseph & Shaw (2011)	5 microsatellites	1	10
Flatback (*Natator depressus*)	Theissinger *et al.* (2009)	4 microsatellites	5	9
Olive ridley (*Lepidochelys olivacea*)	*No publications with repeat females*	*n/a*	*n/a*	*n/a*
Kemp's ridley (*L. kempii*)	*No publications with repeat females*	*n/a*	*n/a*	*n/a*

Chapter 3: Molecular techniques reveal cryptic life history and demographic processes of a critically endangered marine turtle

3.1 Abstract

The concept of 'effective population size' (N_e), which quantifies how quickly a population will lose genetic variation, is one of the most important contributions of theoretical evolutionary biology to practical conservation management. N_e is often much lower than actual population size: how much so depends on key life history and demographic parameters, such as mating systems and population connectivity, that often remain unknown for species of conservation concern. Molecular techniques allow the indirect study of these parameters, as well as estimation of current and historical N_e. Here, we use genotyping to assess the genetic health of an important population of the critically endangered hawksbill turtle *Eretmochelys imbricata*, a slow-to-mature, difficult-to-observe species with a long history of severe overhunting. Our results were surprisingly positive: the study population, located in the Republic of Seychelles, Indian Ocean, has a relatively large N_e, estimated to exceed 1000, and showed no evidence of a recent reduction in N_e (i.e. no genetic bottleneck). Furthermore, molecular inferences suggest the species' mating system is conducive to maintaining large N_e, with a relatively large and widely distributed male population promoting high levels of gene flow among nesting sites across the Seychelles area. This may also be reinforced by the movement of females between nesting sites. Our study underlines how molecular techniques can help to inform conservation biology. In this case our results suggest that this important hawksbill population is starting from a relatively strong position as it faces new conservation challenges, such as global climate change.

3.2 Introduction

Small populations lose genetic variation much more rapidly than large populations, as they are more susceptible to inbreeding and more strongly affected by genetic drift (Wright 1931). Importantly, almost all populations will lose genetic variation more quickly than expected from their census population size N, due to factors that include variation between individuals in reproductive success, fluctuations in population size, unequal sex ratios, and population structure. This greater rate of loss is quantified as the population's effective size N_e (Wright 1931), which is often substantially lower than N (Hartl 1988; Frankham 1995). Given that low genetic diversity increases the risk of population extinction and may reduce adaptability to future environmental change (Franklin & Frankham 1998; Frankham *et al.* 1999), N_e and its implications for genetic diversity are important considerations in the management of species of conservation concern (Frankham *et al.* 2002).

Among conservation-priority species, demography and life history are often not well known enough for their impacts on N_e to be assessed, which restricts the potential for adjusting management plans to aid in solving specific conservation problems (Palstra & Ruzzante 2008; Hare *et al.* 2011). In such situations, molecular techniques are essential tools, allowing mating systems to be assessed, migration and dispersal patterns to be explored, and inbreeding and genetic diversity to be quantified. Of particular value to conservation managers is the utility of molecular methods for inferring connectivity between and/or structure among populations, to identify and measure the breeding contributions of unseen individuals, to derive estimates of N_e directly from molecular data, and to infer past changes in N_e such as population bottlenecks (e.g. Waples 1989; Piry *et al.* 1999; Frankham *et al.* 2002).

Population declines driven by overhunting, habitat loss, and other anthropogenic factors have made marine turtles a global conservation priority (Wallace *et al.* 2011). However, little is known about N_e in most populations of these taxa, or about how N_e might relate to census counts. This makes it difficult to quantify loss of genetic variation, or assess how low levels of variation may slow population recovery and reduce adaptability to future perturbations such as global climate change (see Hawkes *et al.* 2009; Wright *et al.* 2012b). To estimate N_e and adjust conservation management accordingly, we require more information about specific key life history and demographic parameters than is currently available for many marine turtle populations. For example, male reproductive skew is a key parameter influencing effective population size, with N_e being larger the more evenly reproduction is distributed among males within the population (Hartl 1988). In marine turtles, the vast majority of paternity studies have focused on data from a single nesting season (but see Wright *et al.* 2012a), but accurate assessment of skew in such long-lived species requires assessing paternity across years. If the same set of males sires the offspring of a given nesting site across years, skew will be higher and N_e lower than if the number/local turnover of males is greater. Furthermore, the ability to estimate N_e directly from molecular data (e.g. Waples 1989; Wang 2009), and to use these data to infer past changes to N_e such as population bottlenecks (Piry *et al.* 1999; e.g. Garza & Williamson 2001), have rarely been applied to marine turtles (Rivalan *et al.* 2006; Theissinger *et al.* 2009).

The hawksbill turtle (*Eretmochelys imbricata*) occurs throughout the world’s tropical oceans, and is IUCN-listed as Critically Endangered following substantial population declines driven by anthropogenic factors (Mortimer & Donnelly 2008). Many aspects of the hawksbill’s life history are poorly known, and most published genetic work involves hawksbill populations in the Caribbean (Bowen *et al.* 2007a; Blumenthal *et al.* 2009). In the Indo-Pacific, little is known with respect to population genetics beyond the existence of broad-scale structure between several major rookeries (Vargas *et al.* 2013), and gene flow between both juveniles and nesting females of two of the region’s most important populations, those of Seychelles and Chagos (Mortimer & Broderick 1999; Sheppard *et al.* 2012). However, a study of mating systems based on one year’s data from hawksbills in the Republic of Seychelles suggested that the number of males in this population was large, given that the majority of females were fertilised by a single male each but that no male fertilised more than one female (chapter 2 – published as Phillips *et al.* 2013). Here, we use a four-year data set from the same population to quantify N_e and compare it to census data, to test for changes in N_e in the recent past that might indicate genetic effects of population declines, and to assess key processes affecting N_e, such as dispersal and between-year patterns of parentage. Using samples collected from nesting beaches spanning several hundred kilometres across Seychelles, we also assess population genetic structure and consider the implications of our results for N_e and for ongoing hawksbill conservation management in the region. Our results help us move towards a fuller understanding of demographic and life history parameters in a species that is inherently difficult to study, and reiterate the value of molecular techniques to conservation biologists.

3.3 Methods

3.3.1 Field sampling

Sampling was conducted on Cousine Island over four hawksbill nesting seasons (Sep-Apr) spanning Sep 2007 - Apr 2011. Field methods were as described in section 1.2. Over the first three years, sampling of females and nests was near exhaustive. In 2010/11, samples were only collected from previously unsampled adult females, and from hatchlings from the nests of females observed on Cousine in any of the three previous seasons of the study.

For analysis of population structure, tissue samples were collected in the 2010/11 and 2011/12 seasons from females nesting on additional islands across the Seychelles (Fig. 3.1): in the Granitic Seychelles (the region that includes Cousine; Fig. 3.1C), from Frégate (04°35’S, 55°57’E) and North Islands (04°24’S, 55°15’E); and in the Amirantes group (the outer coralline islands; Fig 3.1B), from D’Arros/St Joseph (05°25’S, 53°19’E), Desroches (05°42’S, 53°40’E), and Alphonse/St François (07°04’S, 52°44’E). Additionally, a small number of juvenile hawksbills were hand-captured and sampled in the waters around Aldabra Atoll (09°26’S, 46°23’E). Samples were collected by removing a small section of tissue from the trailing edge of a flipper with a sterile scalpel, ideally during nesting for adult females.

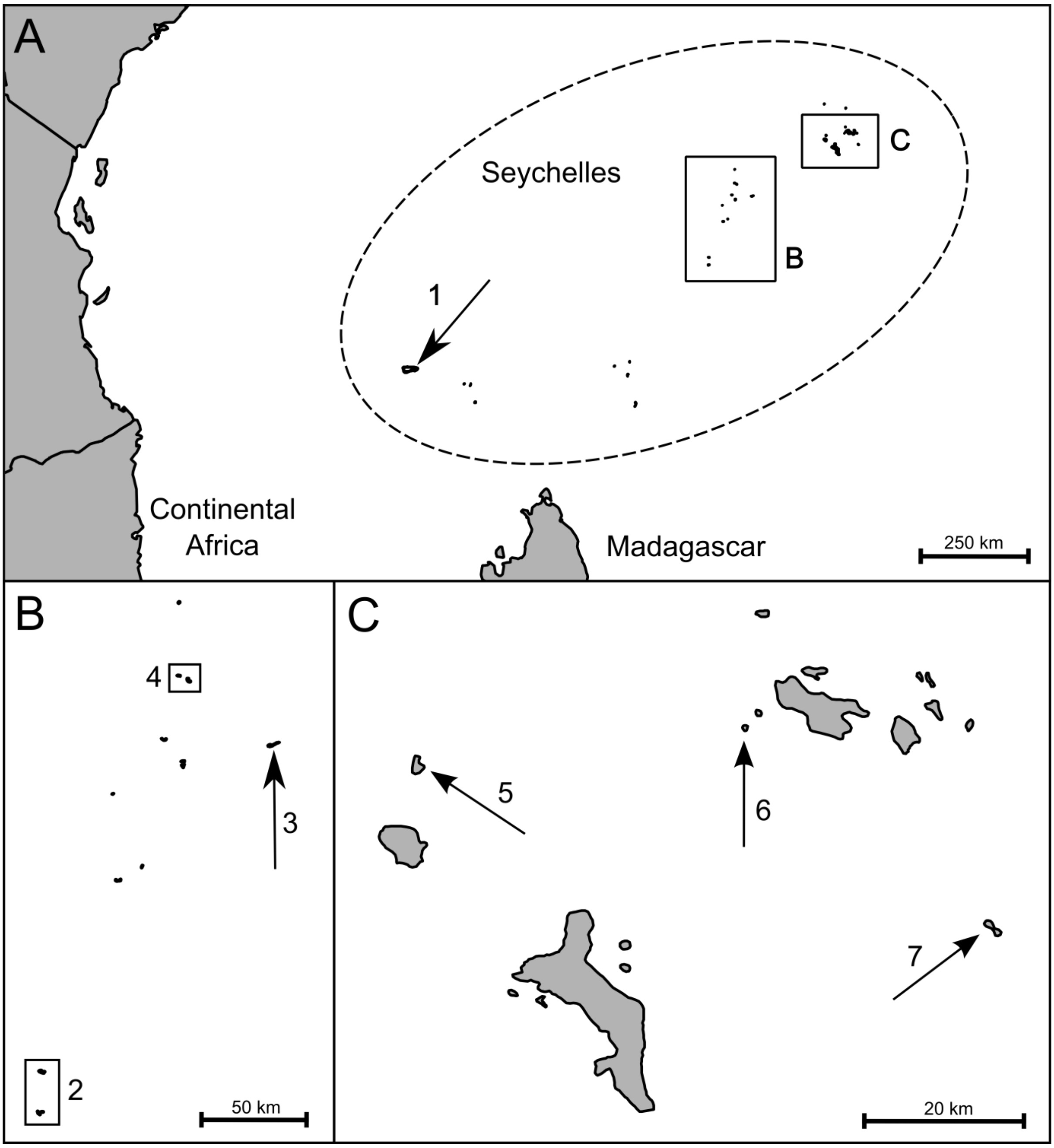

Figure 3.1. Map of the study region, highlighting islands from which samples were collected. A: location of Seychelles archipelago (circled region), with close-ups of the Amirantes (B) and Granitic (C) groups. 1 = Aldabra; 2 = Alphonse and St François; 3 = Desroches; 4 = D’Arros and St Joseph; 5 = North Island; 6 = Cousine Island; 7 = Frégate Island.

3.3.2 Molecular analysis

Laboratory protocols were as described in section 1.2. Where possible, we genotyped at least 20 offspring per female from the 2007/08 and 2008/09 seasons. Time and cost constraints meant that we were unable to do this for 2009/10: instead, we genotyped 3 offspring from every female, and an additional 10-12 offspring from a subsample of 20 families.

3.3.3 Parentage assessment and reconstruction of male genotypes

Parentage analysis and reconstruction of male genotypes were as described in section 1.2. The programme COANCESTRY 1.0 (Wang 2011b) was used to screen the data for related adults prior to running any subsequent analyses, as some population genetics and N_e estimation methods can be adversely affected by the presence of close kin. Allele frequencies for use in COANCESTRY were obtained from three runs of COLONY on the entire data set, with all adult females as candidate mothers and the Aldabra juveniles as offspring.

3.3.4 Population structure

Pairwise F_{ST} values and absolute number of migrants exchanged (*M*; Slatkin 1991) were computed between all population pairs in Arlequin 3.5 (Excoffier *et al.* 2005). The inbreeding coefficient F_{IS} (Wright 1965) was also computed for each population. Male genotypes inferred from Cousine nests were treated as a separate population, in case males and females were not from the same genetic stock (e.g. FitzSimmons *et al.* 1997b). F_{ST} was also calculated after grouping the islands by region (Granitics v. Amirantes). Aldabra juveniles were excluded from this 'regional' analysis because juveniles on feeding grounds may come from multiple rookeries (e.g. Bowen *et al.* 2007a; Blumenthal *et al.* 2009). Cousine-inferred males were also excluded (see above). To test for cryptic population structure, we ran the data through the programme STRUCTURE 2.3 (Pritchard *et al.* 2000), which uses a Bayesian method to cluster samples into groups that minimise deviation from Hardy-Weinberg and linkage equilibria. We ran the programme five times with a burn-in period of 50,000 steps and a sampling period of 100,000, and used three different location priors:

no prior, island, and region. For all STRUCTURE runs, we used an admixture model with correlated allele frequencies, and tested for populations in the range *k* = 1-10 (we chose ten as the upper limit in case any of the eight initial groupings contained substructure, such as if males or juveniles were sourced from multiple discrete stocks).

3.3.5 Sex biased dispersal

To test for sex-biased dispersal, we used the assignment index method of Mossman & Waser (1999), implemented in GENALEX 6 (Peakall & Smouse 2006). The sex with the lower index value is the more dispersive. Note that this method required that we only used genotypes with no missing data. We also used COANCESTRY to compare the mean relatedness *r* (Queller & Goodnight 1989) of female-female to that of male-male dyads using 10,000 bootstraps. A significant result would suggest that the sex with the lower average relatedness is the greater disperser (as dispersal increases, one expects to find fewer relatives within a given area). To aid interpretation of the relatedness test, we ranked all dyads by *r*, including male-female dyads, and calculated the proportions of each dyad class (male-male, female-female, male-female) above increasing thresholds of *r*.

3.3.6 Estimates of effective population size

We used four one-sample N_e estimation methods: the heterozygosity excess method (Robertson 1965; Pudovkin *et al.* 1996), implemented in COLONY; the linkage disequilibrium method (Hill 1981), implemented in LDNE 1.31 (Waples & Do 2008); the sibship method (Wang 2009), implemented by running adults through COLONY as 'offspring' (three runs, no prior allele frequencies); and approximate Bayesian computation (ABC), implemented in DIY ABC 1.0 (prior N_e range 100-10,000; Cornuet *et al.* 2008). DIY ABC was run three times, each using a different random subsample of the Cousine data set to reduce computation times (40 females, 40 males). Each run generated 1,000,000 simulated data sets using an N_e prior range of 100-10,000. We also tried the ABC programme ONeSAMP 1.2 (Tallmon *et al.* 2008), but found its analysis return times for our data set to be too long and unpredictable. We did not apply any multi-sample N_e estimation methods (e.g. Waples & Do 2010) because our sampling period of four years is substantially lower than any marine turtle generation time (see also Miller 1997; 35 years for hawksbills; e.g. Meylan & Donnelly 1999; Spotila 2004), and because it is not possible to age marine turtles accurately in the field. We add the cautionary note that our study system, like most wild systems, is likely to violate some of the assumptions that underlie N_e estimation. Most notably, a sample of adult marine turtles will not represent a single cohort, and generations are likely to overlap. This is discussed later.

3.3.7 Bottleneck testing

We applied three methods to test for genetic evidence of past changes in effective population size. Firstly, the programme BOTTLENECK (Piry *et al.* 1999), which compares a sample's heterozygosity (H_e) at each locus with that expected under mutation-drift equilibrium (H_{eq}). Heterozygosity excess ($H_e > H_{eq}$) suggests a population contraction (i.e. a bottleneck), whereas a heterozygosity deficit suggests a population expansion (Cornuet & Luikart 1996). We applied three mutation models: pure infinite allele, pure single-step, and a two-phase model with parameters recommended by the programme's authors (non-stepwise = 5%, variance = 12). BOTTLENECK does not estimate the timing or magnitude of any detected change in N_e. Secondly, we used a modification of the DIY ABC scenario described in section 3.3.6 to include a 'vary N_e' event at an unspecified time *t* in the population's history. Thus *t* (prior range 1-500 generations) and historical N_e (prior range 100-10,000) were included as parameters for which to estimate posterior probabilities. DIY ABC also allows for comparisons between scenarios using posterior probabilities, enabling us to assess whether the 'vary N_e' scenario had greater support than the constant N_e scenario of section 3.3.6. Thirdly, we took advantage of DIY ABC's significance testing of the Garza-Williamson index, calculated for each microsatellite locus as allelic richness/locus size range (Garza & Williamson 2001). During a population reduction, richness declines more rapidly than size range, and so low values of the index can indicate a bottleneck.

3.3.8 Estimating male population size

Too few males were 'genetically re-sighted' to allow mark-recapture estimation of population size according to standard methods (Greenwood & Robinson 2006). Instead, to provide indicative figures, we conducted simulated sampling from a range of male population sizes (N_{mal}) in R (R Development Core Team 2008). For a given N_{mal}, four samples were drawn, of sizes corresponding to the number of inferred males in each year of our study. We then calculated the mode number of re-sightings for each N_{mal} value, based on 10,000 replicates per value and compared this with the number of actual re-sightings observed within and across years in our wild sample. This basic model assumes a closed population, no mortality or recruitment, and no between-individual or temporal variation in re-sighting probability. This last assumption implies that mating is random, and that a male is as likely to be re-sighted within a year as he is between years. We also ran variations of the model to restrict male re-sightings to between years, to make some males only breed biennially, and to allow some males to be more successful in obtaining paternity than other males.

3.4 Results

3.4.1 Cousine Island parentage patterns

We genotyped 180 adult females and 3162 hatchlings (249 nests), and from this were able to reconstruct 91 male genotypes meeting our confidence criteria. All three runs of COLONY converged on the same result. Genotyped hatchlings represented the progeny of 128 females (1.82 ± 1.18 nests per female (mean ± SD), max. = 6), of which 12 females were unsampled. Four females per year in each of the first three years produced offspring showing multiple paternity within a season. With respect to adult turtles present in multiple years, 12 sampled females laid eggs in two seasons (6 in Y1-Y3, 4 in Y1-Y4, 2 in Y2-Y4), and four males were inferred to have fathered offspring in two seasons (1 in Y1-Y2, 2 in Y1-Y3, and 1 in Y2-Y3). One male-female pair was observed to have reproduced together in two years (Y1 and Y3). All other re-sighted individuals had new partners in each year. For two five-member full-sib clusters, identified by COANCESTRY among the adult females sampled from Cousine, only one member per cluster, selected at random, was retained in downstream analyses.

3.4.2 Population genetics

Sample sizes from the seven sampled islands are given in Table 3.1. No population (island or region) had an F_{IS} significantly different from zero. Between-island pairwise F_{ST} statistics and estimates of number of migrants per generations are summarised in Table 3.2. Mean overall F_{ST} was 0.001 ± 0.006 (SD), with a maximum pairwise value of 0.014 between Desroches and Alphonse/St François. Three F_{ST} values were significant at the $P < 0.05$ level and one at $P < 0.01$, all involving comparisons with Desroches. However, none of these values remained significant following correction for multiple comparisons (*q*-values; Storey 2002). At the region level, F_{ST} between the Granitics and Amirantes was 0.001 ($P = 0.14$), implying a high per-generation migration rate between the groups (M = 648.0). Five runs of STRUCTURE with no location priors all returned $k = 1$ with posterior probability $P > 0.99$. The same was true with island as a location prior. With region as a prior, results of individual runs were more varied, but averaging over 10 runs favoured $k = 1$ with $P = 0.96$. No interpretive differences were made by re-running F_{ST} or STRUCTURE analyses using a random subset of 40 Cousine females and 40 Cousine males to avoid bias arising from the much larger sample from Cousine (data not shown).

Table 3.1. Sample sizes and summary statistics for hawksbill tissue samples collected from seven islands across Seychelles, and for males inferred from parentage analysis on Cousine Island hatchlings. Summaries are also given for regional island groupings (Amirantes: Alphonse/St François, D'Arros/St Joseph, and Desroches; Granitics: Frégate, North, and Cousine females).

Population	**n**	**Alleles per locus (SD)**	**Private alleles**	**H_O (SD)**	**H_E (SD)**	**F_{IS} (SD)**
Aldabra (juveniles)	14	5.8 (2.5)	2	0.66 (0.18)	0.69 (0.16)	0.04 (0.21)
Alphonse/St François	10	5.6 (2.1)	3	0.71 (0.17)	0.71 (0.14)	0.00 (0.18)
D'Arros/St Joseph	38	7.7 (3.5)	3	0.69 (0.15)	0.70 (0.14)	0.01 (0.09)
Desroches	13	5.6 (2.0)	1	0.68 (0.18)	0.67 (0.15)	-0.01 (0.14)
Frégate	32	7.4 (3.3)	3	0.70 (0.18)	0.70 (0.15)	0.00 (0.10)
North	9	5.2 (2.1)	0	0.69 (0.23)	0.69 (0.18)	0.00 (0.19)
Cousine (females)	180	9.3 (4.8)	10	0.69 (0.13)	0.69 (0.13)	-0.01 (0.06)
Cousine (males)	91	8.8 (4.4)	4	0.70 (0.13)	0.69 (0.13)	-0.01 (0.06)
Amirantes (females)	62	8.3 (3.7)	15	0.69 (0.14)	0.70 (0.13)	0.01 (0.08)
Granitics (females)	221	9.5 (5.0)	55	0.69 (0.14)	0.69 (0.14)	-0.01 (0.05)

n = number of genotyped individuals; H_O = observed heterozygosity; H_E = heterozygosity expected under Hardy-Weinberg Equilibrium; F_{IS} = average level of individual inbreeding relative to the subpopulation

Table 3.2. Pairwise F_{ST} between all population pairs (below diagonal) and estimates of migration (numbers of individuals per generation) above diagonal. '*high*' indicates migration rates too high for precise estimation (returned as 'infinite' by Arlequin). Four F_{ST} comparisons against Desroches were significant prior to correction for multiple comparisons (**P* < 0.05; ***P* < 0.01), but these did not remain significant following correction for multiple comparisons (*q*-values; Storey 2002).

	Aldabra	Alphonse & St François	D'Arros & St Joseph	Desroches	Frégate	North	Cousine (females)	Cousine (males)
Aldabra	-	67.7	218.8	46.9	160.8	*high*	151.4	*high*
Alph. & S.F.	0.007	-	204.2	36.5	241.7	*high*	291.3	97.8
D'Arros & S.J.	0.002	0.002	-	71.8	*high*	*high*	658.0	*high*
Desroches	0.011	*0.014	*0.007	-	88.3	*high*	61.9	67.0
Frégate	0.003	0.002	-0.001	0.006	-	*high*	*high*	*high*
North	-0.011	-0.002	-0.003	-0.002	-0.008	-	*high*	*high*
Cousine (f)	0.003	0.002	0.001	**0.008	-0.001	-0.007	-	1054.5
Cousine (m)	-0.001	0.005	0.000	*0.007	-0.002	-0.006	0.000	-

3.4.3 Sex-biased dispersal

Four females and 15 males were excluded from assignment index analysis because of incomplete genotypes. Mean index values were -0.48 ± 2.86 (SD; n = 76) and 0.22 ± 3.04 (n = 166) for males and females respectively. This difference was not significant (Wilcoxon rank sum test: W = 5504, *P* = 0.11). However, males and females differed significantly in their distributions of the index (Kolmogorov-Smirnov test: D = 0.20, *P* = 0.03), possibly due to higher kurtosis in males (1.79 v. 0.03).

Mean relatedness among Cousine Island females was significantly higher than mean relatedness among males, although the effect size of 0.008 was small (females = 0.000 ± 0.108, n = 14,365; males = -0.008 ± 0.105, n = 4095; *P* < 0.001). The effect remained significant when the test was re-run using random subsets of female-female dyads of balanced sample size with the males (n = 4095; three subsets: effect sizes = 0.006-0.009, *P* < 0.001-0.005). The representation of female-female dyads increased rapidly as *r* increased above 0.20, rising from 47.0% to 87.1% over an *r* interval of 0.25 (Fig. 3.2).

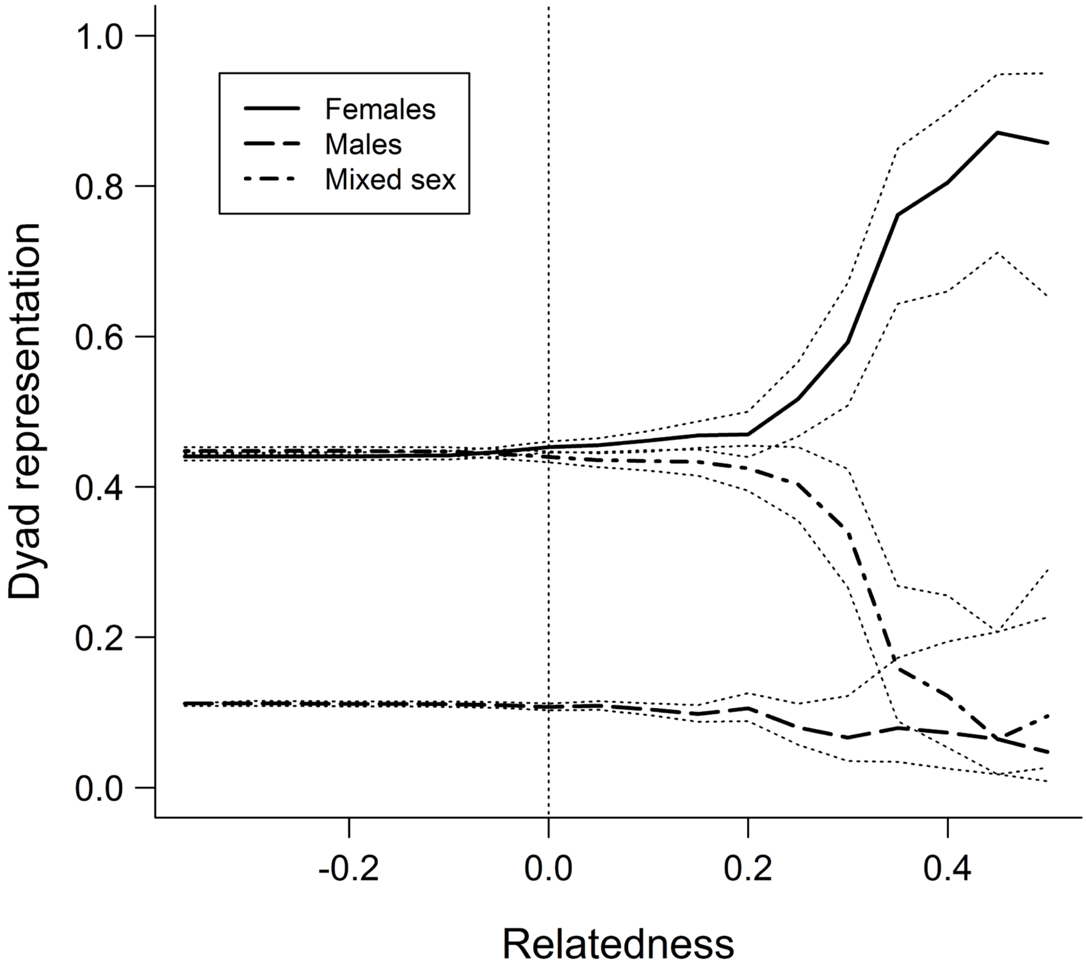

Figure 3.2. Female-female dyads make up an increasingly large proportion of dyads with higher relatedness (*r*) values. Female-female dyads represent 44% (16110/36585) of all dyads, but 87% (27/31) of dyads with $r \geq 0.45$. Dotted lines indicate 95% CIs.

3.4.4 Effective population size

The heterozygosity excess method returned 95% confidence intervals ranging from zero to infinity, which may indicate our population is too large for precise application of this method (Luikart & Cornuet 1999). The mean N_e estimate of three runs of the sibship method was 485 (95% CI = 418-562). The linkage disequilibrium method estimated N_e at 2407 (1578-4872). Results from application of these methods to specific data subsets are given in Table 3.3. Three runs of DIY ABC converged on similar solutions to each other, with median N_e of 1020-1150 (178-8743).

Table 3.3. Estimates of effective population size, N_e (means and 95% CIs) from the sibship and linkage disequilibrium methods applied to data subsets.

Subset	n	Sibship method	Linkage method
Cousine males	91	157 (117-214)	1781 (725-∞)
Cousine females	172*	236 (189-292)	1080 (720-2087)
Non-Cousine females	102	181 (138-242)	1176 (639-6188)
Cousine all	263*	356 (296-426)	1494 (1013-2746)
All adult females	274*	359 (300-427)	1722 (1143-3363)
All adults	365*	485 (418-562)	2407 (1578-4872)

n = number of genotype individuals

* Refer to numbers excluding two five-member full-sib groups, bar one, randomly-selected member per group

3.4.5 Tests for bottlenecks

BOTTLENECK showed a significant heterozygosity excess relative to H_{eq} ($P < 0.001$) under a pure infinite-allele mutation model, but a significant heterozygosity deficiency under a pure single-step model ($P < 0.001$). The two-phase model returned a highly significant heterozygosity deficiency ($P = 0.001$). Re-running the analyses using subsamples of the Cousine data, or on specific data subsets (e.g. Amirantes females, non-Cousine Granitic females) made no interpretative differences (data not shown).

Three runs of DIY ABC allowing a historical change in N_e converged on a scenario of population increase, summarised in Table 3.4. This ‘vary N_e’ scenario returned substantially higher estimates of current N_e than the ‘constant N_e’ scenario summarised above (e.g. medians of 4390-4450 and 1020-1150; Table 3.4), although 95% confidence intervals showed a large overlap (406-9747 v. 178-8743). Comparing the ‘vary N_e’ and ‘constant N_e’ scenarios, DIY ABC’s logistic regression estimate of posterior probabilities did not favour one model over the other: ‘vary’ = 0.49 (95% CI = 0.44-0.54) v. ‘constant’ = 0.51 (0.46-0.56).

Significance testing of the Garza-Williamson (GW) index in DIY ABC indicated that observed index values were on the edge of significance (GW = 0.76-0.77; 'constant N_e': *P* = 0.03-0.06; 'vary N_e': *P* = 0.05-0.06). Note that the whole data set (i.e. including all Cousine samples) had GW = 0.84 ± 0.19 (SD).

Table 3.4. Posterior distribution summary statistics of parameters estimated by DIY ABC simulations that included a 'vary N_e' scenario. Values for medians give the range covered by three runs of the programme; values for 95% CI are the means of the three 95% CI estimates. Estimates of current Ne obtained from the 'constant N_e' scenario are given for comparison.

Parameter	**Median**	**95% CI**
Current N_e ('vary')	4390-4450	406-9747
Historical N_e	901-1110	149-7570
Time since change (generations)	211-220	10-483
Current N_e ('constant')	1020-1150	178-8743

3.4.6 Number of males in the overall population

Four males were observed to father offspring in two years but no males were re-sighted within years. Under our basic simulated sampling model, allowing re-sighting within and between years, male population sizes in the range 2300-2700 had the highest probability of producing four re-sightings. If males are only allowed to be re-sighted in separate years, which is more concordant with our observed data, a simulated male population of 1600-2000 gives a re-sighting mode matching our observed four re-sightings. We treated the single observed incidence of a specific male-female pairing occurring in two years as an independent re-sighting of the male. Removing this pair from the simulation increases estimates of male numbers by approximately 850. Restricting a proportion of males to being biennial did not affect the estimates, but larger population values were indicated if some males were allowed to be more successful in achieving paternity (data not shown).

3.4 Discussion

Our study of hawksbill turtles in Seychelles indicates that population structure over the area sampled was very low. Slightly higher levels of relatedness among Cousine Island females than among males may indicate that dispersal is, to some degree, sex-biased. While the various methods used to estimate effective population size provided differing results, the consensus indicated an N_e of approximately 1000-2000, and there was little evidence of a recent genetic bottleneck. Concordant with this, on Cousine Island, very few males were ‘genetically re-sighted’ within or between years, indicating that a large male population (> 1000) currently exists in the Seychelles area.

The absence of significant genetic population structure between hawksbill nesting beaches across Seychelles suggests that our samples are drawn from a single, panmictic population. In initial testing, several F_{ST} comparisons against Desroches were significant (Table 3.2), but these did not remain significant after correcting for multiple comparisons, and the programme STRUCTURE did separate this island, even when provided with island as a location prior. In terms of breeding females, the genetic population inferred from this data spans at least the 450 km from the inner granitic islands to Alphonse and St François on the Amirante Bank. Juveniles feeding around Aldabra, a further 750 km away, were not genetically separable from adults breeding in the Granitics and Amirantes, potentially suggesting a much wider geographic extent to this single population. However, the long migrations of young turtles, which can span entire oceans (e.g. Bowen *et al.* 1994, 1995), mean that we cannot assume the genetics of these juveniles reflect those of the small number of adult hawksbills nesting on Aldabra. An alternative possibility is that Aldabra is a feeding ground for young turtles hatched on the granitic and coralline islands, a similar process having previously been suggested between Seychelles and Chagos hawksbills (Mortimer & Broderick 1999; Sheppard *et al.* 2012). Such long connections between juvenile feeding grounds and source rookeries are also well known from studies of Caribbean hawksbills

(Bowen *et al.* 2007a; Blumenthal *et al.* 2009), a region where molecular data has featured prominently in discussions on cross-border management of hawksbill populations (Bowen *et al.* 2007a, 2007b; Godfrey *et al.* 2007; Mortimer *et al.* 2007a, 2007b).

Mean genotype assignment index of Cousine Island females was higher than that of inferred males but not significantly so, arguing against sex-biased dispersal. However, the distribution of the index differed significantly between the sexes, which may indicate that males and females are of subtly different genetic origins. This is supported by relatedness among Cousine Island females being significantly higher than among males. The effect size of this test was very small (0.008), but held up to subsampling the female-female dyads to reduce sample size inflation. Is it safe to interpret such a small effect, based on a large sample size, as indicative of meaningful sex-biased dispersal, with males as the more dispersive sex? Were sex-biased dispersal strong, one would expect clearer genetic evidence for a 'non-dispersing' sex. In marine turtles, this is traditionally held to be females returning to breed in their natal areas, a hypothesis that is well-supported by numerous mtDNA studies at coarser geographic scales (reviewed in Bowen & Karl 2007; Lee 2008). However, mtDNA and microsatellites do not give contrasting signatures of population structure between the Granitic Seychelles and the Amirantes: we found no population structure between these island groups at microsatellite loci ($F_{ST} = 0.001$), and Vargas *et al*. (unpublished data) found no mtDNA structure ($\phi_{ST} = -0.018$, $n_{Granitics} = 47$, $n_{Amirantes} = 25$; from a 766 bp section of the mtDNA control region). If the relatedness test is indicative of meaningful sex-biased dispersal, then it must be taking place against a background of high dispersal by both sexes. That female dispersal occurs is supported by hawksbill tagging data from Seychelles: inter-island tag re-sightings are uncommon but not infrequent within the Granitics, and an adult female tagged on Bird Island in the Granitics (03°43'S, 55°12'E) has been observed nesting 280 km away on D'Arros Island (Amirantes group; Mortimer, unpublished data). Such dispersal events need only occur once per generation to reduce population structure to extremely low levels (Wright 1931). Interestingly, high-relatedness dyads ($r > 0.20$) are markedly more frequent among females than among males, and the effect becomes stronger as relatedness increases (Fig. 3.2). This suggests that the overall relatedness effect may be driven by there being more $r \approx 0.25$ (e.g. half-sib) and $r \approx 0.5$ (e.g. full-sib, parent-offspring) dyads among females than among males. It therefore seems that there is some degree of natal homing among females, greater than among males, but that this is not strong enough to generate population genetic structure. Lastly, it is generally presumed that low microsatellite population structure in turtles, as seen in our study, is indicative of sex-biased dispersal, as structure at maternally-inherited mtDNA is often pronounced (Lee 2008). However, explicit tests for sex-biased dispersal are few, and, as far as we know, restricted to two species. In green turtles (*Chelonia mydas*), FitzSimmons *et al.* (1997b) demonstrated male-biased gene flow between two populations along the east coast of Australia, although they attribute this to mating on migration rather than greater dispersal of males *per se* (FitzSimmons *et al.* 1997a). Also in this species, Lee *et al.* (2007) used population assignment indices to demonstrate fine-scale male-biased dispersal among nesting beaches on Ascension Island. In loggerhead turtles (*Caretta caretta*), Bowen *et al.* (2005) reported extremely strong mtDNA structure between rookeries in the south-eastern United States but no significant structure at microsatellites. In loggerheads in the Cape Verde islands, Stiebens *et al.* (2013) report results indicative of sex-biased dispersal, but they present an interpretation that hinges on genetic differences associated with a single island, and do not satisfactorily rule out alternative demographic explanations, such as a bottleneck on this island. Unfortunately it is not possible to make a quantified comparison between these studies and ours, as differences in effect sizes may simply reflect properties of the molecular markers used.

Estimates of N_e based on the pooled adult data set ranged from 485 for the sibship method to *ca* 4400 for the DIY ABC analysis that allowed for a historical change in N_e (see Table 3.4). While it is debatable as how best to form a quantifiable consensus from these estimates, a value in the 1000-2000 range seems a fair summary. This is substantially greater than the figure of 500 often quoted as a minimum for the long-term persistence of current population genetic variation

(Franklin 1980; see also Franklin & Frankham 1998). The wide nature of confidence intervals for most of the methods used may reflect that accuracy and precision of N_e estimation can decrease as N_e increases (Luikart & Cornuet 1999; Waples & Do 2010). Furthermore, both the linkage and sibship methods may be underestimating N_e: the patterns of relatedness observed within the large Cousine Island female dataset may bias both estimates downwards, and, looking at estimates derived from data subsets (Table 3.3), neither method seems to be levelling off as sample size increases. Although marine turtles violate some of the life history and demographic assumptions of N_e estimation (e.g. overlapping generations, which can bias estimates both upwards and downwards; Palstra & Ruzzante 2008; Hare *et al.* 2011), our inferred values are in keeping with census estimates for the region of > 2500 females (> 1000 nesting females per year, with average female re-migration interval of 2.5 years; Mortimer & Bresson 1999), given the genetic evidence that this population is well mixed. If an equal sex ratio is assumed (discussed further below), this gives a relatively high N_e/N ratio of 0.25-0.50 (Frankham 1995).

The Garza-Williamson index was on the edge of significance (P = 0.03-0.06), but the observed values of 0.76-0.84 were far from those that would indicate a recent or strong bottleneck (*ca* 0.6; Garza & Williamson 2001). The mutation model used made a major interpretative difference in the programme BOTTLENECK, but the two-phase model indicated a heterozygosity deficiency, suggesting, if anything, a population expansion. Similarly, DIY ABC returned a scenario of N_e expansion, although the timing of this was ambiguous and the programme did not consider this scenario to be more likely than one of constant population size. We thus conclude there is no clear evidence for a recent or strong population bottleneck. This result is concordant with previous work on leatherbacks (*Dermochelys coriacea*; Rivalan *et al.* 2006) and flatbacks (*Natator depressus*; Theissinger *et al.* 2009), which both found no indication of bottlenecks. Rivalan *et al.* (2006) argue that their population is composed of immigrants from a larger metapopulation of unknown bounds, whereas Theissinger *et al.* (2009) discuss the role of mating systems in maintaining N_e in their population. In the present study on hawksbill turtles, we find evidence that both population connectivity and the mating system play roles in maintaining a high N_e.

Observations on the mating system were consistent with chapter 2: season-long sperm storage, predominance of single paternity, and no males fathering the offspring of more than one female within a breeding season. A small number of males were detected in two seasons: two in consecutive seasons and two on two-year intervals (see also Wright *et al.* 2012a). Note that this includes the single incidence of a particular male-female pairing being observed in two years, which could also arise from inter-year sperm storage by the female (see chapter 5). We have previously speculated on how the absence of males fertilising multiple females within a year, together with infrequent multiple paternity, may be indicative of a large but dispersed male population (chapter 2). The present study, which has extended the sampling across multiple years, supports that conclusion. Even in large, randomly mating populations, one expects occasional re-sightings of males. Our simulations suggest a male population in the 1500-3000 range, which is concordant with the current Seychelles census estimates of > 2500 female hawksbills (Mortimer & Bresson 1999). A potential implication of this is that the widely held view that marine turtles have female-biased sex ratios (see Wright *et al.* 2012b; but see also Lasala *et al.* 2013) may not be the case in Seychelles hawksbills. Although it is possible that this is a systematic difference from other marine turtle populations (there are currently no data available on hatchling sex ratios in the region that might help test this idea; Mortimer, pers. comm.), it must be remembered that, until outlawed in 1994, hawksbill exploitation in Seychelles was intense and female-biased (females are easier to catch and yield more ‘tortoiseshell’; Mortimer 1984). An even or male-biased adult sex ratio may thus represent a hangover from historical over-hunting of females, and may eventually recover, depending upon the severity of illegal poaching.

Within and across years, more males than females contributed to the parentage of Cousine offspring (148 v. 128 for all years; 142 v. 128 for Y1-Y3). Reproductive variance among these males appears to be low: 121/148 inferred males fertilised all the clutches within a single season of a separate, different female. Indeed, males seem to be sufficient in numbers and/or sufficiently widely distributed/mobile to prevent most individual males from gaining matings/fertilisations with multiple females coming to nest on Cousine (see also chapter 2). Were there to be a discrete, repeatable subset of males that dominated paternity, the resulting reproductive skew would significantly reduce N_e. Instead, mating seems likely to be occurring on migration or over a wide geographic area, promoting gene flow and helping keep N_e high.

Hawksbill numbers in Seychelles have declined substantially in the 200+ years since human colonisation (Mortimer 1984, 2004), part of a global reduction that has seen the species listed as critically endangered (Mortimer & Donnelly 2008). However, our work indicates that, in Seychelles, this decline has not left a detectable genetic signature: N_e remains high, and high relative to census counts; the population is not inbred (F_{IS} values were not significantly different from zero); and there is no indication of a population bottleneck. It is possible that the population was not reduced sufficiently, or held low for long enough given the long, overlapping generation times of turtles (*ca* 35 years for hawksbills in the Indo-Pacific; Mortimer & Donnelly 2008), for severe negative genetic effects to occur. If, as is suggested by our results, Seychelles hawksbill turtles from the Granitic islands to the Amirantes are one panmictic population, this will have played an important role in keeping N_e high and preventing loss of genetic diversity to bottlenecks in subdivided populations (Wright 1931; Frankham 1995). That mating appears to involve a large number of widely dispersed males, thereby promoting gene flow and lowering reproductive variance among males, will also help keep N_e high. An alternative explanation for the relatively large N_e and the lack of population bottleneck is that the long generation time of hawksbills has caused a lag in the reduction in N_e one would expect following overhunting. Thus what we perceive as a high N_e, relative to N, is actually closer to the N_e of the larger, historical population.

That hawksbill numbers are now recovering in Seychelles may mean that the population has escaped a serious demographic event with relatively little long-term negative impact on genetic diversity.

Although our results are positive in conservation genetics terms, we should not be complacent with respect to ongoing hawksbill conservation in the region: although population sizes are increasing in protected areas (e.g. Allen *et al.* 2010), the species remains substantially reduced in distribution and numbers compared with recent history (Mortimer 1984; Mortimer 2004). Extension of beach protection may be a productive strategy, given the evidence that females may disperse and lay clutches on different nesting beaches, thereby facilitating the colonisation of currently unoccupied sites. The possibility of a genetic link between juveniles in Aldabra and breeding adults in the Granitic and coralline islands highlights how marine turtle conservation needs to involve the protection of different, potentially very distant, areas for different life stages of a single population (e.g. Bowen *et al.* 1994, 1995, 2007a; Hawkes *et al.* 2006; Blumenthal *et al.* 2009). The future for Seychelles hawksbills is harder to predict. For example, in the face of global climate change, the temperature-dependent sex determination of turtles is an obvious source of vulnerability that may require an adaptive response (Hawkes *et al.* 2009; Wright *et al.* 2012b). However, a large N_e, gene flow spanning a wide geographic area, and no indication of a recent bottleneck suggest that hawksbills in the Seychelles are in a relatively healthy position, genetically speaking, to adapt to the considerable challenges they face.

Finally, this study demonstrates the enduring utility of molecular techniques for providing valuable insight into the life histories and demography, such as mating systems and population connectivity, of difficult-to-study species, and underlines the importance of understanding genetic processes in species of conservation concern. Our results will be of particular interest to biologists and managers focusing on long-lived, slow-to-mature, migratory species, especially marine species and species with wide ranges and low population densities.

Chapter 4: Parental heterozygosity and similarity as predictors of reproductive success in a critically endangered marine turtle

4.1 Abstract

How individual genetic variability relates to fitness is important in understanding evolution and the processes affecting populations of conservation concern. Heterozygosity-fitness correlations (HFCs) have been used to study this link in wild populations, where key parameters that affect both variability and fitness, such as inbreeding, are unknown. Here, we used estimates of parental heterozygosity and parental genetic similarity derived from 32 microsatellite loci to explore the relationship between genetic variation and fitness in a population of the critically endangered hawksbill turtle, *Eretmochelys imbricata*. We found effects of male multilocus heterozygosity (MLH) and parental similarity on the proportion of eggs in a clutch that developed successfully, but no effect of female MLH on either this parameter or the number of eggs laid in a clutch. Critically, the male HFC was not a simple linear effect, but instead showed both positive and negative slopes. The competing models included one with a quadratic effect of male MLH, implying an optimum genetic variability, and one with an interaction between male MLH and parental similarity that can change the direction of selection on variability. Both models could imply a tension between inbreeding and outbreeding depression in this system, a biologically feasible process in turtles, where female natal philopatry has the potential to elevate both inbreeding risk and local adaptation, and both may be subject to disruption by higher male dispersal. Our results show the importance of considering both positive and negative effects when assessing how variation in individual genetic diversity affects fitness in wild systems.

4.2 Introduction

Numerous studies have demonstrated correlations between individual genetic variability and parameters subject to natural selection, such as parasite load, survival and reproductive success (e.g. Chapman *et al.* 2009). How genetic variability relates to individual fitness is therefore a fundamental question in evolutionary biology (Hansson & Westerberg 2002; Coltman & Slate 2003; Szulkin *et al.* 2010; Szulkin & David 2011). It is also an important concept in conservation management, with practical implications for populations of conservation concern that may be facing challenges arising from depleted genetic variation (Crnokrak & Roff 1999; Keller & Waller 2002).

A common method for studying the relationship between individual genetic variability and fitness has been to test for correlations between individual heterozygosity and fitness parameters, so called 'heterozygosity-fitness correlations', or HFCs (Hansson & Westerberg 2002; Balloux *et al.* 2004; Kempenaers 2007; Chapman *et al.* 2009). HFCs have been reported in a variety of taxa (Chapman *et al.* 2009), but the relative roles of the hypothesised explanatory mechanisms behind the correlations remains a topic of debate (Lieutenant-Gosselin & Bernatchez 2006; Hansson & Westerberg 2008; Szulkin *et al.* 2010). With the widespread use of microsatellites for HFC studies, this discussion has focussed on 'local' v. 'general' effects, although it is important to emphasise that these models are not mutually exclusive (e.g. Hansson & Westerberg 2002). A third mechanism, 'direct effects', was applicable to older study techniques such as allozymes (e.g. David 1998), but is not usually considered for microsatellites, which are typically treated as neutral and not directly subject to selection (but see Li *et al.* 2002; Li *et al.* 2004). Under the 'local effects' model, a given marker demonstrates an HFC because it is in linkage disequilibrium, although not necessarily physical linkage, with a functional locus (Hansson & Westerberg 2002; Szulkin *et al.* 2010). This model has the advantage of being less dependent on individual inbreeding coefficients, but there is widespread unease with how to interpret such effects (Szulkin *et al.* 2010). These theoretical concerns are compounded by statistical challenges in testing for single-locus HFCs, and, for many non-model species, a lack of knowledge about the genomic location of the markers in use. In contrast, under the 'general effects' model, average heterozygosity across a number of independent loci is used to estimate genome-wide heterozygosity, which itself is treated as a proxy for an individual's inbreeding coefficient. Individuals with higher inbreeding coefficients are predicted to have lower genome-wide heterozygosity and, consequently, lower fitness due to increased expression of deleterious recessive alleles and loss of heterosis ('inbreeding depression'; Charlesworth & Charlesworth 1987, 1999). However, this interpretation is also controversial, as even when calculated from very large panels of microsatellites, multilocus heterozygosity (MLH) may be a poor correlate of pedigree inbreeding coefficients (e.g. Balloux *et al.* 2004; but see Szulkin *et al.* 2010).

Historically, HFC studies have reported a large number of positive, linear effects (Chapman *et al.* 2009; Küpper *et al.* 2010; Olano-Marin *et al.* 2011a). Such effects may have been overrepresented due to a now-diminishing publication bias (Coltman & Slate 2003; Chapman *et al.* 2009), to biases arising from the properties of genetic markers used (e.g. when first screening loci, a microsatellite involved in a negative 'local effect' HFC might be excluded for lack of variability; Küpper *et al.* 2010), and to a tendency for HFC studies to be conducted on small, founder, or fragmented populations (see Coltman & Slate 2003; Chapman *et al.* 2009; Küpper *et al.* 2010). The other end of the heterozygosity spectrum – negative HFCs – has received much less attention, although this is changing (Szulkin & David 2011). If positive multilocus HFCs indicate inbreeding depression, negative multilocus HFCs may represent outbreeding depression, when population admixture breaks up coadapted gene complexes or disrupts local adaptation (Templeton *et al.* 1986; Waser 1993). This phenomenon has been observed by managers of captive populations (Lacy *et al.* 1993), but its importance among wild populations is less well known (Marshall & Spalton 2000; Szulkin & David 2011). An implication of outbreeding depression is that somewhere between deleterious inbreeding and extreme outbreeding (hybridisation) there should be a level of outcrossing that maximises fitness. This has been well-demonstrated in plants, where offspring produced from crosses at ‘intermediate’ distances have higher fitness than short- or long-distance crosses (Price & Waser 1979; Waser & Price 1989, 1994; Willi & van Buskirk 2005). Animal studies demonstrating both inbreeding and outbreeding depression are few (Marshall & Spalton 2000; Marr *et al.* 2002; Neff 2004; Escobar *et al.* 2008; Olano-Marin *et al.* 2011a, 2011b; see also Knowlton & Jackson 1993; Edmands 2007), but have highlighted important aspects of inbreeding-outbreeding tension. For example, inbreeding and outbreeding may act on the same trait or simultaneously on different traits, and directions of HFC may differ between age classes and sexes (e.g. Marshall & Spalton 2000; Escobar *et al.* 2008; Olano-Marin *et al.* 2011b).

An individual’s genetic variability may affect the fitness of its offspring. In the early stages of a reproductive attempt, variability may correlate with a male’s fertilisation success (Gage *et al.* 2006; Bretman *et al.* 2009; Fitzpatrick & Evans 2009; but see Slate & Pemberton 2006), or the number or hatchability of a female’s eggs (Keller 1998; Cordero *et al.* 2004; Ortego *et al.* 2007). In systems with parental care, variability may correlate with a parent’s quality of provisioning, and thereby result in parental variability correlating with first-year or long-term survival prospects of their offspring (e.g. Richardson *et al.* 2004; Brouwer *et al.* 2007). However, the genetic variability of the offspring themselves may also be important, and determine their short- or long-term survival prospects. Offspring variability is directly related to the genetic similarity of their parents, with more dissimilar parents producing offspring with higher heterozygosity. Thus, the success of a given breeding event could be determined by the heterozygosity of each parent, but also by the average heterozygosity of the offspring that they produce as a result of their similarity (e.g. Bensch *et al.* 1994; van de Casteele *et al.* 2003).

Many studies of correlations between genetic variability and fitness are conducted on species that mature relatively quickly and have relatively short lifespans (Chapman *et al.* 2009), although there are notable exceptions (Amos *et al.* 2001). The HFC literature is also biased towards mammals and birds, and against species of high fecundity (Chapman *et al.* 2009). However, it is important for the general development of any theory in evolutionary biology that it be tested against a wide range of life history backgrounds. Here, we test for correlations between individual genetic variability and fitness in a population of the critically endangered hawksbill turtle (*Eretmochelys imbricata*), a long-lived, slow-to-mature, migratory species. We focus on two readily measurable parameters that relate directly to marine turtle fitness: the number of eggs laid in a clutch and the proportion of those eggs that develop successfully. For number of eggs laid, we test for correlations with female heterozygosity, whereas for successful egg development we test for correlations with parental heterozygosity and similarity. We consider a range of forms a potential effect of variability might take, including linear, quadratic, and interaction effects of multilocus variability. We also compare single-locus and multilocus models. Finally, we assess whether the mortality of developing embryos within nests is biased according to heterozygosity. Because full-siblings have the same inbreeding coefficient, a significant result in this test cannot come about through the general effects HFC model, and are instead indicative of local effects (Hansson *et al.* 2001; but see Szulkin *et al.* 2010). Our study population in the Republic of Seychelles is one of the world's most important populations of hawksbill turtles, but has declined substantially since the islands were colonised by humans 200+ years ago (Mortimer 1984, 2004). Assessing the link between genetic variability and fitness in this population may thus also help conservation managers better understand the processes driving variation in reproductive output in a species of high conservation priority.

4.3 Methods

Sampling was conducted on Cousine Island in the 2007/08 and 2008/09 hawksbill nesting seasons. Field methods, laboratory methods, and parentage analysis were conducted as described in section 1.2.

4.3.1 Independence of locus states

All loci satisfied assumptions of Hardy-Weinberg and linkage equilibria (GENEPOP 4.1; Raymond & Rousset 1995), and had null allele frequencies < 0.1 (CERVUS 3.0; Marshall *et al.* 1998). Following the recommendations of Szulkin *et al.* (2010), we conducted further tests of independence of loci states (heterozygous or homozygous) that indicate how well a given set of loci might reflect an individual's overall level of inbreeding. Firstly, we tested whether states correlate among loci ('identity disequilibrium'; Weir & Cockerham 1973). For a general measure, we calculated the g_2 statistic in RMES (David *et al.* 2007), significant positive values of which indicate identity disequilibrium. For a locus pair by locus pair measure of identity disequilibrium, we calculated the Spearman correlation coefficient (ρ) between within-individual heterozygous states (0 or 1) for all pairs of loci (Küpper *et al.* 2010), controlling for multiple comparisons using q-values (Storey 2002; q-values should be read as corrected P-values). Finally, we tested for heterozygosity-heterozygosity correlations (Balloux *et al.* 2004) by splitting the 32 loci at random into two subsets of 16, calculating multilocus heterozygosity (see below) for all individuals at both subsets, performing a Pearson correlation analysis between the subsets, and repeating this 10,000 times to estimate confidence intervals for the correlation coefficient. A lack of significant identity disequilibrium or heterozygosity-heterozygosity correlation would suggest that multilocus heterozygosity may not be a good indicator of inbreeding in our system.

4.3.2 Genetic predictors

For each genotyped adult hawksbill contributing offspring to our sample, we calculated multilocus heterozygosity (MLH) as standardised heterozygosity (SH), which gives all genotyped loci equal weighting but corrects for missing genotypes (Coltman *et al.* 1999). All the MLH tests that we present were also performed using two alternative MLH metrics: 'internal relatedness' (IR), which gives greater weighting in the MLH summary statistics to loci homozygous for rarer alleles (Amos *et al.* 2001), and 'homozygosity by loci' (HL), which gives greater weighting to loci with higher population-level expected heterozygosity (Aparicio *et al.* 2006). Allele frequencies used for calculating IR and HL were taken from the COLONY parentage output. The outputs of tests using IR and HL barely differed from SH, and we thus present them only in the supplementary material (Tables S1-S6). Data on single-locus heterozygosity (SLH) for each individual were coded as a series of 0's (homozygote loci) and 1's (heterozygote loci), with missing genotypes replaced with the population-level expected heterozygosity for the respective locus (Szulkin *et al.* 2010). Expected heterozygosity of our loci ranged from 0.43-0.89. Because this could distort the comparison between MLH and SLH models due to the functional relationship between SLH partial regression coefficients and marker variability, we standardised individual single-locus heterozygosity entries using the procedure recommended by Szulkin *et al.* (2010).

Genetic similarity between all observed pairings for which we were able to reconstruct male genotype data (henceforth 'multilocus parental similarity') was quantified using the relatedness metric of Queller & Goodnight (1989), calculated in the programme COANCESTRY 1.0 (Wang 2011b) using allele frequencies taken from the COLONY parentage output. On average, pairs with higher similarity produce offspring that are more inbred and have lower MLH. As a measure of single-locus parental similarity, we simply calculated the proportion of a pair's offspring expected to be heterozygotes at a given locus, based on Mendelian inheritance. Missing values were replaced with the population-level expected heterozygosity for the respective locus.

4.3.3 Fitness response variables

All HFC analyses were conducted in the statistical software R (R Development Core Team 2008), using linear mixed models in the package 'nlme' (Pinheiro *et al.* 2013). First, we tested number of eggs laid in a clutch against female heterozygosity, using female identity as a random effects term to account for females contributing multiple nests to the data set. For this analysis, we used every nest for which we knew the female's genotype, including nests that failed to hatch or that were destroyed by flooding or crab predation. Second, we tested proportion of eggs developing successfully against female heterozygosity, male heterozygosity, and parental similarity, with pair identity as a random effect. Using pair identity as the random effect meant that each male-female pair was treated as an independent subject, including the single case of a male that fathered offspring in both study seasons (chapter 3). The response was measured as the number of eggs showing evident development or that hatched, divided by the number of eggs laid, and was logit-transformed to better approximate a normal distribution. We selected this nest success parameter because it accounted for the largest proportion of eggs that did not produce a hatchling, and is less susceptible to noise introduced by crab predation than measures looking at later stages of success. Because the logit function cannot be used on proportions of 0 or 1, we re-calculated the measure for any such nest, adding an extra egg of the opposite success status (e.g. a nest with success of 0/120 would become 1/121). For analyses of this response that used male MLH and parental similarity as predictors, we excluded any nest that completely failed to develop, as all except one of these (which had a sister nest) lacked paternal genotype data. We also excluded multiple-paternity families, as we cannot know the relative contributions of each of the males to the proportion of eggs that fail. However, to test whether our female HFC findings on this smaller data set could be generalised, we re-ran the female HFC analyses, and included multiple-paternity families, nests that failed for unknown reasons (i.e. not inundation or crab predation), and families that produced too few offspring for male genotype reconstruction. The response variable did not differ significantly between multiple paternity and single paternity families (linear mixed model: difference = 0.149 ± 0.261 (SE); likelihood ratio test: d.f. = 3,4, $P = 0.569$).

We used an Akaike Information Criterion (AIC; Akaike 1974) model selection approach, implemented in the R package ‘MuMIn’ (Barton 2013), to compare twelve multilocus models of interest for proportional egg development success. These fell into four classes. Firstly, single predictor models: male MLH, female MLH, and parental similarity, each on its own; secondly, quadratic expressions for each of these predictors; thirdly, pairwise combinations of the three predictors (linear expressions) with no interaction term; and fourthly, each of the three pairwise interactions, with respective main effects still present. Of these, the latter three classes are all capable of showing both positive and negative effects of genetic variability in the same model, whether as opposing slope directions, quadratic curves, or interactions resulting in changes of slope direction. Models, including the null, were ranked by corrected AIC (AIC_C; Hurvich & Tsai 1989). A model was considered the nominal 'best' if it was ≥ 2.00 AIC_C units clear of the next best model. Otherwise, we considered the models comprising the top two units of AIC_C to be equally competitive. The null model was rejected only if it fell outside the top two AIC_C units. This analysis was repeated for all three MLH metrics. Male MLH and parental similarity are correlated to some extent in our data set (Pearson *r*: SH = -0.297), but not enough for collinearity to be a problem in our models after all continuous predictors were zero-centred ($\kappa \leq 2.51$; variance inflation factor ≤ 1.17). For all models, we calculated marginal and conditional R^2 to indicate the amounts of variance explained by the fixed effects and fixed effect-random effect combination respectively (Nakagawa & Schielzeth 2013).

To test for effects of heterozygosity associated with specific loci, we compared two models in a likelihood ratio test: a model with just MLH fitted as a linear expression, and a model with all 32 single loci fitted simultaneously as covariates (Küpper *et al.* 2010; Szulkin *et al.* 2010). We did this for each sex separately. Only if the SLH model was a significant improvement upon the MLH model did we examine the model summary for loci with partial regression slopes significantly different from zero. Unfortunately we were not able to compare the SLH models directly against the more complex MLH models within the AIC framework, as the majority of loci are expected to be of low information value and to result in a swamping of the AIC statistic. However, to offer some form of comparison, we also performed likelihood ratio tests for these models against MLH as a linear expression. We used the same procedure to compare multilocus and single-locus parental similarity.

4.3.4 Non-genetic predictors

Analyses on number of eggs laid were run both before and after controlling for a positive correlation between female body size and clutch size (Table 4.S2). Although there are fitness benefits associated with body size in both adult (Broderick *et al.* 2003) and hatchling turtles (Janzen *et al.* 2000, 2007), and a potential heritable component to body size (Myers *et al.* 2006), we did not test body size as an HFC response because we are unable to control for continuous growth in adult marine turtles (Chaloupka & Limpus 1997). Proportional egg success correlated strongly with clutch lay date (n-shaped quadratic effect) and clutch incubation duration (negative linear effect; Table 4.S2; Figs 4.S1, 4.S2). We thus ran the tests of genetic variability on proportional egg success both before and after controlling for these effects, to establish whether genetic variability explained any unique variance. We henceforth refer to models that include clutch lay date and incubation duration terms as 'D&I' ('date and incubation'), and models without them as 'Base'. Because HFCs and effects of inbreeding depression in other systems have been shown to vary between years (e.g. Brouwer *et al.* 2007; Szulkin & Sheldon 2007; Harrison *et al.* 2011), we initially included an interaction term between heterozygosity and study season when testing multilocus predictors. However, in no model did we find either a significant main effect of or interaction with season (data not shown).

As highlighted in section 1.2.1, relocating marine turtle nests can increase embryo mortality (Limpus *et al.* 1979; Parmenter 1980; Pintus *et al.* 2009). However, because approximately 95% of hawksbill nests laid on Cousine receive this treatment, we do not consider the practice likely to have biased our tests of proportional egg development success.

4.3.5 Biases to offspring heterozygosity

If early embryo mortality is biased according to the genetic variability of individual embryos, the average heterozygosity of offspring emerging from a given nest should differ significantly from expectations based on Mendelian segregation of parental genotypes. To test this, we simulated

5000 offspring genotypes for each full-sibling family for which we had genotype data on both parents, and calculated each family's average expected MLH (SH, HL, IR). The simulation process was Mendelian, and included missing data rates corresponding to those observed in the sample. We used two estimates of per-locus genotyping error, one derived from errors highlighted by the COLONY output, termed 'low' because it is likely to be an underestimate (Appendix 4.1), and one estimated from repeat PCRs (Phillips *et al.* 2013), termed 'high' because it is likely to be an overestimate (Appendix 4.1). For both simulated and observed offspring, only those with genotypes of ≥ 29 loci were included in the analysis. Observed and simulated family-level heterozygosities were then compared using three tests. First, a sign test, with a null hypothesis that half of families are more heterozygous than expected; second, a paired t-test of raw MLH values; third, a one-sample t-test of observed-expected differences standardised by dividing each family's raw difference by the standard deviation of its simulated offspring's MLH, then multiplying by the square root of the number of successfully genotyped offspring. When we applied these methods to entirely simulated data sets, we found no inherent bias in this approach that might inflate Type I error, given our criteria for inclusion of offspring genotypes and reconstructed male genotypes, and our estimates of genotyping error.

To test for single locus biases to offspring heterozygosity, we calculated the difference between the expected and observed numbers of heterozygotes for each family at each locus. We restricted the analysis to only those cases for which 0 < expected heterozygosity < 1, and included per-locus genotyping errors rates in our calculations. Values were then summed within loci and compared to a null hypothesis of no net difference using binomial tests, and controlling for multiple tests using *q*-values.

4.4 Results

We successfully genotyped 95 adult females and 2455 hatchlings over the two nesting seasons. After parentage analysis, and excluding 15 nests attributed to 10 unsampled females, this became a sample of 142 nests produced by 70 genotyped females (mean nests per female = 2.0 ± 1.2 (SD); max. = 5; Table 4.1). We henceforth use 'family' to refer to a nest or group of nests produced by a single female in a given year. All runs of COLONY converged on the same parentage arrangement. Of the 84 males inferred as contributing paternity to the offspring sample, we were able to reconstruct 64 genotypes that met our confidence criteria. Eight of our genotyped families showed multiple paternity (two of these were from unsampled females), and one male achieved paternity in both years of the study. No inferred males achieved paternity with more than one female per year.

Table 4.1. Breakdown by year of nests used in heterozygosity-fitness analysis. 'Nests with male genotype' and 'Male genotypes' give figures only for single-paternity nests/families, as multiple-paternity nests/families were not used in HFC analyses of male genotypes.

Year	Nests with female genotype	Females	Nests with male genotype	Male genotypes
2007/08	80	37	63	30
2008/09	62	33	49*	26
Total	142	70	112*	55**

* Includes one nest that failed completely but which had a sister nest

** One male fathered offspring in both years

4.4.1 Independence of loci

We found no evidence for overall identity disequilibrium among our loci from analysis in RMES (10,000 iterations: g_2 = 0.000, SD = 0.002, P = 0.366), and no cases of significant pairwise identity disequilibrium after controlling for multiple comparisons (maximum Spearman ρ = 0.29, q = 0.092). Similarly, we did not find a significant heterozygosity-heterozygosity correlation: the mean Pearson correlation coefficient, averaged over 10,000 randomisations, was 0.02 ± 0.06 (SD), and was significant (P < 0.05) in only 77 replicates.

4.4.2 Egg production HFC

No female heterozygosity term, MLH (linear or quadratic) or SLH, was a significant improvement upon the null model of the number of eggs laid by a female (Table 4.2). This remained the case after controlling for a significant positive correlation between female body size and number of eggs laid (Table 4.2).

Table 4.2. Model fit statistics for female heterozygosity as a predictor of number of eggs laid. All models are linear mixed models fitted by maximum likelihood, with female identity as a random effect, and are compared using likelihood ratio tests. For the 'Base' column, we give MLH relative to the null model (random effect only), and then compare MLH^2 and SLH models to MLH. For the 'body size' column, we first give the statistics for adding the body size term to the null model, MLH as an addition to the body size model, and MLH^2 and SLH against the 'body size + MLH' model. All analyses were conducted on 140 nests laid by 73 females. Female MLH is given as standardised heterozygosity, as choice of MLH metric did not affect interpretation.

	Base		**Body size**	
Model	***P***	**d.f.**	***P***	**d.f.**
Null	-		< 0.001	3,4
MLH	0.942	3,4	0.882	4,5
MLH^2	0.520	4,5	0.386	5,6
SLH	0.357	4,35	0.081	5,36

4.4.3 Proportional egg development HFCs

Differences between MLH metrics were minor, and we thus present only results for standardised heterozygosity (SH) and append those for the other two metrics in the supplementary material (Tables 4.S3, 4.S4, 4.S5). Male MLH was a significant predictor of proportional egg development success, but female MLH was not (Tables 4.3, 4.4). Parental similarity showed no significant main effect, but showed a significant interaction with male MLH (Tables 4.3, 4.4). In the 'Base' models, two models comprised the top two units of AIC_C: the quadratic model of male MLH (Fig. 4.1), and the model with an interaction between male MLH and parental similarity (Fig. 4.2). Both improved upon the null by ≥ two units, and had a combined Akaike weight of 0.905 (Table 4.3). The quadratic model had a marginal R^2 of 0.086, and the regression slope for the quadratic term was significantly different from zero (Tables 4.3, 4.4). The curve was n-shaped, with its maximum at SH

= 0.981, slightly below the midpoint of the statistic (SH is centred on 1.000). The interaction model had a marginal R^2 of 0.089, with significant regression slopes for the interaction term (positive) and the main effect of male heterozygosity (negative), but not for the main effect of parental similarity (negative) (Table 4.4). In the 'D&I' models, the model with the male MLH × parental similarity interaction term was the outright best, improving upon the model with just main effects of these terms (itself a significant improvement upon the null) by -2.23 AIC_C units (Table 4.3; Fig. 4.2). This interaction model showed a gain in marginal R^2 on the 'D&I' null of 0.077, and significant regression slopes for the interaction term (positive) and male heterozygosity (negative), but not for parental similarity (negative; Table 4.4).

In both the 'Base' and 'D&I' models, the interaction between male MLH and parental similarity means that the slope of male MLH against fitness is negative at low values of parental similarity, and becomes positive above a critical similarity value of 0.09 – approximately 0.88 standard deviations above mean parental similarity in both the 'Base' and 'D&I' models (mean similarity = 0.01, SD = 0.10; Figs 4.2A, 4.2C). Alternatively, the interaction can be read as parental similarity having a negative slope when male MLH is low and a positive slope when MLH is high, with the tipping point occurring at mean male MLH + 0.29 SDs for the 'Base' model and + 0.69 SDs for the 'D&I' model (mean male SH = 1.02, SD = 0.10; Figs 4.2B, 4.2D).

Single-locus models of female heterozygosity and parental similarity did not improve on their respective linear multilocus models, either before or after controlling for effects of lay date and incubation duration (Table 4.5). The interpretation for female heterozygosity did not change when we expanded the analysis to include six families (14 nests) showing multiple paternity, six families (six nests) producing too few offspring for male genotyping, and one nest with no eggs showing development but for which the parental genotypes were known from a sibling nest (we did not observe a single case of paternity changing between a given female's nests within a season; see also chapter 2). However, male SLH improved significantly upon male MLH in both the

'Base' and 'D&I' models (Table 4.5). In the 'Base' models, the SLH model was marginally less significant than the quadratic MLH model, but more significant than the male MLH × parental similarity model. In contrast, in the 'D&I' models, the MLH × parental similarity interaction was emphatically the most significant (Table 4.5). Examination of the 'Base' male SLH model summary showed three loci with slopes significantly different from zero, of which two were negative and one was positive (Table 4.4). In the 'D&I' models, two loci were significant, both negative, although only one of these (D1) was also significant in the base models (Table 4.4).

Table 4.3. Multilocus heterozygosity (MLH) and multilocus parental similarity models of proportional egg development success, assessed by corrected Akaike Information Criterion (AICC). MLH is measured as standardised heterozygosity (SH; Coltman *et al.* 1999). We assess twelve models relative to a null model. All models are linear mixed models with parent pair identity as a random effect. AICC values are expressed as difference from the respective null model. 'Base' models give AICC differences relative to a random effect-only model, whereas 'D&I' models give differences relative to a model that includes clutch lay date and clutch incubation duration. For each model, we also give its Akaike weight, and its marginal and conditional R^2 values (Nakagawa & Schielzeth 2013). AIC_C values in **bold** improve upon their respective null by ≥ two units; underlined values are within two units of the top model.

	Base models					**'D&I' models**			
Model	**AIC_C**	**Weight**	**Marg. R^2**	**Cond. R^2**	**Model**	**AIC_C**	**Weight**	**Marg. R^2**	**Cond. R^2**
M^2	**-4.23**	0.785	0.086	0.194	M × S	**-5.46**	0.878	0.376	0.376
M × S	**-2.35**	0.120	0.089	0.174	M + S	**-3.23**	0.094	0.349	0.349
M	-1.27	0.041	0.036	0.168	S	-0.79	0.008	0.321	0.321
M + S	-0.46	0.018	0.051	0.143	M^2	-0.48	0.006	0.333	0.371
M + F	-0.30	0.015	0.049	0.160	M	-0.27	0.005	0.316	0.354
Null	279.59	0.011	0.000	0.152	Null	248.49	0.004	0.299	0.348
F	+1.22	0.003	0.010	0.136	F + S	+1.11	0.001	0.323	0.323
M × F	+1.47	0.003	0.055	0.177	S^2	+1.34	0.001	0.322	0.322
S	+1.79	0.002	0.005	0.135	M + F	+1.34	0.001	0.321	0.356
F + S	+3.10	0.001	0.014	0.118	F	+1.70	0.001	0.304	0.347
F^2	+3.40	< 0.001	0.011	0.135	M × F	+3.08	< 0.001	0.326	0.367
S^2	+3.98	< 0.001	0.005	0.134	F × S	+3.28	< 0.001	0.324	0.324
F × S	+5.33	< 0.001	0.014	0.119	F^2	+3.59	< 0.001	0.306	0.352

M = male MLH; F = female MLH; S = multilocus parental similarity; '+' indicates two-predictor models with main effects only; '×' indicates two-predictor models with main effects and interaction term.

Table 4.4. Regression slopes for multilocus heterozygosity (MLH) and multilocus parental similarity models of proportional egg development success. MLH is measured as standardised heterozygosity (SH). We also give partial regression coefficients for four loci that were significant in the male single-locus heterozygosity (SLH) model, with coefficients corrected for locus variability. Slopes are estimated from linear mixed models with pair identity as a random effect. ‘Base’ refers to tests with only the genetic terms, whereas ‘D&I’ controls for clutch lay date and clutch incubation duration. We present female MLH results for two datasets: set 1 uses only nests for which a male genotype could be reconstructed, and excludes failing nests and multiple paternity families; set 2 uses all nests with a female genotype available. All non-SLH predictors were mean-centred to minimise collinearity when estimating the male MLH × parental similarity interaction coefficients. Significance is indicated by * (*P* < 0.05) and ** (*P* < 0.01), as calculated in the R package ‘nlme’ (Pinheiro *et al.* 2013).

	Base	**D&I**
Model/Term	**Slope ± SE**	**Slope ± SE**
Male MLH - linear		
Male MLH: SH	-1.58 ± 0.85	-1.11 ± 0.70
Male MLH - quadratic		
Male MLH: SH^2	-15.32 ± 6.74*	-9.05 ± 5.81
Male SLH		
Male SLH: Cc2	-0.22 ± 0.10*	-0.13 ± 0.09
Male SLH: Cm58	-0.14 ± 0.08	-0.18 ± 0.08*
Male SLH: D1	-0.21 ± 0.06**	-0.14 ± 0.06*
Male SLH: Eim31	0.21 ± 0.08*	0.08 ±0.07
Female MLH - linear		
Female MLH: SH – 1	0.81 ± 0.82	0.51 ± 0.68
Female MLH: SH – 2	-0.21 ± 0.94	-0.03 ± 0.78
Female MLH - quadratic		
Female MLH: SH^2 – 1	-0.49 ± 4.47	-2.44 ± 3.80
Female MLH: SH^2 – 2	-1.07 ± 5.07	-2.80 ± 4.18
Parental similarity - linear		
Similarity	-0.55 ± 0.88	-1.23 ± 0.66
Parental similarity - quadratic		
Similarity^2	0.23 ± 6.20	-1.95 ± 4.54
Interaction		
Male MLH: SH	-1.70 ± 0.84*	-1.39 ± 0.68*
Similarity	-0.58 ± 0.89	-1.13 ± 0.69
Male MLH × Similarity	18.78 ± 9.34*	15.76 ± 7.52*

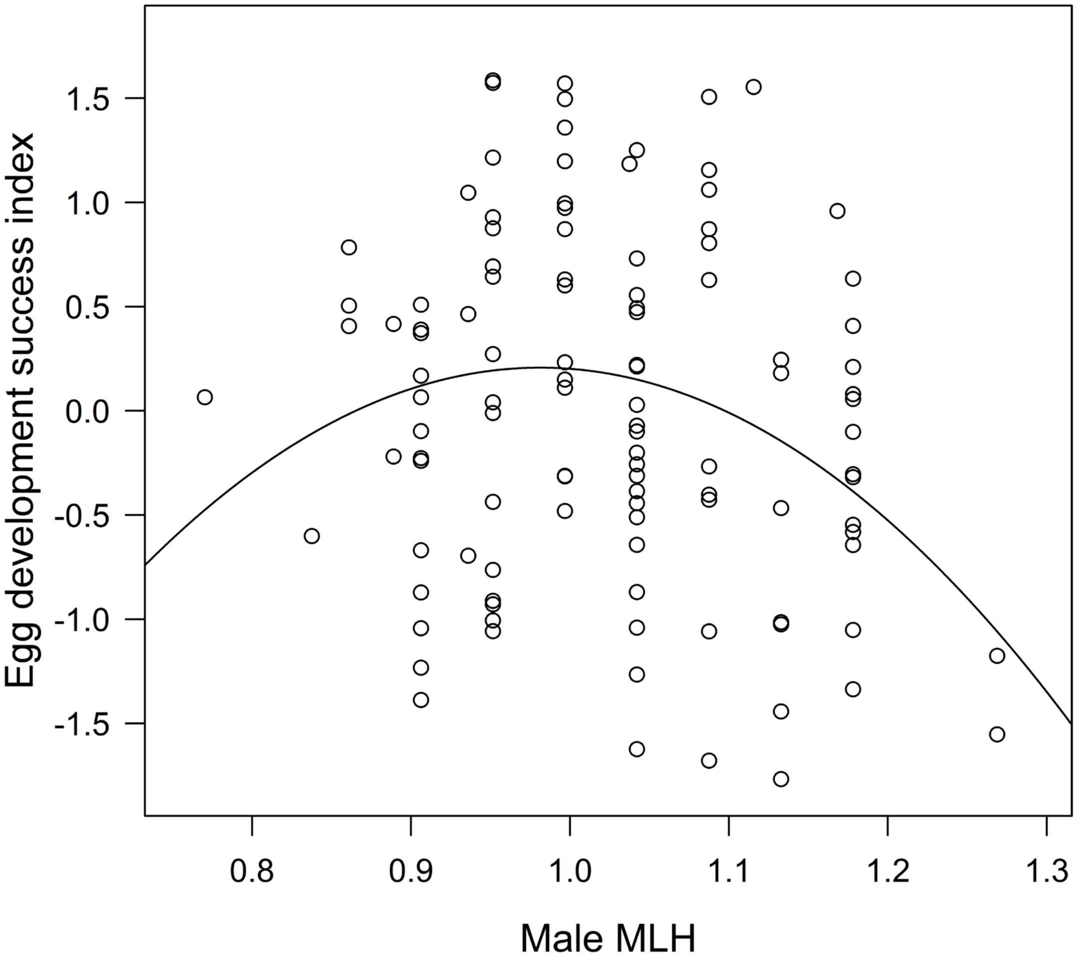

Figure 4.1. Quadratic relationship between male multilocus heterozygosity (MLH) and logit-transformed proportional egg development success in hawksbill turtles, before controlling for significant effects of clutch lay date and incubation duration. MLH is measured as standardised heterozygosity (SH). Model fit assessments are given in tables 3-5.

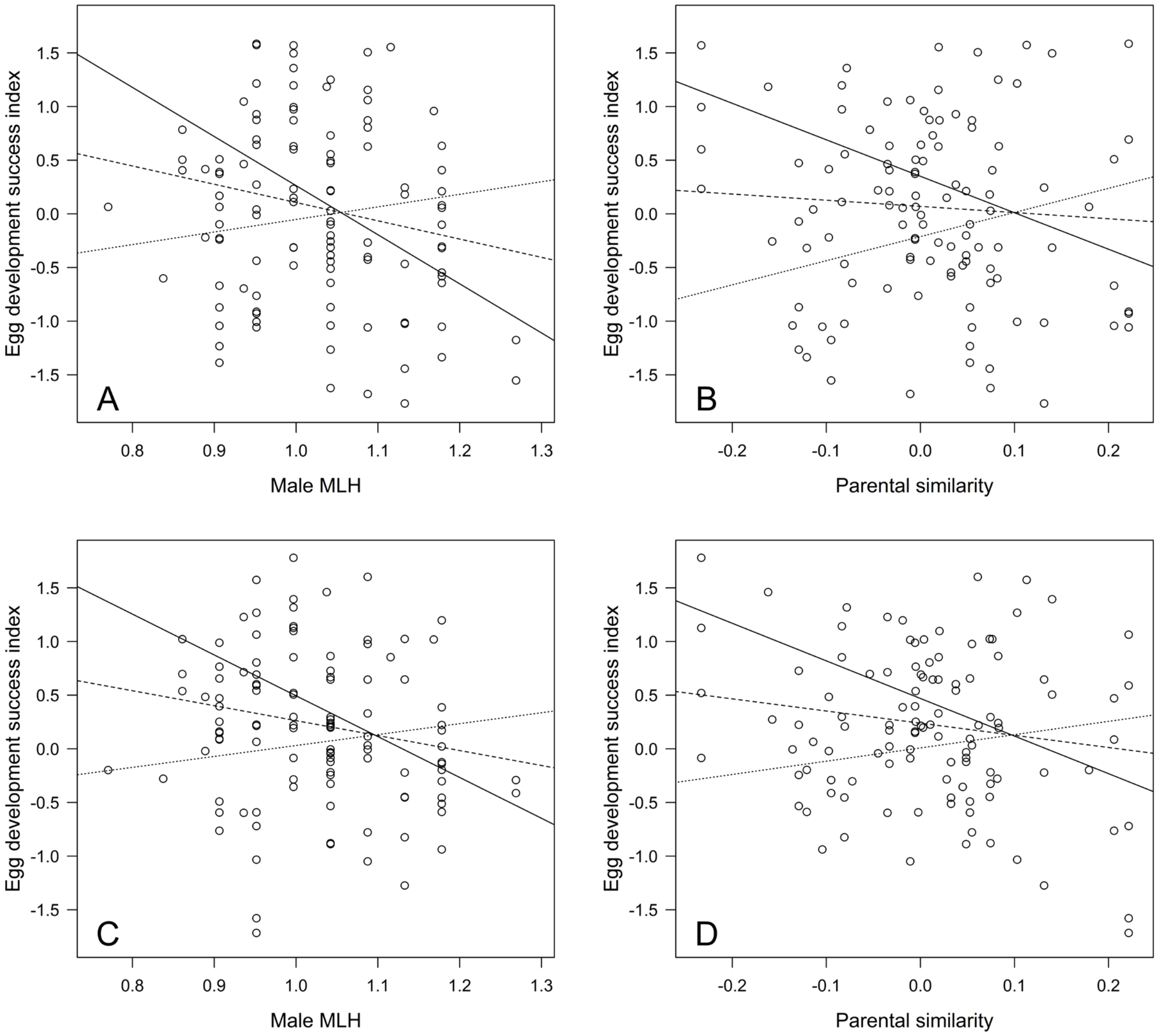

Figure 4.2. Effect of interaction between male multilocus heterozygosity (MLH) and parental similarity on logit-transformed proportional egg development success in hawksbill turtles. MLH is measured as standardised heterozygosity (SH). Model fit assessments are given in tables 3-5. A. Effect of male MLH at three different values of parental similarity *before* controlling for effects of clutch lay date and incubation duration. Solid line = mean parental similarity -1.5 standard deviations; dashed line = mean parental similarity; dotted line = mean parental similarity +1.5 standard deviations. Mean similarity = 0.01, SD = 0.10. B. Effect of parental similarity at three different values of male MLH *before* controlling for effects of clutch lay date and incubation duration. Solid line = mean male MLH -1.5 standard deviations; dashed line = mean male MLH; dotted line = mean male MLH +1.5 standard deviations. Mean male MLH = 1.02, SD = 0.10. C. As A, but *after* controlling for effects of clutch lay date and incubation duration. D. As B, but *after* controlling for effects of clutch lay date and incubation duration.

Table 4.5. Likelihood ratio comparisons of different genetic models of proportional egg development success. Multilocus heterozygosity (MLH) is measured as standardised heterozygosity (SH). Models are linear mixed models with male-female pair as a random effect term. For each focal predictor, we first test a linear expression of the multilocus term against the null model. We then present comparisons of a series of models against this linear MLH model, including the SLH model, a quadratic MLH model, and two-predictor and interaction models. Each model was run both before ('Base') and after ('D&I') controlling for significant effects of clutch lay date and clutch incubation duration.

Model predictors	**'Base'** ***P*** **(d.f.)**	**'D&I'** ***P*** **(d.f.)**
Male heterozygosity		
Male MLH (v. null)	0.064 (3,4)	0.110 (6,7)
SLH	**0.030** (4,35)	**0.026** (7,38)
MLH^2	**0.023** (4,5)	0.111 (7,8)
+ female MLH	0.269 (4,5)	0.399 (7,8)
+ parental similarity	0.238 (4,5)	**0.021** (7,8)
× female MLH	0.430 (4,6)	0.511 (7,9)
MLH × parental similarity	0.064 (4,6)	**0.007** (7,9)
Female heterozygosity		
Female MLH (v. null)	0.334 (3,4)	0.448 (6,7)
SLH	0.100 (4,35)	0.732 (7,38)
MLH^2	0.912 (4,5)	0.510 (7,8)
Female MLH + male MLH	0.054 (4,5)	0.101 (7,8)
+ parental similarity	0.575 (4,5)	0.088 (7,8)
× male MLH	0.124 (4,6)	0.190 (7,9)
× parental similarity	0.854 (4,6)	0.210 (7,9)
Parental similarity		
Multilocus (v. null)	0.546 (3,4)	0.080 (6,7)
Single locus	0.060 (4,35)	0.111 (7,38)
Multilocus $similarity^2$	0.971 (4,5)	0.659 (7,8)
+ male MLH (SH)	**0.035** (4,5)	**0.029** (7,8)
+ female MLH (SH)	0.347 (4,5)	0.513 (7,8)
× male MLH (SH)	**0.014** (4,6)	**0.009** (7,9)
× female MLH (SH)	0.642 (4,6)	0.731 (7,9)

MLH = multilocus heterozygosity; SLH = single-locus heterozygosity; SH = standardised heterozygosity

4.4.4 Heterozygosity of emerging hatchlings

Using the lower genotyping error rate model, significantly more families showed a heterozygosity deficit than expected, and both raw and standardised differences showed observed MLH to be significantly lower than expected (Table 4.6). When the higher genotyping error rate was used, significantly more families still showed a heterozygosity deficit when the sign test was used, although the effect size was reduced relative to that of the lower genotyping error rate (Table 4.6). However, neither t-test of heterozygosity differences was significant.

Table 4.6. Comparisons of observed against expected offspring heterozygosity derived from Mendelian simulations using two different genotyping error parameters ('lower' and 'higher'). Multilocus heterozygosity is measured as standardised heterozygosity. We apply three tests: a sign test (effect size given as percentage of full-sib families showing heterozygosity > 0); a t-test of raw heterozygosity differences (effect size given as mean difference between families' observed and expected heterozygosities); and a t-test of differences standardised by each family's standard deviation of simulated heterozygosity, and by the sample size used to calculate each family's 'observed' measure. For sign tests, N = 65; for t-tests, d.f. = 64.

	Lower error		**Higher error**	
Test	**Effect size**	***P***	**Effect size**	***P***
Sign test	30.8%	0.003	35.4%	0.025
t-test – raw differences	-0.006, t = 2.192	0.032	-0.002, t = 0.583	0.562
t-test – standardised differences	-0.305, t = 2.359	0.021	-0.069, t = 0.528	0.600

Using the lower genotyping error rate, the proportion of loci showing a heterozygosity deficit (22/32; 68.8%) was on the edge of significance (binomial test: $P = 0.050$). Within loci, no locus showed significant deviations from null expectations even without controlling for multiple comparisons (lowest $P = 0.126$). Summed across loci and families there were 222 fewer heterozygotes than expected, but this was not significant (binomial test: n = 66 463, $P = 0.085$). Using the higher genotyping error rate, more loci showed a heterozygote excess than a deficit (18/32; 56.3%), but this was not significant ($P = 0.597$). Within loci, without controlling for multiple comparisons, no locus showed significant deviations from null expectations (lowest P =

0.094). Summed across loci and families there was a heterozygote excess of 116, but this was also not significant (binomial test: n = 66 463, *P* = 0.372).

4.5 Discussion

In this study of the relationship between genetic variability and fitness in hawksbill turtles, we found no link between female heterozygosity and either clutch size or the proportion of eggs in a clutch showing embryo development. This was the case for both single-locus and multilocus heterozygosity models (SLH and MLH), and was unaffected by controlling for female body size. Proportional egg development remained uncorrelated with female heterozygosity when the significant effects of clutch lay date and incubation duration were included in the models. In contrast, several models indicated that male heterozygosity had a significant effect on proportional egg development. Parental similarity was also a significant predictor of proportional egg development, but only in an interaction with male heterozygosity. The precise nature of the male heterozygosity effect was difficult to determine, as several models were competitive. However, a unifying feature was the presence of both positive and negative effects of male genotype: a quadratic expression implying an optimum level of male heterozygosity; an interaction in which the direction of the fitness correlation with male genotype changes with the level of parental similarity; and both positive and negative effects among single-locus HFCs.

In our study, male MLH showed a negative HFC over a greater area of parameter space than it showed a positive HFC. In the model with male MLH as a quadratic term, the maximum of the n-shaped curve occurred to the left of centre. In the model with the male MLH × parental similarity interaction term, the HFC only becomes positive above similarities of 0.88 standard deviations above mean similarity. Negative HFCs are much less widely reported in the literature than positive HFCs (Küpper *et al.* 2010; Mueller *et al.* 2011; Szulkin & David 2011). Part of this relative rarity is likely attributable to study system biases, with many HFC studies being conducted on small, founder or fragmented populations that are potentially more vulnerable to inbreeding (see

Küpper *et al.* 2010). Our study in a large, apparently genetically healthy population (chapter 3) highlights the importance of testing for HFCs against a range of demographic backgrounds. Parental similarity, in the interaction model, also showed a negative effect over the greater part of parameter space, becoming positive at approximately 0.29-0.69 SDs above mean heterozygosity. This net effect is more in line with classic inbreeding models, where parental pairs of high similarity produce offspring of reduced fitness (Charlesworth & Charlesworth 1987; Bensch *et al.* 1994). However, these interpretations should be treated carefully, as it may be taking our results too far out of the context of the interaction between male MLH and parental similarity.

There is considerable debate in the HFC literature as to how well MLH represents individual inbreeding status, a point that has significant ramifications for the relative importance of local and general effects in generating HFCs (Balloux *et al.* 2004; Szulkin *et al.* 2010). Thus all HFC studies are urged to assess the utility of MLH as an inbreeding proxy through either a test for identity disequilibrium or a heterozygosity-heterozygosity correlation, with the former preferred for its greater power (Szulkin *et al.* 2010). In our study, we found no significant identity disequilibrium or heterozygosity-heterozygosity correlation, suggesting that, despite a large panel of markers, MLH in this case is not a good proxy for inbreeding. Without identity disequilibrium, the significant effect associated with MLH is difficult to explain, as one should predict no effect (Chapman & Sheldon 2011). However, for several reasons we are wary of dismissing the MLH-inbreeding link as an explanation for the patterns observed in our study. Firstly, multilocus HFCs underpinned by inbreeding can reach significance before identity disequilibrium is significant (Szulkin *et al.* 2010). Secondly, several authors have argued that HFCs, both multi- and single-locus, are more likely to be detected when conserved markers are used, and marine turtle microsatellite loci are extremely conserved (discussed further below). Thirdly, our population, although large and genetically well-mixed, is not homogeneous, and shows some evidence of female natal philopatry (chapter 3). In large populations, structure creates a greater potential for inbreeding than does full mixing (Olano-Marin *et al.* 2011a, 2011b; see also Szulkin *et al.* 2010). Fourthly, we see variation in genetic similarity between observed male-female pairings, ranging from individuals that look to come from different populations (min. similarity = -0.233) to those approximately equivalent to half-siblings or double-cousins (max. similarity = 0.221). Finally, the rationale behind tests such as identity disequilibrium and heterozygosity-heterozygosity correlations is that they reflect variance in individual inbreeding status. This inbreeding variance helps drive multilocus HFCs, and HFC effect sizes are predicted to be larger with greater variance. However, in meta-analysis, albeit in a self-admittedly coarse test, Chapman *et al.* (2009) did not find empirical support for the link between inbreeding variance and HFC effect size. We therefore discuss MLH in its traditional inbreeding interpretation, but advise this to be treated with caution.

In our study, the male quadratic HFC model implies that fitness is maximised at an optimum amount of genetic variability, with the direction of the HFC changing either side of this point. The model with the male MLH × parental similarity interaction does not imply a single optimum variability, but does result in HFCs that change direction, a process which could exert a stabilising influence on population genetic variability (Neff 2004). If MLH is interpreted in its classic inbreeding context, this could be read as a tension between inbreeding and outbreeding depression, with an optimum level of outcrossing. Studies inferring both inbreeding and outbreeding depression acting on the same fitness trait in the same system are few (Price & Waser 1979; Waser & Price 1989; Marshall & Spalton 2000; Marr *et al.* 2002; Cordero *et al.* 2004; Neff 2004), although further examples of them operating on different traits in the same population exist (Olano-Marin *et al.* 2011a, 2011b). However, their collective implication is that some form of population structure (e.g. isolation-by-distance, philopatry, founder populations) facilitates both local adaptation and elevated inbreeding risk, and that there is a trade-off between reducing inbreeding and avoiding outbreeding depression. Could such a process operate in marine turtles? Potentially, yes. Female green turtles nesting on Ascension Island in the Atlantic show fine-scale local adaptation to between-beach differences in nest incubation conditions

(Weber *et al.* 2012). This local adaptation persists despite evidence for strong male-biased gene flow between Ascension's nesting beaches (Lee *et al.* 2007). More generally, population genetics studies in several marine turtle species have emphasised that females show natal philopatry but that males are more dispersive (reviewed in Bowen & Karl 2007; Lee 2008), which could potentially give rise to an inbreeding-outbreeding tension analogous to that seen in some plants (Waser & Price 1989, 1994). Fine-scale sex-biased natal philopatry has also been implicated in HFCs in blue tits (*Cyanistes caeruleus*), where positive and negative HFCs have been observed in the same population (Olano-Marin *et al.* 2011b; see also Szulkin & David 2011). Is there evidence for such structure in our study system? This is harder to say, as it is not clear how much structure is needed for an inbreeding-outbreeding tension to come about, or how this might vary across ecological contexts. In the western Indian Ocean, maternally-inherited mtDNA shows no structure between hawksbills nesting in the Seychelles and Chagos groups, 2000 km apart (Vargas *et al.* 2013), and microsatellites show no structure within Seychelles on a 200-500 km scale (chapter 3). On the other hand, average pairwise genetic similarity is higher among females nesting on Cousine than among the males that have fertilised them, implying male-biased dispersal and some degree of population structuring (chapter 3). Unfortunately we do not have comparable data from other sites in Seychelles (i.e. fewer female samples than from Cousine, and no male genotypes) that would allow us to test the spatial scale and strength of this inference.

Several authors have argued for the importance of marker type in HFC studies. MLH measured using microsatellites located in expressed or otherwise conserved regions may be more likely to yield HFCs than MLH using anonymous/non-conserved loci by virtue of, on average, being closer to polymorphic loci under selection (Küpper *et al.* 2010; Olano-Marin *et al.* 2011a, 2011b; Szulkin & David 2011). The effect is statistically still a 'general' one, as it is the net, cumulative effect of multiple small effects (Szulkin & David 2011). Interestingly, the vast majority of marine turtle microsatellite loci characterised to date show a remarkably high degree of conservation. Almost all primers designed from a given hard-shelled species (family Cheloniidae) have amplified across other hard-shelled species (e.g. FitzSimmons *et al.* 1995; Aggarwal *et al.* 2004; Lin *et al.* 2008) – indeed, 18/32 loci used in our study were first characterised in members of this family other than the hawksbill (chapter 1). The deepest internal split in Cheloniidae has been dated at approximately 63 MYA (Naro-Maciel *et al.* 2008). Furthermore, a large number of these loci amplify in leatherbacks (Dermochelyidae: *Dermochelys coriacea*; e.g. Shamblin *et al.* 2007), which diverged from Cheloniidae approximately 110-120 MYA (Bowen *et al.* 1993b; Naro-Maciel *et al.* 2008), and some loci even amplify in freshwater species (e.g. FitzSimmons *et al.* 1995; Lin *et al.* 2008). Our findings of significant multilocus HFCs may thus not be out of keeping with prevailing HFC theory, given the highly conserved nature of our markers. However, it is important to note that marine turtle sequence evolution is known to be particularly slow (Avise *et al.* 1992; Bowen *et al.* 1993b; FitzSimmons *et al.* 1995; but see Lourenço *et al.* 2013), which may affect the comparability between our 'conserved' markers and those explicitly selected to be conserved in taxa with much faster sequence evolution rates (e.g. Küpper *et al.* 2010; Olano-Marin *et al.* 2011a,2011b; see also Dawson *et al.* 2013).

The male SLH model was significantly better than a linear expression of male MLH, suggesting that some loci may be exerting a particularly strong influence on fitness. In the 'Base' model, three loci had significant effects, two negative and one positive, while in the 'D&I' models, two loci had significant negative effects (Table 4.4). Only one locus (D1) was significant in both models. However, given our sample size relative to the number of loci, it is probably unsafe to speculate on whether the change between the two models reflects relationships between heterozygosity at specific loci and lay date or incubation, as we are not aware of a statistical approach that would enable us to test this without either severely overstressing the model or severely inflating the risk of Type I error. That three out of these four single-locus HFCs were negative may help explain the net negative effect of male multilocus heterozygosity, but robustly assessing how much each locus contributes to the inbreeding-outbreeding dynamic suggested by our MLH models would require much greater sample sizes. Also, although the SLH model was competitive among the

'Base' models, its improvement on the linear male MLH model in the 'D&I' set was notably less significant than the improvement of adding parental similarity or the male MLH × parental similarity interaction term, encouraging scepticism over how much meaning can be safely attached to the single-locus effects.

Negative single-locus HFCs are more difficult to explain than positive single-locus HFCs (Lieutenant-Gosselin & Bernatchez 2006; Küpper *et al.* 2010; Mueller *et al.* 2011). A single-locus equivalent to outbreeding depression can come about through underdominance, when co-adapted gene complexes are broken up (Mueller *et al.* 2011). Such HFCs are predicted to be extremely rare, as they will be unstable in the absence of negative frequency-dependent selection against homozygotes (Mueller *et al.* 2011). Interestingly, our multilocus interaction effect demonstrates the potential for an HFC to change direction depending on genomic context, which might, if operating at the single-locus level, allow an underdominance-driven negative HFC to persist in the population. Negative HFCs may also come about in loci exhibiting codominance and directional selection (Küpper *et al.* 2010; Mueller *et al.* 2011). These correlations are predicted to be transient, to occur only when the 'beneficial' allele has a frequency > 0.5, and to have an effect size related to the frequency of the beneficial allele. This model may have relevance to our system, as 12 of our loci have an allele of frequency > 0.5. However, the three loci with significant negative regression slopes were not among these. Furthermore, even if a microsatellite is in close physical linkage and/or close identity disequilibrium with a functional locus, caution is still needed before using the microsatellite allele frequencies as a proxy for those of the functional locus in such a way. Recessive allele advantage can also lead to negative single-locus HFCs, but it is not clear how we can assess this with microsatellite data (Küpper *et al.* 2010; Mueller *et al.* 2011).

We found no female HFC with either tested fitness parameter. Examples of differences between sexes in HFCs and effects of inbreeding are common, and females seem more likely to be affected than males (reviewed in Olano-Marin *et al.* 2011b). These effects vary in form from early-life survival of females (Coulson *et al.* 1999; Olano-Marin *et al.* 2011b) to maternally-transmitted effects to the next generation (Richardson *et al.* 2004; Brouwer *et al.* 2007). In our case, the absence of any female HFC may be a consequence of the female hawksbill breeding strategy. Like all marine turtle females, hawksbills are capital breeders: they accumulate an energetic reserve ('capital') with which to produce and provision offspring, in contrast with 'income breeders' that use a continuous energetic intake to support breeding (e.g. Stephens *et al.* 2009). Accumulating reproductive capital seems to be a slow and variable process in this species, as female hawksbills typically only breed every 2-4 years. Individual rates of capital accumulation may be heavily influenced by environmental conditions (e.g. Limpus & Nicholls 1988; Broderick *et al.* 2001, 2003). Differences in quality between the sites visited by different females between breeding episodes may thus mean that females vary substantially in their breeding condition, and thus their ability to produce/provision eggs, for reasons unrelated to heterozygosity. Correlations between female heterozygosity and egg number/quality may thus be difficult to detect in marine turtles without controlling for this capital-associated variance. An implication of our detecting significant effects of male MLH and parental similarity is that any systematic between-female effect is not strong enough, after including a random effects term, to mask these other effects. With sufficient long-term data, it might be possible to test for an HFC with female remigration interval, with the hypothesis that heterozygosity affects efficiency at accumulating energetic capital, and thus remigration frequency.

A prominent difficulty with our overall result on male MLH and parental similarity is the uncertainty as to how the interaction between these might operate mechanistically. In our study, fitness is reduced when an egg is either unfertilised or an embryo dies very early. A simple effect of male heterozygosity might imply an effect of sperm/ejaculate quality, although previous work showing season-long sperm storage without evidence of depletion in this population suggest that sperm quantity is unlikely to be limiting (chapter 2). Alternatively, an effect of parental similarity on its own might imply issues arising from parental genetic compatibility (Jennions 1997; Zeh &

Zeh 1997). One possible explanation for the interaction between male MLH and similarity could be that if parents are dissimilar, and thus are already producing offspring of high heterozygosity, the addition of extra variability from a particularly heterozygous male may be deleterious (outbreeding depression). If the parents are similar, producing offspring of low variability, additional variability from paternal heterozygosity may be beneficial. How this might apply in practice will be affected by the heritability of heterozygosity (Mitton *et al.* 1993; Garcia-Navas *et al.* 2009) and the relationship between heterozygosity and similarity (Roberts *et al.* 2006). We should add that although male MLH and parental similarity are correlated in our data set, collinearity was not problematically high (see section 4.3.3).

An important caveat to our results is that reconstructing male genotypes applies a minimum quality threshold to a nest with respect to the response variable – if a clutch does not produce enough offspring for paternal genotype reconstruction, it cannot be included. Thus our findings with respect to male heterozygosity and parental similarity may only be applicable to nests that are already ‘not very bad’. Unfortunately there is no way to eliminate these difficulties in a system where males cannot be sampled directly, except to extend sampling in time and space, hoping to reconstruct the male genotype from another nest. However, our findings on female heterozygosity were not limited in this way and were thus more generalisable, with interpretation not differing between the data set that had male genotypes and the data set that incorporated every nest for which female genotype was known.

Heterozygosity of offspring was lower than expected when simulations used low estimates of genotyping error (likely to be underestimates). When higher per-locus error rates (likely to be overestimates) were used, more families than expected still showed a net homozygosity excess, but the overall difference between observed and expected heterozygosity was no longer significant. If taken as a real effect, this deficit of heterozygotes would suggest either a bias to embryo mortality (more heterozygous offspring being more likely to die early), or a bias to fertilisation (sperm-egg combinations resulting in more homozygous offspring occurring more frequently). If the former were true, one would predict a positive effect of multilocus parental similarity on proportional egg development success, assuming random union of gametes. We saw no such effect: if anything, the net effect of parental similarity was marginally negative. If the latter mechanism were true, it is difficult to find a sensible mechanistic explanation for the bias in the wider literature on sperm competition and cryptic female choice: although it is well established that females of some species can use post-copulatory selection to bias paternity when inseminated by multiple males (Snow & Andrade 2005; Løvlie *et al.* 2013), we are aware of no clear evidence for choice *within* the sperm of a male. Furthermore, in chapter 2 we did not find any evidence for female hawksbills engaging in any kind of mate choice on genetic grounds. Thus, although there is a possibility of heterozygosity being slightly lower than expected, we are wary of separating this from the low background rate of allelic dropout occurring in our offspring genotypes.

We have shown an effect of male heterozygosity on reproductive fitness in hawksbill turtles, but one that is complex and likely to be composed of both negative and positive effects. We also found a significant effect of parental genetic similarity on reproductive fitness, as an interaction term with male heterozygosity. Collectively, these models imply that the HFCs in our system are not simple linear processes. Instead, they may change according to genetic context, and lead to a stabilised level of genetic diversity. If we accept the multilocus HFC models and treat MLH as a proxy of inbreeding, our results highlight a potential tension between inbreeding depression and outbreeding depression, which may arise from female natal philopatry and male dispersal. However, we are cautious of doing so due a lack of identity disequilibrium in our test, and have highlighted single-locus HFCs, both positive and negative, that might be contributing to the effect of variability on fitness. How well MLH reflects individual inbreeding could be better tested by using additional markers, potentially including next generation sequencing approaches, such as restriction-site associated DNA (e.g. Miller *et al.* 2007; Pavey *et al.* 2012). We also report a potential effect of parental similarity, an effect that was only present as an interaction term, but which, given that it is a proxy for average offspring variability, also implied an inbreeding-outbreeding tension.

A potentially informative extension to our study would be to examine whether the effects we observed in males and parental similarity apply to candidate loci known to exhibit HFCs, such as immune genes (e.g. major-histocompatibility complex and Toll-like receptors (e.g. Piertney & Oliver 2006; Grueber *et al.* 2012)). It would also be informative to extend the study into additional years, partly to expand the sample size, but also because previous studies have highlighted that HFC can change according to prevailing conditions in a given breeding season, being more likely to be expressed in 'bad' years that might expose effects of inbreeding (or outbreeding) depression (e.g. Brouwer *et al.* 2007; Harrison *et al.* 2011). More generally, our interpretation of both MLH and SLH testing could be aided by a fully annotated marine turtle genome, as such a resource would allow us to assess the proximity of our loci to functional regions. At the time of writing, a draft green turtle (*Chelonia mydas*) has been published (Wang *et al.* 2013), but its annotations (predicted genes) are not yet available in a form amenable to such testing.

To summarise, our results emphasise the importance of looking for fitness effects of both high and low levels of genetic variation within a system, and even on the same fitness trait. Such studies are relatively few, but play an important role in understanding how genetic variation is maintained in wild populations and how this might affect individual fitness. Our study is also of value to the HFC literature for the characters of the species, as being long-lived, slow-to-mature, fecund, and a reptile are all traits that have been under-examined by HFC studies (Chapman *et al.* 2009). From a conservation perspective, our results suggest that both inbreeding and outbreeding may affect fitness in the Seychelles population of hawksbill turtles. Whether these effects have been altered by the substantial population declines caused by two centuries of overhunting is impossible to say from our study, but their mutual presence may highlight an important balance that could be disturbed by anthropogenic processes

4.6 Appendices

4.6.1 Appendix 6.1: Explanations of ‘low’ and ‘high’ genotyping error rates used for generating simulated offspring for comparison with observed family heterozygosity.

‘Low’ genotyping error rates were taken from the output of the COLONY parentage analysis, which identifies genotypes (parental and offspring) that it suspects of containing errors. We reviewed all highlighted errors and classified them as ‘dropout’ (homozygous for a parental allele) or ‘other’ (heterozygous with a non-parental allele, arising from mutation, allele sizing error, or human error when naming alleles). This measure will underestimate dropout. This is because if parents share an allele at a locus, dropout resulting in an offspring being homozygous for the shared allele will not be separable from real homozygosity. For example, dropout occurring in the AB offspring on an AB-AB parental cross will produce genotypes appearing to be AA or BB, indistinguishable from the real AA and BB offspring that this cross will produce.

‘High’ genotypes error rates were estimated by repeating PCR on 96 randomly-selected samples and comparing scored genotypes (see section 1.2.4). We also compared the genotypes of all offspring of known parentage to the genotypes of their respective mother. These error rates will be overestimates because we took steps to resolve all highlighted mismatches by re-checking raw genotype electropherograms and, where necessary, repeating PCRs .

Table 4.S1. Pearson correlation coefficients between different metrics for multilocus heterozygosity using 32 microsatellite loci. Correlations are derived from adult genotypes used in the main analysis, which were only used if they contained ≥ 29/32 loci. 'H' is uncorrected MLH (number of heterozygous loci/number of amplifying loci); 'SH' is standardised heterozygosity, which standardises the scale to control for missing loci (Coltman *et al.* 1999); 'HL' is homozygosity by loci, which weights loci by their expected heterozygosity (Aparicio *et al.* 2006); 'IR' is internal relatedness, which gives greater weight to genotypes homozygous for rare alleles (Amos *et al.* 2001). Raw HL and IR are negatively correlated with genetic variability – individuals homozygous at all loci have a score of 1 for both metrics.

	SH	HL	IR
H	0.9999	-0.9770	-0.9663
SH	-	-0.9766	-0.9663
HL	-	-	0.9547

Table 4.S2. Significant regression terms for non-genetic predictors controlled for in our HFC analyses. Body size is controlled for when testing HFCs with clutch size, whereas incubation duration and lay date are controlled for when testing proportional egg development success. All models are linear mixed models, with parental identity as a random effects term.

Model/term	**Slope**	**SE**	**Overall *P***	**(d.f.)**	**ΔAIC_C**	**Marginal R^2**	**Conditional R^2**
Clutch size							
Female body size	2.967	0.777	< 0.001	(3,4)	-10.44	0.116	0.289
Egg development							
Lay date	0.028	0.010					
Lay date^2	-1.80 E-4	4.96 E-5					
Incubation duration	-0.088	0.019	< 0.001	(3,6)	-31.10	0.299	0.348

Table 4.S3. Fit qualities of multilocus heterozygosity (MLH) and multilocus parental similarity models of proportional egg development success, assessed by corrected Akaike Information Criterion (AIC_C). For each of three MLH metrics (SH, HL, IR) we assess twelve models relative to a null model. All models are linear mixed models with parent pair identity as a random effect. AIC_C values are expressed as difference from the respective null model. ‘Base’ models give AIC_C differences relative to a random effect-only model, whereas ‘D&I’ models give differences relative to a model that includes clutch lay date and clutch incubation duration. For each model, we also give its Akaike weight, and its marginal and conditional R^2 values (Nakagawa & Schielzeth 2013). AIC_C values in **bold** improve upon their respective null by ≥ two units; underlined values are within two units of the top model.

	Base models					**‘D&I’ models**			
Model	**AIC_C**	**Weight**	**Marg. R^2**	**Cond. R^2**	**Model**	**AIC_C**	**Weight**	**Marg. R^2**	**Cond. R^2**
MLH: SH									
M^2	**-4.23**	0.785	0.086	0.194	M × S	**-5.46**	0.878	0.376	0.376
M × S	**-2.35**	0.120	0.089	0.174	M + S	**-3.23**	0.094	0.349	0.349
M	-1.27	0.041	0.036	0.168	S	-0.79	0.008	0.321	0.321
M + S	-0.46	0.018	0.051	0.143	M^2	-0.48	0.006	0.333	0.371
M + F	-0.30	0.015	0.049	0.160	M	-0.27	0.005	0.316	0.354
Null	279.59	0.011	0.000	0.152	Null	248.49	0.004	0.299	0.348
F	+1.22	0.003	0.010	0.136	F + S	+1.11	0.001	0.323	0.323
M × F	+1.47	0.003	0.055	0.177	S^2	+1.34	0.001	0.322	0.322
S	+1.79	0.002	0.005	0.135	M + F	+1.34	0.001	0.321	0.356
F + S	+3.10	0.001	0.014	0.118	F	+1.70	0.001	0.304	0.347
F^2	+3.40	< 0.001	0.011	0.135	M × F	+3.08	< 0.001	0.326	0.367
S^2	+3.98	< 0.001	0.005	0.134	F × S	+3.28	< 0.001	0.324	0.324
F × S	+5.33	< 0.001	0.014	0.119	F^2	+3.59	< 0.001	0.306	0.352
MLH: HL									
M × S	**-3.41**	0.407	0.097	0.178	M × S	**-5.06**	0.791	0.374	0.374
M^2	**-3.19**	0.327	0.076	0.191	M + S	**-3.52**	0.170	0.351	0.351
M	**-2.13**	0.113	0.044	0.167	S	-0.79	0.011	0.321	0.321
M + F	-1.57	0.065	0.061	0.160	M	-0.66	0.010	0.318	0.352
M + S	-1.34	0.051	0.059	0.142	Null	248.49	0.005	0.299	0.348
M × F	-0.16	0.016	0.069	0.180	M^2	+0.20	0.004	0.328	0.365
Null	279.59	0.013	0.000	0.152	M + F	+0.20	0.004	0.329	0.354
F	+1.33	0.004	0.009	0.139	F + S	+0.87	0.002	0.325	0.325
S	+1.79	0.002	0.005	0.135	S^2	+1.34	0.001	0.322	0.322
F + S	+3.23	0.001	0.013	0.122	F	+1.41	0.001	0.306	0.347
F^2	+3.52	< 0.001	0.009	0.140	M × F	+2.25	0.001	0.331	0.361
S^2	+3.98	< 0.001	0.005	0.134	F × S	+3.23	< 0.001	0.325	0.325
F × S	+5.34	< 0.001	0.014	0.128	F^2	+3.24	< 0.001	0.308	0.354

Continued on next page

Continued from previous page

MLH: IR									
M × S	**-2.52**	0.523	0.090	0.173	M × S	**-7.50**	0.956	0.387	0.387
M^2	-1.51	0.190	0.063	0.193	M + S	**-4.24**	0.037	0.355	0.355
M	-1.09	0.125	0.034	0.163	M^2	-1.77	0.003	0.34	0.371
M + S	-0.16	0.049	0.048	0.137	M	-1.08	0.002	0.321	0.356
M + F	-0.02	0.043	0.047	0.155	S	-0.79	0.001	0.321	0.321
Null	279.59	0.042	0.000	0.152	Null	248.49	0.001	0.299	0.348
F	+1.40	0.010	0.008	0.137	M + F	+0.41	< 0.001	0.327	0.358
S	+1.79	0.007	0.005	0.135	F + S	+1.14	< 0.001	0.323	0.323
M × F	+1.90	0.006	0.050	0.168	S^2	+1.34	< 0.001	0.322	0.322
F + S	+3.30	0.002	0.012	0.120	F	+1.70	< 0.001	0.304	0.346
F^2	+3.57	0.001	0.008	0.139	M × F	+2.57	< 0.001	0.329	0.365
S^2	+3.98	0.001	0.005	0.134	F × S	+3.00	< 0.001	0.326	0.326
F × S	+5.42	< 0.001	0.014	0.115	F^2	+3.42	< 0.001	0.307	0.353

M = male MLH; F = female MLH; S = multilocus parental similarity; ‘+’ indicates two-predictor models with main effects only; ‘×’ indicates two-predictor models with main effects and interaction term.

Table 4.S4. Regression slopes for multilocus heterozygosity (MLH; three metrics) and multilocus parental similarity models of proportional egg development success. We also give partial regression coefficients for four loci that were significant in the male single-locus heterozygosity (SLH) model, with coefficients corrected for locus variability. Slopes are estimated from linear mixed models with pair identity as a random effect. 'Base' refers to tests with only the genetic terms, whereas 'D&I' controls for clutch lay date and clutch incubation duration. For males, we present three multilocus heterozygosity metrics (SH, HL, IR), as model rankings differed subtly between metrics (Table S3). HL and IR slopes have been sign-swapped from their raw test outputs. For females, there were no such differences, and we thus present only SH. However, we present female MLH results for two datasets: set 1 uses only nests for which a male genotype could be reconstructed, and excludes failing nests and multiple paternity families, whereas set 2 uses all nests with a female genotype available, and where the nest was neither preyed upon nor flooded. All non-SLH predictors were centred to minimise collinearity when estimating the male MLH × parental similarity interaction coefficients. Significance is indicated by * ($P < 0.05$) and ** ($P < 0.01$), as calculated in the R package 'nlme' (Pinheiro *et al.* 2013).

	Base	**D&I**
Term	**Slope ± SE**	**Slope ± SE**
Male MLH - linear		
Male MLH: SH	-1.58 ± 0.85	-1.11 ± 0.70
Male MLH: HL	-2.71 ± 1.30*	-1.84 ± 1.08
Male MLH: IR	-1.83 ± 1.01	-1.50 ± 0.83
Male MLH - quadratic		
Male MLH: SH^2	-15.32 ± 6.74*	-9.05 ± 5.81
Male MLH: HL^2	-26.19 ± 14.61	-14.76 ± 12.49
Male MLH: IR^2	-14.33 ± 8.93	-12.53 ± 7.37
Male SLH		
Male SLH: Cc2	-0.22 ± 0.10*	-0.13 ± 0.09
Male SLH: Cm58	-0.14 ± 0.08	-0.18 ± 0.08*
Male SLH: D1	-0.21 ± 0.06**	-0.14 ± 0.06*
Male SLH: Eim31	0.21 ± 0.08*	0.08 ±0.07
Female MLH - linear		
Female MLH: SH – 1	0.81 ± 0.82	0.51 ± 0.68
Female MLH: SH – 2	-0.21 ± 0.94	-0.03 ± 0.78
Female MLH - quadratic		
Female MLH: SH^2 – 1	-0.49 ± 4.47	-2.44 ± 3.80
Female MLH: SH^2 – 2	-1.07 ± 5.07	-2.80 ± 4.18
Parental similarity - linear		
Similarity	-0.55 ± 0.88	-1.23 ± 0.66
Parental similarity - quadratic		
Similarity^2	0.23 ± 6.20	-1.95 ± 4.54

Continued on next page

Continued from previous page

Interaction		
Male MLH: SH	-1.70 ± 0.85*	-1.39 ± 0.68*
Similarity	-0.58 ± 0.89	-1.13 ± 0.69
Male MLH × Similarity	18.78 ± 9.34*	15.76 ± 7.52*
Male MLH: HL	-2.80 ± 1.29*	-2.19 ± 1.05*
Similarity	-0.57 ± 0.88	-1.17 ± 0.68
Male MLH × Similarity	28.92 ± 14.06*	22.17 ± 11.48
Male MLH: IR	-2.05 ± 1.00*	-1.90 ± 0.79*
Similarity	-0.40 ± 0.90	-1.03 ± 0.15
Male MLH × Similarity	23.06 ± 10.84*	20.10 ± 8.64*

Table 4.S5. Likelihood ratio comparisons of different genetic models of proportional egg development success. All models are linear mixed models with male-female pair as a random effect term. For each focal predictor, we first test a linear expression of the multilocus term against the null model. We then present comparisons of a series of models against this linear MLH model, including the SLH model, a quadratic MLH model, and two-predictor and interaction models. For males, we present three MLH metrics (SH, HL, IR), due to subtle differences in model ranking. For females, we present only results for the SH metric, but also test a second, larger dataset for models not requiring the use of male genotypes. Each model was run both before ('Base') and after ('D&I') controlling for significant effects of clutch lay date and clutch incubation duration.

	Base models				**'D&I' models**			
Male heterozygosity	**SH *P***	**HL *P***	**IR *P***	**d.f.**	**SH *P***	**HL *P***	**IR *P***	**d.f.**
Male MLH (v. null)	0.064	**0.039**	0.072	3,4	0.110	0.086	0.067	6,7
SLH	**0.030**	**0.036**	**0.029**	4,35	**0.026**	**0.029**	**0.031**	7,38
MLH^2	**0.023**	0.071	0.106	4,5	0.111	0.227	0.084	7,8
+ female MLH	0.269	0.201	0.289	4,5	0.399	0.227	0.361	7,8
+ parental similarity	0.238	0.236	0.260	4,5	**0.021**	**0.023**	**0.019**	7,8
× female MLH	0.430	0.292	0.485	4,6	0.511	0.409	0.593	7,9
MLH × parental similarity	0.064	0.058	0.053	4,6	**0.007**	**0.011**	**0.004**	7,9

MLH = multilocus heterozygosity; SLH = single-locus heterozygosity; SH = standardised heterozygosity; HL = homozygosity by loci; IR = internal relatedness.

Table 4.S6. Comparisons of observed against expected offspring heterozygosity derived from Mendelian simulations using two different genotyping error parameters (‘lower’ and ‘higher’) and three metrics of multilocus heterozygosity (SH, HL, IR). We apply three tests: a sign test (effect size given as percentage of full-sib families showing heterozygosity > 0); a t-test of raw heterozygosity differences (effect size given as mean difference between families’ observed and expected heterozygosities); and a t-test of differences standardised by each family’s standard deviation of simulated heterozygosity, and by the sample size used to calculate each family’s ‘observed’ measure. For sign tests, N = 65; for t-tests, d.f. = 64. HL and IR outputs were sign-swapped to aid interpretation.

	Lower error		**Higher error**	
Test	**Effect size**	***P***	**Effect size**	***P***
SH				
Sign test	30.8%	0.003	35.4%	0.025
t-test – raw differences	-0.006, t = 2.192	0.032	-0.002, t = 0.583	0.562
t-test – standardised differences	-0.305, t = 2.359	0.021	-0.069, t = 0.528	0.600
HL				
Sign test	33.8%	0.013	36.9%	0.046
t-test – raw differences	-0.004, t = 1.969	0.053	0.000, t = 0.175	0.861
t-test – standardised differences	-0.277, t = 2.127	0.037	-0.012, t = 0.092	0.927
IR				
Sign test	33.8%	0.013	41.5%	0.215
t-test – raw differences	-0.006, t = 2.244	0.028	-0.001, t = 0.324	0.747
t-test – standardised differences	-0.309, t = 2.384	0.020	-0.026, t = 0.198	0.844

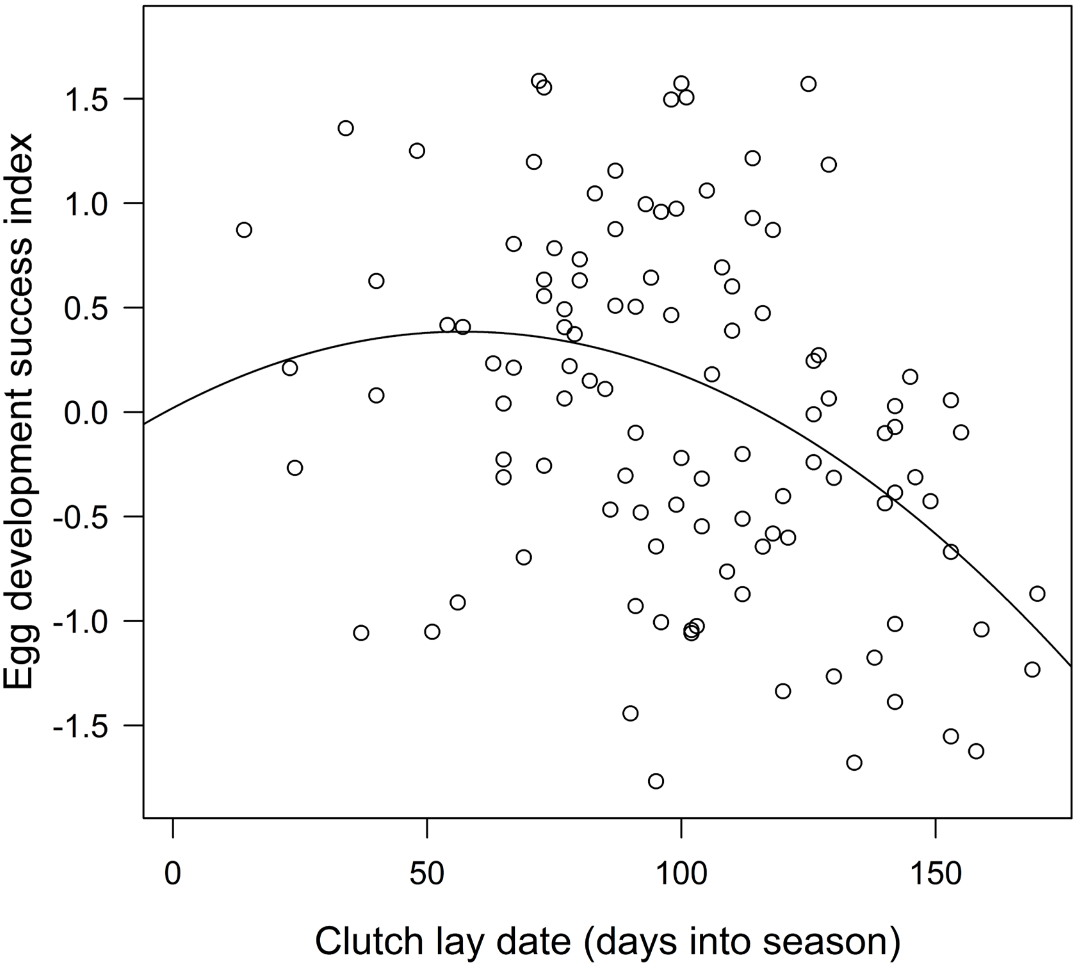

Figure 4.S1. Quadratic relationship between clutch lay date (days from 1st September) and logit-transformed proportional egg development success.

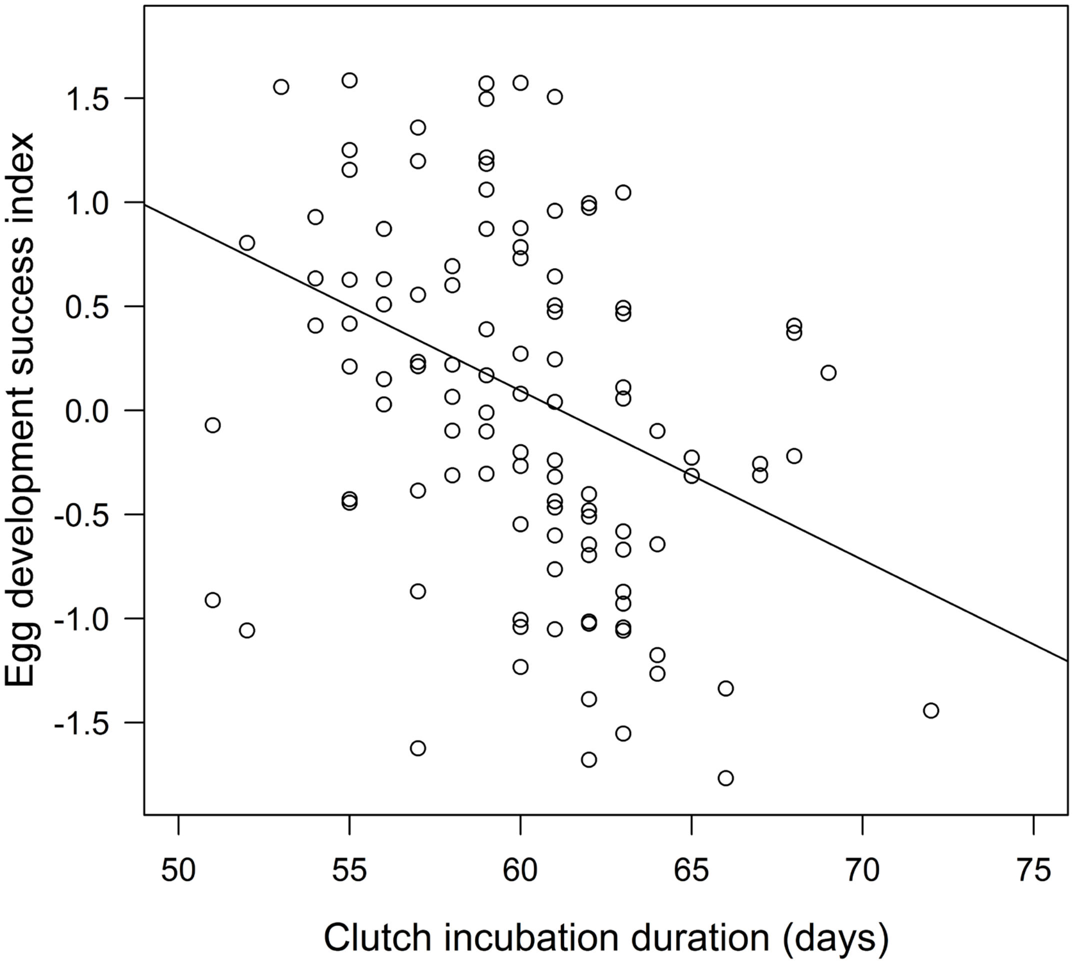

Figure 4.S2. Negative relationship between clutch incubation duration and logit-transformed proportional egg development success.

Chapter 5: Potential inter-season sperm storage by a female hawksbill turtle

Female Testudines can store viable sperm for a long time. Among marine species, a single insemination is often enough to sire a female's entire reproductive output for a nesting season, extending to hundreds of offspring laid over a period exceeding two months (e.g. Phillips *et al.* 2013). For some terrestrial species, the standard reproductive tactic is for females to mate prior to hibernation, store sperm over the winter, and then use this to fertilise their eggs in the spring (e.g. Johnston *et al.* 2006; Loy & Cianfrani 2010). However, several terrestrial and freshwater species in captivity have been recorded laying viable eggs after periods of isolation from males extending well beyond a single breeding season (e.g. Ewing 1943; Whitaker 2006; Murphy *et al.* 2007), raising the question as to whether longer-term sperm storage, spanning more than one breeding episode, occurs in wild populations.

As part of a study into paternity patterns in hawksbill turtles (*Eretmochelys imbricata*), we sampled tissue from nesting females and emerging hatchlings on Cousine Island, Republic of Seychelles, in the 2007/08 and 2008/09 nesting seasons. We generated DNA profiles of these samples using an extremely powerful array of 32 variable microsatellite loci (non-exclusion probability = 9.95×10^{-31}), and used mother and offspring data to reconstruct the paternal genotypes. Over the following two seasons, 12 of these females were observed returning to nest on Cousine (re-migration intervals of 2-3 years), and we again sampled their offspring. The fathers of the offspring of 11 of these returning females were new males. However, the offspring of the twelfth female (first seen in 2007/08, and re-sighted in 2009/10) were all sired by the exact same male as in her previous visit two years earlier (we genotyped 79 of this female's offspring in her first year and 32 in her second). Paternity analyses were performed using the programme COLONY 2.0 (Wang & Santure 2009).

However one interprets this finding, it is remarkable. A chance re-encounter with the same male is possible but seems unlikely, given the rarity of male re-sightings in our study (three other males in this data set were seen twice) and the conclusion from this that the number of available male mates is likely very large and/or highly mobile. A repeat encounter may be more likely if individuals use the same, idiosyncratic migration routes across years (e.g. Broderick *et al.* 2007), but testing this hypothesis would require tracking individuals of both sexes over several remigration periods. The alternative explanation is that this single female stored viable sperm over two years. This raises the possibility that all females store unused sperm from one season as a means of ensuring fertility, utilising it if they don't manage to mate successfully during their next fertile period. Should they re-mate, females presumably eject their store from the previous season, as otherwise we might expect to see a higher rate of multiple paternity resulting from stored sperm mixing with new sperm. We saw relatively few cases of multiple paternity (< 10% of females), and in the one case of multiple paternity in our 12 re-migrant females, neither male was the father of her offspring in her previous season. Interestingly, if our focal female has stored sperm for two years, her reproductive output did not significantly change between seasons (number of hatchlings per nest (mean ± SE) = 164 ± 14 v. 146 ± 24, n_{nests} = 4 and 5), suggesting that the viability of the sperm remained high – although 66% of eggs in her final observed nest failed to develop, compared with an average of 1-9% over her previous eight nests.

Our inferences are necessarily speculative, but the basic finding should be of interest to marine (and non-marine) turtle biologists, however they choose to interpret it, and we urge other researchers to keep a look out for similar patterns. However, if one does choose to interpret this case as inter-season sperm storage, we should not get too carried away: in social insects, such as ants, a single mating will often supply a queen with sufficient sperm to last decades, fertilising many thousands of offspring.

Chapter 6: General conclusions and discussion

Molecular techniques are invaluable to the study of cryptic life histories and cryptic evolutionary processes, and allow for both descriptive and hypothesis-driven studies. In this thesis, I have applied microsatellite genotyping on a large scale to study a population of hawksbill turtles (*Eretmochelys imbricata*) in the Republic of Seychelles. The hawksbill is an international conservation priority, but many components of its biology and ecology are poorly known, and especially so outside of the Caribbean population. The twin aims of the project have been to better characterise aspects of this species' life history, with the hope of better informing its conservation, and to test certain evolutionary hypotheses against the novel ecological context offered by marine turtles (e.g. long-lived, slow-to-mature, migratory, fecund). To that end, I have studied the hawksbill's mating system (chapter 2), population genetics (chapter 3), effective population size (chapter 3), and how individual genetic diversity relates to reproductive fitness (chapter 4). Each of these components offers information essential to the aims stated above. Here, I will draw together the more general conceptual strands relating to evolutionary biology, and consider their collective implications for the study population, the species, and beyond.

In chapter 2, I found that the majority (approximately 90%) of females were fertilised by a single male each, and that they then utilised sperm stored from this mating to fertilise all their clutches in a nesting season. The consistency of paternity across a given female's clutches could also be explained if females faithfully re-mated after laying each nest. However, I considered this extremely unlikely for two reasons. Firstly, females with mixed-paternity clutches showed the same pattern of consistency, i.e. the same two males across all clutches. Secondly, no changes in paternity between nests were observed at all, despite the potential for error or sexually-selected changing of partner that the presence of a large number of nearby males should offer. I observed no cases of males obtaining paternity with more than one female in a season, with only a very few cases between seasons, and inferred from this that a large number of males was potentially available to females. However, I found no evidence for sexually selected benefits to females in the system, which, because males provide no parental care, can only come about through indirect, genetic means. Females fertilised by two males did not lay clutches that were more successful than those fertilised by one male (chapter 4), and paternity was not biased towards males with particular genotypic properties (variability or similarity to the female – see chapter 2), despite evidence from chapter 4 that both male variability and parental similarity can affect nest success. A possible explanation for why mate choice on genetic grounds has not evolved in marine turtles is that it would be too costly relative to the potential benefits obtainable. Although in chapter 4, I reported that 8-9% of variance in nest success was explainable with parental genetic properties, none of these models were simple, with no universal ‘rule of thumb’ such as ‘more variable is better’. Further, if the low rate of multiple paternity in this study is indicative of a low encounter rate between males and females (see next paragraph), females may not be presented with sufficient opportunities to encounter a male that would represent a substantially beneficial ‘trade up’. In broader evolutionary terms, the potential benefits obtainable through biasing paternity towards males with particular genotypic properties do not outweigh the costs or constraints associated with obtaining those benefits, such as seeking additional matings when males are at low density, or developing a sufficiently discriminatory recognition system that then allowed paternity to be biased.

With respect to the lack of detected benefits from multiple paternity, my results are consistent with previous studies on green turtles (Lee & Hays 2004; Wright *et al.* 2013). Interpreting these results as females not benefitting from multiple paternity could be confounded if re-mating was restricted to only those females initially paired with a poor-quality male, with re-mating being used to recover fitness that would otherwise be lost (e.g. Price *et al.* 2010; Michalczyk *et al.* 2011). This would also explain why, if multiple paternity is beneficial, it is not more common in marine turtles. Unfortunately, in our study, the males with the lower shares of paternity in multiple-paternity families only rarely sired enough offspring for us to be able to compare their genotypic properties to those of the more dominant males. However, in a recent study, Wright *et al.* (2013) showed that female green turtles that were fertilised by multiple males laid significantly smaller clutches than singly-fertilised females. This suggests that multiple mating may cost female turtles reproductive capital, although it has also been argued that green turtles, which are herbivorous, are more sensitive to resource depletion than carnivorous species (Broderick *et al.* 2003). However, whether restricted to green turtles or not, this finding raises the predominant alternative explanation for multiple paternity in marine turtles, that of 'convenience polyandry' (see sections 1.1.2 and 1.1.5). Under this model, additional matings are costly, but less so than the harassment females receive from males if they refuse mating. Multiple paternity is thus an expression of a sexual conflict rather than a sexually-selected benefit. In marine turtles, and in reptiles in general, convenience polyandry predicts that the frequency of multiple paternity correlates with population density at the time of mating (e.g. Uller & Olsson 2008; see also sections 1.1.2, 1.1.5, and chapter 2). Testing the density-dependent convenience polyandry hypothesis was beyond the scope of this thesis, but, if the model is correct, it implies that individuals in this population are thinly dispersed at the time of mating – indeed, the frequency of multiple paternity in this study was among the lowest documented in marine turtles (see chapter 2). Further paternity studies within and across marine turtle species will help these taxa better contribute to the wider evolutionary discussion on the selective forces driving multiple mating, including convenience polyandry. One priority would be to test the generality of the finding of Jensen *et al.* (2006) within the genus *Lepidochelys*, i.e. are *arribada* nesting sites systematically associated with higher rates of multiple paternity than sites where females emerge singly. Another priority would be to test whether my detection of a correlation between male genetic variability and nest success is applicable in other populations and species, as it hints at a potential route whereby a female could increase her number of grandchildren by producing offspring with particular genetic properties. However, it must always be remembered that a large component of marine turtle fitness remains virtually impossible to study, as we cannot yet track individuals from hatching to recruitment to the breeding population.

With respect to how female turtles benefit from sperm storage within a season, I concluded that the primary benefit was enabling multiple clutches to be fertilised from a single insemination, and thereby allowing a female to avoid the costs associated with seeking additional matings to fertilise subsequent clutches. I reached this judgement based upon the absence of detectable sexual selection (no biases to paternity according to male genetic properties) and the lack of evidence for sperm stratification (i.e. if a female was using sperm from two males, that sperm seemed to be mixed rather than used sequentially). In some non-marine testudines the benefit of separating mating and fertilisation is more obvious, allowing females to mate in autumn and fertilise their eggs immediately after emerging from hibernation in spring (e.g. Gist *et al.* 1990; Johnston *et al.* 2006; Loy & Cianfrani 2010). Interestingly, we observed a single case of a female that nested in two of our study seasons, whose offspring were fertilised by the same male in both seasons (chapter 5). This is potentially the first documented case of long-term sperm storage in a wild marine turtle. Long-term sperm storage may benefit a female as an ‘insurance policy’, should she fail to encounter a male during her subsequent fertile period. How frequently such occasions arise will likely also be linked to population density, but I could only assess a very small number of remigrating females, and I thus emphasise the preliminary and cautious nature of this interpretation. In the broader context, these results emphasise the value of sperm storage in allowing a female to separate mating and fertilisation in time. The utility of sperm storage in allowing some female testudines to mate in autumn and commence egg production immediately upon emerging from hibernation in spring is also seen in bats (Chiroptera; Hosken 1997). In hornbills (Bucerotidae), sperm storage facilitates the extraordinary adaptation whereby females seal themselves inside a nest hole after mating, and then use their stored sperm to fertilise their eggs while their partner feeds them through a small aperture (e.g. Stanback *et al.* 2002). The utility reaches its apogee in the eusocial Hymenoptera, where the inseminations a ‘queen’ receives on her nuptial flight (i.e. after emerging from her natal nest) can last for decades and fertilise millions of eggs (Cole 1983; Hölldobler & Wilson 1990; Boomsma *et al.* 2005; Boomsma

2009). In the case of the hawksbill turtle, the primary value may be in allowing a female to avoid the energetic expenditures and risks associated with finding a new mate.

In chapter 4 I highlighted a link between individual genetic variability and reproductive fitness in my study population. Although this suggests a potential vulnerability to inbreeding depression, which conservation managers need to be aware of, in chapter 3 I showed that the population was not systematically inbred. The presence of both positive and negative effects of genetic variability in this system may be linked to female natal philopatry and male dispersal in marine turtles. However, the relative magnitudes of these processes in this system are unclear, as although I found evidence that males disperse more than females, there was no signficant population structure across 450 km of the Seychelles (chapter 3), and a previous study on maternally-inherited mitochondrial DNA also found no structure over this area (Vargas *et al.* 2013 & unpublished data). I encourage researchers working on other marine turtle populations to test whether any of these observed patterns or implied processes are applicable beyond this system. This would contribute to better understanding the link between neutral genetic variability and individual fitness, which remains a poorly understood topic in evolutionary biology. It is important, if this link is to be better understood, that it be assessed against a range of ecological and life-history backgrounds. Certain traits, such as being long-lived, slow-to-mature, highly fecund, or a reptile, remain under-represented by such studies. Thus, by presenting data on the population genetics and fitness of a marine turtle, I hope that this study will contribute to the broader understanding of how genetic variability relates to fitness in wild populations.

In chapter 3 I found that the effective population size of hawksbill turtles in Seychelles to comfortably exceed the often-quoted figure of 500 required for the long-term maintenance of population genetic variation, and to be relatively large as a ratio to census estimates when compared with the wider effective population size literature (see references in chapter 3). This is extremely positive from a conservation genetics perspective, although it is possible that this is not the equilibrium effective population size for the current census population. It may, instead, be closer to that of the historical, much larger census population, and is lagging behind because of the long generation time of the hawksbill. In support of this lag effect, I found no evidence of a genetic bottleneck. However, my findings on both the mating system (chapter 2) and population genetics (chapter 3) suggest other processes that might have contributed to the maintenance of genetic variation, processes which are applicable beyond marine turtles.

On the mating system, reproductive skew, whereby a few males obtain a disproportionately large amount of paternity, can substantially reduce effective population size. However, I found no skew in this system within years, across years, and across a subset of females seen in two separate years. Indeed, slightly more males than females contributed to the families sampled on Cousine. It is probably unsafe to extrapolate this to a regional scale, as the same group of males could be fertilising females over a very wide area (Wright *et al.* 2012a, 2012b), although the rarity with which I re-sighted a male in the dataset does indicate a large male population. A recent paternity study on loggerhead turtles (*Caretta caretta*) came to a similar conclusion after observing similar patterns, going as far as arguing for a male-biased effective sex ratio in their system (Lasala *et al.* 2013). The effective population size will also be influenced by how frequently these males remigrate. Of the three males that were definitely remigrants (i.e. not including the potential case of inter-year sperm storage), two were observed in consecutive years. It is difficult to assess male breeding periodicity from such a sample size. However, annual remigration has previously been demonstrated for male loggerhead turtles fitted with satellite trackers (Hays *et al.* 2010). In a paternity study of green turtles (*Chelonia mydas*) in the Mediterranean, Wright *et al.* (2012a) observed a similar frequency of remigrant males to this study, and a similar remigration interval pattern. However, they interpreted their rarity of remigrants as indicative of males being on long remigration intervals, on the grounds that independent estimates of male numbers meant that they should have seen more remigrants if males were on shorter intervals. Comparable independent data on male numbers is not available for Seychelles hawksbills, and it thus seems more parsimonious at present to conclude that there are a large number of males in Seychelles that mostly remigrate annually.

With regard to population genetics, I found no significant genetic differentiation between island pairs ranging from 40 km to 250 km, or between the Amirantes and Granitic Seychelles treated as groups. Vargas *et al.* (2013 & unpublished data) also report no population structure between these groups using mitochondrial DNA. This suggests that there is gene flow occurring through both sexes. As outlined above, though, males may be more dispersive than females, on account of similarity (relatedness) being higher among females on Cousine than among the males that fertilised their eggs. Were the hawksbills of Seychelles instead to consist of a set of smaller, genetically discrete subpopulations, the overall effective population size would be much smaller, and the population much more vulnerable to loss of genetic variation when overhunted. Gene flow also hints at a potential for occasional dispersal that could facilitate the colonisation of beaches where hawksbills do not currently nest.

The findings of this genetic study are extremely positive with respect to the conservation status of hawksbill turtles in the Republic of Seychelles (see also Thesis Appendix 2). The population seems to be reasonably large and of roughly even sex ratio, spread over a wide geographic area, and genetically diverse and well-mixed. However, although the data indicate that this population should be seen as a single genetic unit, this should not be taken as an argument for management relaxation or complacency at the island level – the population is greatly reduced from historical levels, and the target should still be to have more hawksbill turtles. This requires that current protection be continued, and extended where possible. Extension of protection to suitable beaches that don’t currently have nesting hawksbills on them may be particularly fruitful, given the potential for dispersal mentioned in the previous paragraph. Unfortunately, despite an upwards population trend in Seychelles, the hawksbill’s future is far from guaranteed: deliberate and accidental killings remain a problem, and the threats posed by climate change are a serious cause for concern. However, a large, dispersed, genetically diverse population puts it in as good a position to face these future challenges.

In terms of sample size, this study represents one of the most comprehensive genetic studies of marine turtles to date. Nonetheless, to address the questions raised in this thesis more fully would require additional sampling, in more years and from more islands. A stronger sampling regime would allow, for example, better resolution of male remigration intervals, whether females store sperm between years, and whether the fathers in multiple-paternity families differ in their genotypic properties. It would also extend the capacity for applying capture-mark-recapture-type analyses for estimation of the number of males. Although the sample size was large, few individual of either sex were re-sighted within the study. Furthermore, additional samples would enable assessment of how well the observation that relatedness among females on Cousine is higher than among the males that fertilised their eggs applies to other islands. It would also be possible to compare within-island female relatedness to between-island female relatedness, and also for males, to assess the spatial scale over which these processes apply. For studying the link between genetic variability and fitness, it might enable comparison of effect sizes between years of different quality, as the effects of variability may be more exposed to selection in poorer quality years. It would also be possible to observe the success of remigrants of both sexes that have mated with different individuals, which would enable better control of random effects variance. Satellite tracking of both sexes would also substantially advance knowledge of migration patterns, and provide a valuable context against which to interpret molecular data. There has been very little satellite tracking of female hawksbills in the region (Mortimer & Balazs 2000), and globally very little of males (but see van Dam *et al.* 2008).

There is also scope for using alternative molecular markers. For example, immune genes such as the major histocompatibility complex (e.g. Løvlie *et al.* 2013) and Toll-like receptors (e.g. Kawai & Akira 2010) could be informative for verifying the findings on mate choice and the genetic variability-fitness effects. This would likely require the use of next-generation sequencing techniques, as obtaining sufficient offspring genotypes for paternal genotype reconstruction using Sanger sequencing would likely be restrictively expensive. Next-generation sequencing, such as using RAD-tags (restriction-site associated DNA) to identify large numbers of single-nucleotide polymorphisms (Miller *et al.* 2007; Baird *et al.* 2008), would also help address questions founded upon neutral markers. For example, it should be remembered that 32 microsatellites, although still a relatively large number for an HFC study (Chapman *et al.* 2009), will barely scratch the surface of representing variation across the hawksbill genome (56 chromosomes; Bickham 1981). This is a general criticism of HFC studies, but one which may be addressable using the much larger number of markers and much greater genome coverage available from the RAD approach. The larger number of markers, coupled with the greater tractability of mutation models for sequence data relative to that of microsatellites, would also allow better resolution of fine-scale population structure.

I have used microsatellite genotyping to study the life history and demography of the hawksbill turtle, and have attempted to place my results in the contexts of both evolutionary biology and conservation genetics. The hawksbill, like all marine turtles, will never be a model species for testing evolutionary concepts, due to its long lifespan and the logistical difficulties associated with following it at sea. However, it is essential to the development of such general concepts that they be tested against a wide range of life histories and ecologies to assess the breadth of their applicability. Beyond marine turtles, the results of this study, and of others on marine turtles, may be relevant to understanding the biology of species with similar life history attributes, especially long-lived, slow-to-mature, migratory marine vertebrates, such as cetaceans, seabirds, and certain fish. To conclude, I hope that this thesis has reiterated the enduring value of molecular techniques to the study of cryptic processes, cryptic species, and how to conserve them. Advances in laboratory and computational techniques will continue to expand the range of questions that can be asked and the quality of answers that can be obtained, and thereby obtain better insights into the ecological and evolutionary forces that shape biodiversity.

References

Aggarwal RK, Lalremruata A, Velavan TP, Sowjanya AP, Singh L (2008) Development and characterization of ten novel microsatellite markers from olive ridley sea turtle (*Lepidochelys olivacea*). *Conservation Genetics*, **9:** 981-984.

Aggarwal RK, Velavan TP, Udaykumar D, Hendre PS, Shanker K, Choudhury BC, Singh L (2004) Development and characterization of novel microsatellite markers from the olive ridley sea turtle (*Lepidochelys olivacea*). *Molecular Ecology Notes*, **4:** 77-79.

Akaike H (1974) A new look at the statistical model identification. *IEEE Transactions on Automatic Control*, **19:** 716-723.

Alcock J (2005) *Animal Behavior: An Evolutionary Approach.* Eighth edition. Sinauer Associates, Sunderland, MA, USA.

Allan TM (1963) Hirszfeld and ABO blood groups. *British Journal of Preventive & Social Medicine*, **17:** 166-171.

Allen ZC, Shah NJ, Grant A, Derand GD, Bell D (2010) Hawksbill turtle monitoring in Cousin Island Special Reserve, Seychelles: an eight-fold increase in annual nesting numbers. *Endangered Species Research*, **11:** 195-200.

Amos W, Wilmer JW, Fullard K, Burg TM, Croxall JP, Bloch D, Coulson T (2001) The influence of parental relatedness on reproductive success. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **268:** 2021-2027.

Andrew RL, Bernatchez L, Bonin A, Buerkle CA, Carstens BC, Emerson BC, Garant D, Giraud T, Kane NC, Rogers SM, Slate J, Smith H, Sork VL, Stone GN, Vines TH, Waits L, Widmer A, Rieseberg LH (2013) A road map for molecular ecology. *Molecular Ecology*, **22:** 2605-2626.

Aparicio JM, Ortego J, Cordero PJ (2006) What should we weigh to estimate heterozygosity, alleles or loci? *Molecular Ecology*, **15:** 4659-4665.

Arnqvist G, Kirkpatrick M (2005) The evolution of infidelity in socially monogamous passerines: the strength of direct and indirect selection on extrapair copulation behavior in females. *American Naturalist*, **165:** S26-S37.

Arnqvist G, Nilsson T (2000) The evolution of polyandry: multiple mating and female fitness in insects. *Animal Behaviour*, **60:** 145-164.

Avise JC, Bowen BW, Lamb T, Meylan AB, Bermingham E (1992) Mitochondrial DNA evolution at a turtle's pace: evidence for low genetic variability and reduced microevolutionary rate in the Testudines. *Molecular Biology and Evolution*, **9:** 457-473.

Avise JC, Nelson WS (1989) Molecular genetic relationships of the extinct dusky seaside sparrow. *Science*, **243:** 646-648.

Baird NA, Etter PD, Atwood TS, Currey MC, Shiver AL, Lewis ZA, Selker EU, Cresko WA, Johnson EA (2008) Rapid SNP discovery and genetic mapping using sequenced RAD markers. *PLoS ONE*, **3:** e3376.

Balloux F, Amos W, Coulson T (2004) Does heterozygosity estimate inbreeding in real populations? *Molecular Ecology*, **13:** 3021-3031.

Balloux F, Brunner H, Lugon-Moulin N, Hausser J, Goudet J (2000) Microsatellites can be misleading: An empirical and simulation study. *Evolution*, **54:** 1414-1422.

Barton K (2013) MuMIn: Multi-model inference. R package version 1.9.5. http://CRAN.R-project.org/package=MuMIn.

Bateman AJ (1948) Intra-sexual selection in *Drosophila*. *Heredity*, **2:** 349-368.

Beebee T, Rowe G (2004) *An Introduction to Molecular Ecology.* Second edition. Oxford University Press, Oxford.

Bensch S, Hasselquist D, Vonschantz T (1994) Genetic similarity between parents predicts hatching failure - nonincestuous inbreeding in the great reed warbler. *Evolution*, **48:** 317-326.

Bickham JW (1981) Two-hundred-million-year-old chromosomes - deceleration of the rate of karyotypic evolution in turtles. *Science*, **212:** 1291-1293.

Birkhead TR, Hall J, Schut E, Hemmings N (2008) Unhatched eggs: methods for discriminating between infertility and early embryo mortality. *Ibis*, **150:** 508-517.

Birkhead TR, Hunter FM (1990) Mechanisms of sperm competition. *Trends in Ecology & Evolution*, **5:** 48-52.

Birkhead TR, Møller AP (1992) *Sperm Competition in Birds: Evolutionary Causes and Consequences.* Academic Press, London.

Birkhead TR, Møller AP (1993) Sexual selection and the temporal separation of reproductive events - sperm storage data from reptiles, birds and mammals. *Biological Journal of the Linnean Society*, **50:** 295-311.

Birkhead TR, Møller AP (1998) *Sperm Competition and Sexual Selection.* Academic Press, San Diego, California.

Bjorndal K, Jackson JBC (2002) Roles of Sea Turtles in Marine Ecosystems: Reconstructing the Past. In: *The Biology of Sea Turtles vol. II* (eds Lutz PL, Musick JA, Wyneken J), pp. 259-274. CRC Press, Boca Raton, FL.

Bjorndal KA, Reich KJ, Bolten AB (2010) Effect of repeated tissue sampling on growth rates of juvenile loggerhead turtles *Caretta caretta*. *Diseases of Aquatic Organisms*, **88:** 271-273.

Blumenthal JM, Abreu-Grobois FA, Austin TJ, Broderick AC, Bruford MW, Coyne MS, Ebanks-Petrie G, Formia A, Meylan PA, Meylan AB (2009) Turtle groups or turtle soup: dispersal patterns of hawksbill turtles in the Caribbean. *Molecular Ecology*, **18:** 4841-4853.

Bolten AB, Bjorndal KA, Martins HR, Dellinger T, Biscoito MJ, Encalada SE, Bowen BW (1998) Transatlantic developmental migrations of loggerhead sea turtles demonstrated by mtDNA sequence analysis. *Ecological Applications*, **8:** 1-7.

Boomsma JJ (2009) Lifetime monogamy and the evolution of eusociality. *Philosophical Transactions of the Royal Society B-Biological Sciences*, **364:** 3191-3207.

Boomsma JJ, Baer B, Heinze J (2005) The evolution of male traits in social insects. In: *Annual Review of Entomology*, pp. 395-420. Annual Reviews, Palo Alto.

Booth J, Peters JA (1972) Behavioural studies on the green turtle (*Chelonia mydas*) in the sea. *Animal Behaviour*, **20:** 808-812.

Booth W, Million L, Reynolds RG, Burghardt GM, Vargo EL, Schal C, Tzika AC, Schuett GW (2011) Consecutive virgin births in the New World boid snake, the Colombian rainbow boa, *Epicrates maurus*. *Journal of Heredity*, **102:** 759-763.

Bowen B, Avise JC, Richardson JI, Meylan AB, Margaritoulis D, Hopkinsmurphy SR (1993a) Population structure of loggerhead turtles (*Caretta caretta*) in the northwestern Atlantic Ocean and Mediterranean Sea. *Conservation Biology*, **7:** 834-844.

Bowen BW, Abreu-Grobois FA, Balazs GH, Kamezaki N, Limpus CJ, Ferl RJ (1995) Trans-Pacific migrations of the loggerhead turtle (*Caretta caretta*) demonstrated with mitochondrial DNA markers. *Proceedings of the National Academy of Sciences of the United States of America*, **92:** 3731-3734.

Bowen BW, Bass AL, Soares L, Toonen RJ (2005) Conservation implications of complex population structure: lessons from the loggerhead turtle (*Caretta caretta*). *Molecular Ecology*, **14:** 2389-2402.

Bowen BW, Grant WS, Hillis-Starr Z, Shaver DJ, Bjorndal A, Bolten AB, Bass AL (2007a) Mixed-stock analysis reveals the migrations of juvenile hawksbill turtles (*Eretmochelys imbricata*) in the Caribbean Sea. *Molecular Ecology*, **16:** 49-60.

Bowen BW, Grant WS, Hillis-Starr Z, Shaver DJ, Bjorndal KA, Bolten AB (2007b) The advocate and the scientist: debating the commercial exploitation of endangered hawksbill turtles. *Molecular Ecology*, **16:** 3514-3515.

Bowen BW, Kamezaki N, Limpus CJ, Hughes GR, Meylan AB, Avise JC (1994) Global phylogeography of the loggerhead turtle (*Caretta caretta*) as indicated by mitochondrial DNA haplotypes. *Evolution*, **48:** 1820-1828.

Bowen BW, Karl SA (1997) Population Genetics, Phylogeography, and Molecular Evolution. In: *The Biology of Sea Turtles* (eds Lutz PL, Musick JA), pp. 29-50. CRC Press, Boca Raton, FL.

Bowen BW, Karl SA (2007) Population genetics and phylogeography of sea turtles. *Molecular Ecology*, **16:** 4886-4907.

Bowen BW, Nelson WS, Avise JC (1993b) A molecular phylogeny for marine turtles - trait mapping, rate assessment, and conservation relevance. *Proceedings of the National Academy of Sciences of the United States of America*, **90:** 5574-5577.

Box GEP, Cox DR (1964) An analysis of transformations. *Journal of the Royal Statistical Society Series B-Statistical Methodology*, **26:** 211-252.

Bretman A, Newcombe D, Tregenza T (2009) Promiscuous females avoid inbreeding by controlling sperm storage. *Molecular Ecology*, **18:** 3340-3345.

Bretman A, Tregenza T (2005) Measuring polyandry in wild populations: a case study using promiscuous crickets. *Molecular Ecology*, **14:** 2169-2179.

Broderick AC, Coyne MS, Fuller WJ, Glen F, Godley BJ (2007) Fidelity and over-wintering of sea turtles. *Proceedings of the Royal Society B-Biological Sciences*, **274:** 1533-1538.

Broderick AC, Glen F, Godley BJ, Hays GC (2003) Variation in reproductive output of marine turtles. *Journal of Experimental Marine Biology and Ecology*, **288:** 95-109.

Broderick AC, Godley BJ, Hays GC (2001) Trophic status drives interannual variability in nesting numbers of marine turtles. *Proceedings of the Royal Society B-Biological Sciences*, **268:** 1481-1487.

Brouwer L, Barr I, van de Pol M, Burke T, Komdeur J, Richardson DS (2010) MHC-dependent survival in a wild population: evidence for hidden genetic benefits gained through extra-pair fertilizations. *Molecular Ecology*, **19:** 3444-3455.

Brouwer L, Komdeur J, Richardson DS (2007) Heterozygosity-fitness correlations in a bottlenecked island species: a case study on the Seychelles warbler. *Molecular Ecology*, **16:** 3134-3144.

Brown JL (1997) A theory of mate choice based on heterozygosity. *Behavioral Ecology*, **8:** 60-65.

Burke T, Davies NB, Bruford MW, Hatchwell BJ (1989) Parental care and mating-behavior of polyandrous dunnocks *Prunella modularis* related to paternity by DNA fingerprinting. *Nature*, **338:** 249-251.

Campbell LM (2002) Contemporary Culture, Use, and Conservation of Sea Turtles. In: *The Biology of Sea Turtles vol. II* (eds Lutz PL, Musick JA, Wyneken J), pp. 307-338. CRC Press, Boca Raton, FL.

Carr AF (1967) *So Excellent a Fishe: a Natural History of Sea Turtles.* Schribner, New York.

Carreras C, Pont S, Maffucci F, Pascual M, Barcelo A, Bentivegna F, Cardona L, Alegre F, SanFelix M, Fernandez G, Aguilar A (2006) Genetic structuring of immature loggerhead sea turtles (*Caretta caretta*) in the Mediterranean Sea reflects water circulation patterns. *Marine Biology*, **149:** 1269-1279.

Chaloupka MY, Limpus CJ (1997) Age, Growth, and Population Dynamics. In: *The Biology of Sea Turtles* (eds Lutz PL, Musick JA), pp. 233-276. CRC Press, Boca Raton, FL.

Chapman JR, Nakagawa S, Coltman DW, Slate J, Sheldon BC (2009) A quantitative review of heterozygosity-fitness correlations in animal populations. *Molecular Ecology*, **18:** 2746-2765.

Chapman JR, Sheldon BC (2011) Heterozygosity is unrelated to adult fitness measures in a large, noninbred population of great tits (*Parus major*). *Journal of Evolutionary Biology*, **24:** 1715-1726.

Charlesworth B, Charlesworth D (1999) The genetic basis of inbreeding depression. *Genetical Research*, **74:** 329-340.

Charlesworth D, Charlesworth B (1987) Inbreeding depression and its evolutionary consequences. *Annual Review of Ecology and Systematics*, **18:** 237-268.

Cohas A, Yoccoz NG, Da Silva A, Goossens B, Allaine D (2006) Extra-pair paternity in the monogamous alpine marmot (*Marmota marmota*): the roles of social setting and female mate choice. *Behavioral Ecology and Sociobiology*, **59:** 597-605.

Cole BJ (1983) Multiple mating and the evolution of social behavior in the Hymenoptera. *Behavioral Ecology and Sociobiology*, **12:** 191-201.

Collet J, Richardson DS, Worley K, Pizzari T (2012) Sexual selection and the differential effect of polyandry. *Proceedings of the National Academy of Sciences of the United States of America*, **109:** 8641-8645.

Coltman DW, Pilkington JG, Smith JA, Pemberton JM (1999) Parasite-mediated selection against inbred Soay sheep in a free-living, island population. *Evolution*, **53:** 1259-1267.

Coltman DW, Slate J (2003) Microsatellite measures of inbreeding: A meta-analysis. *Evolution*, **57:** 971-983.

Cordero PJ, Aparico JM, Veiga JP (2004) Parental genetic characteristics and hatching success in the spotless starling, *Sturnus unicolor*. *Animal Behaviour*, **67:** 637-642.

Cornuet JM, Luikart G (1996) Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics*, **144:** 2001-2014.

Cornuet JM, Santos F, Beaumont MA, Robert CP, Marin JM, Balding DJ, Guillemaud T, Estoup A (2008) Inferring population history with DIY ABC: a user-friendly approach to approximate Bayesian computation. *Bioinformatics*, **24:** 2713-2719.

Coulson T, Albon S, Slate J, Pemberton J (1999) Microsatellite loci reveal sex-dependent responses to inbreeding and outbreeding in red deer calves. *Evolution*, **53:** 1951-1960.

Crandall KA, Bininda-Emonds ORP, Mace GM, Wayne RK (2000) Considering evolutionary processes in conservation biology. *Trends in Ecology & Evolution*, **15:** 290-295.

Crim JL, Spotila LD, Spotila JR, O'Connor M, Reina R, Williams CJ, Paladino FV (2002) The leatherback turtle, *Dermochelys coriacea*, exhibits both polyandry and polygyny. *Molecular Ecology*, **11:** 2097-2106.

Crnokrak P, Roff DA (1999) Inbreeding depression in the wild. *Heredity*, **83:** 260-270.

Csilléry K, Johnson T, Beraldi D, Clutton-Brock T, Coltman D, Hansson B, Spong G, Pemberton JM (2006) Performance of marker-based relatedness estimators in natural populations of outbred vertebrates. *Genetics*, **173:** 2091-2101.

Cutuli G, Cannicci S, Vannini M, Fratini S (2013) Influence of mating order on courtship displays and stored sperm utilization in Hermann's tortoises (*Testudo hermanni hermanni*). *Behavioral Ecology and Sociobiology*, **67:** 273-281.

David P (1998) Heterozygosity-fitness correlations: new perspectives on old problems. *Heredity*, **80:** 531-537.

David P, Pujol B, Viard F, Castella V, Goudet J (2007) Reliable selfing rate estimates from imperfect population genetic data. *Molecular Ecology*, **16:** 2474-2487.

Dawson DA, Ball AD, Spurgin LG, Martin-Galvez D, Stewart IRK, Horsburgh GJ, Potter J, Molina-Morales M, Bicknell AWJ, Preston SAJ, Ekblom R, Slate J, Burke T (2013) High-utility conserved avian microsatellite markers enable parentage and population studies across a wide range of species. *Bmc Genomics*, **14:** 176.

Dawson DA, Horsburgh GJ, Krupa AP, Stewart IRK, Skjelseth S, Jensen H, Ball AD, Spurgin LG, Mannarelli M-E, Nakagawa S, Schroeder J, Vangestel C, Hinten GN, Burke T (2012) Microsatellite resources for Passeridae species: a predicted microsatellite map of the house sparrow Passer domesticus. *Molecular Ecology Resources*, **12:** 501-523.

Dawson DA, Horsburgh GJ, Kupper C, Stewart IRK, Ball AD, Durrant KL, Hansson B, Bacon I, Bird S, Klein A, Krupa AP, Lee J-W, Martin-Galvez D, Simeoni M, Smith G, Spurgin LG, Burke T (2010) New methods to identify conserved microsatellite loci and develop primer sets of high cross-species utility - as demonstrated for birds. *Molecular Ecology Resources*, **10:** 475-494.

Diamond AW (1976) Breeding biology and conservation of hawksbill turtles, *Eretmochelys imbricata* L., on Cousin Island, Seychelles. *Biological Conservation*, **9:** 199-215.

Double MC, Cockburn A (2003) Subordinate superb fairy-wrens (*Malurus cyaneus*) parasitize the reproductive success of attractive dominant males. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **270:** 379-384.

Dutton PH, Frey A (2009) Characterization of polymorphic microsatellite markers for the green turtle (*Chelonia mydas*). *Molecular Ecology Resources*, **9:** 354-356.

Eberhard WG (1996) *Female Control: Sexual Selection by Cryptic Female Choice.* Princeton University Press, Princeton.

Edmands S (2007) Between a rock and a hard place: evaluating the relative risks of inbreeding and outbreeding for conservation and management. *Molecular Ecology*, **16:** 463-475.

Encalada SE, Bjorndal KA, Bolten AB, Zurita JC, Schroeder B, Possardt E, Sears CJ, Bowen BW (1998) Population structure of loggerhead turtle (*Caretta caretta*) nesting colonies in the Atlantic and Mediterranean as inferred from mitochondrial DNA control region sequences. *Marine Biology*, **130:** 567-575.

Escobar JS, Nicot A, David P (2008) The different sources of variation in inbreeding depression, heterosis and outbreeding depression in a metapopulation of *Physa acuta*. *Genetics*, **180:** 1593-1608.

Ewing HE (1943) Continued fertility in female box turtles following mating. *Copeia*, **1943:** 112-114.

Excoffier L, Laval G, Schneider S (2005) Arlequin (version 3.0): An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics*, **1:** 47-50.

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes - application to human mitochondrial DNA restriction data. *Genetics*, **131:** 479-491.

Faria CMA, Zarza E, Reynoso VH, Emerson BC (2010) Predominance of single paternity in the black spiny-tailed iguana: conservation genetic concerns for female-biased hunting. *Conservation Genetics*, **11:** 1645-1652.

Fitzpatrick JL, Evans JP (2009) Reduced heterozygosity impairs sperm quality in endangered mammals. *Biology Letters*, **5:** 320-323.

FitzSimmons NN, Limpus CJ, Norman JA, Goldizen AR, Miller JD, Moritz C (1997a) Philopatry of male marine turtles inferred from mitochondrial DNA markers. *Proceedings of the National Academy of Sciences of the United States of America*, **94:** 8912-8917.

FitzSimmons NN, Moritz C, Limpus CJ, Pope L, Prince R (1997b) Geographic structure of mitochondrial and nuclear gene polymorphisms in Australian green turtle populations and male-biased gene flow. *Genetics*, **147:** 1843-1854.

FitzSimmons NN, Moritz C, Moore SS (1995) Conservation and dynamics of microsatellite loci over 300 million years of marine turtle evolution. *Molecular Biology and Evolution*, **12:** 432-440.

Frankham R (1995) Effective population size/adult population size ratios in wildlife - a review. *Genetical Research*, **66:** 95-107.

Frankham R, Ballou JD, Briscoe DA (2002) *Introduction to Conservation Genetics.* Cambridge University Press, Cambridge, UK.

Frankham R, Lees K, Montgomery ME, England PR, Lowe EH, Briscoe DA (1999) Do population size bottlenecks reduce evolutionary potential? *Animal Conservation*, **2:** 255-260.

Franklin IR (1980) Evolutionary changes in small populations. In: *Conservation Biology: an Evolutionary Ecological Perspective* (eds Soulé ME, Wilcox BA), pp. 135-149. Sinauer & Associates, Sunderland, MA.

Franklin IR, Frankham R (1998) How large must populations be to retain evolutionary potential? *Animal Conservation*, **1:** 69-70.

Frazier J (1971) Observations on sea turtles at Aldabra Atoll. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences*, **260:** 373-410.

Frazier J (2002) Critical Approaches To Sex Determination In Sea Turtles. In: *The Biology of Sea Turtles vol. II* (eds Lutz PL, Musick JA, Wyneken J), pp. 1-38. CRC Press, Boca Raton, FL.

Freckleton RP (2000) Phylogenetic tests of ecological and evolutionary hypotheses: checking for phylogenetic independence. *Functional Ecology*, **14:** 129-134.

Freeman S, Herron JC (2004) *Evolutionary Analysis.* Third edition. Pearson Education, Upper Saddle River, NJ.

Fromhage L, Kokko H, Reid JM (2009) Evolution of mate choice for genome-wide heterozygosity. *Evolution*, **63:** 684-694.

Futuyma DJ (1998) *Evolutionary Biology.* Third edition. Sinauer Associates, Sunderland, MA.

Gage MJG, Surridge AK, Tomkins JL, Green E, Wiskin L, Bell DJ, Hewitt GM (2006) Reduced heterozygosity depresses sperm quality in wild rabbits, *Oryctolagus cuniculus*. *Current Biology*, **16:** 612-617.

Garcia-Navas V, Ortego J, Sanz JJ (2009) Heterozygosity-based assortative mating in blue tits (*Cyanistes caeruleus*): implications for the evolution of mate choice. *Proceedings of the Royal Society B-Biological Sciences*, **276:** 2931-2940.

Garza JC, Williamson EG (2001) Detection of reduction in population size using data from microsatellite loci. *Molecular Ecology*, **10:** 305-318.

Gilpin ME, Soulé ME (1986) Minimum viable populations: the process of species extinctions. In: *Conservation Biology: The Science of Scarcity and Diversity* (ed. Soulé ME), pp. 19-34. Sinauer Associates, Sunderland, MA.

Gist DH, Congdon JD (1998) Oviductal sperm storage as a reproductive tactic of turtles. *Journal of Experimental Zoology*, **282:** 526-534.

Gist DH, Jones JM (1989) Sperm storage within the oviduct of turtles. *Journal of Morphology*, **199:** 379-384.

Gist DH, Michaelson JA, Jones JM (1990) Autumn mating in the painted turtle, *Chrysemys picta*. *Herpetologica*, **46:** 331-336.

Glenn TC (2011) Field guide to next-generation DNA sequencers. *Molecular Ecology Resources*, **11:** 759-769.

Godfrey MH, Webb GJW, Manolis SC, Mrosovsky N (2007) Hawksbill sea turtles: Can phylogenetics inform harvesting? *Molecular Ecology*, **16:** 3511-3513.

Godley BJ, Blumenthal JM, Broderick AC, Coyne MS, Godfrey MH, Hawkes LA, Witt MJ (2008) Satellite tracking of sea turtles: Where have we been and where do we go next? *Endangered Species Research*, **4:** 3-22.

Goudet J, Perrin N, Waser P (2002) Tests for sex-biased dispersal using bi-parentally inherited genetic markers. *Molecular Ecology*, **11:** 1103-1114.

Greenwood JD, Robinson RA (2006) General census methods. In: *Ecological Census Techniques: A Handbook* (ed. Sutherland WJ), pp. 87-185. Cambridge University Press, Cambridge, UK.

Griffith SC (2007) The evolution of infidelity in socially monogamous passerines: neglected components of direct and indirect selection. *American Naturalist*, **169:** 274-281.

Griffith SC, Immler S (2009) Female infidelity and genetic compatibility in birds: the role of the genetically loaded raffle in understanding the function of extrapair paternity. *Journal of Avian Biology*, **40:** 97-101.

Griffiths R, Double MC, Orr K, Dawson RJG (1998) A DNA test to sex most birds. *Molecular Ecology*, **7:** 1071-1075.

Grueber CE, Wallis GP, Jamieson IG (2013) Genetic drift outweighs natural selection at toll-like receptor (TLR) immunity loci in a re-introduced population of a threatened species. *Molecular Ecology*, **22:** 4470-4482.

Grueber CE, Wallis GP, King TM, Jamieson IG (2012) Variation at innate immunity Toll-like Receptor Genes in a bottlenecked population of a New Zealand robin. *PLoS ONE*, **7**.

Haas F, Pointer MA, Saino N, Brodin A, Mundy NI, Hansson B (2009) An analysis of population genetic differentiation and genotype-phenotype association across the hybrid zone of carrion and hooded crows using microsatellites and MC1R. *Molecular Ecology*, **18:** 294-305.

Halliday TR (1983) The study of mate choice. In: *Mate Choice* (ed. Bateson P), pp. 3-32. Cambridge University Press, Cambridge, UK.

Hamann M, Godfrey MH, Seminoff JA, Arthur K, Barata PCR, Bjorndal KA, Bolten AB, Broderick AC, Campbell LM, Carreras C, Casale P, Chaloupka M, Chan SKF, Coyne MS, Crowder LB, Diez CE, Dutton PH, Epperly SP, FitzSimmons NN, Formia A, Girondot M, Hays GC, Cheng IJ, Kaska Y, Lewison R, Mortimer JA, Nichols WJ, Reina RD, Shanker K, Spotila JR, Tomas J, Wallace BP, Work TM, Zbinden J, Godley BJ (2010) Global research priorities for sea turtles: informing management and conservation in the 21st century. *Endangered Species Research*, **11:** 245-269.

Hansson B, Bensch S, Hasselquist D, Akesson M (2001) Microsatellite diversity predicts recruitment of sibling great reed warblers. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **268:** 1287-1291.

Hansson B, Jack L, Christians JK, Pemberton JM, Akesson M, Westerdahl H, Bensch S, Hasselquist D (2007) No evidence for inbreeding avoidance in a great reed warbler population. *Behavioral Ecology*, **18:** 157-164.

Hansson B, Westerberg L (2002) On the correlation between heterozygosity and fitness in natural populations. *Molecular Ecology*, **11:** 2467-2474.

Hansson B, Westerberg L (2008) Heterozygosity-fitness correlations within inbreeding classes: local or genome-wide effects? *Conservation Genetics*, **9:** 73-83.

Hansson B, Westerdahl H, Hasselquist D, Akesson M, Bensch S (2004) Does linkage disequilibrium generate heterozygosity-fitness correlations in great reed warblers? *Evolution*, **58:** 870-879.

Hare MP, Nunney L, Schwartz MK, Ruzzante DE, Burford M, Waples RS, Ruegg K, Palstra F (2011) Understanding and estimating effective population size for practical application in marine species management. *Conservation Biology*, **25:** 438-449.

Harrison XA, Bearhop S, Inger R, Colhoun K, Gudmundsson GA, Hodgson D, McElwaine G, Tregenza T (2011) Heterozygosity-fitness correlations in a migratory bird: an analysis of inbreeding and single-locus effects. *Molecular Ecology*, **20:** 4786-4795.

Hartl DL (1988) *A Primer of Population Genetics.* 2nd edition. Sinauer Associates, Sunderland, MA.

Hawkes LA, Broderick AC, Coyne MS, Godfrey MH, Lopez-Jurado LF, Lopez-Suarez P, Merino SE, Varo-Cruz N, Godley BJ (2006) Phenotypically linked dichotomy in sea turtle foraging requires multiple conservation approaches. *Current Biology*, **16:** 990-995.

Hawkes LA, Broderick AC, Godfrey MH, Godley BJ (2009) Climate change and marine turtles. *Endangered Species Research*, **7:** 137-154.

Hays GC, Fossette S, Katselidis KA, Schofield G, Gravenor MB (2010) Breeding periodicity for male sea turtles, operational sex ratios, and implications in the face of climate change. *Conservation Biology*, **24:** 1636-1643.

Heppell SS, Snover ML, Crowder LB (2002) Sea Turtle Population Ecology. In: *The Biology of Sea Turtles vol. II* (eds Lutz PL, Musick JA, Wyneken J), pp. 275-306. CRC Press, Boca Raton, FL.

Hewitt GM (1990) Divergence and speciation as viewed from an insect hybrid zone. *Canadian Journal of Zoology*, **68:** 1701-1715.

Hill WG (1981) Estimation of effective population size from data on linkage disequilibrium. *Genetical Research*, **38:** 209-216.

Hitchins PM, Bourquin O, Hitchins S (2004) Nesting success of hawksbill turtles (*Eretmochelys imbricata*) on Cousine Island, Seychelles. *Journal of Zoology*, **264:** 383-389.

Holland B, Rice WR (1998) Perspective: Chase-away sexual selection: antagonistic seduction versus resistance. *Evolution*, **52:** 1-7.

Hölldobler B, Wilson EO (1990) *The Ants.* Harvard University Press, Cambridge, MA.

Holleley CE, Geerts PG (2009) Multiplex Manager 1.0: a cross-platform computer program that plans and optimizes multiplex PCR. *Biotechniques*, **46:** 511-517.

Hosken DJ (1997) Sperm competition in bats. *Proceedings of the Royal Society B-Biological Sciences*, **264:** 385-392.

Hubby JL, Lewontin RC (1966) A molecular approach to the study of genic heterozygosity in natural populations .I. Number of alleles at different loci in *Drosophila pseudoobscura*. *Genetics*, **54:** 577-594.

Hudson ME (2008) Sequencing breakthroughs for genomic ecology and evolutionary biology. *Molecular Ecology Resources*, **8:** 3-17.

Hurvich CM, Tsai CL (1989) Regression and time-series model selection in small samples. *Biometrika*, **76:** 297-307.

Ireland JS, Broderick AC, Glen F, Godley BJ, Hays GC, Lee PLM, Skibinski DOF (2003) Multiple paternity assessed using microsatellite markers, in green turtles *Chelonia mydas* (Linnaeus, 1758) of Ascension Island, South Atlantic. *Journal of Experimental Marine Biology and Ecology*, **291:** 149-160.

IUCN (2013) The IUCN Red List of Threatened Species. Version 2013.1. . http://www.iucnredlist.org. Downloaded on 02 July 2013.

Jamieson IG, Taylor SS, Tracy LN, Kokko H, Armstrong DP (2009) Why some species of birds do not avoid inbreeding: insights from New Zealand robins and saddlebacks. *Behavioral Ecology*, **20:** 575-584.

Janzen FJ, Tucker JF, Paukstis GL (2000) Experimental analysis of an early life-history stage: avian predation selects for larger body size of hatchling turtles. *Journal of Evolutionary Biology*, **13:** 947-954.

Janzen FJ, Tucker JK, Paukstis GL (2007) Experimental analysis of an early life-history stage: direct or indirect selection on body size of hatchling turtles? *Functional Ecology*, **21:** 162-170.

Jeffreys AJ, Wilson V, Thein SL (1985) Hypervariable 'minisatellite' regions in human DNA. *Nature*, **314:** 67-73.

Jennions MD (1997) Female promiscuity and genetic incompatibility. *Trends in Ecology & Evolution*, **12:** 251-253.

Jennions MD, Petrie M (2000) Why do females mate multiply? A review of the genetic benefits. *Biological Reviews*, **75:** 21-64.

Jensen MP, Abreu-Grobois FA, Frydenberg J, Loeschcke V (2006) Microsatellites provide insight into contrasting mating patterns in arribada vs. non-arribada olive ridley sea turtle rookeries. *Molecular Ecology*, **15:** 2567-2575.

Johnson WE, Onorato DP, Roelke ME, Land ED, Cunningham M, Belden RC, McBride R, Jansen D, Lotz M, Shindle D, Howard J, Wildt DE, Penfold LM, Hostetler JA, Oli MK, O'Brien SJ (2010) Genetic restoration of the Florida panther. *Science*, **329:** 1641-1645.

Johnston EE, Rand MS, Zweifel SG (2006) Detection of multiple paternity and sperm storage in a captive colony of the central Asian tortoise, *Testudo horsfieldii*. *Canadian Journal of Zoology*, **84:** 520-526.

Joseph J, Shaw PW (2011) Multiple paternity in egg clutches of hawksbill turtles (*Eretmochelys imbricata*). *Conservation Genetics*, **12:** 601-605.

Kawai T, Akira S (2010) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nature Immunology*, **11:** 373-384.

Keller LF (1998) Inbreeding and its fitness effects in an insular population of song sparrows (*Melospiza melodia*). *Evolution*, **52:** 240-250.

Keller LF, Waller DM (2002) Inbreeding effects in wild populations. *Trends in Ecology & Evolution*, **17:** 230-241.

Kempenaers B (2007) Mate choice and genetic quality: A review of the heterozygosity theory. In: *Advances in the Study of Behavior, Vol 37* (eds Brockmann HJ, Roper TJ, Naguib M*, et al.*), pp. 189-278. Elsevier Academic Press Inc, San Diego.

Kempenaers B, Verheyen GR, Vanderbroeck M, Burke T, Vanbroeckhoven C, Dhondt AA (1992) Extra-pair paternity results from female preference for high-quality males in the blue tit. *Nature*, **357:** 494-496.

Kenta T, Gratten J, Haigh NS, Hinten GN, Slate J, Butlin RK, Burke T (2008) Multiplex SNP-SCALE: a cost-effective medium-throughput single nucleotide polymorphism genotyping method. *Molecular Ecology Resources*, **8:** 1230-1238.

Kleven O, Fossoy F, Laskemoen T, Robertson RJ, Rudolfsen G, Lifjeld JT (2009) Comparative evidence for the evolution of sperm swimming speed by sperm competition and female sperm storage duration in passerine birds. *Evolution*, **63:** 2466-2473.

Knowlton N, Jackson JBC (1993) Inbreeding and outbreeding in marine invertebrates. In: *The Natural History of Inbreeding and Outbreeding* (ed. Thornhill NW), pp. 200-249. University of Chicago Press, Chicago.

Kokko H, Brooks R, Jennions MD, Morley J (2003) The evolution of mate choice and mating biases. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **270:** 653-664.

Kokko H, Brooks R, McNamara JM, Houston AI (2002) The sexual selection continuum. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **269:** 1331-1340.

Krebs JR, Davies NB (1993) *An Introduction to Behavioural Ecology. 3rd edition.* Blackwell Science, Oxford.

Küpper C, Kosztolanyi A, Augustin J, Dawson DA, Burke T, Székely T (2010) Heterozygosity-fitness correlations of conserved microsatellite markers in Kentish plovers *Charadrius alexandrinus*. *Molecular Ecology*, **19:** 5172-5185.

Lacy RC (1987) Loss of genetic diversity from managed populations: interacting effects of drift, mutation, immigration, selection, and population subdivision. *Conservation Biology*, **1:** 143-158.

Lacy RC, Petric A, Warneke M (1993) Inbreeding and outbreeding in captive populations of wild animal species. In: *The Natural History of Inbreeding and Outbreeding* (ed. Thornhill NW), pp. 352-374. University of Chicago Press, Chicago.

Landry C, Garant D, Duchesne P, Bernatchez L (2001) 'Good genes as heterozygosity': the major histocompatibility complex and mate choice in Atlantic salmon (*Salmo salar*). *Proceedings of the Royal Society of London Series B-Biological Sciences*, **268:** 1279-1285.

Lank DB, Smith CM, Hanotte O, Ohtonen A, Bailey S, Burke T (2002) High frequency of polyandry in a lek mating system. *Behavioral Ecology*, **13:** 209-215.

Lasala JA, Harrison JS, Williams KL, Rostal DC (2013) Strong male-biased operational sex ratio in a breeding population of loggerhead turtles (*Caretta caretta*) inferred by paternal genotype reconstruction analysis. *Ecology and Evolution***:** DOI: 10.1002/ece1003.1761.

Laurent L, Lescure J, Excoffier L, Bowen B, Domingo M, Hadjichristophorou M, Kornaraki L, Trabuchet G (1993) Genetic studies of relationships between Mediterranean and Atlantic populations of loggerhead turtle *Caretta caretta* with a mitochondrial marker. *Comptes Rendus De L Academie Des Sciences Serie III-Sciences De La Vie*, **316:** 1233-1239.

Lee PLM (2008) Molecular ecology of marine turtles: new approaches and future directions. *Journal of Experimental Marine Biology and Ecology*, **356:** 25-42.

Lee PLM, Hays GC (2004) Polyandry in a marine turtle: females make the best of a bad job. *Proceedings of the National Academy of Sciences of the United States of America*, **101:** 6530-6535.

Lee PLM, Luschi P, Hays GC (2007) Detecting female precise natal philopatry in green turtles using assignment methods. *Molecular Ecology*, **16:** 61-74.

Leon YM, Bjorndal KA (2002) Selective feeding in the hawksbill turtle, an important predator in coral reef ecosystems. *Marine Ecology Progress Series*, **245:** 249-258.

Lewontin RC, Hubby JL (1966) A molecular approach to the study of genic heterozygosity in natural populations. II. Amount of variation and degree of heterozygosity in natural populations of *Drosophila pseudoobscura*. *Genetics*, **54:** 595-&.

Li YC, Korol AB, Fahima T, Beiles A, Nevo E (2002) Microsatellites: genomic distribution, putative functions and mutational mechanisms: a review. *Molecular Ecology*, **11:** 2453-2465.

Li YC, Korol AB, Fahima T, Nevo E (2004) Microsatellites within genes: Structure, function, and evolution. *Molecular Biology and Evolution*, **21:** 991-1007.

Lieutenant-Gosselin M, Bernatchez L (2006) Local heterozygosity-fitness correlations with global positive effects on fitness in threespine stickleback. *Evolution*, **60:** 1658-1668.

Limpus CJ (1993) The green turtle, *Chelonia mydas*, in Queensland: breeding males in the southern Great Barrier Reef. *Wildlife Research*, **20:** 513-523.

Limpus CJ, Baker V, Miller JD (1979) Movement induced mortality of loggerhead eggs. *Herpetologica*, **35:** 335-338.

Limpus CJ, Nicholls N (1988) The Southern Oscillation regulates the annual numbers of green turtles (*Chelonia mydas*) breeding around northern Australia. *Australian Wildlife Research*, **15:** 157-161.

Lin G, Chang A, Yap HW, Yue GH (2008) Characterization and cross-species amplification of microsatellites from the endangered Hawksbill turtle (*Eretmochelys imbricate*). *Conservation Genetics*, **9:** 1071-1073.

Lourenço JM, Glémin S, Chiari Y, Galtier N (2013) The determinants of the molecular substitution process in turtles. *Journal of Evolutionary Biology*, **26:** 38-50.

Løvlie H, Gillingham MAF, Worley K, Pizzari T, Richardson DS (2013) Cryptic female choice favours sperm from major histocompatibility complex-dissimilar males. *Proceedings of the Royal Society B-Biological Sciences*, **280:** 20131296.

Loy A, Cianfrani C (2010) The ecology of *Eurotestudo h. hermanni* in a mesic area of southern Italy: first evidence of sperm storage. *Ethology Ecology & Evolution*, **22:** 1-16.

Luikart G, Cornuet JM (1999) Estimating the effective number of breeders from heterozygote excess in progeny. *Genetics*, **151:** 1211-1216.

Lutcavage ME, Plotkin P, Witherington B, Lutz PL (1997) Evolution, Phylogeny, and Current Status. In: *The Biology of Sea Turtles* (eds Lutz PL, Musick JA), pp. 387-410. CRC Press, Boca Raton, FL.

Lyon BE, Eadie JM (2008) Conspecific brood parasitism in birds: a life-history perspective. In: *Annual Review of Ecology Evolution and Systematics*, pp. 343-363.

Madsen T, Shine R, Olsson M, Wittzell H (1999) Conservation biology - Restoration of an inbred adder population. *Nature*, **402:** 34-35.

Madsen T, Ujvari B, Olsson M (2004) Novel genes continue to enhance population growth in adders (*Vipera berus*). *Biological Conservation*, **120:** 145-147.

Marr AB, Keller LF, Arcese P (2002) Heterosis and outbreeding depression in descendants of natural immigrants to an inbred population of song sparrows (*Melospiza melodia*). *Evolution*, **56:** 131-142.

Marshall TC, Slate J, Kruuk LEB, Pemberton JM (1998) Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology*, **7:** 639-655.

Marshall TC, Spalton JA (2000) Simultaneous inbreeding and outbreeding depression in reintroduced Arabian oryx. *Animal Conservation*, **3:** 241-248.

Martinez-Cruz B, Godoy JA, Negro JJ (2007) Population fragmentation leads to spatial and temporal genetic structure in the endangered Spanish imperial eagle. *Molecular Ecology*, **16:** 477-486.

May B (1992) Starch gel electrophoresis of allozymes. In: *Molecular Genetic Analysis of Populations: a Practical Approach* (ed. Hoelzel AR), pp. 1-27.

McKay JK, Latta RG (2002) Adaptive population divergence: markers, QTL and traits. *Trends in Ecology & Evolution*, **17:** 285-291.

Meirmans PG, Hedrick PW (2011) Assessing population structure: F-ST and related measures. *Molecular Ecology Resources*, **11:** 5-18.

Metzgar D, Bytof J, Wills C (2000) Selection against frameshift mutations limits microsatellite expansion in coding DNA. *Genome Research*, **10:** 72-80.

Metzker ML (2010) Sequencing technologies - the next generation. *Nature Reviews Genetics*, **11:** 31-46.

Meylan AB, Bowen BW, Avise JC (1990) A genetic test of the natal homing versus social facilitation models for green turtle migration. *Science*, **248:** 724-727.

Meylan AB, Donnelly M (1999) Status justification for listing the hawksbill turtle (*Eretmochelys imbricata*) as Critically Endangered on the 1996 IUCN Red List of Threatened Animals. *Chelonian Conservation and Biology*, **3:** 200-224.

Michalczyk Ł, Millard AL, Martin OY, Lumley AJ, Emerson BC, Chapman T, Gage MJG (2011) Inbreeding promotes female promiscuity. *Science*, **333:** 1739-1742.

Miller JD (1997) Reproduction in Sea Turtles. In: *The Biology of Sea Turtles* (eds Lutz PL, Musick JA), pp. 51-82. CRC Press, Boca Raton, FL.

Miller MR, Dunham JP, Amores A, Cresko WA, Johnson EA (2007) Rapid and cost-effective polymorphism identification and genotyping using restriction site associated DNA (RAD) markers. *Genome Research*, **17:** 240-248.

Miro-Herrans AT, Velez-Zuazo X, Acevedo JP, McMillan WO (2008) Isolation and characterization of novel microsatellites from the critically endangered hawksbill sea turtle (*Eretmochelys imbricata*). *Molecular Ecology Resources*, **8:** 1098-1101.

Mitton JB, Schuster WSF, Cothran EG, Defries JC (1993) Correlation between the individual heterozygosity of parents and their offspring. *Heredity*, **71:** 59-63.

Møller AP, Alatalo RV (1999) Good-genes effects in sexual selection. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **266:** 85-91.

Monzón-Argüello C, Munoz J, Marco A, López-Jurado LF, Rico C (2008) Twelve new polymorphic microsatellite markers from the loggerhead sea turtle (*Caretta caretta*) and cross-species amplification on other marine turtle species. *Conservation Genetics*, **9:** 1045-1049.

Moritz C (1995) Uses of molecular phylogenies for conservation. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences*, **349:** 113-118.

Mortimer JA (1984) Marine turtles in the Republic of Seychelles: status and management. In: *Report on WWF project 1809, 1981-1984*, p. 80. IUCN/WWF, Gland, Switzerland.

Mortimer JA (2004) Seychelles Marine Ecosystem Management Project (GEF-SEYMEMP): Turtle Component. Final Report.

Mortimer JA, Balazs GH (2000) Post-nesting migrations of hawksbill turtles in the granitic Seychelles and implications for conservation. *Proceedings of the 19th Annual Symposium on Sea Turtle Biology and Conservation*. US Department of Commerce, Miami, FL. NOAA Technical Memorandum NMFS-SEFSC-443, 22-26.

Mortimer JA, Bresson R (1999) Temporal distribution and periodicity in hawksbill turtles (*Eretmochelys imbricata*) nesting at Cousin Island, Republic of Seychelles, 1971-1997. *Chelonian Conservation and Biology*, **3:** 318-325.

Mortimer JA, Broderick D (1999) Chapter14: Population genetic structure and developmental migrations of sea turtles in the Chagos Archipelago and adjacent regions inferred from mtDNA sequence variation. In: *Ecology of the Chagos Archipelago.* (eds Sheppard CRC, Seaward MRC), pp. 185-194. Linnean Society Occasional Publications 2.

Mortimer JA, Donnelly M (2008) Marine Turtle Specialist Group 2007 IUCN Red List Status Assessment Hawksbill Turtle (*Eretmochelys imbricata*). http://www.iucn-mtsg.org/red_list/ei/index.shtml, p. 121.

Mortimer JA, Donnelly M, Meylan AB, Meylan PA (2007a) Critically endangered hawksbill turtles: molecular genetics and the broad view of recovery. *Molecular Ecology*, **16:** 3516-3517.

Mortimer JA, Meylan PA, Donnelly M (2007b) Whose turtles are they, anyway? *Molecular Ecology*, **16:** 17-18.

Mossman CA, Waser PM (1999) Genetic detection of sex-biased dispersal. *Molecular Ecology*, **8:** 1063-1067.

Mueller JC, Hermisson J, Olano-Marin J, Hansson B, Kempenaers B (2011) Linking genetic mechanisms of heterozygosity-fitness correlations to footprints of selection at single loci. *Evolutionary Ecology*, **25:** 1-11.

Mullis KB, Faloona FA (1987) Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain-reaction. *Methods in Enzymology*, **155:** 335-350.

Murphy RW, Berry KH, Edwards T, McLuckie AM (2007) A genetic assessment of the recovery units for the Mojave population of the desert tortoise, *Gopherus agassizii*. *Chelonian Conservation and Biology*, **6:** 229-251.

Musick JA, Limpus CJ (1997) Habitat Utilization and Migration in Juvenile Sea Turtles. In: *The Biology of Sea Turtles* (eds Lutz PL, Musick JA), pp. 137-164. CRC Press, Boca Raton, FL.

Myers EM, Janzen FJ, Adams DC, Tucker JK (2006) Quantitative genetics of plastron shape in slider turtles (Trachemys scripta). *Evolution*, **60:** 563-572.

Nakagawa S, Schielzeth H (2013) A general and simple method for obtaining R2 from generalized linear mixed-effects models. *Methods in Ecology and Evolution*, **4:** 133-142.

Naro-Maciel E, Le M, FitzSimmons NN, Amato G (2008) Evolutionary relationships of marine turtles: A molecular phylogeny based on nuclear and mitochondrial genes. *Molecular Phylogenetics and Evolution*, **49:** 659-662.

Neff BD (2004) Stabilizing selection on genomic divergence in a wild fish population. *Proceedings of the National Academy of Sciences of the United States of America*, **101:** 2381-2385.

Newcombe RG (1998) Two-sided confidence intervals for the single proportion: Comparison of seven methods. *Statistics in Medicine*, **17:** 857-872.

Nicholls JA, Double MC, Rowell DM, Magrath RD (2000) The evolution of cooperative and pair breeding in thornbills *Acanthiza* (Pardalotidae). *Journal of Avian Biology*, **31:** 165-176.

Olano-Marin J, Mueller JC, Kempenaers B (2011a) Correlations between heterozygosity and reproductive success in the blue tit (*Cyanistes caeruleus*): an analysis of inbreeding and single locus effects. *Evolution*, **65:** 3175-3194.

Olano-Marin J, Mueller JC, Kempenaers B (2011b) Heterozygosity and survival in blue tits (*Cyanistes caeruleus*): contrasting effects of presumably functional and neutral loci. *Molecular Ecology*, **20:** 4028-4041.

Ortego J, Calabuig G, Cordero PJ, Aparicio JM (2007) Egg production and individual genetic diversity in lesser kestrels. *Molecular Ecology*, **16:** 2383-2392.

Owens DW (1980) The comparative reproductive physiology of sea turtles. *American Zoologist*, **20:** 549-563.

Palmer KS, Rostal DC, Grumbles JS, Mulvey M (1998) Long-term sperm storage in the desert tortoise (*Gopherus agassizii*). *Copeia*, **3:** 702-705.

Palsboll PJ, Berube M, Allendorf FW (2007) Identification of management units using population genetic data. *Trends in Ecology & Evolution*, **22:** 11-16.

Palstra FP, Ruzzante DE (2008) Genetic estimates of contemporary effective population size: what can they tell us about the importance of genetic stochasticity for wild population persistence? *Molecular Ecology*, **17:** 3428-3447.

Parker GA (1970) Sperm competition and its evolutionary consequences in insects. *Biological Reviews of the Cambridge Philosophical Society*, **45:** 525-567.

Parker GA (1990) Sperm competition games - raffles and roles. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **242:** 120-126.

Parker GA, Pizzari T (2010) Sperm competition and ejaculate economics. *Biological Reviews*, **85:** 897-934.

Parmenter CJ (1980) Incubation of the eggs of the green sea turtle, *Chelonia mydas*, in Torres Strait, Australia - the effect of movement on hatchability. *Australian Wildlife Research*, **7:** 487-491.

Pavey SA, Bernatchez L, Aubin-Horth N, Landry CR (2012) What is needed for next-generation ecological and evolutionary genomics? *Trends in Ecology & Evolution*, **27:** 673-678.

Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, **6:** 288-295.

Pearse DE, Janzen FJ, Avise JC (2001) Genetic markers substantiate long-term storage and utilization of sperm by female painted turtles. *Heredity*, **86:** 378-384.

Pearse DE, Janzen FJ, Avise JC (2002) Multiple paternity, sperm storage, and reproductive success of female and male painted turtles (*Chrysemys picta*) in nature. *Behavioral Ecology and Sociobiology*, **51:** 164-171.

Phillips KP, Jorgensen TH, Jolliffe KG, Jolliffe S-M, Henwood J, Richardson DS (2013) Reconstructing paternal genotypes to infer patterns of sperm storage and sexual selection in the hawksbill turtle. *Molecular Ecology*, **22:** 2301-2312.

Piertney SB, Lambin X, Maccoll ADC, Lock K, Bacon PJ, Dallas JF, Leckie F, Mougeot F, Racey PA, Redpath S, Moss R (2008) Temporal changes in kin structure through a population cycle in a territorial bird, the red grouse *Lagopus lagopus scoticus*. *Molecular Ecology*, **17:** 2544-2551.

Piertney SB, MacColl ADC, Lambin X, Moss R, Dallas JF (1999) Spatial distribution of genetic relatedness in a moorland population of red grouse (*Lagopus lagopus scoticus*). *Biological Journal of the Linnean Society*, **68:** 317-331.

Piertney SB, Oliver MK (2006) The evolutionary ecology of the major histocompatibility complex. *Heredity*, **96:** 7-21.

Pinheiro J, Bates D, DebRoy S, Sarkar D, R Development Core Team (2013) nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1-111. .

Pintus KJ, Godley BJ, McGowan A, Broderick AC (2009) Impact of clutch relocation on green turtle offspring. *Journal of Wildlife Management*, **73:** 1151-1157.

Piry S, Luikart G, Cornuet JM (1999) BOTTLENECK: a computer program for detecting recent reductions in the effective size using allele frequency data. *Journal of Heredity*, **90:** 502-503.

Pitcher TE, Neff BD, Rodd FH, Rowe L (2003) Multiple mating and sequential mate choice in guppies: females trade up. *Proceedings of the Royal Society B-Biological Sciences*, **270:** 1623-1629.

Pizzari T, Cornwallis CK, Lovlie H, Jakobsson S, Birkhead TR (2003) Sophisticated sperm allocation in male fowl. *Nature*, **426:** 70-74.

Plotkin P (2002) Adult Migrations and Habitat Use. In: *The Biology of Sea Turtles vol. II* (eds Lutz PL, Musick JA, Wyneken J), pp. 225-242. CRC Press, Boca Raton, FL.

Price MV, Waser NM (1979) Pollen dispersal and optimal outcrossing in *Delphinium nelsoni*. *Nature*, **277:** 294-297.

Price TAR, Hurst GDD, Wedell N (2010) Polyandry prevents extinction. *Current Biology*, **20:** 471-475.

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics*, **155:** 945-959.

Pritchard PCH (1997) Evolution, Phylogeny, and Current Status. In: *The Biology of Sea Turtles* (eds Lutz PL, Musick JA), pp. 1-28. CRC Press, Boca Raton, FL.

Prugnolle F, de Meeus T (2002) Inferring sex-biased dispersal from population genetic tools: a review. *Heredity*, **88:** 161-165.

Pudovkin AI, Zaykin DV, Hedgecock D (1996) On the potential for estimating the effective number of breeders from heterozygote-excess in progeny. *Genetics*, **144:** 383-387.

Queller DC, Goodnight KF (1989) Estimating relatedness using genetic markers. *Evolution*, **43:** 258-275.

Queller DC, Strassmann JE, Hughes CR (1993) Microsatellites and kinship. *Trends in Ecology & Evolution*, **8:** 285-&.

R Development Core Team (2008) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Ralls K, Ballou J (1982) Effects of inbreeding on infant mortality in captive Primates. *International Journal of Primatology*, **3:** 491-505.

Ralls K, Ballou JD, Templeton A (1988) Estimates of lethal equivalents and the cost of inbreeding in mammals. *Conservation Biology*, **2:** 185-193.

Raymond M, Rousset F (1995) GENEPOP (Version-1.2) - population-genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86:** 248-249.

Richardson DS, Jury FL, Blaakmeer K, Komdeur J, Burke T (2001) Parentage assignment and extra-group paternity in a cooperative breeder: the Seychelles warbler (*Acrocephalus sechellensis*). *Molecular Ecology*, **10:** 2263-2273.

Richardson DS, Komdeur J, Burke T (2004) Inbreeding in the Seychelles warbler: Environment-dependent maternal effects. *Evolution*, **58:** 2037-2048.

Richardson DS, Komdeur J, Burke T, von Schantz T (2005) MHC-based patterns of social and extra-pair mate choice in the Seychelles warbler. *Proceedings of the Royal Society B-Biological Sciences*, **272:** 759-767.

Rivalan P, Dutton PH, Baudry E, Roden SE, Girondot M (2006) Demographic scenario inferred from genetic data in leatherback turtles nesting in French Guiana and Suriname. *Biological Conservation*, **130:** 1-9.

Roberts SC, Hale ML, Petrie M (2006) Correlations between heterozygosity and measures of genetic similarity: implications for understanding mate choice. *Journal of Evolutionary Biology*, **19:** 558-569.

Robertson A (1965) Interpretation of genotypic ratios in domestic animal populations. *Animal Production*, **7:** 319-324.

Rogers DW, Denniff M, Chapman T, Fowler K, Pomiankowski A (2008) Male sexual ornament size is positively associated with reproductive morphology and enhanced fertility in the stalk-eyed fly *Teleopsis dalmanni*. *BMC Evolutionary Biology*, **8:** 236.

Rousset F (2008) GENEPOP'007: a complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources*, **8:** 103-106.

Sanger F, Coulson AR (1975) A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. *Journal of Molecular Biology*, **94:** 441-448.

Scott R, Marsh R, Hays GC (2012) Life in the really slow lane: loggerhead sea turtles mature late relative to other reptiles. *Functional Ecology*, **26:** 227-235.

Scribner KT, Petersen MR, Fields RL, Talbot SL, Pearce JM, Chesser RK (2001) Sex-biased gene flow in spectacled eiders (Anatidae): Inferences from molecular markers with contrasting modes of inheritance. *Evolution*, **55:** 2105-2115.

Seddon PJ, Armstrong DP, Maloney RF (2007) Developing the science of reintroduction biology. *Conservation Biology*, **21:** 303-312.

Shamblin BM, Faircloth BC, Dodd M, Wood-Jones A, Castleberry SB, Carroll JP, Nairn CJ (2007) Tetranucleotide microsatellites from the loggerhead sea turtle (*Caretta caretta*). *Molecular Ecology Notes*, **7:** 784-787.

Shamblin BM, Faircloth BC, Dodd MG, Bagley DA, Ehrhart LM, Dutton PH, Frey A, Nairn CJ (2009) Tetranucleotide markers from the loggerhead sea turtle (*Caretta caretta*) and their cross-amplification in other marine turtle species. *Conservation Genetics*, **10:** 577-580.

Sheldon BC (1994) Male phenotype, fertility, and the pursuit of extra-pair copulations by female birds. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **257:** 25-30.

Sheppard CRC, Ateweberhan M, Bowen BW, Carr P, Chen CA, Clubbe C, Craig MT, Ebinghaus R, Eble J, Fitzsimmons N, Gaither MR, Gan CH, Gollock M, Guzman N, Graham NAJ, Harris A, Jones R, Keshavmurthy S, Koldewey H, Lundin CG, Mortimer JA, Obura D, Pfeiffer M, Price ARG, Purkis S, Raines P, Readman JW, Riegl B, Rogers A, Schleyer M, Seaward MRD, Sheppard ALS, Tamelander J, Turner JR, Visram S, Vogler C, Vogt S, Wolschke H, Yang JMC, Yang SY, Yesson C (2012) Reefs and islands of the Chagos Archipelago, Indian Ocean: why it is the world's largest no-take marine protected area. *Aquatic Conservation-Marine and Freshwater Ecosystems*, **22:** 232-261.

Slate J, David P, Dodds KG, Veenvliet BA, Glass BC, Broad TE, McEwan JC (2004) Understanding the relationship between the inbreeding coefficient and multilocus heterozygosity: theoretical expectations and empirical data. *Heredity*, **93:** 255-265.

Slate J, Pemberton J (2006) Does reduced heterozygosity depress sperm quality in wild rabbits (*Oryctolagus cuniculus*)? *Current Biology*, **16:** R790-R791.

Slatkin M (1991) Inbreeding coefficients and coalescence times. *Genetical Research*, **58:** 167-175.

Slatyer RA, Mautz BS, Backwell PRY, Jennions MD (2012) Estimating genetic benefits of polyandry from experimental studies: a meta-analysis. *Biological Reviews*, **87:** 1-33.

Snow LSE, Andrade MCB (2005) Multiple sperm storage organs facilitate female control of paternity. *Proceedings of the Royal Society B-Biological Sciences*, **272:** 1139-1144.

Spotila JR (2004) *Sea Turtles: A Complete Guide To Their Biology, Behavior, And Conservation.* The Johns Hopkins University Press, Baltimore.

Stanback M, Richardson DS, Boix-Hinzen C, Mendelsohn J (2002) Genetic monogamy in Monteiro's hornbill, *Tockus monteiri*. *Animal Behaviour*, **63:** 787-793.

Stephens PA, Boyd IL, McNamara JM, Houston AI (2009) Capital breeding and income breeding: their meaning, measurement, and worth. *Ecology*, **90:** 2057-2067.

Stewart KR, Dutton PH (2011) Paternal genotype reconstruction reveals multiple paternity and sex ratios in a breeding population of leatherback turtles (*Dermochelys coriacea*). *Conservation Genetics*, **12:** 1101-1113.

Stiebens VA, Merino SE, Roder C, Chain FJJ, Lee PLM, Eizaguirre C (2013) Living on the edge: how philopatry maintains adaptive potential. *Proceedings of the Royal Society B-Biological Sciences*, **280:** DOI: 10.1098/rspb.2013.0305.

Storey JD (2002) A direct approach to false discovery rates. *Journal of the Royal Statistical Society Series B-Statistical Methodology*, **64:** 479-498.

Storfer A (1996) Quantitative genetics: A promising approach for the assessment of genetic variation in endangered species. *Trends in Ecology & Evolution*, **11:** 343-348.

Szulkin M, Bierne N, David P (2010) Heterozygosity-fitness correlations: a time for reappraisal. *Evolution*, **64:** 1202-1217.

Szulkin M, David P (2011) Negative heterozygosity-fitness correlations observed with microsatellites located in functional areas of the genome. *Molecular Ecology*, **20:** 3949-3952.

Szulkin M, Sheldon BC (2007) The environmental dependence of inbreeding depression in a wild bird population. *PLoS ONE*, **2:** e1027.

Szymura JM, Barton NH (1986) Genetic analysis of a hybrid zone between the fire-bellied toads, *Bombina bombina* and *Bombina variegata*, near Cracow in southern Poland. *Evolution*, **40:** 1141-1159.

Tallmon DA, Koyuk A, Luikart G, Beaumont MA (2008) ONeSAMP: a program to estimate effective population size using approximate Bayesian computation. *Molecular Ecology Resources*, **8:** 299-301.

Tautz D, Renz M (1984) Simple sequences are ubiquitous repetitive components of eukaryotic genomes. *Nucleic Acids Research*, **12:** 4127-4138.

Templeton AR, Hemmer H, Mace G, Seal US, Shields WM, Woodruff DS (1986) Local adaptation, coadaptation, and population boundaries. *Zoo Biology*, **5:** 115-125.

The MathWorks Inc. (2010) MATLAB 7.11. The MathWorks Inc., Natick, MA, USA.

Theissinger K, FitzSimmons NN, Limpus CJ, Parmenter CJ, Phillott AD (2009) Mating system, multiple paternity and effective population size in the endemic flatback turtle (*Natator depressus*) in Australia. *Conservation Genetics*, **10:** 329-346.

Tóth G, Gáspári Z, Jurka J (2000) Microsatellites in different eukaryotic genomes: Survey and analysis. *Genome Research*, **10:** 967-981.

Tregenza T, Wedell N (1998) Benefits of multiple mates in the cricket *Gryllus bimaculatus*. *Evolution*, **52:** 1726-1730.

Turtle Expert Working Group (2000) Assessment update for the Kemp's ridley and loggerhead sea turtle populations in the western north Atlantic, p. 115. U.S. Department of Commerce.

Uller T, Olsson M (2008) Multiple paternity in reptiles: patterns and processes. *Molecular Ecology*, **17:** 2566-2580.

van Dam RP, Diez CE, Balazs GH, Colon LAC, McMillan WO, Schroeder B (2008) Sex-specific migration patterns of hawksbill turtles breeding at Mona Island, Puerto Rico. *Endangered Species Research*, **4:** 85-94.

van de Casteele T, Galbusera P, Schenck T, Matthysen E (2003) Seasonal and lifetime reproductive consequences of inbreeding in the great tit *Parus major*. *Behavioral Ecology*, **14:** 165-174.

van de Pol MV, Wright J (2009) A simple method for distinguishing within- versus between-subject effects using mixed models. *Animal Behaviour*, **77:** 753-758.

Vargas SM, Jensen MP, Mobaraki A, Santos FR, Broderick D, Mortimer JA, Limpus CJ, Whiting S, FitzSimmons NN (2013) Phylogeography of the hawksbill turtle (*Eretmochelys imbricata*) from the Indo-Pacific region. *Proceedings of the 30th Annual Symposium on Sea Turtle Biology and Conservation*. US Department of Commerce, Miami, FL. NOAA Technical Memorandum NMFS-SEFSC-640: 177, 155.

Varian-Ramos CW, Webster MS (2012) Extrapair copulations reduce inbreeding for female red-backed fairy-wrens, *Malurus melanocephalus*. *Animal Behaviour*, **83:** 857-864.

Vos P, Hogers R, Bleeker M, Reijans M, Vandelee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research*, **23:** 4407-4414.

Wallace BP, DiMatteo AD, Bolten AB, Chaloupka MY, Hutchinson BJ, Abreu-Grobois FA, Mortimer JA, Seminoff JA, Amorocho D, Bjorndal KA, Bourjea J, Bowen BW, Briseño Dueñas R, Casale P, Choudhury BC, Costa A, Dutton PH, Fallabrino A, Finkbeiner EM, Girard A, Girondot M, Hamann M, Hurley BJ, López-Mendilaharsu M, Marcovaldi MA, Musick JA, Nel R, Pilcher NJ, Troëng S, Witherington B, Mast RB (2011) Global conservation priorities for marine turtles. *PLoS ONE*, **6:** e24510.

Wang J (2011a) A new likelihood estimator and its comparison with moment estimators of individual genome-wide diversity. *Heredity*, **107:** 433-443.

Wang J (2012) On the measurements of genetic differentiation among populations. *Genetics Research*, **94:** 275-289.

Wang J, Santure AW (2009) Parentage and sibship inference from multilocus genotype data under polygamy. *Genetics*, **181:** 1579-1594.

Wang JL (2009) A new method for estimating effective population sizes from a single sample of multilocus genotypes. *Molecular Ecology*, **18:** 2148-2164.

Wang JL (2011b) COANCESTRY: a program for simulating, estimating and analysing relatedness and inbreeding coefficients. *Molecular Ecology Resources*, **11:** 141-145.

Wang Z, Pascual-Anaya J, Zadissa A, Li WQ, Niimura Y, Huang ZY, Li CY, White S, Xiong ZQ, Fang DM, Wang B, Ming Y, Chen Y, Zheng Y, Kuraku S, Pignatelli M, Herrero J, Beal K, Nozawa M, Li QY, Wang J, Zhang HY, Yu LL, Shigenobu S, Wang JY, Liu JN, Flicek P, Searle S, Kuratani S, Yin Y, Aken B, Zhang GJ, Irie N (2013) The draft genomes of soft-shell turtle and green sea turtle yield insights into the development and evolution of the turtle-specific body plan. *Nature Genetics*, **45:** 701-706.

Waples RS (1989) A generalized approach for estimating effective population size from temporal changes in allele frequency. *Genetics*, **121:** 379-391.

Waples RS, Do C (2008) LDNE: a program for estimating effective population size from data on linkage disequilibrium. *Molecular Ecology Resources*, **8:** 753-756.

Waples RS, Do C (2010) Linkage disequilibrium estimates of contemporary N-e using highly variable genetic markers: a largely untapped resource for applied conservation and evolution. *Evolutionary Applications*, **3:** 244-262.

Waser NM (1993) Population structure, optimal outbreeding, and assortative mating in angiosperms. In: *The Natural History of Inbreeding and Outbreeding* (ed. Thornhill NW), pp. 173-199. University of Chicago Press, Chicago.

Waser NM, Price MV (1989) Optimal outcrossing in *Ipomopsis aggregata* - seed set and offspring fitness. *Evolution*, **43:** 1097-1109.

Waser NM, Price MV (1994) Crossing-distance effects in *Deplhinium nelsonii* - outbreeding and inbreeding depression in progeny fitness. *Evolution*, **48:** 842-852.

Weber SB, Broderick AC, Groothuis TGG, Ellick J, Godley BJ, Blount JD (2012) Fine-scale thermal adaptation in a green turtle nesting population. *Proceedings of the Royal Society B-Biological Sciences*, **279:** 1077-1084.

Wedell N (1994) Variation in nuptial gift quality in bush-crickets (Orthoptera, Tettigoniidae). *Behavioral Ecology*, **5:** 418-425.

Weeks AR, Sgro CM, Young AG, Frankham R, Mitchell NJ, Miller KA, Byrne M, Coates DJ, Eldridge MDB, Sunnucks P, Breed MF, James EA, Hoffmann AA (2011) Assessing the benefits and risks of translocations in changing environments: a genetic perspective. *Evolutionary Applications*, **4:** 709-725.

Weigensberg I, Fairbairn DJ (1994) Conflicts of interest between the sexes - a study of mating interactions in a semiaquatic bug. *Animal Behaviour*, **48:** 893-901.

Weir BS, Cockerham CC (1973) Mixed self and random mating at two loci. *Genetical Research*, **21:** 247-262.

While GM, Uller T, Wapstra E (2011) Variation in social organization influences the opportunity for sexual selection in a social lizard. *Molecular Ecology*, **20:** 844-852.

Whitaker N (2006) Immaculate conception, incubation protocols, and egg characteristics of the Ganges softshell turtle (*Aspideretes gangeticus*). *Contemporary Herpetology*, **2006:** 1-6.

Willi Y, van Buskirk J (2005) Genomic compatibility occurs over a wide range of parental genetic similarity in an outcrossing plant. *Proceedings of the Royal Society B-Biological Sciences*, **272:** 1333-1338.

Wilson EB (1927) Probable inference, the law of succession, and statistical inference. *Journal of the American Statistical Association*, **22:** 209-212.

Wood JR, Wood FE (1980) Reproductive biology of captive green sea turtles *Chelonia mydas*. *American Zoologist*, **20:** 499-505.

Wright LI, Fuller WJ, Godley BJ, McGowan A, Tregenza T, Broderick AC (2012a) Reconstruction of paternal genotypes over multiple breeding seasons reveals male green turtles do not breed annually. *Molecular Ecology*, **279:** 2122-2127.

Wright LI, Fuller WJ, Godley BJ, McGowan A, Tregenza T, Broderick AC (2013) No benefits of polyandry to female green turtles. *Behavioral Ecology*, **24:** 1022-1029.

Wright LI, Stokes KL, Fuller WJ, Godley BJ, McGowan A, Snape R, Tregenza T, Broderick AC (2012b) Turtle mating patterns buffer against disruptive effects of climate change. *Proceedings of the Royal Society B: Biological Sciences*, **21:** 3625-3635.

Wright S (1931) Evolution in Mendelian populations. *Genetics*, **16:** 97-159.

Wright S (1965) The interpretation of population structure by F-statistics with special regard to systems of mating. *Evolution*, **19:** 395-420.

Zbinden JA, Largiader AR, Leippert F, Margaritoulis D, Arlettaz R (2007) High frequency of multiple paternity in the largest rookery of Mediterranean loggerhead sea turtles. *Molecular Ecology*, **16:** 3703-3711.

Zeh JA, Zeh DW (1997) The evolution of polyandry II: Post-copulatory defences against genetic incompatibility. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **264:** 69-75.

Thesis appendices

Thesis appendix 1. DNA extractions from blood/tissue – ammonium acetate precipitation method applied to plates, and 'recipes' for reagents. Written by technical support staff of the NERC Biomolecular Analysis Facility, Department of Animal and Plant Sciences, University of Sheffield.

1) Add 250 µl Digsol buffer and 10 µl Proteinase K (10 mg/ml) to each tube in TWO racked 8 × 12 microtube plates (Starlab catalogue code: E1760-8400). Dry samples and add them to the tubes.

2) Close tubes with microtube caps (Starlab code: E1702-8400). Label tube-columns on one side to avoid potential errors when handling single tube columns. Place a folded piece of blue roll into the lids. Secure lid with 2-3 strong rubber bands and place plates in rotating oven at 55°C for between 3 hrs and overnight.

3) Spin in plate centrifuge (1000 rpm for 1 min). Add 300 µl 4 M ammonium acetate to each sample and recap the tubes with the same microtube caps. Mix samples several times by inversion over a period of at least 15 min at room temperature to precipitate the proteins. NOTE: keep an eye on the centrifuge when you first try spinning plates. If the centrifuge is unbalanced with two full plates, try splitting each plate in half, and then using single empty tubes to try to improve the balance.

4) Centrifuge for 15 min at 4000 rpm.

5) Take 2 new microtube plates and new caps.

6) Label new tube-columns (Starlab catalogue code: E1720-8000) and place these into new microtube plates. Aspirate the supernatant (clear liquid containing the DNA) into the new tubes. Best done using the electronic multichannel pipette with P1000 tips. Also, best to take out a single tube-column and place it into a modified (cut open at one side) empty plate so that you can easily see how deep the tips are reaching (the blue plates from Qiagen extraction kits are good for this).

7) Add 500 µl 100% ethanol to each tube. Invert tubes gently over 5-10 mins to precipitate DNA. Again, line the lid with a folded piece of blue roll to soak up any minor seepage that may occur. Check each tube that the DNA did not get stuck to the microcap (if so, you get it off with a single vigorous shaking, but note that DNA is often not visible). Small amounts of DNA sticking to the microcap are fine.

8) Balance plates and centrifuge for 15 min at 4000rpm.

9) Keep microcaps, pour off ethanol, blot tubes on tissue paper, and add 500 µl 70% ethanol and invert several times (again, check that the DNA did not get stuck to the microcaps). Centrifuge for 15 min at 4000 rpm.

10) Take off microcaps and keep them somewhere clean (e.g. lay them out upside down on blue roll then cover them with another sheet). Pour off ethanol and blot tubes. Places tubes into an empty microtube plate and stand this on its side in incubator (up to 65°C) for 30- 60 min.

11) Once fully dry, add T_{10} $E_{0.1}$. The amount added is dependent on the size of the pellet – we usually recommend 100 µl, but trial, error, and the Nanodrop will help determine what is best for your samples. Secure microcaps (lid + folded tissue, possibly 1.5-2 folded pieces) and turn plates upside down to dissolve potential DNA remains that are stuck to the microcaps. Make sure that TE is at the "bottom" covering the microcaps. Leave plates like this for 30 min.

12) Balance plates, centrifuge for 1 min at 1000 rpm. Place in incubator for 30-60 min at 65°C, flicking tubes every 10-15mins, to dissolve DNA.

13) Store at -20°C (long term) or 4°C (short term)

DIGSOL

To make 1L:

Final concentration required	Volume/amount required of stock reagents
20 mM EDTA	40 ml of 0.5 M EDTA (pH 8.0)
120 mM NaCl	6.85 g solid Sodium Chloride (NaCl)
50 mM Tris.HCl	50 ml of 1 M, Tris.HCl (pH 8.0)
Water	810 ml of double distilled autoclaved water

Ensure all constituents are dissolved.

Autoclave at 15 psi for 20 minutes

add 50 ml of 20% SDS (Sodium dodecyl/lauryl sulphate)
pH to 8.0 with concentrated HCl or NaOH if needed

If SDS precipitates, warm the solution in an incubator, or place in microwave for several 5-10 sec bursts, swirling the solution between bursts.
Do not autoclave once SDS has been added.

$TE_{10}E_{0.1}$ (pH 7.5-8.0)

To make 400 ml:

Concentration required	Volume of stock reagents required
10 mM Tris.HCl	4 ml of 1M Tris.HCl (pH 8.0)
0.1 mM EDTA	80 µl (NB MICROLITRES!) of 0.5 M EDTA (pH 8.0)
Water	396 ml of double distilled autoclaved water

Thesis appendix 2. Implications of this thesis for hawksbill turtle conservation biology in the Republic of Seychelles – summary for managers and stakeholders. This report is intended to facilitate dissemination of my results to a non-scientific audience, including governmental and non-governmental organisations, and to act as a form of knowledge exchange and public outreach.

1. Background

When seeking to conserve a species, some threats are easy to identify and, in theory, to manage. An obvious example is poaching. However, almost every species will also be vulnerable to less targeted but no-less damaging processes, such as habitat degradation or disturbance, that may have subtle but serious long-term impacts. A lack of awareness of such threats may, at least partially, stem from insufficient knowledge about a species' ecology and life history. Thus scientists urgently need to study endangered species in order to understand their basic biology and provide the information that can help drive their effective long-term conservation. Unfortunately, as marine turtles illustrate, many endangered species do not lend themselves well to direct study. Endangered species are, by definition, limited in number, and may have complicated life-history traits or behaviours hidden from easy observation. However, it is possible to make inferences about the life history and demography of these species using modern molecular tools, such as DNA profiling. The breadth of this inferential power can be striking to the uninitiated: that one can use DNA to determine mating patterns, or the extent of an individual's home range from its territory marking is more intuitive to a general audience than population genetics, demographic inferences, and how an individual's genetic variability might affect its survival or reproductive success (e.g. Frankham *et al.* 2002; Beebee & Rowe 2004).

Over the last six years (2007-2013) we have applied DNA-based techniques to the study of the hawksbill turtle (*Eretmochelys imbricata*), a species internationally listed as critically endangered, and a conservation priority for many of the countries in whose seas it occurs. DNA techniques have already addressed a wide range of questions about turtle biology more generally, including testing one of the oldest hypotheses of turtle behaviour (Carr 1967), that of natal philopatry of nesting females (for reviews, see Bowen & Karl 2007; Lee 2008). However, despite the hawksbill occurring throughout the tropics and being an international conservation priority, it has remained poorly studied compared with many of its relatives, and there has been relatively little application of molecular techniques to populations other than that of the Caribbean. We studied the hawksbill turtle in the Republic of Seychelles, one of the world's most important populations of this species. Here, we summarise what our genetic study has revealed about hawksbill breeding behaviour and demography, and consider the implications of our findings for ongoing hawksbill conservation.

2. Mating systems

Marine turtles mate at sea, perhaps far from the nesting grounds, and it is therefore rarely possible to know which, or how many, males are gaining paternity and contributing to the future gene pool. As a result, a very basic piece of demographic data – the size of the male breeding population – is normally unknown. Furthermore, little is known about other key behaviours in the hawksbill's mating system, such as how many males a female turtle typically mates with, whether females practice any kind of mate choice, and whether any males are able to dominate a particular area and monopolise females and paternity. To answer these questions, we collected DNA samples from nesting females (6 mm biopsy from trailing edge of flipper) and from 20 hatchlings per nest (2 mm biopsy from marginal scute) on Cousine Island, Seychelles, over three consecutive hawksbill nesting seasons (Sep 2007-Apr 2010). We then conducted parentage analyses on these samples to determine how many males had fertilised the females in our study, and to reconstruct the DNA profiles of these males. For a detailed description of the method, see chapter 1. Over the three seasons we sampled 156 females and approximately 5000 hatchlings. We also sampled offspring in a fourth season (Sep 2010-Apr 2011) from six re-migrating females that had been sampled in the first two seasons of our study.

Our results showed that more males contributed to the offspring in our sample than females. This came about by virtue of approximately 10% of females having offspring in a nest sired by two different males, but only 2% of males gaining paternity with more than one female. All three cases where a male fertilised multiple females involved females in two separate seasons – we never saw a male obtain paternity twice in the same season. In every case where a female laid multiple nests within a season, the identity of the father/s that sired her offspring never changed. This indicates that females normally mate once, occasionally twice, prior to nesting, store the sperm they obtain, and then use it to fertilise up to five clutches of 50-250 eggs (mean = 135, SD = 35) over the nesting season. Importantly, there was no apparent drop in the success rate of the females' eggs (measured as proportion of eggs showing development) over the season that would have indicated that the stored sperm was being used up or deteriorating in quality over time. For twelve females we were able to sample their offspring in two seasons, and found that they almost always mated with a new male in their next nesting season. However, one female, remigrating after two years, produced offspring all fathered by the same one male. This may represent a case of long-term sperm storage, although we cannot rule out the possibility that this male-female pair re-encountered each other and re-mated (chapter 5).

From a conservation perspective, our results are extremely positive: the number of nesting female hawksbills in Seychelles is on the increase (e.g. Allen *et al.* 2010), and it seems that there is a large number of males out there for them to mate with. The frequency of multiple paternity in a marine turtle population is thought to be related to male density (e.g. see Jensen *et al.* 2006), with higher density resulting in more male-female encounters and therefore more multiple paternity. Consequently, the low rate of mixed paternity observed in our study, among the lowest reported in wild marine turtles, suggests that the hawksbill males are thinly spread over a large area. Furthermore, that no male sired the offspring of more than one female in a season suggests mating is taking place far enough from nesting beaches to prevent any given male from being able to fertilise multiple females heading for a given beach. It is more difficult to comment on generalities with respect to males fathering offspring in more than one year of our study, save that two males contributed to the sample in consecutive years, suggesting that male re-migration intervals may be shorter than those for females.

Marine turtle sex ratios are generally held to be female-biased (e.g. see Wright *et al.* 2012b). However, our extrapolations from the rarity of any given male fertilising multiple females estimate the male population size in the 1600-2700 range. This corresponds strikingly well with the census of female hawksbill numbers (upwards of 2500; Mortimer *et al.*, unpublished data). A possible explanation for a more even sex ratio is that hawksbill hunting in Seychelles (illegal since 1994) was heavily female-biased, as females are easier to catch and have thicker scutes that yield more ‘tortoiseshell’. Thus we may be witnessing a hangover demographic consequence of decades of sex-biased mortality, one that, given the long generation times of marine turtles, may take some time to correct. This speculation could be tested by repeating our study in fifty years – although this would assume no long-term trends in offspring sex ratios over that time, an assumption that may not hold given the potential for climate change to influence the sex of hatchlings.

3. Population genetics

A common question when faced with conserving a species with a wide or scattered distribution is ‘is this one big population or several small ones’. The answer can substantially alter management strategies because a set of separate small populations will face very different challenges from a single large population. Indeed, each population may require a separate, distinct management plan. In marine turtles, satellite tracking and capture-mark-recapture studies have helped enormously in the study of individual movements and migrations. However, tags and trackers cannot be fitted to hatchlings, and studies involving marking hatchlings' shells require considerable time to produce results. Thus we cannot yet track large numbers of individual hatchlings from birth to sexual maturity, limiting our ability to determine where hatchlings born at a particular site will eventually end up breeding. However, if two populations of a species rarely, or never, interbreed, they will, over time, acquire a distinct population-level 'genetic fingerprint'. The magnitude of difference between these fingerprints will relate to the duration and magnitude of their isolation. Examining such differences can help biologists infer which populations are sufficiently isolated to justify separate management plans, and to highlight vulnerable connections between populations that, if disturbed, may lead to population fragmentation. An extension of this analysis, comparing key sections of DNA that are passed down either only through the female line (mitochondrial DNA) or through both sexes, allows for the testing of whether the dispersal patterns of males and females differ. It is also possible to compare foraging juveniles to adults, and to infer which nesting grounds these juveniles might have come from. This latter technique has already informed hawksbill conservation in the Caribbean (see e.g. Mortimer *et al.* 2007b; but see Godfrey *et al.* 2007). In our study, we tested for genetic differentiation between females nesting on six islands (including Cousine) separated by between 40-450 km in the Granitic and Amirantes island groups of Seychelles. We also tested for genetic differentiation between the females nesting on Cousine Island and the males that fathered their offspring. We found no evidence that females nesting on different islands were genetically distinct, even between the pair of islands that was the furthest apart (Alphonse/St François v. Cousine, 450 km). From this we can conclude that the hawksbills of the Seychelles comprise a single, genetically-mixed population. However, we did find evidence that males and females are not equal dispersers: there were more close relatives (e.g. full- and half-siblings) among the females nesting on Cousine than among the males that mated with them. This shows that females breed in the areas where they were born more often than males do, which is perhaps not surprising given what we know about the strong female natal philopatry seen in other marine turtles. That this philopatry has not led to strong inter-island genetic differences may be because dispersing males act to 'spread' genetic diversity across a wider area. It could also be because females do disperse, but to a lesser degree than males. Within Seychelles, previous work found no genetic structure at maternally-inherited mitochondrial DNA, even at the 250+ km scale between the Amirantes and Granitic island groups (Vargas *et al.* 2013; Vargas *et al.*, unpublished data). This suggests that sufficient dispersal of females occurs to keep separate islands genetically indistinguishable at mitochondrial DNA, an implication supported by occasional re-sightings of tagged individuals nesting on islands separated by considerable distance (Mortimer, unpublished data; see also chapter 3). Finally, we also analysed genetic data from a sample of juvenile hawksbills feeding in the waters around Aldabra Atoll, 750 km from the nearest island to feature in our study of adults. These individuals were found to be part of the same genetic population as our studied adults. This suggests that the limits of the Seychelles genetic population may extend well beyond the geographic range of our study of nesting females, and/or that different geographic areas may be important at different life stages of hawksbills in this region.

From a conservation perspective there is much that is positive in our results. By being a single, large, genetically well-mixed population, the hawksbills of Seychelles are less vulnerable to demographic fluctuations, systematic inbreeding, and long-term environmental change than if the region were comprised of many smaller, isolated populations. Also, that females are not overly strict in returning to breed on their natal beaches offers hope for the re-colonisation of areas of Seychelles from where they have been extirpated. If these presently unused beaches are rendered suitable and disturbance-free, they should eventually be nested upon by a female. On the other hand, our finding that juvenile hawksbills foraging around Aldabra are part of the same Seychelles population raises a cautionary point: that this is a wide-ranging population/species for which different areas are important at different stages of its life, and that effective management plans, which may need to be international, cannot afford to ignore this.

4. Genetic variability and fitness

Inbreeding reduces individual fitness (survival, reproductive output), and is therefore an important consideration in conservation management. Inbreeding is difficult to study in wild populations without data on pedigrees, but because inbreeding also reduces individual genetic variability, biologists can use this variability as a proxy. However, fitness does not increase indefinitely with genetic variability, as matings between individuals from separate populations may disrupt coadapted gene complexes or local adaptation. If this happens, it can result in ‘outbreeding depression’ – outbred offspring experience reduced fitness because of *higher* individual genetic variability.

We tested for correlations between individual genetic variability and fitness in the hawksbills nesting on Cousine. Specifically, we tested whether the number of eggs a female laid was affected by how genetically variable she was, and whether the proportion of eggs that successfully developed was affected by female genetic variability, male genetic variability, and the genetic similarity between the male and female (an index of how related they are, and thus how inbred their offspring will be). Our results were somewhat surprising, with two competitive scenarios. One of these was a quadratic effect of male genetic variability, where egg development success is maximised at intermediate values of male variability. The second was an interaction between male variability and parental similarity, which implied that mating with a more variable male had a negative effect if the pair were genetically dissimilar but positive if the pair were similar. An alternative way of representing this is to say that more male variability is bad if the parents are already producing variable offspring (parents dissimilar), but good if the parents are producing less variable offspring (parents similar). Both the quadratic and the interaction scenarios explained 8-9% of variance in egg development success. We found no effects of female genetic variability.

Whichever one of the above interpretations one goes with, the relationship between genetic variability and fitness can be both positive and negative in the same statistical model, which may exert a long-term stabilising effect on population genetic variability. Potentially, this may imply a tension between inbreeding and outbreeding depression. One aspect of marine turtle life-history that could contribute to both positive and negative effects of genetic variability is female philopatry. This creates potential for both local adaptation (good) and inbreeding (bad), both of which might be disrupted by mating with a male of different genetic origins. Interestingly, we do not find evidence that females engage in mate choice to optimise offspring variability, suggesting the potential reproductive benefits from such selection might be outweighed by the costs of evolving an appropriate mechanism.

We see no immediate cause of concern for turtle conservation managers in our findings regarding genetic variability. An optimum genetic variability may simply be a natural consequence of the dynamic between female homing and male dispersal in turtles. It is also not clear what we could do in terms of conservation management even if we wanted to, as it would be impossible to control who mates with whom, or even know pairings until after a nest has hatched. All that can be said for these results in an immediate practical context is that they help us better understand the sources of variation affecting nest success. The broader conservation implications are uncertain, for our results highlight a vulnerability to the fitness-reducing effects of changes in genetic variability, but also that the population has not been sufficiently negatively affected by overhunting to be experiencing a serious problem with systematic inbreeding.

5. Effective population size

The larger a population, the more readily it retains genetic diversity and future adaptive potential. The census population of Seychelles hawksbill turtles is upwards of 2500 females, and the implications of our paternity study are that the number of males is comparable (see above). Furthermore, our study also indicates that the hawksbill turtles spread across the islands of Seychelles are a single mixed population rather than many separate smaller populations associated with specific island(s). Thus the population appears to be widely distributed, genetically healthy, and not in danger of systematic inbreeding. However, as biologists we should be wary of judging vulnerability to loss of genetic diversity from census counts, as a variety of processes such as skewed sex ratios, variation between individuals in reproductive output, and fluctuations in population size increase the rate of loss. A measure termed 'effective population size' is a revision of census data to take account of such processes. It is rarely possible to estimate this directly from demographic data, not least because the relevant data are not normally available, but several statistical techniques have been developed to estimate effective population size directly from DNA data. It is also possible to use these techniques to look for past changes in effective population size that might indicate whether population has experienced a recent or strong reduction or expansion in numbers.

Using our DNA dataset, we estimated the effective size of the Seychelles hawksbill turtle population to be approximately 1000-2000. Although substantially lower than the census population size, this is above the figure of 500 often cited as the minimum for long-term preservation of genetic diversity (Franklin 1980), and is higher as a ratio to census size (0.19-0.48 – see estimates of males given above) than many populations of conservation concern (0.11; Frankham 1995). We also looked for evidence of past changes to effective population size that might indicate whether the decline in Seychelles hawksbill numbers that has occurred since humans colonised these islands has left a detectable genetic signature. However, we found no indication of a recent (in evolutionary terms) change in effective population size, despite the substantial reductions in the number of nesting females over the 250+ years since humans arrived in Seychelles.

The hawksbill's long generation time may partly explain why the Seychelles hawksbill population has maintained its genetic diversity despite a population reduction. Although numbers have been reduced substantially from historic highs, they have not been held at their lower level for very long (hunting was banned in Seychelles 1994) when measured in hawksbill generations (approximately 35 years; Mortimer & Donnelly 2008). Perhaps we might see the effective population size estimate come down if the population were held at its current level for several generations, but given that the number of nesting females is increasing, it is possible that the Seychelles hawksbill population will escape its historic reduction in size with minimal consequences for population genetic diversity. Interestingly, the mating system (reproduction is not skewed to a few males) and population connectivity (high) that we have inferred in this population may also have helped keep genetic diversity high. Looking to the future, a large effective population size that keeps genetic diversity could help the population retain the potential to adapt to future environmental changes. This may be particularly important in militating against deleterious effects of global climate change, such as perturbations to hatchling sex ratios.

6. Summary

Overall, the findings of our genetic study on hawksbills in the Republic of Seychelles are extremely positive with respect to this population's conservation status. The population seems to be reasonably large and of roughly even sex ratio, spread over a wide geographic area, and genetically diverse and well-mixed. We have also shed light on several aspects of hawksbill life history and demography that were previously poorly understood, including its mating system. Our data indicate that this population should be seen as a single genetic unit, but this should not be taken as an argument for management relaxation or complacency at the island level – the population is greatly reduced from historical levels, and the target should still be to have more hawksbill turtles. This requires that current protection be continued, and extended where possible. Extension of protection to suitable beaches that don't currently have nesting turtles may be particularly fruitful, as our results highlight a potential for dispersal that could assist the re-colonisation of such areas from which turtles have been extirpated. Unfortunately, despite their upwards population trend in Seychelles, the hawksbill's future is far from guaranteed: deliberate and accidental killings remain a problem, and the threats posed by climate change are a serious cause for concern. However, a large, dispersed, genetically diverse population puts it in as good a position as might be hoped for to face these future challenges.